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Leptospira Seroprevalence in Companion Animals in Tennessee

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To the Graduate Council:

I am submitting herewith a thesis written by Kellie Anne McCreight entitled "Leptospira Seroprevalence in Companion Animals in Tennessee." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Comparative and Experimental Medicine.

Dr. Sreekumari Rajeev, Major Professor

We have read this thesis and recommend its acceptance:

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Seroprevalence of *Leptospira* in Companion Animals in Tennessee

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ABSTRACT

Leptospirosis is a re-emerging zoonotic disease in humans and animals. The bacteria *Leptospira spp.* causing this disease is maintained in the kidneys of animals such as rodents and cattle as well as in the environment. Animals harboring *Leptospira spp*. in the kidneys frequently shed the bacteria in their urine, contaminating the environment. Contact with contaminated soil and water may result in infection. Animals and humans may develop serious life threatening disease from *Leptospira* infection. Approximately 1 million new human cases and over 50,000 deaths are reported worldwide. Numerous animal species including rodents, cattle, and dogs may serve as reservoir hosts and can act as carriers for the infection. Our objective was to determine the *Leptospira* seroprevalence in dogs, horses, and cats in Tennessee.

In this study, we collected convenient serum samples from dogs (n = 376), horses (n = 88), and cats (n = 169) submitted to The University of Tennessee College of Veterinary Medicine clinical pathology diagnostic laboratory. We tested the serum for *Leptospira* using the Microscopic Agglutination Test (MAT) against 12 *Leptospira* serovars. Seroprevalence was recorded as 29.41% (110/376) in dogs, 47.73% (42/88) in horses, and 12.35% (21/169) in cats. The seroprevalence in our study was higher in dogs and cats than previously reported in the Cumberland region. The highest seroprevalence was observed for serovar Autumnalis (82/110; 74.55%) in dogs, Bratislava (40/42; 95.24%) in horses, and Bratislava (9/21; 42.86%) in cats. We found a significant cross-reactivity between multiple *Leptospira* serovars tested, specifically among serovars Autumnalis, Canicola, Copenhageni, Grippotyphosa, Mankarso and Pomona. We also found that vaccinated dogs had a significantly higher seroprevalence (45.92%) compared to unvaccinated dogs (16.28%; $p < 0.001$). A significant difference in seroprevalence was observed in vaccinated and unvaccinated dogs to all of the serovars included in the canine vaccine; Canicola, Grippotyphosa, Icterohaemorrhagiae, and Pomona ($p < 0.001$).

This study provides critical knowledge on *Leptospira* seroprevalence in companion animals allowing for a wider picture of the impact of the disease in this state.

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CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Leptospirosis is an emerging infectious disease, caused by the bacteria *Leptospira*, that has gained global recognition due to large outbreaks, occurring mainly in tropical countries. This disease is most commonly seen in poor, tropical regions but is not restricted to those places (Costa, Hagan, et al., 2015; Torgerson et al., 2015). In 1886, leptospirosis was first described by Dr. Adolph Weil as a disease manifested as jaundice with splenomegaly, renal dysfunction, conjunctivitis, and skin rashes, which was then named Weil's disease (Alston & Brown, 1937). Now, over one million leptospirosis cases occur annually, with case fatality rates around 5% overall (Munoz-Zanzi et al., 2020).

1.2 *Leptospira spp.*

Leptospira are thin, spiral shaped bacteria that are highly motile. There are both pathogenic and saprophytic species, with the former being able to infect a vast majority of mammals, and survive in the environments as well. The structure of the bacterial cell is similar to gram-negative bacteria, with an outer membrane composed of lipopolysaccharides (LPS), an inner membrane, and a peptidoglycan-containing inner space. *Leptospira* is one of the few spirochetes that contain a LPS on the surface of the outer membrane (Bonhomme & Werts, 2020). Like other gram-negative bacteria, *Leptospira* get their firmness, shape, and strength from the peptidoglycan layer, however this layer in the *Leptospira* cell is closer to the cytoplasmic membrane than in a traditional gram-negative bacterial cell. The shape of *Leptospira* is independent of the periplasmic flagella, with the peptidoglycan layer and cytoskeletal proteins adding to the spiral shape. The periplasmic flagella also allows pathogens to rapidly cross mammalian cell barriers, disseminate hematogenously, and infect the target organ, while avoiding recognition from the host innate immune system, fleeing immune attacks (Picardeau, 2020).

1.3 Taxonomy

The family *Leptospiraceae*, was defined in 1979, and includes the genera *Leptonema* and *Leptospira*. The species of the *Leptospira* genera are clustered into three groups: a pathogenic, a saprophytic, and an intermediate group. The pathogenic group is further divided into two subgroups, P1 and P2. P1 is the original virulent lineage and P2 is intermediate and low-virulence. The saprophytic group is also further divided into two subgroups, S1 and S2. S1 is the original saprophytic lineage and S2 is a newer subgroup (Vincent et al., 2019). **Figure 1** shows the phylogenic tree and **Table 1** shows the 68 valid and currently published species in the *Leptospira* genus (Levett & Picardeau, 2021). The members of *Leptospira spp.* that are antigenically similar are classified into serogroups. The serogroups can then be further classified into serovars based on surfaced antigens. The sergrouping classification does not have any taxonomical significance (Zhang et al., 2015).

1.4 *Leptospira* Epidemiology and Transmission

1.4.1 Humans

The bacteria enter through compromised skin or mucous membranes. Direct contact can occur through an infected animal and indirect contact can occur through a contaminated source, such as water and soil sources (Haake & Levett, 2015), while many infections are caused by walking barefoot in damp conditions or from gardening without gloves (Douglin et al., 1997). Due to the large scale risks of direct and indirect contact, occupational risks are a problem for veterinarians, abattoir workers, farmers, animal shelter workers, and those that work in a laboratory setting (Campagnolo et al., 2000; Chan et al., 1987; Looke, 1986). Leptospirosis is endemic in warmer tropical countries, mostly due to the prolonged survival in wet and humid areas, but also because those climates are often seen in tropical countries and developing countries (Felzemburgh et al., 2014; Levett, 2001). However, due to factors such as climate change and human migration, leptospirosis is now seen in temperate regions as well (Costa, Hagan, et al., 2015; Desai et al., 2009). The incidence of leptospirosis has been increasing in non-endemic regions due to the increasing popularity of water sports, such as rafting

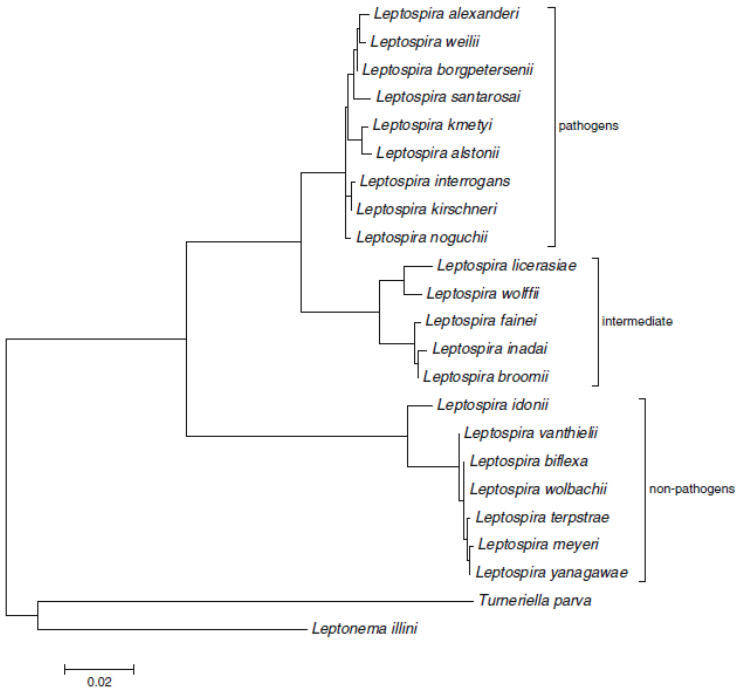

Figure 1 *A representative phylogenic tree of the Leptospiraceae 16S rRNA gene sequences showing positions of various Leptospira species. Image taken from Tamura et al. (2011).*

Table 1 *Species within the Leptospiraceae family.*

Species	**Valid Publication**
L. abararensis	Korba et al. (2021)
L. adleri	Thibeaux et al. (2018)
L. ainazelensis	Korba et al. (2021)
L. ainlahdjerensis	Korba et al. (2021)
L. alexanderi	Brenner et al. (1999)
L. alstonii	Smythe et al. (2013)
L. andrefontaineae	Vincent et al. (2019)
L. bandrabouensis	Vincent et al. (2019)
L. barantonii	Thibeaux et al. (2018)
L. biflexa	Noguchi (1918)
L. borgpetersenii	Yasuda et al. (1987)
L. bourretii	Vincent et al. (2019)
L. bouyouniensis	Vincent et al. (2019)
L. brenneri	Thibeaux et al. (2018)
L. broomii	Levett et al. (2006)
L. chreensis	Korba et al. (2021)
L. congkakensis	Vincent et al. (2019)
L. dzianensis	Vincent et al. (2019)

Table 1 Continued

L. dzoumogneensis	Vincent et al. (2019)
L. ellinghausenii	Masuzawa et al. (2019)
L. ellisii	Thibeaux et al. (2018)
L. fainei	Perolat et al. (1998)
L. fletcheri	Vincent et al. (2019)
L. gomenesis	Vincent et al. (2019)
L. haakeii	Thibeaux et al. (2018)
L. harrisiae	Thibeaux et al. (2018)
L. hartskeerlii	Thibeaux et al. (2018)
L. idonii	Saito et al. (2013)
L. ilyithenensis	Vincent et al. (2019)
L. inadai	Yasuda et al. (1987)
L. interrogans	Faine and Stallman (1982)
L. johnsonii	Masuzawa et al. (2019)
L. kanakyensis	Vincent et al. (2019)
L. kemamanensis	Vincent et al. (2019)
L. kirschneri	Ramadass et al. (1992)
L. kmetyi	Slack et al. (2009)
L. kobayashii	Vincent et al. (2019)
L. koniambonensis	Vincent et al. (2019)
L. langatensis	Vincent et al. (2019)
L. levettii	Thibeaux et al. (2018)
L. licerasiae	Matthias et al. (2008)
L. mayottensis	Bourhy et al. (2014)
L. meyeri	Yasuda et al. (1987)
L. montravelensis	Vincent et al. (2019)
L. mtsangambouensis	Vincent et al. (2019)
L. neocaledonica	Thibeaux et al. (2018)
L. noguchii	Yasuda et al. (1987)
L. noumeaensis	Vincent et al. (2019)
L. ognonensis	Vincent et al. (2019)
L. parva	Hovind-Hougen et al. (1981)
L. perdikensis	Vincent et al. (2019)
L. perolatii	Thibeaux et al. (2018)
L. putramalaysiae	Vincent et al. (2019)
L. ryugenii	Masuzawa et al. (2019)
L. saintgironsiae	Thibeaux et al. (2018)
L. sanjuanensis	Fernandes et al. (2022)
L. santarosai	Yasuda et al. (1987)
L. sarikeiensis	Vincent et al. (2019)
L. selangorensis	Vincent et al. (2019)
L. semungkisensis	Vincent et al. (2019)
L. stimsonii	Casanovas-Massana et al. (2020)

Table 1 Continued

L. terpstrae	Smythe et al. (2013)
L. tipperaryensis	Vincent et al. (2019)
L. vanthielii	Smythe et al. (2013)
L. venezuelensis	Puche et al. (2018)
L. weilii	Yasuda et al. (1987)
L. wolbachii	Yasuda et al. (1987)
L. wolffii	Slack et al. (2008)
L. yanagawae	Smythe et al. (2013)
L. yasudae	Casanovas-Massana et al. (2020)

Table taken from Parte, A.C., et al., (2020)

(Agampodi et al., 2014; Wilkins et al., 1988), canoeing (Philipp et al., 1992), and triathlons (Morgan et al., 2002).

In a study by Costa, Hagan, et al. (2015) on the global morbidity and mortality of leptospirosis, the Monte Carlo model estimated the annual morbidity and mortality to be 14.77 cases per 100,000 population and 0.84 deaths per 100,000 population, respectively. The model also estimated that there are 1,030,000 cases and 58,900 deaths worldwide, per year. Seventy-three percent of the cases and deaths occur between the Tropics of Cancer and Capricorn. The highest morbidity occurred in males 20-29 years of age, with the highest mortality being in males 50-59 years of age. Based on the 10 studies that reported age and gender data, adult males had the highest risk of leptospirosis, with men being the most at risk at ages 20-29. The highest risk for death was adult males, ages 50-59. The authors have concluded that this disease is among the top zoonotic causes of morbidity and mortality worldwide. Whether it is due to gender occupational differences or due to exposure bias, Costa hypothesized that adult males were most typically infected, which the study confirmed.

Three patterns of the epidemiology of leptospirosis were described by Faine (1999). The first pattern is described as temperate climates where human infection almost always occurs by direct contact with infected farming animals, such as cattle or pigs, with only a few serovars causing infection. The second pattern is described as a wet, tropical area with more reservoir species and with a broader variety of serovars causing infection than the first pattern. The third pattern describes a rodent-borne infection in urban environments, becoming especially infectious when infrastructure is disarranged by poor social conditions or natural disasters, which have been confirmed by other literature (Felzemburgh et al., 2014; Haake & Levett, 2015).

1.4.2 Animals

Leptospira can be found in almost any region of the world and can infect almost every species of animal. There are many animals that are reservoirs of infection, pathogenic *Leptospira* have been isolated from both wild and domestic animals (Ellis, 2015), as well as reptiles (Ebani, 2017). Animals can be considered maintenance or incidental hosts. A maintenance host maintains the pathogen by chronic infection of the

renal tubules without experiencing symptoms. Incidental hosts, also called “accidental hosts”, show severe clinical signs, but have a shorter renal infection time. Specific species of animals are known to host many different serovars around the globe (Hartskeerl & Terpstra, 1996). Rodents are commonly known to be a maintenance host for serogroups Icterhaemorrhagiae and Ballum, dogs tend to harbor Canicola, dairy cattle tend to harbor Grippotyphosa, Hardjo, and Pomona, and pigs tend to harbor Bratislava, Pomona, or Tarassovi (Levett, 2001).

Infection in incidental hosts or with non-host adapted serovars is typically acquired through indirect contact and results in mild to severe clinical disease that can lead to life-threatening renal, hepatic, and/or pulmonary disease (Ellis, 2015). Like humans, animals are more prone to disease in warm, wet climates. Transmission typically occurs from contact with *Leptospira* contaminated water or soil, or from direct contact through infected urine. *Leptospira* infection has been linked to infection through the genital tract and through sexual transmission in cattle, horses, and pigs (Fernandes et al., 2020; Hamond et al., 2014; Loureiro et al., 2017; Masri et al., 1997). One to two days after initial infection, a bacteremia phase occurs, and lasts as long as a week. This stage is typically associated with acute clinical disease and at this stage of infection *Leptospira* can be isolated from blood, most organs, and cerebral spinal fluid. Antibodies appear at the end of this stage, around 10 to 14 days after initial infection, and reach peak level three to six weeks later. The highest titers can be observed for six weeks but vary substantially. Following this period, *Leptospira* will colonize the proximal renal tubules where they will accumulate and be shed through the urine. The time and magnitude of the *Leptospira* shedding varies between species. In pregnant females, *Leptospira* may also colonize in the reproductive tract, resulting in abortions, stillbirth, or neonatal disease caused by intrauterine infections during late gestation. There are more asymptomatic infections than realized, which can be explained by the large difference found in seroprevalence versus disease prevalence (Ellis, 2015; Mori et al., 2017).

