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Detecting Enzootic Leishmaniasis and American Trypanosomiasis in Stray Dogs in El Paso County, Texas and the Potential for Autochthonous Transmission to Humans

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Abstract

Cutaneous leishmaniasis and Chagas disease (American trypanosomiasis) are two vector-borne, protozoal zoonoses whose emergence into the southern United States is a public health problem of increasing significance. Cutaneous leishmaniasis is caused by several species of intracellular protozoa in the genus *Leishmania* and is most often characterized by the formation of large, ulcerative skin lesions that can result in considerable scarring and permanent disfigurement. Infection with *Leishmania* is prevalent throughout the world in tropical and sub-tropical regions and in areas where people are regularly exposed to the hematophagous sand fly vectors that transmit the disease. Chronic infection with *Trypanosoma cruzi*, the causative agent of Chagas disease, is a well-known cause of heart failure, arrhythmia, and enlargement of the esophagus and colon. Between 6 and 8 million people currently living in Latin America and the United States are believed to be chronically infected with *Trypanosoma cruzi* with complications from these infections resulting in approximately 45,000 deaths annually. In the southern United States, enzootic transmission of both *Leishmania mexicana* and *Trypanosoma cruzi* has been documented in addition to sporadic reports of autochthonous human cases of cutaneous leishmaniasis and Chagas disease. Future climate change may lead to the emergence of these pathogens into new foci across the southern United States and could possibly result in an increase in the incidence of human infections.

Domestic dogs (*Canis familiaris)* have been implicated as potential reservoirs or incidental hosts of both *Leishmania* and *Trypanosoma cruzi* in endemic foci throughout the Americas. This study among a sample of stray canines provides evidence that transmission of these pathogens is occurring in El Paso County, Texas. From July 2014 to May 2015, skin, spleen, and heart biopsies from 159 stray canines were collected. Genomic DNA of sufficient quality for use in PCR analyses was successfully extracted from these biopsies in 156 of the stray canines surveyed. PCR-amplification of *Leishmania spp*. or *Trypanosoma cruzi* DNA was attempted using extracted genomic DNA from these biopsies to screen for evidence of infection.

Using agarose gel electrophoresis, *Leishmania spp*. DNA was detected in 41 stray dogs and *Trypanosoma cruzi* DNA was detected in 21 stray dogs, representing 26.3% and 13.5% of the total sample, respectively. From the group of animals identified as PCR-positive for *Leishmania spp*., 18 biopsy PCR products were randomly chosen for bidirectional sequencing. Analysis of sequenced PCR products showed that all infections were caused by *Leishmania mexicana*. A multiple sequence alignment was created to show homology between sequenced PCR products and known reference strains of *Leishmania mexicana* and a distantly related species, *Leishmania major*.

Collectively, these results suggest that enzootic transmission of *Leishmania mexicana* and *Trypanosoma cruzi* is occurring over a wide portion of El Paso County and nearby densely populated areas in the city of El Paso. The majority of stray dogs infected with *Leishmania mexicana* were found during the late summer and early fall, and the majority of those infected with *Trypanosoma cruzi* were found during the late fall and winter, suggesting that these may be periods during which increased transmission is occurring. The behaviors of many stray canines and their relatively close associations with humans could potentially put people in El Paso County at greater risk for infection. Whether the finding of *Leishmania mexicana* and *Trypanosoma cruzi* in these stray canines is evidence of a relatively new emergence of these pathogens warrants additional investigation. Further research is also needed to better understand the epidemiology and transmission of these pathogens in order to better assess any potential for autochthonous transmission to people living in the region.

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Chapter 1: Background and Significance

1.1 Leishmaniasis

Protozoal parasites of the genus *Leishmania* represent one of the more ancient and genetically diverse taxa of all eukaryotic human pathogens. Belonging to class Kinetoplastida, the *Leishmania* are intracellular, haemoflagellate, multi-host protozoans that belong to the same family (i.e., the Trypanosomatide) as the important human parasites *Trypanosoma cruzi* and *Trypanosoma brucei* (Stevens et al, 2001). The disease caused by *Leishmania* protozoa, termed leishmaniasis, can range from relatively benign, ulcerative skin lesions to severe hepatomegaly and splenomegaly that is almost invariably fatal if left untreated.

The genus *Leishmania* is known to comprise an estimated 35 genetically distinct species, over 20 of which have been linked to human disease (World Health Organization, 2014). This substantial diversity among the *Leishmania* is due to their lengthy evolutionary history (likely established over 50 million years ago) that has allowed for both the distribution of these parasites across the world and for their divergence and speciation within several ecological settings (Tuon et al, 2008). The genus *Leishmania* can be taxonomically subdivided into two major subgenera containing all *Leishmania* species known to parasitize humans and cause disease, and two putative subgenera with species not recognized as human pathogens (Figure 1). The two major subgenera, *Leishmania Leishmania* and *Leishmania Viannia*, divide the genus based on ancient evolutionary origins in either the Old World or New World, respectively, and contain all known leishmanial pathogens of humans. Species in the *Leishmania Leishmania* subgenus can be categorized into three major species complexes, the *L. donovani* complex, the *L. tropica* complex, and the *L. mexicana* complex (Britto et al, 1998). Although part of the *Leishmania Leishmania* subgenus (with evolutionary origins in the Old World), the *L. mexicana* complex now contains parasites found exclusively in the Americas (Bates, 2007). Species of the *Leishmania Viannia* subgenus are exclusively found in the New World, distributed throughout South and Central America (Lainson and Shaw, 2005). Two other subgenera, *Leishmania*

Sauroleishmania and *Leishmania Endotrypanum* contain parasites of lizards and sloths, respectively, and are not of medical importance to humans (Croan and Ellis, 1996) (Figure 1).