1.5 *Leptospira* Disease and Pathogenesis

1.5.1 Humans

The spectrum of symptoms of leptospirosis in humans is extremely broad. Symptoms typically begin with fever, chills, jaundice, and headache that can be observed with other causes of acute febrile syndrome. The symptoms are non-specific and could be misdiagnosed as many different illnesses such as the flu, dengue, or malaria (Haake & Levett, 2015). Headaches typically occur with retro-orbital pain and muscle pain is most common in the legs and lower back (Silva et al., 2011). Symptoms of severe cases of leptospirosis may be associated with multiple organs including liver, kidneys, lungs, and brain. In cases of severe leptospirosis, bleeding can occur, ranging from mild petechiae to pulmonary hemorrhage. Neurologic symptoms can occur, including altered mental status, hemiplegia, transverse myelitis, and Guillain-Barré syndrome (Saeed et al., 2018). The clinical presentation of leptospirosis is biphasic. The acute phase is around a week long, followed by the immune phase. In the immune phase, antibodies are produced and *Leptospira* can be excreted in the urine. During the immune phase *Leptospira* can localize in the tissues and can cause complications (Levett, 2001; Philip et al., 2020).

1.5.2 Animals

1.5.2.1 Rodents

The primary reservoir hosts for *Leptospira* are rodents. Rats were identified as the first hosts of *Leptospira*, and It was discovered that rats can carry *Leptospira* in the kidneys while being asymptomatic, which was the first observation of an asymptomatic carrier status (Adler & de la Pena Moctezuma, 2010). Rodents were first recognized as the reservoir host when Ido et al. (1917) discovered that after infecting them with *Leptospira*, they produced no clinical signs, even when shedding occurred. Animals being in close proximity to infected rodents is a recognized risk factor for *Leptospira* infection (Costa, Wunder, et al., 2015), with peaks in rodent abundance being studied as a link to peaks in *Leptospira* infection (Holt et al., 2006).

1.5.2.2 Domestic Animals

Canines are the most extensively studied companion animal, due to the close relation and contact they share with humans. Worldwide, the most commonly reported serovar in canines is Canicola, however, infections from serovars in the Icterohaemorrhagiae serogroup is becoming increasingly prevalent globally. Ten distinct serovars have been associated with clinical disease serologically, the main ones being Autumnalis, Bratislava, Canicola, Grippotyphosa, and Icterohaemorrhagiae (Gautam, Wu, et al., 2010). The seroprevalence of canine leptospirosis is decreasing in many countries, which is thought to be caused by the use of vaccines (Ellis, 2010). Leptospirosis can present symptoms ranging from mild subclinical disease to a severe disease paired with renal failure. Most canines are infected by contact with infected urine or contaminated water that enter through broken skin. Chronic carrier status can occur in canines, making them a source of infection as well as a public health concern. The forms of clinical disease in canines are anicteric and icteric illness. The icteric form presents with jaundice and liver involvement and is a severe illness, typically coupled with multiorgan involvement, or failure. The anicteric form of the disease is typically a self-limited, flu-like illness. The anicteric phase is typically a sudden onset with a bi-phasic fever. This phase accounts for 80-90% of cases and can be coupled with kidney failure. The first phase occurs during the same time as the bacteremic phase and can last for a week. A secondary phase can occur after about three or four days, and happens at the same time the immune response occurs. The pyretic phases usually occurs with a wide array of symptoms. The common symptoms can include anorexia, vomiting, nausea, prostration, and sever myalgia. The icteric phase is more severe, accounting for 5-10% of cases, with liver, kidney, or vascular symptoms, and progression is rapid (Ellis, 2015; Karpagam & Ganesh, 2020; Levett, 2001).

In felines, leptospirosis is extremely understudied. Felines are reported to be resistant to the bacteria and therefore typically show no clinical signs, with early attempts of experimental infections failing (Larsson et al., 1985). Felines are able to be infected and are able to be carriers, although the transmission routes have yet to be studied in depth, with rodents being thought to play a role (Shophet & Marshall, 1980). The ability of cats to transmit *Leptospira* and the role as maintenance or incidental hosts is still being

studied, and is not known at this time. The most common symptoms in felines are those similar to renal issues, such as vomiting, fever, and increased thirst (Alashraf et al., 2020; Arbour et al., 2012; Rodriguez et al., 2014). In a study done by Rodriguez et al. (2014), seropositivity was greater in cats with kidney disease and urinary shedding was observed. In a study done by Arbour et al. (2012), renal insufficiency was noticed but liver involvement was not. In a study done by Markovich et al. (2012), the cats captured had no symptoms of *Leptospira* infection, but 3 of the animals tested were MAT positive for one or more *Leptospira* serovar.

Most of the infections in horses are sub-clinical, but acute infections can resemble those of severe infection in other animals, such as jaundice, depression, and hemoglobinuria, while fatigue and pulmonary hemorrhage following exercise have also been reported. Although those types of cases are possible, the most common cases are associated either with abortion (Hamond et al., 2015; Pinna et al., 2014) or with equine recurrent uveitis (ERU) (Ellis, 2015). These cases are typically associated with the presence of specific antibodies in the aqueous and vitreous humor, with persistence of *Leptospira* in the eye. A period of blindness is suggested to occur as a result of an auto-immune response, reacting to two *Leptospira* proteins, LruA and LruB, which are indicated in those cases by the eyes (Ellis, 2015). ERU is mostly associated with specific serovars, particularly Kennewicki in the USA and Grippotyphosa in Europe (Hartskeerl et al., 2004). Serovar Kennewicki is one of the predominant serovars in horses, but has been associated with various host species, like pigs or skunks (Timoney et al., 2011). Since most clinical signs are nonspecific, diagnosing equine leptospirosis is hard without the help of diagnostic tests. The most common serovars associated with equine leptospirosis are Pomona and Grippotyphosa (Divers et al., 2019).

1.5.2.3 Ruminants

Cattle are considered one of the main *Leptospira* maintenance hosts. *L. borgpetersenii* serovar Hardjo is the most common strain maintained in cattle, while *L. interrogans* serovar Hardjo can also be seen, with both strains being able to persist and colonize in the genital tract, meaning venereal transmission could occur. Open herds, co-grazing with sheep, and contaminated water can all be risk factors for Hardjo infection

(Ryan et al., 2012). Another serovar that causes infection is Kennewicki, which is typically only seen South America, Australia, and New Zealand, and very rarely North America. Other serovars in the Australis, Autumnalis, Canicola, Grippotyphosa, Pomona, or Tarassovi serogroups are able to infect cattle, but are typically only incidental infections. Clinical signs of severe leptospirosis in cattle include pyrexia, hemolytic anemia, hemoglobinuria, jaundice, death, and blood tinged milk is seen in lactating cows. Leptospirosis in cattle can be attributed with large economic losses, mostly occurring with abortions, stillbirths, premature births, and reduced birth weight (Ellis et al., 1985; Martins et al., 2012; Yatbantoong & Chaiyarat, 2019).

Sheep were considered to be resistant to *Leptospira*, but have emerged as alternative maintenance hosts for serovar Hardjo (Martins et al., 2012; Vermunt et al., 1994). Infections are mostly subclinical, while abortion, stillbirth, and agalactia may occur (da Silva et al., 2021; Ellis et al., 1983). Leptospirosis has also been linked to infertility in sheep and goats (Lilenbaum et al., 2009).

Like cattle, leptospirosis in swine can lead to major economic losses. Serovars Australis, Pomona, and Tarassovi are most commonly linked to infection, but incidental infections can be caused by serovars Canicola, Grippotyphosa, and Icterohaemorrhagiae. Symptoms include hemorrhage, hematuria, jaundice, and signs of renal failure. Adults that are not pregnant can be carriers, resulting in a large amount of *Leptospira* shed in the urine. If pregnant, abortion, stillbirths, or birthing weak piglets are common symptoms of disease (Ellis, 2015).

1.6 Diagnosis

The diagnosis of *Leptospira* infection is often times overlooked or misdiagnosed due to the broad spectrum of symptoms. Due to this, clinicians may not recognize the symptoms as *Leptospira* infection, and therefore, rely on laboratory testing for confirmation of diagnosis. Many diagnostic tests are available, depending on the stage of the disease. It is important to know the course of infection in order to know which diagnostic test will be the most beneficial (Reagan & Sykes, 2019). The types of diagnostic tests used can be separated into two different categories: agent detection and host response. Agent detection are those that are used to culture *Leptospira*, detect

Leptospira DNA, or to see live *Leptospira* under the microscope. Host response tests include those that test for an immune response, such as microscopic agglutination test (MAT) and the enzyme-linked immunoassay test (ELISA).

1.6.1 Agent Detection

1.6.1.1 Microscopy and Culture

Leptospira can be seen under the microscope by dark-field microscopy. Dark-field microscopy uses samples such as blood, urine, or cerebral spinal fluid. Dark-field microscopy requires 10^4 *Leptospira*/ml for one cell per field to be seen, but you can increase the reactivity by using a staining method, such as silver staining (Verma et al., 2020). Disadvantages include false positives, skilled personnel needed to confirm diagnosis, and both the sensitivity and specificity of dark-field microscopy are around 60% when used alone (Pinto et al., 2022; Sharma & Kalawat, 2008).

A direct immunofluorescence assay (DFA) can be used with urine or tissue samples. The assay is prepared with a smear, fixed in acetone, then treated with polyclonal antibodies conjugated with fluorescein isothiocyanate. The fluorescing organisms with a morphology consistent with *Leptospira* will be seen when positive.

Culture is considered a standard technique and is typically grown using urine, blood, or cerebral spinal fluid. The samples are grown in Ellinghousen-McCullough-Johnson-Harris (EMJH) media or Fletcher's medium enriched with vitamins, long-chain fatty acids, and ammonium salts (Adler & de la Pena Moctezuma, 2010). *Leptospira* can survive in blood culture media for a few days, with nothing specific influencing the viability of the *Leptospira* in cultures. Isolation and identification requires an incubation period of 6-8 weeks before diagnosis is confirmed. It has been studied and thought that the combination of aerobic culture and incubation temperature at 30^{o}C increases the viability, increasing the chances of isolation (Palmer & Zochowski, 2000). Cultures should be examined weekly by dark-field microscopy for up to 13 weeks before throwing away (Levett, 2001). Once cultured and isolated, *Leptospira* are either identified by serological testing or molecular techniques. The sensitivity of *Leptospira* culture is <10%, but has a 100% specificity (Maze et al., 2019).

1.6.1.2 Molecular Diagnostics

A study published recently used five different PCR methods, culture, and immunofluorescence for recognition of Hardjo. Primers used from rRNA genes were the least specific, with none of the tests being 100% sensitive (Levett, 2001).

One of the most common ways to confirm the presence of *Leptospira* is by polymerase chain reaction (PCR). PCR is typically performed on blood or urine samples but tissue, kidney, or lung samples can be used (Reagan & Sykes, 2019) and this test is highly sensitive. PCR is a widely used method to detect *Leptospira* in clinical samples and is beneficial for an early diagnosis, where other testing requires hours to days (Pinto et al., 2022). When performed, PCR amplifies specific gene fragments, usually targeting the pathogen-specific genes *lipL32*, *lig*, or *lfb1*, allowing it to lengthen the DNA. However, during the late-acute phase once the bacteria starts to disappear from the bloodstream, PCR can yield a negative result. Researchers use sequencing and analysis of PCR products for further confirmation and characterization of samples. However, this test cannot be used for serovar identification (Marquez et al., 2017). PCR is widely used due to the high sensitivity and specificity and rapid turnaround time. The cost of the machine is very high, preventing some countries from being able to use this method. The sensitivity of PCR is around 50% and the specificity is around 80% (Mullan & Panwala, 2016)

1.6.1.3 Serological Diagnosis

Most strains of *Leptospira* are confirmed by serology. Antibodies are detectable between five and seven days after symptoms are noticeable. However, due to the wide range of clinical signs, diagnosis of leptospirosis is very difficult and depends on an array of laboratory diagnoses to detect specific antibodies (Adler & de la Pena Moctezuma, 2010).

The enzyme-linked immunoassay test (ELISA) is used to detect specific IgM and/or IgG antibodies in patient serum samples to detect the presence of antibodies in the infected patients to *Leptospira*. ELISA can detect antibodies between 5-7 days after symptoms occur. If testing occurs before 7 days, it can fail to detect IgM antibodies and therefore cannot detect the infecting serovar. The modification of this test detects for IgM against LipL32, aiding in earlier diagnosis (Pinto et al., 2022). The commercial IgG ELISA

has a sensitivity around 30% to 50% and specificity around 85% to 95%. The commercial IgM ELISA has a sensitivity of 80% and a specificity of 93% (Niloofa et al., 2021).

The microscopic agglutination test (MAT) is considered the gold standard for diagnosis. The test uses agglutination of live *Leptospira* serovars with patient serum to detect antibodies developed in the host against *Leptospira*. However, there are often false-positive and/or false-negative results that appear, based on stage of infection, previous exposure, and vaccination status. Strains common to the area or region are used. A titer equal to or above 1:50 is considered seropositive (Niloofa et al., 2015). In a clinical case of *Leptospira* a paired titer testing acute and convalescent serum and observation of a fourfold rise increase in MAT titer is considered as diagnostic for leptospirosis. The best time to perform this test is 5-7 days after the onset of symptoms. MAT detects both IgM and IgG agglutinating antibodies, but does not aid in patient management since diagnosis is not immediate. Disadvantages to MAT testing include requiring live organisms, cross-reactions occurring, inability to detect for current infection, and false positives (Pinto et al., 2022).

1.7 Treatment

1.7.1 Humans

The majority of leptospirosis cases are mild with early detection being key in diagnosis. Early detection followed by antimicrobial therapy can stop the disease from progressing into a severe case that may require hospitalization. Diagnosis often depends on suspicion based on the patient's previous exposure and symptoms. *Leptospira* are susceptible to β-lactams, macrolides, tetracyclines, fluoroquinolones, and streptomycin (Alexander & Rule, 1986). Antibiotic therapy for patients with severe leptospirosis includes intravenous penicillin, ampicillin, ceftriaxone, or cefotaxime. Adult outpatients should be given doxycycline or azithromycin (Hospenthal & Murray, 2003; Ressner et al., 2008). Doxycycline was shown to have reduced the intensity of the anicteric phase and has also been known to prevent shedding in the urine (Haake & Levett, 2015; Levett, 2001).

1.7.2 Domestic Animals

The treatment of leptospirosis is a controversial topic in the veterinary community, mostly due to the lack of complete understanding of pathogenesis. For canines and felines, treatment occurring after four to seven days post infection has been seen as less effective in recovery (Pappas & Cascio, 2006). The treatment of leptospirosis in canines and felines is usually a combined approach of antibiotics and supportive symptomatic treatment. Intensive supportive therapy may be required for domestic animals, along with fluid therapy and occasionally blood transfusions and dialysis. Acute leptospirosis is typically treated by doxycycline, but can also be treated by penicillin, ampicillin, or amoxycillin, as long as treatment begins early, with treatment lasting for at least two weeks (Ellis, 2015). In horses, acute disease is often treated by antibiotics, typically penicillin, ampicillin, enrofloxacin, tetracyclines, and doxycycline. Treatment of ERU is different; corticosteroids are given to decrease inflammation, but is not useful long-term (Gagnon et al., 2021). However, third generation cephalosporins can also be used and have been useful in treating ERU (Dixon & Coppack, 2002).