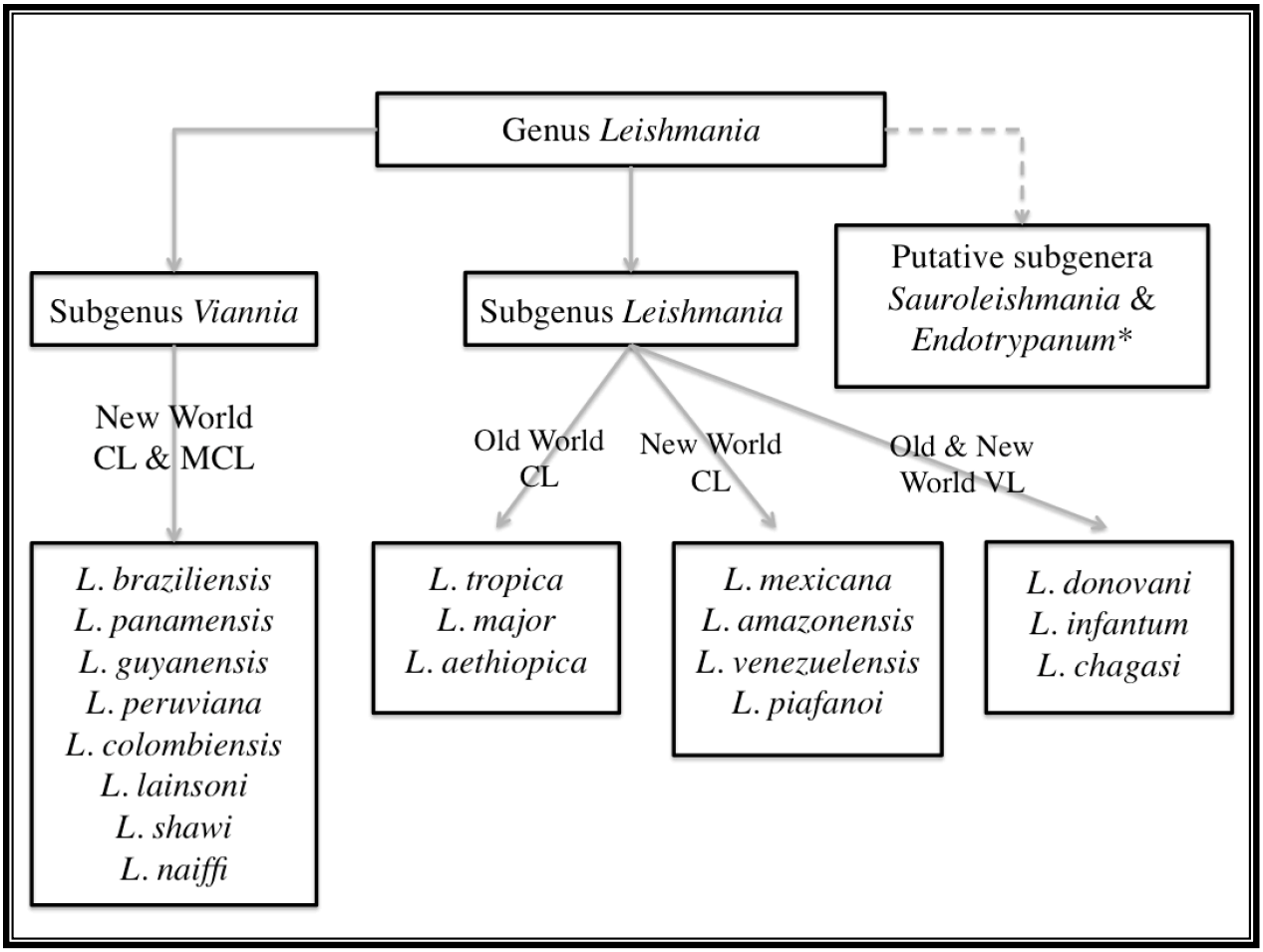

Figure 1: Diagrammatical taxonomic breakdown of medically relevant species in Genus *Leishmania*. Species within the subgenera *L. Leishmania* and *L. Viannia* are subdivided based on their geographic occurrences and the major clinical disease they cause. (CL: cutaneous leishmaniasis; MCL: mucocutaneous leishmaniasis; VL: visceral leishmaniasis) *Species of these subgenera are not known to infect humans. Figure by E. Kipp (Adapted from Bates, 2007)

Collectively referred to as leishmaniases, the disease caused by *Leishmania* infection can present in a variety of clinical forms in people and is responsible for significant morbidity and mortality in tropical and sub-tropical nations throughout the world. In people, clinical disease

caused by *Leishmania* typically presents in one of three major forms: visceral leishmaniasis, cutaneous leishmaniasis, or mucocutaneous leishmaniasis. Development of one of these three disease phenotypes is primarily determined by the species of *Leishmania* causing the infection, but can also be influenced by the ecological setting in which infection occurs, by the strength of the host's immune response, and by other complex host-parasite interactions (McCall and McKerrow, 2014).

Visceral leishmaniasis (VL), the most serious manifestation of the disease, is typically caused by the parasites *L. donovani, L. infantum,* and *L. chagasi* and is almost invariably fatal if left untreated. Onset of symptoms can be sudden or gradual, and usually occurs approximately 3 to 6 months after initial exposure (Despommier et al, 2000). Patients with VL often present with fever, abdominal discomfort, cachexia, diarrhea, and hepatosplenomegaly (Guerin et al, 2002). Involvement and enlargement of the spleen and liver is a defining feature of the disease, often resulting in significant abdominal distension, and is due to the preferential metastasis of *Leishmania*-infected macrophages to these organs (Meddeb-Garnaoui et al, 2008). Replication of *Leishmania* parasites in VL patients can also result in bone marrow suppression, pancytopenia, potential secondary infection, and ultimately leads to death if left untreated (McGwire and Satoskar, 2013).

The majority of known pathogenic *Leishmania* throughout the world cause a form of the disease known as cutaneous leishmaniasis (CL) in which a sometimes large, ulcerative lesion will erupt on the skin near the site of the sand fly bite wound. These cutaneous lesions often become apparent between two to eight weeks after initial exposure to the pathogen and begin as small, painless, erythematous papules that progressively become larger and eventually ulcerate and depress to reveal a sizeable crateriform lesion that can often be over 5 cm in diameter. (Despommier et al, 2000). Full progression from papule to ulcer can take anywhere between two weeks to nearly six months and can vary significantly in size, outward appearance and in the amount of time needed for healing to occur (Reithinger et al, 2007). CL will often spontaneously resolve, even in the absence of antiprotozoal chemotherapy, but may take anywhere between 2

and 15 months depending on numerous host factors and the species of parasite causing the infection (Bailey and Lockwood, 2007). In cases of spontaneous healing, individuals are often protected against future infection from the same *Leishmania sp*. but may still be susceptible to infection by other *Leishmania spp*. (Reithinger et al, 2007). Even after healing, significant scarring and permanent disfigurement may result which may have a serious negative social or economic impact on the part of the afflicted individual. In many settings where CL is endemic, children are at greater risk of being infected than their adult counterparts and also have a greater risk of developing lesions on the exposed and hairless portions of their head or face where the resulting scar can be severely disfiguring (Armijos et al, 1997). For people living in endemic areas these scars can also have a lifelong psychological impact. Large scars on an individual's face or another visible part of the body can have a significant negative psychosocial impact and can greatly reduce an individual's self-esteem. These negative psychosocial impacts are especially common among women in endemic areas who often report that having scars as a result of CL lowers their perceived attractiveness, reduces their marriage prospects, and results in significant social stigmatization within their respective communities (Weigel et al, 1994). Adult males, on the other hand, living in endemic areas are more likely to view CL as a disease that can have a significant economic impact, as they are more likely to develop lesions and scars on their hands, feet, or limbs that interfere with their daily occupational activities (Weigel et al, 1994).

Mucocutaneous leishmaniasis (MCL) is a form of the disease limited to Central and South America and typically caused by members of the *L. Viannia* subgenus, most often *L. Vianna braziliensis* and *L. Vianna panamensis*. In this form of the disease, *Leishmania*-infected macrophages preferentially metastasize to the mucosa of the oral cavity and nose, ultimately leading to the progressive erosion of these body parts and severe disfigurement and morbidity (Lainson and Shaw, 2005). In a large majority of cases, MCL is secondary to the cutaneous form of the disease, often erupting many months or years after the apparent spontaneous resolution of the initial cutaneous lesion. In these cases of MCL secondary to CL, *Leishmania*-infected macrophages may survive for extended periods of time within the human host without causing

noticeable disease and ultimately reactivating and metastasizing to the oral and nasal mucosa in a small percentage of those infected (i.e., less than one percent of those initially infected with MCL prone *Leishmania spp*.) (Lainson and Shaw, 2005; Lessa et al, 2007). Appropriate treatment of initial CL can significantly reduce an individual's risk of developing secondary MCL. There have been numerous reports, however, of latent CL infections following antiprotozoal reactivating months or years later as MCL, although this reactivation is far more likely to occur in those that receive no treatment or do not adhere to the entire treatment regimen (Weigle and Saravia, 1996). Complications due to MCL can be very severe and debilitating and can include blockage of the nasal passage leading to respiratory distress, a greater likelihood of developing potentially lethal pulmonary infections, and significant facial disfigurement that arises as a result of the erosion of the nasal and oral mucosa (Lainson and Shaw, 2005).

Another less common manifestation of leishmaniasis in humans is known as diffuse cutaneous leishmaniasis. This diffuse (or disseminated) form of the disease can be caused by multiple species of *Leishmania* parasites but is most often due to *L. mexicana* infection in immunocompromised individuals. Cases of diffuse cutaneous leishmaniasis are characterized by the development of small and nonulcerative papules that may erupt across the entirety of the host's skin (Reithinger et al, 2007). In these cases of diffuse cutaneous leishmaniasis, *Leishmania* parasites proliferate widely throughout the body and are consistently resistant to any antiprotozoal chemotherapy, making cure of this form of the disease often unachievable (Convit et al, 1972).

1.1.1 Pathobiology of Human Cutaneous Leishmaniasis

All species of *Leishmania*, including those that cause CL, have obligate life cycle stages in a phlebotomine sand fly vector and in a mammalian host. Transmission of the protozoa between vector and host occurs when the female sand fly takes a blood meal from a human or other mammalian species capable of harboring the parasite.

Humans are initially exposed to a flagellated form of the parasite, known as the promastigote that gets injected into the subcutaneous tissue when an infected female sand fly takes a blood meal (Figure 2). Tissue macrophages encounter the promastigote and proceed to engulf it, initiating a series of biochemical changes that result in its transformation into an aflagellate and intracellular form, known as the amastigote (Figure 2). These intracellular amastigotes replicate rapidly via binary fission within the macrophage (inside a specialized organelle called the phagolysosome) ultimately causing it to burst and release amastigotes into the surrounding tissue where they are free to infect new macrophages (Despommier et al, 2000). In CL, infected macrophages are often only found in areas of the skin immediately surrounding the raised edge of the cutaneous lesion or in the adjacent lymphatic drainage. Only in VL, MCL or diffuse cutaneous leishmaniasis do the parasites travel to distant sites or infect the body's deeper organs (Bailey and Lockwood, 2007).