1.7.3 Ruminants

Penicillin is known to be inhibitory against bovine leptospirosis, but, in action, is primarily bacteriostatic. Streptomycin, chlortetracycline, chloramphenicol, erythromycin, and more recently, tulathromycin have been used for antibiotic treatment (Cortese et al., 2007; Hanson, 1976). In more recent studies, tulathromycin and Excede have been shown to clear *Leptospira* in the urine and kidney tissues of cattle (Cortese et al., 2007).

1.8 Prevention and Control

1.8.1 Humans

The most effective ways to prevent the spread of leptospirosis involves awareness of the disease along with its epidemiology. Leptospirosis has been associated with specific occupations, which makes protective measures and taking steps to reduce exposure extremely important strategies (Haake & Levett, 2015).

Occupations such as slaughterhouse workers, paddy workers, and those who work closely with animals are the most likely to become infected (Blackmore & Schollum, 1982;

Colavita & Paoletti, 2007). Wearing the proper personal protective equipment (PPE) is essential for prevention of leptospirosis. Because of the option to wear PPE, indirect contact is more likely to occur in human disease. People who participate in water sports and triathlons also experience an increase in potential infections (Haake & Levett, 2015).

1.8.2 Animals

Prevention and control of leptospirosis in animals is similar to that in humans. The first step for prevention would be vaccination. Vaccines are available for canine, equine, and bovine species. Vaccinations are fairly common in canines, and the vaccine contains killed preparations of serovars Canicola, Icterohaemorrhagiae, Grippotyphosa, and Pomona (Levett, 2001). Feline species are considered resistant and do not currently have a vaccine available. Control of the disease is important, with many factors contributing to the spread of the disease. These factors include location, transmission, risk factors, and maintenance hosts, with these factors also aiding to the zoonotic risk (Ellis, 2015).

The decision of which control factor and tools to use depends on the objective, which can vary from control using vaccination, to rodent control, to removal of infected animals. Vaccination is considered the easiest option, but there is a limit to the use based on availability, expenses, and quality. The vaccines typically contain one to five serovars, with the cattle vaccine showing good microbiological protection for up to a year (Bolin et al., 1991) and the four way canine vaccine also producing similar results (Ellis, 2015; Klaasen et al., 2013).

Proper rodent and livestock management is crucial in prevention and control of leptospirosis. By preventing the contact of rodents with other animals and using rodenticides, the transmission route between rodents will be cut off. For livestock management, keeping a closed herd is the most efficient way to prevent disease, but is difficult to obtain most of the time. The best way to prevent leptospirosis in ruminants is herd vaccination, with both booster and primary vaccinations. Most North American bovine vaccines typically contain serovars Hardjo, Pomona, Canicola, Grippotyphosa, and Icterohaemorrhagiae (Levett, 2001). Each new animal that is to be introduced into the herd should be given antibiotics and all common water sources should be cleaned regularly (Ellis, 2015).

1.9 *Leptospira* Prevalence in Dogs, Cats, and Horses in the United States

There have been studies done regarding the seropositivity in dogs between 1983 until recent, but the early studies were mostly limited to small geographic areas and did not utilize PCR results, but rather MAT only (Moore et al., 2006; Smith et al., 2021; Ward et al., 2002). Ward (2002) estimated the prevalence in dogs that had presented to veterinary hospitals between 1970 and 1998 in the U.S and Canada that were available in the veterinary medicine database (VMDB). Between 1970 and 1982, the prevalence decreased, but the prevalence increased significantly between 1983 and 1998. During this time, cases were increasing at a rate of 1.2 cases per 100,000 annually. The increase in cases could be caused by many reasons, including changes in testing, an increased awareness, or new *Leptospira* transmission patterns. They found that middle-aged, male, herding and working dogs were the most at risk for the disease. Moore et al. (2006) studied canine leptospirosis in the U.S. from 2002 to 2004. This study found that the most common positive serovar was Autumnalis, but it has been considered that Autumnalis cross-reacts with serovar Pomona (Prescott et al., 2002). They found that there was no geographical pattern in the positive cases, but the seropositivity was greater in the Midwest, south-central, and northwest regions. Smith et al. (2021) looked at canine leptospirosis in the U.S from 2009 to 2016. They had at least one test from every state, and only 3 states (Utah, North Dakota, and Alaska) did not have a positive test submitted. They found 7 significant clusters in the upper Midwest, south-central, and northeast areas. One of the clusters found in the study from March 2015 to July 2015 included west Tennessee.

Equine studies in the United States are not as broad as canine studies, but still give us useful information. One of the earliest studies performed was by Smith et al. (1976). This study found that 68 of the 1,346 equine serum samples were MAT positive. Donahue et al. (1991) conducted a study evaluating the prevalence of *Leptospira* in aborted and stillborn horses. They found that 2.5% (15/594) of the submissions tested positive by fluorescent antibody test (FAT) and/or MAT. Poonacha et al. (1993) performed a study on *Leptospira* in equine fetuses, placentas, and stillborn foals. They detected antibodies 55 aborted fetuses and 16 stillborn foals. Andersen-Ranberg et al. (2016)

studied the global patterns of *Leptospira* prevalence in vertebrate hosts. For horses, the mean prevalence was calculated to be 16.4% ± 4.28%.

Feline studies in the United States only consist of literature reviews, seropositivity, and seroprevalence studies. These studies range from 2019-2020, using MAT for all studies and only utilizing PCR for 1 study. The prevalence in cats in the United States ranges from 4% to 33% (Murillo et al., 2020; Palerme et al., 2019; Spangler et al., 2020).

1.10 *Leptospira* Prevalence in Dogs, Cats, and Horses in Tennessee

Tennessee is located in the southwestern region of the United States, with very few previous prevalence studies done throughout this region. The literature search performed resulted in only one article discussing the prevalence of canine leptospirosis specifically in Tennessee. This study was conducted in the Cumberland Gap region (CGR). The GCR is located within the Cumberland Gap National Historic Park. It is close to the intersection of Kentucky, Tennessee, and Virginia. It is primarily rural, has hot and humid summer seasons, mild winters, high precipitation annually, and has dense forest cover, making it an ideal place for occurrence of leptospirosis infection (Verma et al., 2019). Richardson et al. (1995) did a study on the prevalence of leptospirosis in dogs and cats in the GCR area. Overall, they found that 26/198 (13.13%) dogs were positive for leptospiral DNA in urine by qPCR and 38/211 (18%) were seropositive by MAT. Out of those animals, 19 dogs were from shelters in Tennessee. Of these 19 dogs, one dog was positive for MAT, but none were positive for qPCR. Out of the 38 positive MAT samples, serovar Icterohaemorrhagiae had the most reactions.

There have been no equine studies done for Tennessee, to the authors knowledge, and based on only one previous study available of the seroprevalence of *Leptospira* in cats in Tennessee, all 50 cats in that study tested negative by both qPCR and MAT (Spangler et al., 2020).

Emerging zoonotic diseases increase the concern for public health, due to the vast majority of animals that are able to become reservoirs and carriers. Since there has only been one previous study done to determine the threat in Tennessee, this study will serve to help increase knowledge and to lessen the impact of future potential outbreaks. This

study focuses on the seroprevalence of leptospirosis in companion animals in Tennessee only, allowing for a wider picture of the impact of the disease in this state.

1.11 Hypothesis

The overall *Leptospira* seroprevalence in companion animals is higher than that reported in the Cumberland region.

1.12 Study Objective

To determine the seroprevalence of *Leptospira* in dogs, horses, and cats in Tennessee.

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CHAPTER 2: MATERIALS AND METHODS

2.1 *Leptospira* Seroprevalence Study

2.1.1 Sample Size

Convenient serum samples from dogs, cats and horses were tested in this study. The University of Tennessee College of Veterinary Medicine clinical pathology diagnostic laboratory receives serum samples from the UTCVM veterinary teaching hospital and referring local veterinarians. After the requested testing, samples are considered as biowaste and discarded. All serum samples were collected between January 2022 and August 2022 in the state of Tennessee. We used the sample size determination formula, $\frac{Z_a^2 pq}{L^2}$. Z_a is the value required for confidence, which is 1.96 since a 95% confidence interval was used. The p value is the priori estimate of the proportion, or the exposed prevalence value, and q is 1 minus p. L is the margin of error, where 5% was used. Based on a 15% prevalence in canines, we would need total of 196 samples. Based on a 9% prevalence in felines, we would need a total of 126 feline samples. Based on a 45% prevalence in equines, we would need a total of 381 equine samples (Andersen-Ranberg et al., 2016) to allow for a more precise estimate of the results and for a lower margin of error. For this study, 376 canine samples, 169 feline samples, and 88 equine samples were collected and stored at -20°C until use.

2.1.2 Microscopic Agglutination Test

The MAT is considered the gold standard for *Leptospira* diagnosis but worldwide standardization is yet to be achieved, due to the different devices used and also the testing conditions (Oyamada et al., 2021). Samples are screened for the presence of antigen-antibody interaction, measured in a score of agglutination to 6-12 *Leptospira* serovars. If a patient is considered positive due to agglutination, a titration using a serial dilution is performed. MAT is considered complex and difficult to interpret. In the early stages of the disease, there is a lack of specific antibodies and can produce false negative results. If the patient has been vaccinated, false positives can occur, due to the

vaccination producing protection. The specificity of this test is very high, with a fairly high sensitivity as well ("Leptospirosis: an emerging public health problem," 2011).

2.1.3 Reagent, Supplies, and Equipment List

Reagents

• Liquid Ellinghausen-McMullough-Johnson-Harris (EMJH) medium

• *Leptospira* cultures

• Positive Control (PAC): Homologous high titered (usually >3200) antisera prepared in rabbits for each serovar used in the assay. Antisera are purchased from KIT or from the National Veterinary Services Laboratory in Ames, Iowa.

• Negative Control (NAC): PBS Buffer (0.01M Phosphate Buffered Saline, pH 7.2-7.4)

• McFarland Standard 0.5

Supplies

• Nitrile or latex gloves

• Racks for micro tubes and glass tubes

• Microcentrifuge tubes 2mL,

• Micropipette and tips

• Individually-wrapped sterile pipets (5 and 10 mL)

• Kim-Wipe tissues

• Plastic reagent boats

• 96-well, flat bottom plate

• 10% bleach

Equipment

• Biological safety cabinet, Class II

• Vortex mixer

• Single channel micropipettes

• Multi-channel pipette, 10-100 µL

• Pipet filler

• Dark-field microscope with an optional 5X long-working-distance objective

2.1.4 Methodology

The test was performed by mixing the patient serum with 12 commonly infective *Leptospira* serovars. The serovars used are representative of the endemic strains most commonly seen in this area (Fagre et al., 2020; Gautam, Wu, et al., 2010; Moore et al., 2006; Murillo et al., 2020; Sonrier et al., 2000). All of the *Leptospira* serovars were maintained in the laboratory through continuous passage in Ellinghausen-McMullough-Johnson-Harris (EMJH) medium and incubated at 28°C. The concentration of cultures was standardized using a 0.5% McFarland standard and then confirmed by a transmittance value between 75% and 80% on the spectrophotometer. *Leptospira* subcultures for MAT were used between the 4^{th} and 7^{th} day of incubation. Homologous antisera prepared from rabbits for each serovar used in the assay served as a positive control and phosphate buffered saline (PBS) was used as the negative control. Serum samples were diluted in a 1.5 mL microcentrifuge tube to a 1:25 dilution adding 40 µL of sera to 960 µl of PBS. Fifty µL of the positive control sera were added horizontally along row A, and 50 µL of the negative control sera were added horizontally along row B. Fifty microliters of each diluted sera were used to screen for agglutination against each of the twelve serovars. Each patient serum sample was added horizontally in rows C-H, respectively and fifty µL of each standardized culture goes in each column (1-Autumnalis, 2-Ballum, etc.). The plate layout for MAT screening is shown in **Figure 2**. The sera and serovars in each well were mixed by gently tapping on each side of the plate. The plate was then incubated for 1.5 hours at 28-29°C. All serum samples were tested for the presence of antibodies against twelve *Leptospira* serovars, using the standard operating protocol from our *Leptospira* reference laboratory. The list of serovars used for MAT is in **Table 2**.

2.1.5 Screening for *Leptospira* Antibodies

The plates were read using a dark-field microscope, using a 5X long working distance objective. The agglutination reaction was scored as 1+, 2+, 3+ or 4+ **(Figure 3)**. A 1+ score was given when some, but <50% agglutination is present. A 2+ score was given when 50% agglutination is present. A 3+ score was given when more than 50%

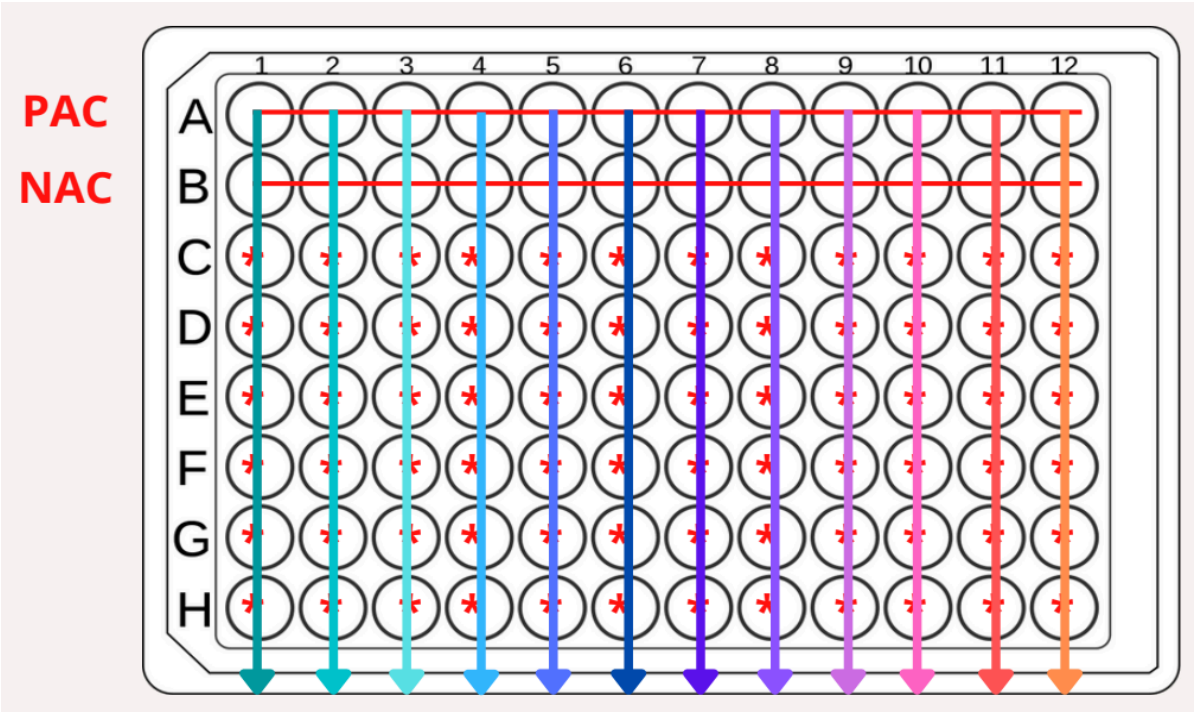
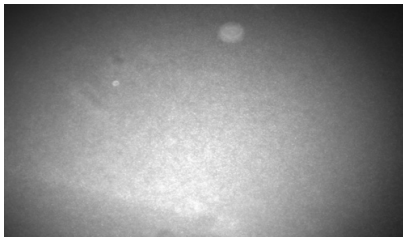


Figure 2 *Microscopic Agglutination Test (MAT) plate layout for screening. PAC is the positive control (homologous rabbit antisera), NAC is the negative control (phosphate buffered saline). Diluted patient serum goes horizontally down rows C, D, E, F, G, and H, and the standardized culture goes down each column (1-Autumnalis, 2-Ballum, etc.).*

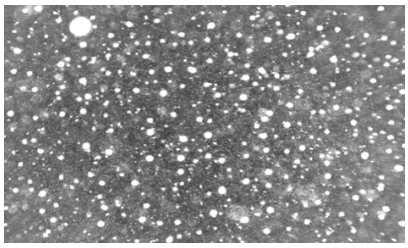
Table 2 *List of the 12 serovars and their respective species and serogroups used in this study.*

Serovar	Species	Serogroup
Autumnalis	*L. interrogans*	Autumnalis
Ballum	*L. borgpetersenii*	Ballum
Bataviae	*L. interrogans*	Bataviae
Bratislava	*L. interrogans*	Australis
Canicola	*L. interrogans*	Canicola
Copenhageni (NVSL2019)	*L. interrogans*	Icterohaemorrhagiae
Grippotyphosa	*L. interrogans*	Grippotyphosa
Hardjo	*L. interrogans*	Sejroe
Icterohaemorrhagiae	*L. interrogans*	Icterohaemorrhagiae
Mankarso	*L. interrogans*	Icterohaemorrhagiae
Pomona (NVSL2019)	*L. interrogans*	Pomona
Tarassovi	*L. interrogans*	Tarassovi

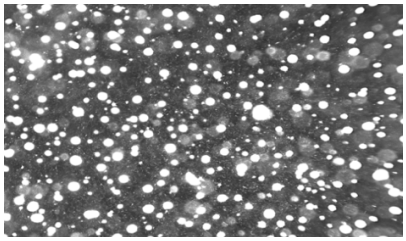
(A)



(B)



(C)



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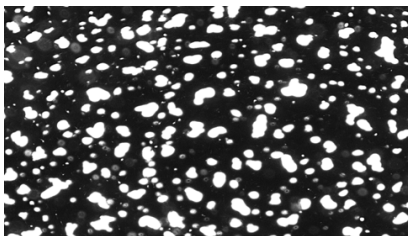

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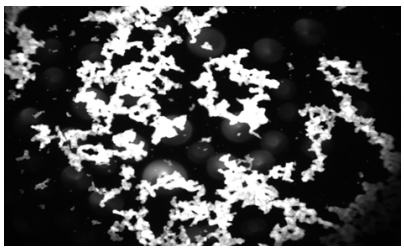

Figure 3 *Image A and represents a negative agglutination score. Image B represents a 1+ agglutination score. Images C represents a 2+ agglutination score. Image D represents a 3+ agglutination score. Inage E represents a 4+ agglutination score.*

agglutination was present, but some free *Leptospira* were still seen in the background. A 4+ score was given if 100% agglutination was seen, with no free *Leptospira* were seen in the background. Samples with a reaction value equal to 2+ and above were considered positives and tittered.

2.1.6 Determination of Antibody Titer

To quantify the antibody, the samples that showed positive agglutination during screening were subjected to serum titration to determine the antibody titer. One hundred µL of the patient serum was serially diluted using PBS until the concentration was 1:6400, then 50 µL of the specific serovar to be titrated was added to the plate. After gentle mixing, the plate was incubated for 1.5 hours at 28-29°C. The plates were read using a dark field microscope, using a 5X long working distance objective. The last dilution showing 50% agglutination was recorded as the titer. The plate layout for MAT titration is shown in **Figure 4.**

2.1.7 Data Analysis

IBM SPSS Statistics (Version 27 and 28, IBM, New York) was used to estimate prevalence. A T-Test test was used to determine the 95% confidence interval for each serovar tested and for all 3 species. A 2-tailed Spearman rank-order correlation test was performed for the 12 serovars used in the study to determine if a correlation of cross-reactivity was present among them for both dogs and horses. The significance was set at $p < 0.05$ and $p < 0.001$. We deiced to use a spearman rank-order correlation rather than Pearson due to the ability of the spearman correlation to work with both linear and monotonic relationships. In dogs, a comparison of seroprevalence between the vaccinated positive and unvaccinated positive groups as well as association between serovars included in the vaccine were done using Fishers exact test, due to the small sample sizes of vaccinated versus unvaccinated dogs used and due to it being an exact test to show if there is a nonrandom association. The significance was set at $p < 0.001$. A p value below 0.005 is considered highly statistically significant.

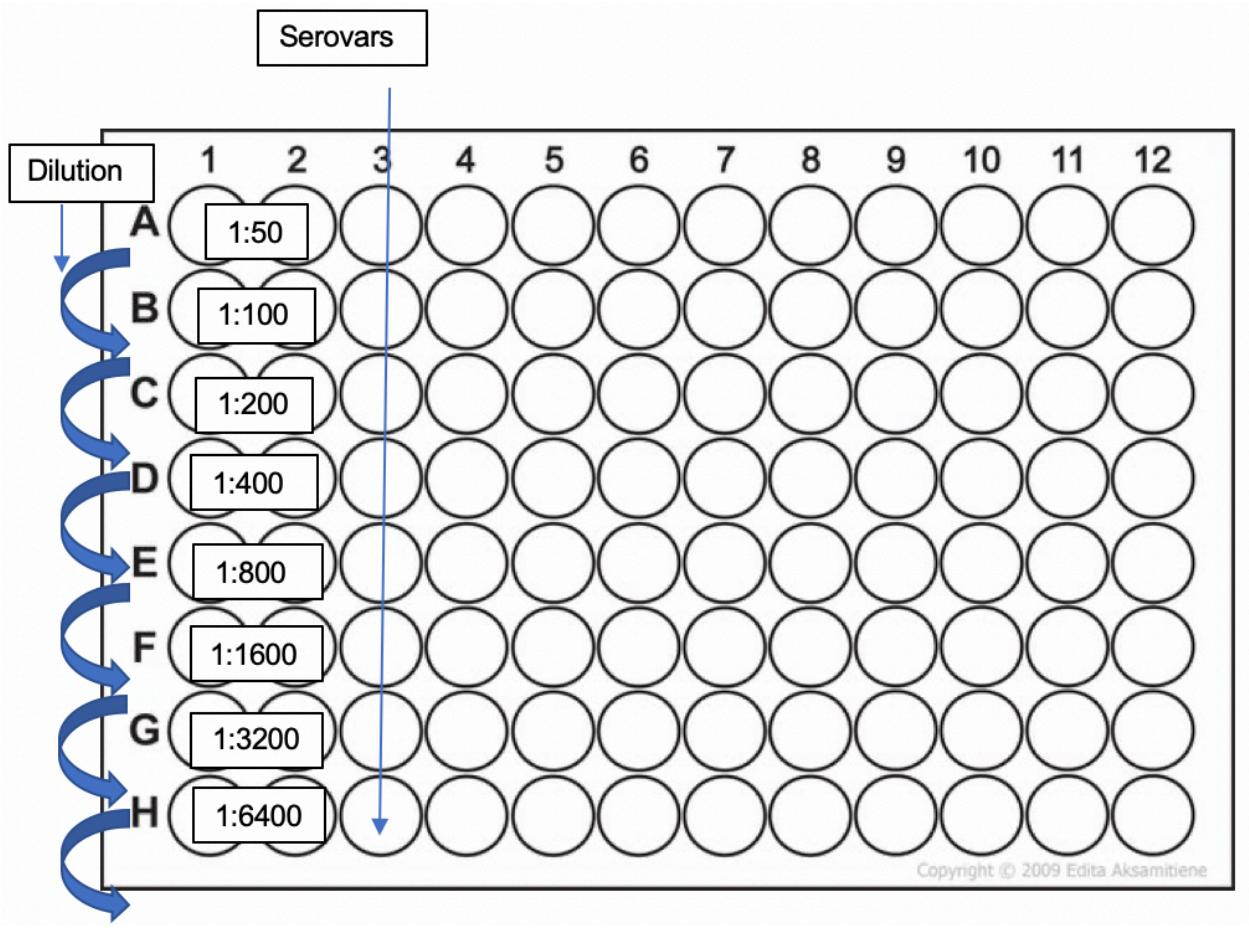

Figure 4 *Microscopic Agglutination Test (MAT) titration plate layout. Serial dilution of patient serum performed vertically, then 50 µL of the respective standardized culture is added to each well.*

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CHAPTER 3: RESULTS

3.1 *Leptospira* Seroprevalence in Dogs

We tested the serum of 374 dogs against twelve *Leptospira* serovars using Microscopic Agglutination Test (MAT) to assess the *Leptospira* seroprevalence. The serum samples were collected between January 2022 and August 2022 and all samples are from animals in Tennessee. A total of 110 (29.41%) samples were MAT positive for one or more serovar **(Figure 5)**. Out of the 12 total serovars tested by MAT, we observed detectable agglutinating antibody response to 10 serovars. Among the serovars tested, the highest seroprevalence was observed for the serovar Autumnalis (82/110; 74.55%) followed by Grippotyphosa (44/110; 40.0%) and the lowest seroprevalence was observed for serovar Hardjo (3/110; 2.73%). Seroprevalence to serovars Bataviae and Tarassovi was not observed in the samples tested. A summary of the seroprevalence observed to individual serovars is shown in **Figure 6** and **Table 3**.

3.2 MAT Reactivity to Multiple Serovars Observed in Canine Samples

Since many of the serum samples had positive reactivity to multiple serovars tested, we examined the potential level of cross-reactivity in serum samples to multiple serovars. The serum samples that had positive reactivity to serovar Autumnalis also reacted with 8 other serovars; the highest cross-reactivity was observed for the serovar Grippotyphosa (33/82; 40.24%), followed by Pomona (30/82; 36.59%), Copenhageni (28/82; 34.15%), Canicola (27/82; 32.93%), Icterohaemorrhagiae (27/82; 32.93%), Mankarso (18/82; 21.95%), Bratislava (7/82; 8.54%), and Hardjo (1/82; 1.22%). The serum samples that had positive reactivity to serovar Bratislava also reacted with 7 other serovars; the highest cross-reactivity was with Autumnalis (7/10; 70.0%), followed by Copenhageni (6/10; 60.0%), Grippotyphosa (5/10; 50.0%), Canicola (4/10; 40.0%), Icterohaemorrhagiae (4/10; 40.0%), Mankarso (3/10; 30.0%), and Pomona (3/10; 30.0%). The serum samples that had positive reactivity to serovar Canicola also reacted with 9 other serovars; the highest cross-reactivity was with Autumnalis (27/34; 79.41%), followed by Grippotyphosa (17/34; 50.0%), Copenhageni (14/34; 41.18%),

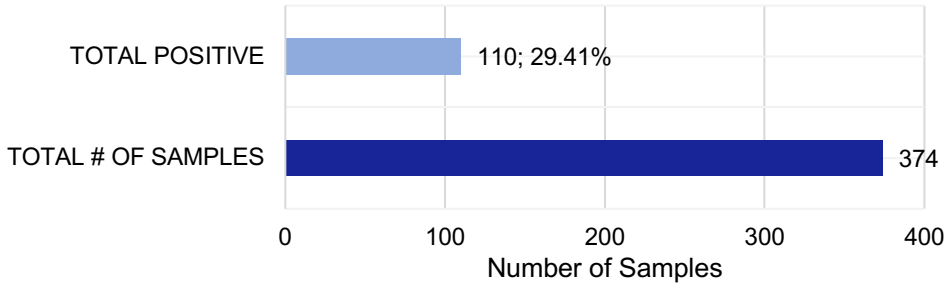

Figure 5 *Summary of Leptospira seroprevalence in dogs. A total of 110/374 samples were positive for antibodies, totaling 29.41%.*

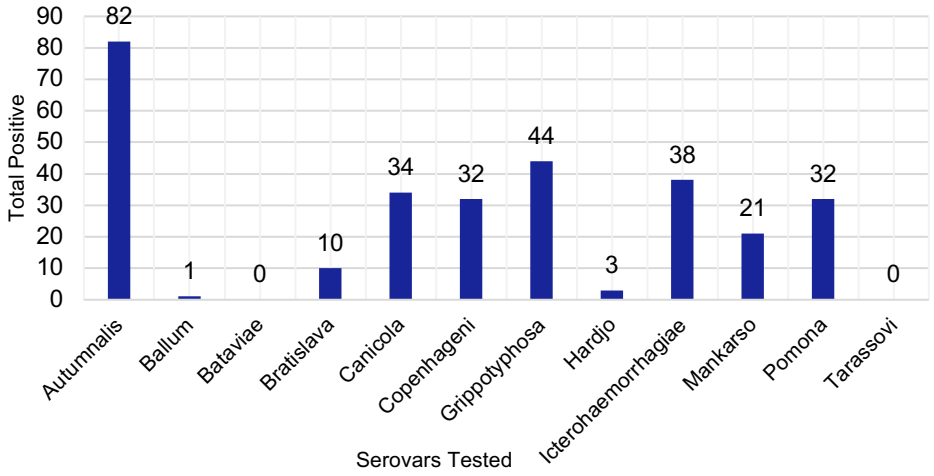

Figure 6 *Summary of Leptospira seroprevalence to 12 serovars tested in dogs.*

Table 3 *Canine Leptospira seroprevalence and 95% confidence intervals for each serovar in dogs.*

	Number Positive	Prevalence	Confidence Interval
Autumnalis	82/110	74.55%	0.588-0.805
Ballum	1/110	0.90%	0.003-0.021
Bataviae	-	-	0.00-0.011
Bratislava	10/110	9.09%	0.088-0.179
Canicola	34/110	30.91%	0.249-0.396
Copenhageni	32/110	29.09%	0.227-0.369
Grippotyphosa	44/110	40.0%	0.375-0.531
Hardjo	3/110	2.73%	0.021-0.072
Icterohaemorrhagiae	38/110	34.55%	0.278-0.460
Mankarso	21/110	19.09%	0.163-0.289
Pomona	32/110	29.09%	0.291-0.425
Tarassovi	-	-	0.00-0.00

Icterohaemorrhagiae (14/34; 41.18%), Mankarso (11/34; 32.35%), Pomona 11/34; 32.35%), Bratislava 4/34; 11.76%), Ballum (1/34; 2.94%), and Hardjo (1/34; 2.94%). The serum samples that had positive reactivity to serovar Copenhageni also reacted with 7 other serovars; the highest cross-reactivity was with Autumnalis (28/32; 87.50%), followed by Icterohaemorrhagiae (20/32; 62.50%), Grippotyphosa (19/32; 59.38%), Mankarso (16/32; 50.0%), Canicola (14/32; 43.75%), Pomona (14/32; 43.75%), and Bratislava (6/32; 18.75%). The serum samples that had positive reactivity to serovar Grippotyphosa reacted with 8 other serovars; the highest cross-reactivity was with Autumnalis (33/44; 75.0%), followed by Icterohaemorrhagiae (20/44; 45.45%), Copenhageni (19/44; 43.18%), Pomona (18/44; 40.91%), Canicola (17/44; 38.64%), Mankarso (13/44; 29.55%), Bratislava (5/44; 11.36%), and Hardjo (1/44; 2.27). The serum samples that had positive reactivity to serovar Icterohaemorrhagiae also reacted with 7 other serovars; the highest cross-reactivity was with Autumnalis (27/38; 71.05%), followed by Copenhageni (20/38; 52.63%), Grippotyphosa (20/38; 52.63%), Mankarso (18/38; 47.37%), Pomona (16/38; 42.11%), Canicola (14/38; 36.84%), and Bratislava (4/38; 10.53%). The serum samples that had positive reactivity to serovar Mankarso also reacted with 7 other serovars; the highest cross-reacting serovars were Autumnalis (18/21; 85.71%), Icterohaemorrhagiae (18/21; 85.71%), and Copenhageni (18/21; 85.71%), followed by Grippotyphosa (13/21; 61.94%), Canicola (11/21; 52.38%), Pomona (11/21; 52.38%), and Bratislava (3/21; 14.29%). The serum samples that had positive reactivity to serovar Pomona also reacted with 7 other serovars; the highest cross-reactivity was with Autumnalis (30/32; 93.75%), followed by Grippotyphosa (18/32; 56.25%), Icterohaemorrhagiae (16/32; 50.0%, Copenhageni (14/32; 43.75%), Canicola (11/32; 34.38%), Mankarso (11/32; 34.38%), and Bratislava (3/32; 9.38%). A summary of the pattern of cross reactivity among serovars showing high level cross reactivity is shown in **Figure 7**. A Spearman rank-order correlation was performed against all 12 serovars to determine the correlations between the cross reactivity between serovars **(Table 4)**. There were 62 correlations found at the $p < 0.001$ level and 8 correlations found at the $p < 0.05$ level. Tarassovi was the only serovar with no significant association.