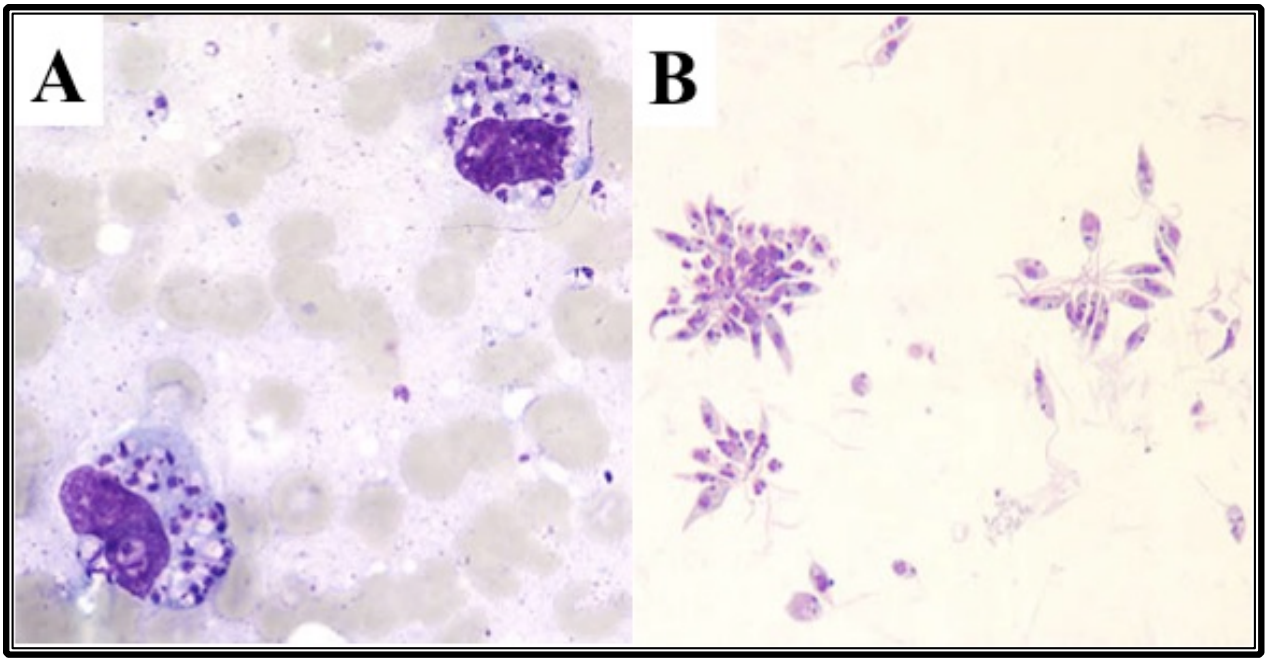

Figure 2: *Leishmania* parasites under light microscopy. (A) Amastigotes visible as darkly stained granules within Giemsa-stained macrophages. (B) Promastigotes in liquid culture. Images adopted from CDC Public Health Image Library

A female sand fly becomes infected with *Leishmania* when it ingests free amastigotes in the blood or infected tissue macrophages containing intracellular amastigotes. In the gut tract of the sand fly, chemical cues cause the parasite to change into its flagellated promastigote form in a period of about a week. Eventually these new, infective metacyclic promastigotes will migrate to the sand fly's salivary glands. The cycle is completed when the female sand fly takes a second blood meal from a human or other mammal and injects infective promastigotes into the bite wound along with its salivary secretions (Despommier et al, 2000) (Figure 3).