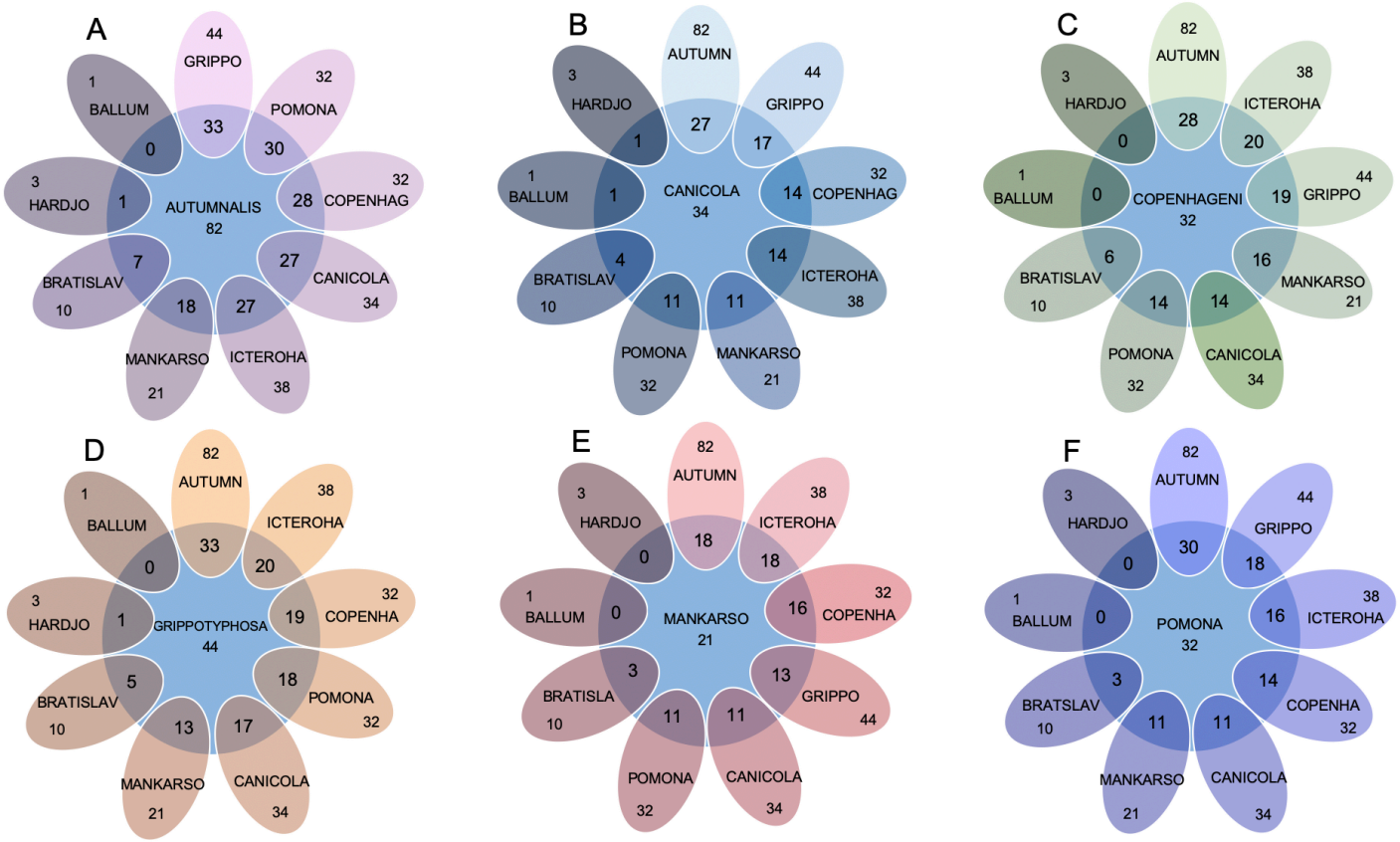

Figure 7 *A Venn diagram showing the 6 highest cross-reacting Leptospira serovars shown in relation to the 9 other cross-reacting serovars. A: Autumnalis. B: Canicola. C: Copenhageni. D: Grippotyphosa. E: Mankarso. F: Pomona*

Table 4 *Spearman rank-order correlation of cross-reactivity between the 12 Leptospira serovars in dogs.*

	Autumnalis	Ballum	Bataviae	Bratislava	Canicola	Copenhageni	Grippotyphosa	Hardjo	Icterohaemorrhagiae	Mankarso	Pomona	Tarassovi
Autumnalis	1.00 -	0.039 (0.449)	0.071 (0.171)	0.265 (<0.001)	0.505 (<0.001)	0.625 (<0.001)	0.584 (<0.001)	0.020 (0.697)	0.397 (<0.001)	0.540 (<0.001)	0.731 (<0.001)	- -
Ballum	0.039 (0.449)	1.00 -	-0.004 (0.942)	0.105 (0.043)	.173 (<0.001)	0.065 (0.207)	0.042 (0.414)	-0.014 (0.780)	0.050 (0.332)	0.070 (0.176)	0.053 (0.306)	- -
Bataviae	0.071 (0.171)	-0.004 (0.942)	1.00 -	0.149 (0.004)	0.094 (0.071)	0.118 (0.023)	-0.035 (0.504)	-0.010 (0.844)	-0.024 (0.640)	0.120 (0.020)	0.106 (0.041)	- -
Bratislava	0.265 (<0.001)	0.105 (0.043)	0.149 (0.004)	1.00 -	0.158 (0.002)	0.359 (<0.001)	0.190 (<0.001)	0.068 (0.187)	0.238 (<0.001)	0.323 (<0.001)	0.203 (<0.001)	- -
Canicola	0.505 (<0.001)	0.173 (<0.001)	0.094 (0.071)	0.158 (0.002)	1.00 -	0.591 (<0.001)	0.416 (<0.001)	0.013 (0.802)	0.398 (<0.001)	0.520 (<0.001)	0.447 (<0.001)	- -
Copenhageni	0.625 (<0.001)	0.065 (0.207)	0.118 (0.023)	0.359 (<0.001)	0.591 (<0.001)	1.00 -	0.552 (<0.001)	0.008 (0.877)	0.645 (<0.001)	0.818 (<0.001)	0.608 (<0.001)	- -
Grippotyphosa	0.584 (<0.001)	0.042 (0.414)	-0.035 (0.504)	0.190 (<0.001)	0.416 (<0.001)	0.552 (<0.001)	1.00 -	0.040 (0.438)	0.414 (<0.001)	0.529 (<0.001)	0.539 (<0.001)	- -
Hardjo	0.020 0.697	-0.014 (0.780)	-0.010 (0.844)	0.068 (0.187)	0.013 (0.802)	0.008 (0.877)	0.040 (0.438)	1.00 -	0.152 (0.003)	0.082 (0.113)	0.037 (0.471)	- -
Icterohaemorrhagiae	0.397 (<0.001)	0.050 (0.332)	-0.024 (0.640)	0.238 (<0.001)	0.398 (<0.001)	0.645 (<0.001)	0.414 (<0.001)	0.152 (0.003)	1.00 -	0.642 (<0.001)	0.452 (<0.001)	- -
Mankarso	0.540 (<0.001)	0.070 (0.176)	0.120 (0.020)	0.323 (<0.001)	0.520 (<0.001)	0.818 (<0.001)	0.529 (<0.001)	0.082 (0.113)	0.642 (<0.001)	1.00 -	0.596 (<0.001)	- -
Pomona	0.731 (<0.001)	0.053 (0.306)	0.106 (0.041)	0.203 (<0.001)	0.447 (<0.001)	0.608 (<0.001)	0.539 (<0.001)	0.037 (0.471)	0.452 (<0.001)	0.596 (<0.001)	1.00 -	- -
Tarassovi	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	1.00 -

The blue values indicate a significant correlation at the p < 0.001 level. The green values indicate a significant correlation at the p < 0.05 level.

3.3 MAT Antibody Titers in Dogs

Overall, MAT titers ranged from 1:50 to 1:1600 in the samples tested . The highest titers were observed in Bratislava (1:1600) followed by Autumnalis (1:800), Grippotyphosa (1:800), and Pomona (1:800). A summary of the titer distribution among serovars is shown in **Figure 8**.

Since *Leptospira* vaccination is practiced in dogs, we evaluated the difference in seroprevalence in vaccinated vs. unvaccinated population. We separated the MAT results from samples for vaccinated and unvaccinated patients using clinical history obtained from hospital records. The vaccination data was available for 184 of the 374 canine patients. Of the 184 serum samples collected, 98 (53.26%) had been from vaccinated dogs and 86 (46.74%) from non-vaccinated dogs. Of the 98 samples from vaccinated dogs, 53 (54.08%) were negative for antibodies and 45 (45.92%) were positive to one or more *Leptospira* serovars tested. The 86 samples from unvaccinated dogs were also further categorized as positive or negative samples, with 72 (83.72%) testing negative and 14 (16.28%) testing positive **(Figure 9)**. Included in the vaccine are serovars Canicola, Grippotyphosa, Icterohaemorrhagiae, and Pomona. Meanwhile, in dogs that were vaccinated and tested positive, the most common serovars with reactivity were Autumnalis (36/45; 80.0%), followed by Grippotyphosa (22/45; 48.89%), Icterohaemorrhagiae (20/45; 44.44%), and Canicola (18/45; 40.0%). The seroprevalence in both vaccinated and unvaccinated dogs are shown in **Figure 10**. In dogs that are not vaccinated but tested positive, the most common serovar was also Autumnalis (12/14; 85.71%), followed by Pomona (6/14; 42.86%) Copenhageni (5/14; 35.71%) and Grippotyphosa (5/14; 35.71%). We also evaluated if there was a significant difference between the overall seroprevalence between vaccinated and unvaccinated dogs. The vaccinated dogs had a significantly higher seroprevalence compared to the unvaccinated dogs ($p < 0.001$). We then tested to see if there was a significant difference in seroprevalence to the 4 serovars present in the canine vaccine between the vaccinated and unvaccinated dogs. A significant difference was observed for all of the serovars included in the vaccine; Canicola, Grippotyphosa, Icterohaemorrhagiae, and Pomona ($p < 0.001$). A summary of the data is shown in **Table 5** and **Table 6**.

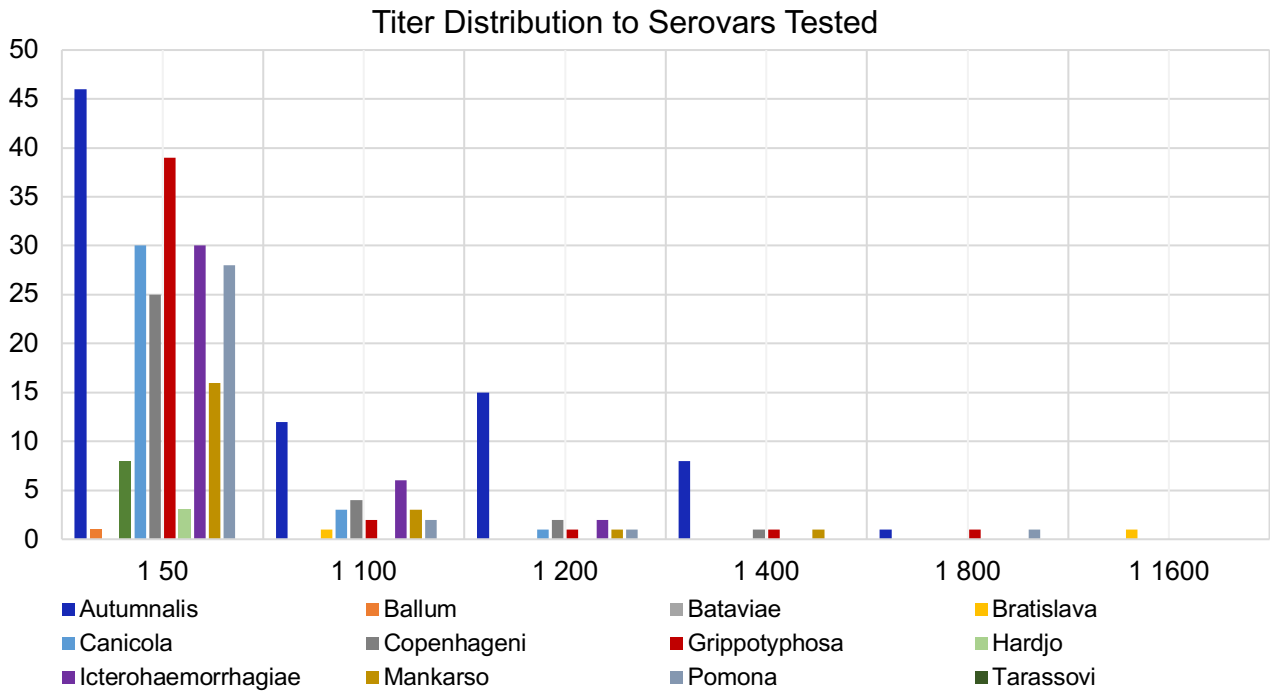

Figure 8 *Titer distribution of the microscopic agglutination test (MAT) titers in dogs. The starting titer was 1:50 and the highest titer was 1:1600.*

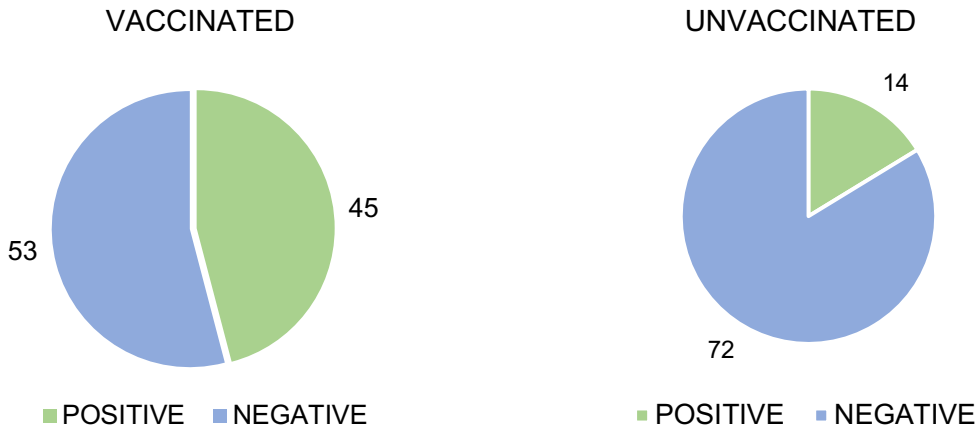

Figure 9 *Unvaccinated and vaccinated canine patients separated into positive and negative groups. Of the 98 vaccinated dogs, 45 tested positive and 53 tested negative. Of the 86 unvaccinated dogs, 14 tested positive and 72 tested negative.*

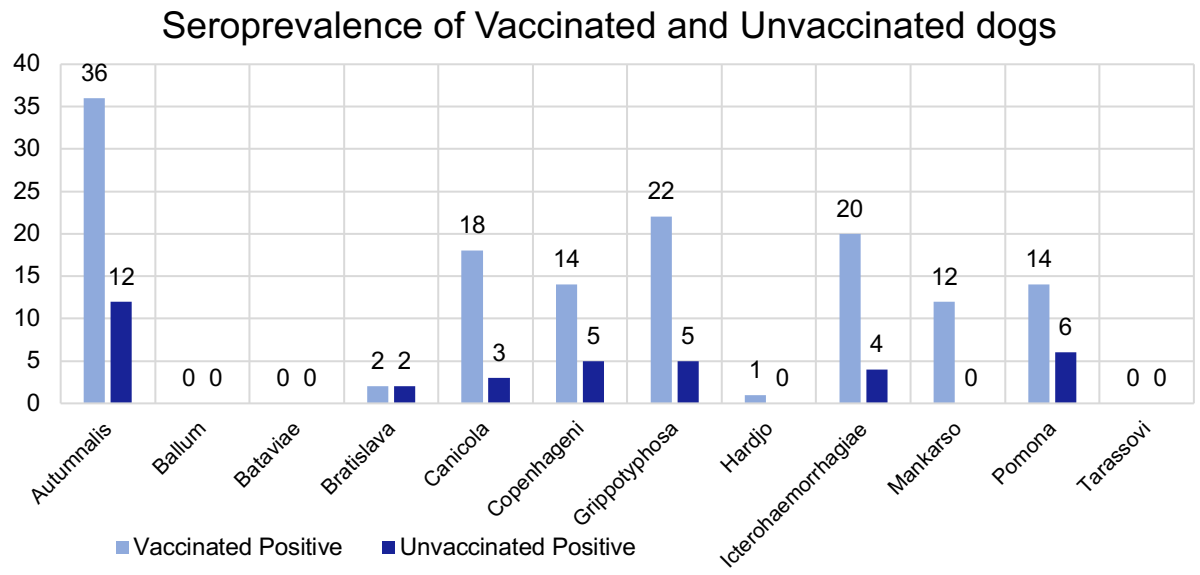

Figure 10 *Seroprevalence in vaccinated and unvaccinated dogs.*

Table 5 *Seroprevalence in dogs based on vaccination status.*

	Number of Samples	Number of Positive Samples	Seroprevalence (%)	95% Confidence Interval	P value (Fishers exact)
Vaccinated	98	45	45.92	45.7 – 60.3	<0.001
Unvaccinated	86	14	16.28	39.7 – 54.3	

Table 6 *Seroprevalence in vaccinated and unvaccinated dogs to serovars present in the canine vaccine.*

	Prevalence in Vaccinated Group (%) [95% Confidence Interval]	**Prevalence in Unvaccinated Group (%) [95% Confidence Interval]**	**P value (Fishers exact)**
Canicola	18.37 [0.038-0.289]	3.49 [0-0.255]	<0.001
Grippotyphosa	22.68 [-0.098-.0131]	5.81 [0-0.103]	<0.001
Icterohaemorrhagiae	20.41 [-0.082-0.191]	4.65 [0-0.174]	<0.001
Pomona	14.29 [-0.005-0.213]	6.98 [0-0.190]	<0.001

3.4 Age, Sex, Breed, and County Distribution in Dogs

We also examined age, sex, breed, distribution and geographic information related to the samples tested. The majority of the samples came from dogs in the 6-year to 10-year age range, with the least amount of sample were from dogs in the 16-year to 20-year range **(Figure 11).** Of the 374 samples, 191 (51.07%) samples were from female dogs and 183 (48.93%) were from male dogs **(Figure 12)**. For breed distribution, the population was overrepresented by mixed breed (97/374; 25.9%), followed by Terrier (31/374; 8.3%), Labrador (21/374; 5.61%), German Shepherd (14/374; 3.74%), Golden Retriever (13/374; 3.48%), Shih-Tzu (13/374; 3.48%), Spaniel (12/374; 3.21%), Yorkie (11/374; 2.94%), Australian Shepherd (10/374; 2.67%), Boxer (10/374; 2.67%), Chihuahua (10/374; 2.67%), Dachshund (10/374; 2.67%), and Wolfhounds (10/374; 2.67%). In this study, we were able to obtain at least one sample from 43 of the 95 counties in Tennessee. We classified sample origin to 4 regions of Tennessee **(Figure 13)**. The majority of the samples collected were from Region 1 (304 samples; 81.28%). Fifty-nine (15.78%) were from Region 2, and 11 (2.94%) from Region 3, and no samples were received from Region 4. A map of the regions is and the detailed sample size of each county is shown in **Table 7.**

3.5 *Leptospira* Seroprevalence in Horses

The Microscopic Agglutination Test (MAT) was used to assess the seroprevalence in 88 horses to twelve *Leptospira* serovars. A total of 42 (47.73%) samples were MAT positive for one or more serovar **(Figure 14)**. Out of the 12 total serovars, all had detectable agglutinating antibody response. Among the serovars tested, the highest seroprevalence was observed in Bratislava (40/42; 95.24%), followed by Copenhageni (24/42; 57.14%) and the lowest seroprevalence was observed in Tarassovi (1/42; 2.38%). A summary of the seroprevalence observed to individual serovars is shown in **Figure 15** and **Table 8.**

3.6 MAT Reactivity to Multiple Serovars Observed in Equine Samples

Since many of the serum samples were reacting to multiple serovars tested, we examined the cross-reaction among serovars. The serum samples that had positive reactivity to

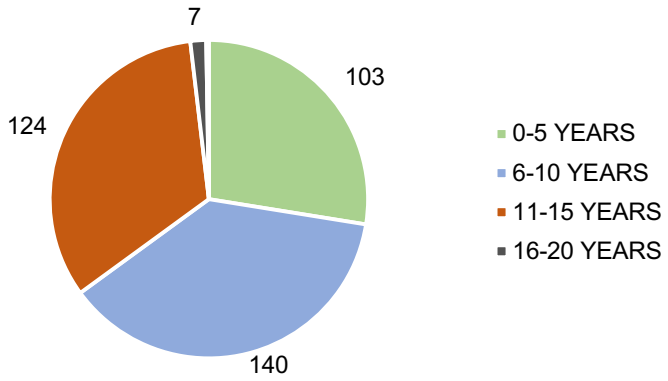

Figure 11 *Age distribution of the 374 canine samples tested.*

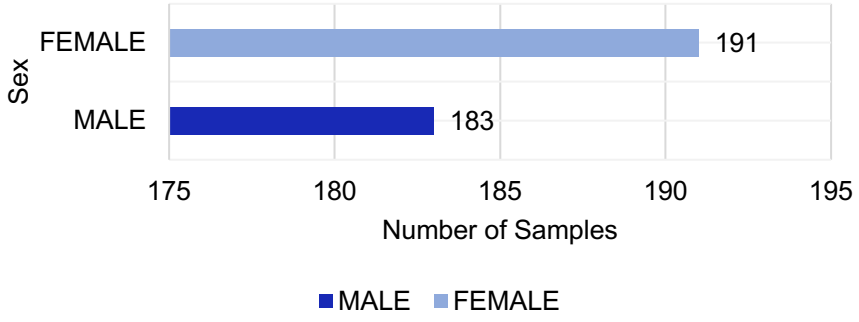

Figure 12 *Sex distribution of the 374 canine samples tested.*

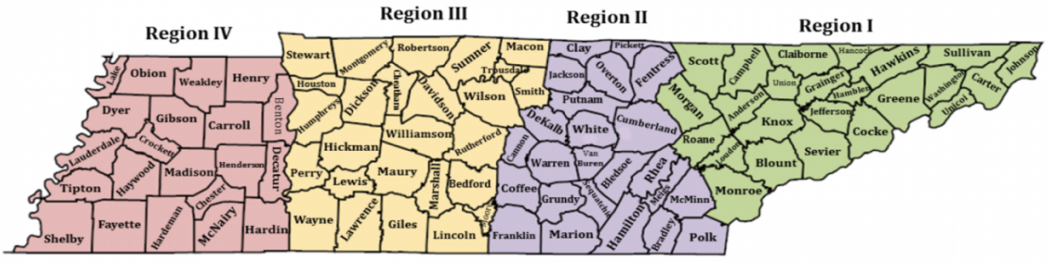

Figure 13 *A map of regions I-IV in Tennessee presented by the Tennessee County Highway Officials Association (TCHOA).*

Table 7 *Total number of samples and total number of positive samples by Tennessee region in dogs.*

Region	Number of Positive Samples	Total Number of Samples
Region 1	84	304
Region 2	26	59
Region 3	0	11
Region 4	0	0

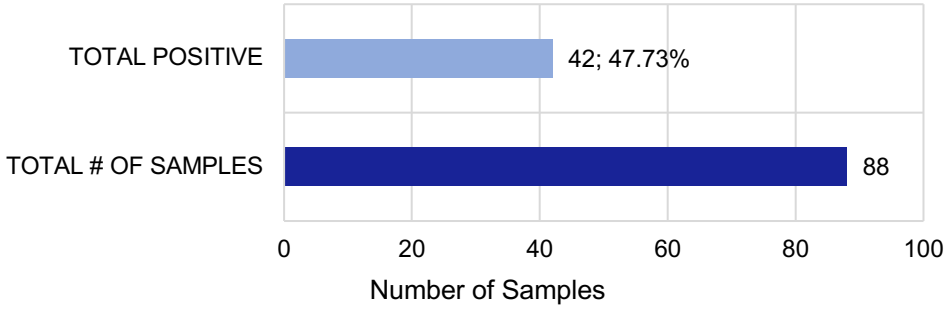

Figure 14 *Summary of Leptospira seroprevalence in horses. A total of 42/88 samples were positive for antibodies, totaling 47.73%.*

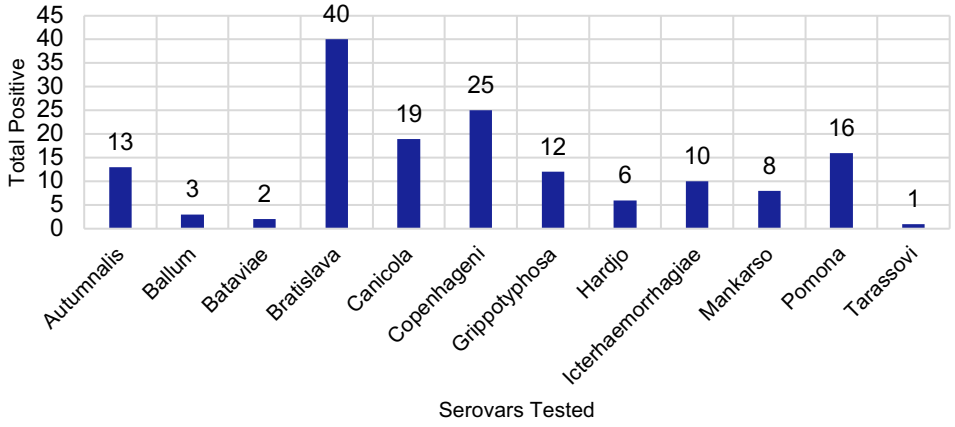

Figure 15 *Summary of Leptospira seroprevalence to 12 serovars tested in horses.*

Table 8 *Seroprevalence and 95% confidence intervals for each serovar in horses.*

	Number Positive	Prevalence	Confidence Interval
Autumnalis	13/42	30.59%	0.198-0.642
Ballum	3/42	7.14%	0.025-0.247
Bataviae	2/42	4.76%	0.025-0.173
Bratislava	40/42	95.24%	1.062-1.630
Canicola	17/42	40.48%	0.296-0.716
Copenhageni	24/42	57.14%	0.432-0.864
Grippotyphosa	12/42	28.57%	0.148-0.469
Hardjo	6/42	14.29%	0.049-0.296
Icterohaemorrhagiae	10/42	23.81%	0.148-0.568
Mankarso	8/42	19.05%	0.074-0.358
Pomona	16/42	38.1%	0.309-0.790
Tarassovi	1/42	2.38%	0.037-0.148

serovar Autumnalis also reacted with 7 other serovars; the highest cross-reactivity was with Bratislava (11/13; 84.62%), followed by Copenhageni (9/13; 69.23%), Pomona (9/13; 69.23%), Icterohaemorrhagiae (5/13; 38.46%), Grippotyphosa (4/13; 30.77%), Mankarso (3/13; 23.08%), and Canicola (2/13; 15.38%). The serum samples that had positive reactivity to serovar Bratislava also reacted with 10 other serovars; the highest cross-reactivity was with Copenhageni (15/40; 37.5%), followed by Canicola (12/40; 30.0%), Autumnalis (11/40; 27.5%), Pomona (10/40; 25.0%), Icterohaemorrhagiae (8/40; 20.0%), Grippotyphosa (7/40; 17.5%),Mankarso (6/40; 15.0%), Hardjo (3/40; 7.50%), Ballum (1/40; 2.50%), and Bataviae (1/40; 2.50%). The serum samples that had positive reactivity to serovar Canicola also reacted with 10 other serovars; the highest cross-reactivity was with Bratislava (12/17; 70.59%) and Copenhageni (12/17; 70.59%), followed by Mankarso (7/17; 41.18%), Icterohaemorrhagiae (5/17; 29.41%), Pomona (4/17; 23.53%), Ballum (3/17; 17.65%), Grippotyphosa (3/17; 17.65%), Autumnalis (2/17; 11.76%), Bataviae (2/17; 11.76%), and Hardjo (2/17; 11.76%). The serum samples that had positive reactivity to serovar Copenhageni also reacted with 11 other serovars; the highest cross-reactivity was with Bratislava (15/24; 62.5%), followed by Canicola (12/24; 50.0%), Icterohaemorrhagiae (10/24; 41.67%), Pomona (10/24; 41.67%), Autumnalis (9/24; 37.50%), Mankarso (8/24; 33.33%), Grippotyphosa (6/24; 25.0%), Hardjo (4/24; 16.67%), Ballum (2/24; 8.33%),Bataviae (2/24; 8.33%), and Tarassovi (1/24; 4.17%). The serum samples that produced agglutinating response to serovar Grippotyphosa also reacted with 8 other serovars; the highest cross-reactivity was with Bratislava (7/12; 58.33%), followed by Copenhageni (6/12; 50.0%), Autumnalis (4/12; 33.33%), Canicola (3/12; 25.0%), Pomona (3/12; 25.0%), Hardjo (1/12; 8.33%), Mankarso (1/12; 8.33%), and Tarassovi (1/12; 8.33%). The serum samples that produced agglutinating response to serovar Pomona also reacted with 9 other serovars; the highest cross-reactivity was with Bratislava (10/16; 62.5%) and Copenhageni (10/16; 62.5%), followed by Autumnalis (9/16; 56.25%), Icterohaemorrhagiae (7/16; 43.75%), Canicola (4/16; 25.0%), Mankarso (4/16; 25.0%), Grippotyphosa (3/16; 18.75%), Bataviae (1/16; 6.25%), and Hardjo (1/16; 6.25%). A spearman rank-order correlation was performed against all 12 serovars to determine the correlations between the cross-reactivity between serovars **(Table 9)**.