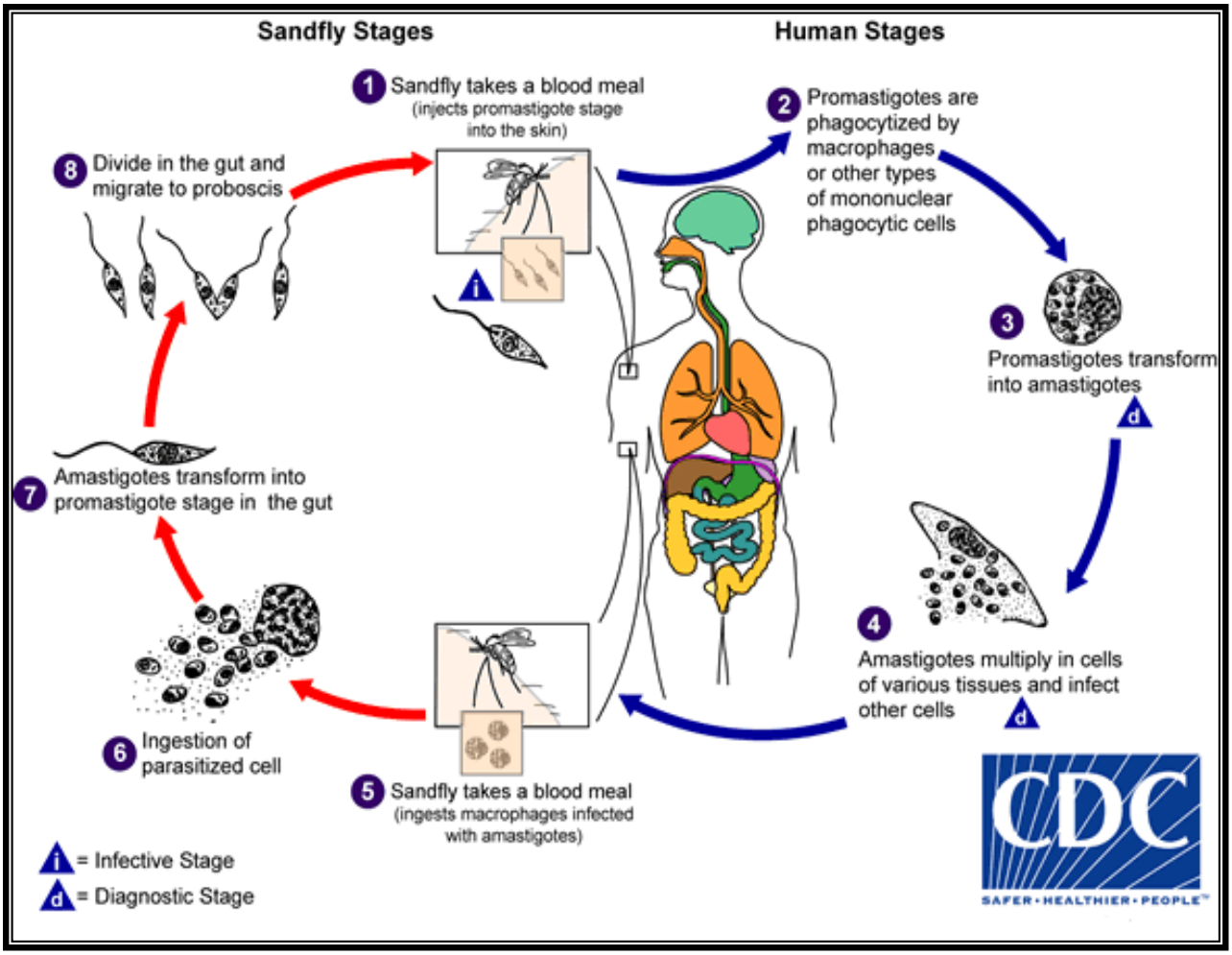

Figure 3: Lifecycle of *Leishmania* within the human host. Figure adopted from the CDC Division of Parasitic Diseases and Malaria

1.1.2 Biology of Phlebotomine Vectors

All forms of human leishmaniasis are transmitted via the bite of a female phlebotomine sand fly (Diptera: Psychodidae: Phlebotominae) belonging to either the genus *Phlebotomus* or the genus *Lutzomyia*. *Phlebotomus spp*. are responsible for transmission of Old World leishmaniasis throughout Europe, the Middle East, Africa and Asia, and *Lutzomyia spp*. are responsible for transmission of the disease throughout the Americas (Reithinger et al, 2007). Approximately 700 species of sand fly have been described to date, only about 10% of which are known to be competent vectors of human leishmaniasis (Bates, 2007). In some settings, sand flies have also been identified as competent vectors of *Bartonella* bacteria and numerous arboviruses (Young and Duncan, 1994).

The sand fly life cycle begins when a female sand fly lays her eggs in a humid habitat often with ample decaying organic material (e.g., forest leaf litter, rock crevices, animal burrows). About ten days after oviposition, the sand fly eggs will hatch into small caterpillar-like larvae that feed on available organic material. After a period of a few weeks to months, these larvae will transform into pupae and attach themselves to a solid object such as a stone or dead leaf. Adult sand flies then emerge about 7 to 12 days later. Both male and female sand flies require a carbohydrate energy source that they may obtain from floral nectar, fruits, honeydew from aphids, or other plant material. Female sand flies require an additional vertebrate blood meal for the maturation of their eggs. Biting preferences differ substantially between different sand fly subgenera and species and can include a wide variety of mammalian, avian, amphibian, or reptilian species (Young and Duncan, 1994). Transmission of *Leishmania* parasites between mammalian hosts requires a female sand fly to take at least two blood meals, one in which *Leishmania* amastigotes are taken up from an infected animal and a second blood meal in which infectious promastigotes are inoculated into a naïve mammalian host (Despommier et al, 2000).