There were 34 correlations found at the $p < 0.001$ level and 12 correlations found at the $p < 0.05$ level. Tarassovi was the only serovar with no significant association.

3.7 MAT Antibody Titers in Horses

The titers ranged from 1:50 to 1:400. The highest titer was observed in both Bratislava and Pomona (1:400). The seroprevalence and the confidence intervals of all serovars tested are shown in **Table 8.** A summary of the distribution among serovars is shown in **Figure 16**.

3.8 Age, Sex, Breed, and County Distribution in Horses

We also examined the distributions of age, sex, breed, and county were looked at. For age, the majority of the samples were in the 0-5 year age range, with the least amount of samples in the > 30 year range (**Figure 17**). Of the 88 samples, 26 were from females and 61 were from males (**Figure 18**). The breeds represented were American Quarter Horse (20/88; 22.73%), followed by Donkey (7/88; 7.95%), American Standard Horse (6/88; 6.82%), mixed breed (6/88; 6.82%), Tennessee Walking Horse (6/88; 6.82%), miscellaneous breed (5/88; 5.68%), and Warmblood (5/88; 5.68%). In this study we were able to obtain at least one sample from 25/95 (26.32%) of the counties in Tennessee. There are 4 regions in Tennessee, with the majority of the samples collected from Region 1. We had 65 samples (73.86%) from Region 1, 18 (20.45%) from Region 2, 4 (4.55%) from Region 3, no samples from Region 4., and 1 (1.1%) did not have a county listed. A map of the regions is in **Figure 13** and the detailed sample size of each county is shown in **Table 10**.

3.9 *Leptospira* Seroprevalence in Cats

The Microscopic Agglutination Test (MAT) was used to assess the seroprevalence of the serum of 170 cats to twelve *Leptospira* serovars. A total of 21 (12.35%) samples were MAT positive for one or more serovar **(Figure 19)**. Out of the 12 total serovars, 10 had detectable agglutinating antibody response in MAT. Among the serovars tested, the highest seroprevalence was observed in Bratislava (9/21; 42.86%) followed by Hardjo

Table 9 *Spearman rank-order correlation of cross-reactivity between the 12 Leptospira serovars in horses.*

	Autumnalis	Ballum	Bataviae	Bratislava	Canicola	Copenhageni	Grippotyphosa	Hardjo	Icterohaemorrhagiae	Mankarso	Pomona	Tarassovi
Autumnalis	1.00 -	-0.085 (0.448)	-0.069 (0.539)	0.440 (<0.001)	-0.055 (0.625)	0.390 (<0.001)	0.182 (0.105)	-0.123 (0.273)	0.350 (0.001)	0.205 (0.067)	0.608 (<0.001)	-0.049 (0.666)
Ballum	-0.085 (0.448)	1.00 -	0.374 (<0.001)	-0.039 (0.728)	0.334 (0.002)	0.144 (0.201)	-0.082 (0.468)	0.197 (0.078)	-0.073 (0.515)	-0.065 (0.565)	-0.097 0.391	-0.022 (0.846)
Bataviae	-0.069 (0.539)	0.374 (<0.001)	1.00 -	0.024 (0.829)	0.271 (0.014)	0.228 (0.041)	-0.066 (0.556)	-0.045 (0.690)	0.191 (0.088)	0.214 (0.055)	0.132 (0.239)	-0.018 (0.875)
Bratislava	0.440 (<0.001)	-0.039 (0.728)	0.024 (0.829)	1.00 -	0.179 (0.110)	0.239 (0.032)	0.133 (0.237)	0.043 (0.701)	0.318 (0.004)	0.250 (0.024)	0.285 (0.010)	-0.104 (0.355)
Canicola	-0.055 (0.625)	0.334 (0.002)	0.271 (0.014)	0.179 (0.110)	1.00 -	0.476 (<0.001)	0.023 (0.838)	0.148 (0.186)	0.292 (0.008)	0.554 (<0.001)	0.088 (0.435)	-0.061 (0.586)
Copenhageni	0.390 (<0.001)	0.144 (0.201)	0.228 (0.041)	0.239 (0.032)	0.476 (<0.001)	1.00 -	0.182 (0.104)	0.210 (0.060)	0.584 (<0.001)	0.551 (<0.001)	0.365 (<0.001)	0.160 (0.154)
Grippotyphosa	0.182 (0.105)	-0.082 (0.468)	-0.066 (0.556)	0.133 (0.237)	0.023 (0.838)	0.182 (0.104)	1.00 -	0.013 (0.908)	-0.156 (0.164)	-0.023 (0.839)	0.078 (0.488)	0.268 (0.015)
Hardjo	-0.123 (0.273)	0.197 (0.078)	-0.045 (0.690)	0.043 (0.701)	0.148 (0.186)	0.210 (0.060)	0.013 (0.908)	1.00 -	0.050 (0.660)	0.072 (0.523)	-0.038 (0.739)	0.390 (<0.001)
Icterohaemorrhagiae	0.350 (0.001)	-0.073 (0.515)	0.191 (0.088)	0.318 (0.004)	0.292 (0.008)	0.584 (<0.001)	-0.156 (0.164)	0.050 (0.660)	1.00 -	0.662 (<0.001)	0.467 (<0.001)	-0.042 (0.711)
Mankarso	0.205 (0.067)	-0.065 (0.565)	0.214 (0.055)	0.250 (0.024)	0.554 (<0.001)	0.551 (<0.001)	-0.023 (0.839)	0.072 (0.523)	0.662 (<0.001)	1.00 -	0.260 (0.019)	-0.037 (0.743)
Pomona	0.608 (<0.001)	-0.097 (0.391)	0.132 (0.239)	0.285 (0.010)	0.088 (0.435)	0.365 (<0.001)	0.078 (0.488)	-0.038 (0.739)	0.467 (<0.001)	0.260 (0.019)	1.00 -	-0.055 (0.625)
Tarassovi	-0.049 (0.666)	-0.022 (0.846)	-0.018 (0.875)	-0.104 (0.355)	-0.061 (0.586)	0.160 (0.154)	0.268 (0.016)	0.390 (<0.001)	-0.042 (0.711)	-0.037 (0.743)	-0.055 (0.625)	1.00 -

The blue values indicate a significant correlation at the p < 0.001 level. The green values indicate a significant correlation at the p < 0.05 level.

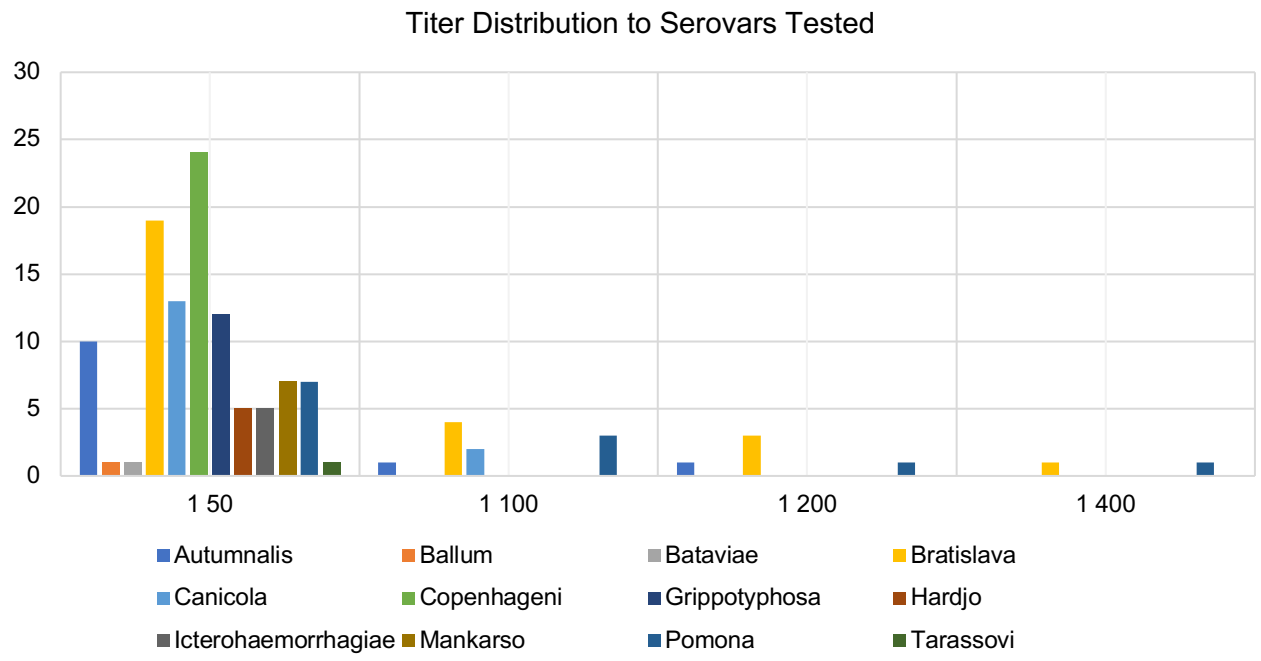

Figure 16 *Titer distribution of the microscopic agglutination test titers (MAT) in horses. The starting titer was 1:50 and the highest titer was 1:400.*

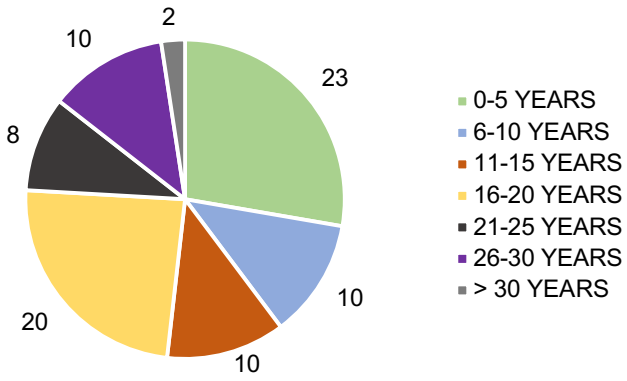

Figure 17 *Age distribution of the 88 equine patients tested.*

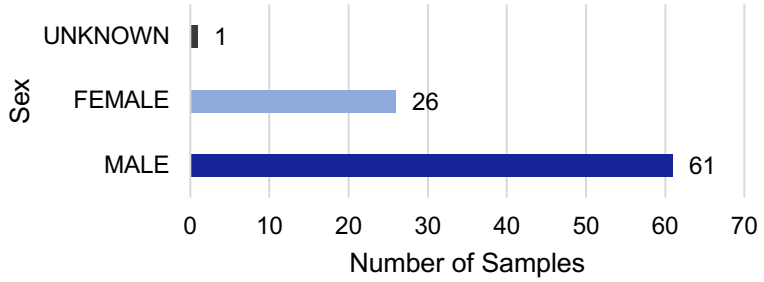

Figure 18 Sex distribution of the 88 equine samples tested.

Table 10 *Total number of samples and total number of positive samples by Tennessee region in horses.*

Region	Number of Positive Samples	Total Number of Samples
Region 1	32	65
Region 2	8	18
Region 3	2	4
Region 4	0	0

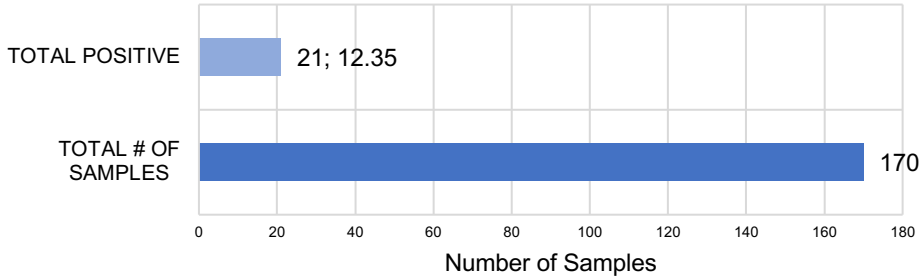

Figure 19 *Summary of Leptospira seroprevalence in cats. A total of 21/170 samples were positive for antibodies, totaling 12.35%.*

(8/21; 38.10%), and the lowest seroprevalence was observed for serovars Bataviae (1/21; 4.76%) and Mankarso (1/21; 4.76%). Seroprevalence to Ballum and Tarassovi were not observed. A summary of the seroprevalence observed to positive serovars is shown in **Figure 20.**

3.10 MAT Reactivity to Multiple Serovars Observed in Feline Samples

There were not enough positive samples for a Spearman rank-order correlation to be performed.

3.11 MAT Antibody Titers in Cats

The titers ranged from 1:50 to 1:3200. The highest titer was observed in Hardjo (1:3200), but the majority of the samples only had a 1:50 titer. The seroprevalence and the confidence intervals of all serovars tested are shown in **Table 12**. A summary of the distribution among serovars is shown in **Figure 21**.

3.12 Age, Sex, and Breed, and County Distribution in Cats

We also evaluated age, sex, breed, and county the distributions from the samples tested. For age, the majority of the samples were in the 6-year to 10-year age range, with the least number of samples in the 21-year to 25-year range (**Figure 22**). Of the 170 samples, 78 (45.88%) were from females, 91 (53.53%) were from males, and 1 (0.59%) was unknown. (**Figure 23**). For breed distribution, the population was overrepresented by Domestic Shorthair (126/170; 74.18%), followed by Domestic Longhair (13/170; 7.65%), Domestic Medium-hair (10/170; 5.88%), mixed breed (4/170; 2.35%), Siamese (3/170; 1.76%), and Sphynx (2/170; 1.18%). In this study we were able to obtain at least one sample from 27.37% (26/95) of the counties in Tennessee. There are 4 regions in Tennessee, with the majority of the samples collected from Region 1, which skewed our data. We had 145 samples (85.29%) from Region 1, 14 (8.24%) from Region 2, 11 (6.47%) from Region 3, and no samples from Region 4. A map of the regions is in **Figure 13,** and the detailed sample size of each county is shown in **Table 12**.