1.1.3 Mammalian Reservoirs of Cutaneous Leishmaniasis and Leishmaniasis in Canines

Leishmania parasites have been isolated from a variety of non-human mammalian hosts in different settings throughout the world, likely acting in widely different capacities as reservoirs. Mammalian hosts include numerous rodent species, marsupials, sloths, armadillos, domestic and wild canines, cats, and various other carnivorous mammals (Reithinger et al, 2007; Esch and Petersen, 2013). Within these mammalian hosts, infection with *Leishmania spp*. may cause asymptomatic infection or result in clinical disease that may or may not mirror the clinical forms of leishmaniasis in humans. For example, dogs infected with *Leishmania infantum* present with a distinct viscero-dermic form of disease in which the parasite can be detected in visceral organs as well as in intact and apparently healthy skin. Additionally, rodents infected with *Leishmania spp*. responsible for the cutaneous form of the disease in humans can have detectable parasites in their deep visceral organs (Roque and Jansen, 2014).

Mammalian reservoirs for leishmaniasis have been classically divided into one of three categories: primary reservoirs, secondary reservoirs, and incidental hosts. Primary reservoirs are defined as those that can harbor the infection and transmit it back to phlebotomine vectors in endemic settings in such a way that the amplification and transmission of the parasite is largely favored (i.e., with a basic reproduction number greater than one) (Reithinger and Davies, 1999). Additionally, an effective primary reservoir must have a natural history that puts them in regular contact with the hematophagous sand fly vector, must be able to carry the parasite for long periods of time while being asymptomatic or without developing debilitating disease, and must be known to carry the parasite in areas adjacent to where human cases have been reported (González et al, 2010). Secondary reservoirs are those that can significantly aid in the transmission of the parasite in the presence of other mammalian reservoirs but that are unable to act as primary reservoirs in the absence of these other host species. Finally, incidental hosts are those whose infection with the parasite may cause clinical illness but are unable to facilitate long-term transmission back to the phlebotomine vector population (Reithinger and Davies, 1999).

The role of domestic dogs (*Canis familiaris)* in the transmission of CL in the Americas is of particular interest. In endemic foci throughout the Americas, it has been established that numerous species of rodent, sloth, and other sylvatic mammalian species can act as primary reservoirs for *Leishmania*. Infection in domestic dogs is well-documented with positive dogs having been identified in Bolivia, Brazil, Colombia, Ecuador, Panama, Peru, Venezuela, and the United States (Reithinger and Davies, 1999). Whether these infected dogs are acting as secondary reservoirs or only as incidental hosts in these settings is still a topic of debate. However, dogs may be playing a very interesting epidemiological role in some of these settings through their close associations with humans. It remains a possibility that dogs could be acting as something of a "bridge reservoir", bringing *Leishmania* from sylvatic, enzootic cycles into areas more densely populated with humans and where transmission to people is more likely to occur (Esteve-Gassent et al, 2014).

1.1.4 Diagnosis and Treatment of Human Cutaneous Leishmaniasis

Human CL can be very difficult to appropriately diagnose in many rural settings or endemic areas with limited medical infrastructure. Physicians throughout the Americas should consider CL in their differential diagnosis in cases of problematic ulcerative skin lesions that do not respond to topical antibiotic treatment. Unfortunately, the clinical presentation of CL can be similar to that of some skin cancers, leprosy, cutaneous tuberculosis, and various fungal infections. A more specific diagnosis not based on clinical presentation alone is needed to confirm the diagnosis of CL and to begin antiprotozoal treatment (Reithinger et al, 2007).

A definitive diagnosis can be obtained through the observation of Giemsa-stained *Leishmania* parasites under microscopic examination, either through tissue smears or parasitological culture. Direct diagnosis of CL through microscopic examination has a somewhat low sensitivity and often only works well only in the early stages of the infection when *Leishmania* amastigotes are widely circulating in the area surrounding the skin lesion.

Parasitological culture has a greater sensitivity than microscopic observation, but is often not an available tool in many rural clinics in endemic settings and also has a lower sensitivity in cases of chronic CL (Reithinger et al, 2007). For diagnosis of CL infections that have persisted for many months, PCR detection of *Leishmania* from ulcer biopsies can greatly improve diagnostic sensitivity but also is not a readily available diagnostic tool in rural endemic areas that lack adequate medical and laboratory infrastructure (Reithinger et al, 2007). Another diagnostic method, known as the Montenegro skin test, is used in some endemic areas in which a small amount of leishmanial antigen is injected subcutaneously and observed for localized inflammatory response (i.e., any swelling, itching, redness, or heat) indicative of previous exposure to the pathogen. The Montenegro skin test has proved itself a useful diagnostic tool for identifying cases of MCL and chronic CL due to the strong host immune response that is elicited. While this can be both a sensitive and specific method for diagnosis, it is rarely used outside of epidemiologic studies in endemic areas, and it is unable to distinguish between a current *Leishmania* infection or previous exposure to the pathogen (Reithinger et al, 2007; Calvopina et al, 2004).