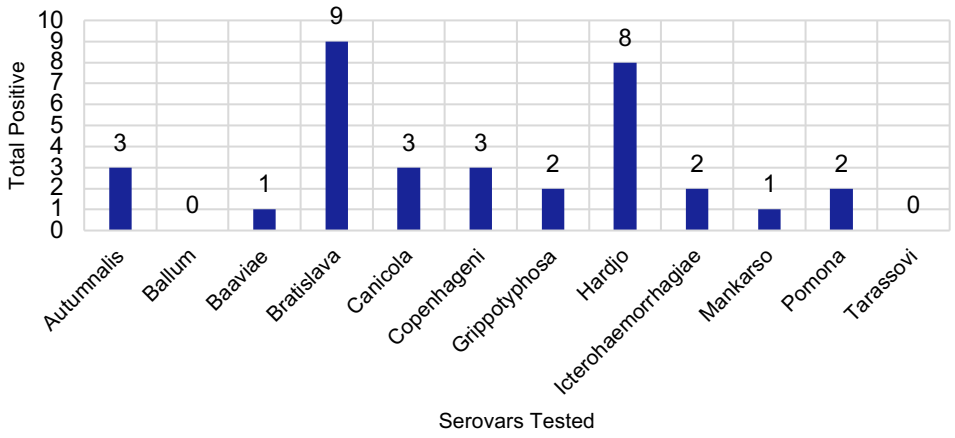

Figure 20 *Summary of Leptospira seroprevalence to 12 serovars tested in cats.*

Table 11 *Seroprevalence and 95% confidence intervals for each serovar in cats.*

	Number Positive	Prevalence	Confidence Interval
Autumnalis	3/21	14.29%	0.018-0.129
Ballum	-	-	-
Bataviae	1/21	4.76%	0.006-0.065
Bratislava	9/21	42.86%	0.165-0.329
Canicola	3/21	14.29%	0.012-0.106
Copenhageni	3/21	14.29%	0.029-0.129
Grippotyphosa	2/21	9.52%	0.035-0.129
Hardjo	8/21	38.10%	0.082-0.241
Icterohaemorrhagiae	2/21	9.52%	0.006-0.118
Mankarso	1/21	4.76%	0.006-0.047
Pomona	2/21	9.52%	0.041-0.153
Tarassovi	-	-	-

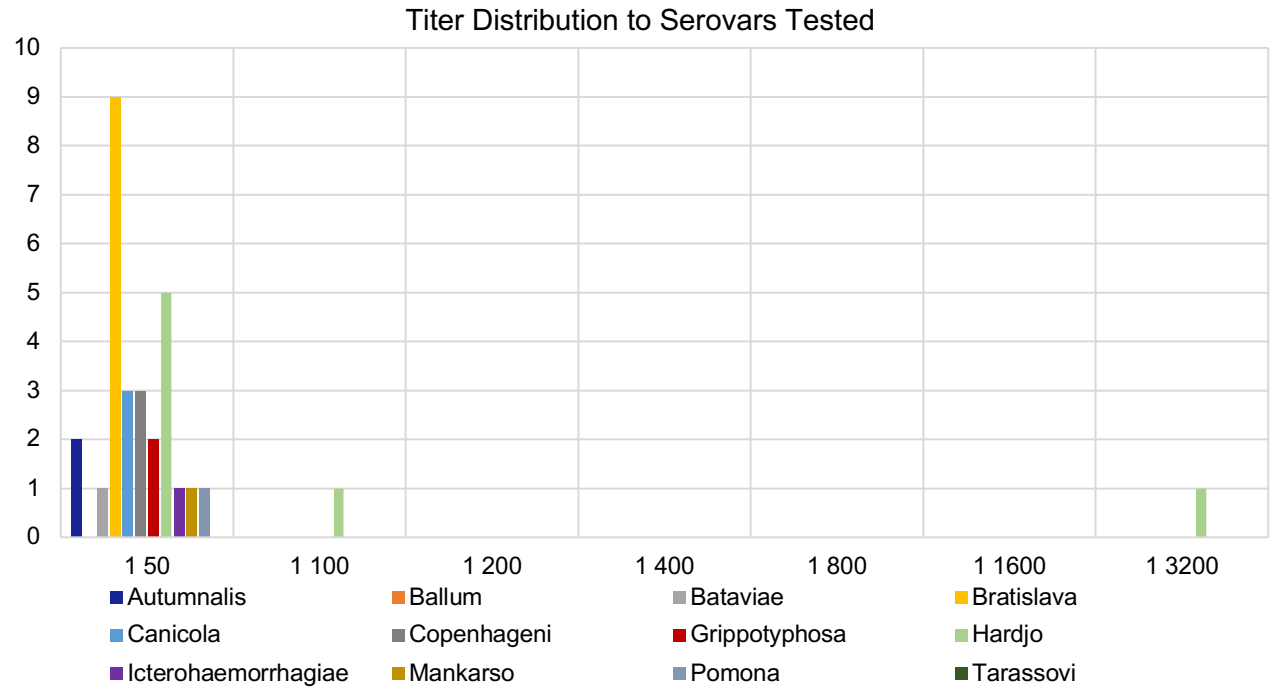

Figure 21 *Titer distribution of the microscopic agglutination test (MAT) titers in cats. The starting titer was 1:50 and the highest titer was 1:3200.*

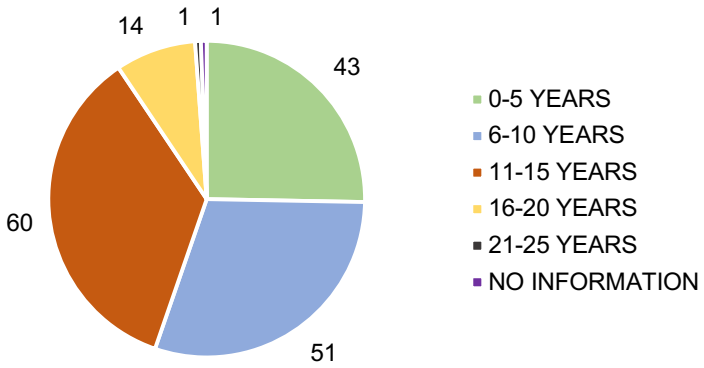

Figure 22 *Age distribution of the 170 feline samples tested.*

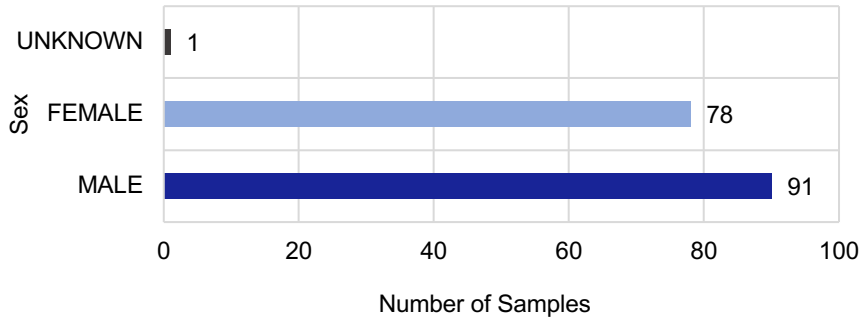

Figure 23 *Sex distribution of the 170 feline samples tested.*

Table 12 *Total number of samples and total number of positive samples by Tennessee region in cats.*

Region	Number of Positive Samples	Total Number of Samples
Region 1	19	145
Region 2	1	14
Region 3	1	11
Region 4	0	0

CHAPTER 4: DISCUSSION AND CONCLUSION

4.1 Discussion

The goal of this study was to evaluate *Leptospira* seroprevalence in dogs, horses, and cats in Tennessee. We obtained convenient samples from patients of our clinical pathology lab submitted for other evaluations, not specifically for *Leptospira* testing. This type of sampling might have decreased the possibility of selection bias, potentially provided an equal chance of selection, and offered a lower margin of error in assessing the overall *Leptospira* seroprevalence. However, it is disadvantageous for its inability to guarantee that the data collected is reflective of the entire community and the lack of samples from other regions of the state will do the opposite of decreasing the selection bias. The MAT test detects antibodies to *Leptospira* using live cultures and has acceptable specificity since antibodies to *Leptospira* do not cross-react with other bacterial species. However, cross-reactivity is observed between *Leptospira* serovars that may or may not be in the same serogroup (Levett, 2003). The sensitivity, or the ability of the diagnostic test to detect the pathogen, of MAT is low when compared to other tests (Limmathurotsakul et al., 2012) and this test is not especially useful in detecting antibodies in animals with chronic infection or in reservoir animals when they have a low level of antibodies (Ellis, 2015).

Among the three companion animals tested, the highest prevalence was observed in horses (47.73%), followed by dogs (29.41%) and cats (12.35%). Literature regarding the prevalence and risk factors of *Leptospira* infection in dogs and horses in the United States are available, however, literature in cats is very limited. Literature regarding *Leptospira* infection in dogs range from those evaluating the seasonal patterns, to spatial and spatio-temporal clustering, to seroprevalence throughout the United States (Gautam, Guptill, et al., 2010; Lee et al., 2014; Raghavan et al., 2011; White et al., 2017). The prevalence of leptospirosis in dogs in the United States ranges from 4.48% to 12.03% (Andersen-Ranberg et al., 2016; Lee et al., 2014). Based on only one previous study available of the prevalence of leptospirosis in dogs and cats in Tennessee, the prevalence in dogs was 18% (Spangler et al., 2020). Since the prevalence of *Leptospira* infection is widely understudied, our study could provide an improved understanding of the

prevalence in this area. The MAT positive results may typically indicate the presence of antibodies from current infection or previous exposure, or recent vaccination against *Leptospira.* This could cause an overestimation of prevalence due to the patients that had a previous infection.

In routine MAT testing, a typical cutoff titer is 1:100, but titers as low as 1:10 can be used for increasing the test sensitivity. However, higher titers such as ≥1:1600 can be associated with recent natural infection (Lopez et al., 2019). Our results show that the highest titers in dogs were observed for serovars Autumnalis, Bratislava, Grippotyphosa, and Pomona. The seroprevalence observed for serovars Autumnalis, Grippotyphosa, and Pomona were consistent with other previous findings (Gautam, Wu, et al., 2010; Moore et al., 2006). For dogs, there was a significant correlation of cross-reactivity between multiple serovars, specifically Autumnalis, Canicola, Copenhageni, Grippotyphosa, Mankarso and Pomona. Some of the serovars that cross-reacted belong to the same serogroup, such as Copenhageni and Mankarso belonging to serogroup Icterohaemorrhagiae, which may explain the cross-reactivity between those serovars, as described earlier.

Vaccination is commonly practiced in dogs using a quadrivalent bacterin vaccine that consists of serovars Canicola, Grippotyphosa, Icterohaemorrhagiae and Pomona. The significant difference of vaccinated and unvaccinated dogs to serovars Canicola, Grippotyphosa, Icterohaemorrhagiae, and Pomona are thought to be related to the vaccine. Serovar Autumnalis is not included in the canine vaccine, but the positivity and cross-reactivity observed in this group to was significantly higher. This could be attributed to cross-reactivity between serovars or could be an indication of that serovar is potentially being the infecting serovar. However, there has not been any publications in relation to serovar Autumnalis isolations in dogs in the US.

As for breed, *Leptospira* infection is not directly linked to any specific breed of animal, but literature suggests that for dogs, small breeds and hunting breeds could become infected more often than others (Birnbaum et al., 1998; Hennebelle et al., 2014; Rentko et al., 1992; Ward et al., 2002). Out of the 12 dog breeds identified in our results, half of the positive samples came from hunting dogs (6/12; 50.0%) followed by working

dogs (3/12; 25.0%) and small breed dogs (3/12; 25.0%), which was expected based on previous literature mentioned above.

Equine studies are available and range from serologic evidence or diagnosis of leptospirosis in the United States (Poonacha et al., 1993; Smith et al., 1976) to challenges of establishing *Leptospira* infection (Zilch et al., 2021). The prevalence in horses in the United States ranges from 2.5% to 16.4% (Andersen-Ranberg et al., 2016; Donahue et al., 1991). In horses, a bacterin vaccine containing *L. interrogans* serovar Pomona is used. In horses, the highest seroprevalence was with serovars Bratislava, Copenhageni, and Canicola. Serovars Bratislava and Canicola have shown seropositivity in horses in previous studies in the United States (Fagre et al., 2020; Williams et al., 1994), agreeing with our findings. No studies in the United States have been done to assess the risk of equine breeds and *Leptospira* infection.

Feline studies range from literature reviews aiding in diagnoses (Murillo et al., 2020), to shedding, seropositivity, and seroprevalence (Palerme et al., 2019; Spangler et al., 2020). The prevalence in cats in the United States ranges from 4% to 33% (Murillo et al., 2020). Based on only one previous study available of the prevalence of *Leptospira* in dogs and cats in Tennessee, and all of the cats in that study tested negative (Spangler et al., 2020). In our study, the serovars that had the highest number of positive samples were serovars Bratislava and Hardjo, and the highest titer seen in cats was against serovar Hardjo (1:3200). Positivity to serovar Bratislava agrees with other feline studies both inside and outside of the United States (Donato et al., 2022; Lehtla et al., 2020; Markovich et al., 2012; Palerme et al., 2019), while a limited number of studies reported positive samples against serovar Hardjo (Lehtla et al., 2020; Murillo et al., 2020). No studies in the United States have been done to assess the risk of feline breeds and *Leptospira* infection.

Although our study described *Leptospira* seroprevalence in three companion animal species in Tennessee, there were limitations in this study. We calculated a required number of samples we needed for each species in this study using a sample size formula. However, we were not able collect the proposed number of equine samples, resulting in a less precise estimate and an added potential for both Type I and Type II errors, such as the mistaken rejection of the actually true null (type I) and the failure to

reject an actually false null (type II). The MAT test has limitations, such as the difficulty in reading the results and management of cultures, but is still considered the gold standard and is the most widely used method of assessing the prevalence of *Leptospira*. *Leptospira* vaccination status in dogs was only available for 49% of the samples collected, and *Leptospira* vaccination status in horses was not available to us so we could not assess potential effect of vaccination in horses. The majority of our samples were from East Tennessee. The small number of samples from the other regions made it difficult to assess the prevalence in these regions accurately but, in future studies, using a national database would be a feasible solution to this problem by being able to use data from more areas, allowing for a more accurate assessment. When researching the prevalence, risk factors, and overall number of literature available for dogs, horses, and cats, there was very little information on horses, and minimal information on cats resulting in difficulty in comparing the results obtained in our study to other previous studies.

4.2 Conclusion

The aim of this study was to estimate the *Leptospira* seroprevalence in Tennessee. We confirmed positive serological reactivity to many different *Leptospira* serovars. We found a 29.41% prevalence in dogs, a 47.73% prevalence in horses, and a 12.35% prevalence in cats, and we found that there were multiple instances of cross-reactivity between the *Leptospira* serovars used in the MAT test. We also found that vaccinated dogs were significantly more likely to test positive for 1 or more *Leptospira* serovar, which is potentially associated with vaccination.

Future research should focus on the significant associations found in this study and pair the MAT with a highly specific test, such as real-time PCR, and isolation of *Leptospira* from animal samples to better address the potential carrier status and shedding of these animals. In addition, sequencing to characterize the *Leptospira* isolated should also be considered. This will benefit veterinarians and public health workers to focus on circulating *Leptospira* serovars and species and to help future vaccine development. Potential risk factors and associations with sex, breed, and age are other areas that need further studies.

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