Despite the tendency of many CL lesions to spontaneously heal, treatment of the disease is often necessary, especially in cases of large, persistent, or debilitating lesions. Rapid diagnosis and treatment is especially necessary in children with CL, as the cutaneous lesions are more likely to be in the head or face area and have a tendency to grow rapidly due to their small body size (Armijos et al, 1997; Armijos et al, 1998). Additionally, treatment of CL is often necessary to reduce the risk of recurrent CL, known as leishmaniasis recidivans, in which cutaneous lesions can erupt months or years after an apparent spontaneous cure of CL near the site of the original lesion (Bailey and Lockwood, 2007).

Treatment of CL usually involves administration of a pentavalent antimonial compound, the most common of which is sodium stibogluconate. The Word Health Organization recommends intralesional, intramuscular or intravenous administration of sodium stibogluconate (20 mg/kg) for 20 to 28 consecutive days (World Health Organization, 2014; Reithinger et al,

2007). For most *Leishmania spp*., cure rates of between 85 and 95% are observed after administration. Despite its efficacy, sodium stibogluconate treatment can be associated with a number of serious adverse side effects including severe headache, rash, arthralgia, myalgia, pancreatitis, and liver damage (Despommier et al, 2000).

Numerous other alternative treatments against CL are also available with varying degrees of efficacy. Amphotericin B is an antifungal drug that is often administered in cases of VL (caused by *L. infantum*), MCL, or in cases of CL where sodium stibogluconate administration is contraindicated (Reithinger et al, 2007). Amphotericin B is most often delivered within lipid formulations or encapsulated in liposomes in order to improve its overall efficacy and to increase its selectivity at killing protozoal cells. Clinical studies have shown liposomal Amphotericin B to be very efficacious for the treatment of leishmaniasis and despite some toxic side effects (especially to the renal system) it may ultimately offer a more effective and safe alternative to the traditional antimonial treatment regimen (Amato et al, 2004).

Currently there are no readily available human vaccines against any form of leishmaniasis, although numerous vaccine candidates have shown success in human and animal models. Leishmanization, a process in which live and virulent parasites are intentionally inoculated into an inconspicuous area of the body to cause CL and provide long lasting immunity, has been practiced for centuries in some endemic areas (Dunning, 2009). More recent studies using attenuated or killed parasite vaccines, recombinant protein vaccines, and third generation DNA vaccines have shown experimental efficacy, raising the prospect that a human vaccine against CL will become available in the future (Dunning, 2009; Armijos et al, 2004).

1.1.5 Epidemiology of Leishmaniasis

Human leishmaniasis is widely distributed throughout the world with autochthonous transmission having been described in 98 countries on all continents, excluding Australia and Antarctica (Alvar et al, 2012). The World Health Organization estimates that over 350 million

people around the world are at risk for contracting at least one form of leishmaniasis. The vast majority of leishmaniasis-associated mortality is due to VL, primarily caused by *L. donovani*, *L. infantum*, and *L. chagasi*. Up to 500,000 people are affected by VL each year and between 40,000 and 59,000 deaths are recorded annually (Ready, 2014; Alvar et al, 2006). Among all parasitic diseases, VL is second only to malaria in number of annual deaths. Collectively, leishmaniasis also has a significant economic impact worldwide. Approximately 2.1 million disability-adjusted life years are lost annually due to all forms of leishmaniasis and with 44,000 of these lost life years coming from Latin American nations alone (Hotez et al, 2008).

Although not a significant cause of mortality, CL is a major infectious cause of morbidity throughout the world's tropics and subtropics. Worldwide, CL has an estimated annual incidence of between 700,000 and 1.3 million cases, with 95% of those cases occurring in the Americas, the Middle East, the Mediterranean basin, and Central Asia (World Health Organization, 2014). In the Americas, 59 million people live at risk of contracting CL and there is an estimated annual incidence of approximately 59,300, though this figure may be significantly higher as the disease often goes unreported in many areas where access to medical care is limited (Weigle and Saravia, 1996). The distribution of CL in the New World extends throughout South and Central America, into Mexico and even into the southern United States (Lainson and Shaw, 2005).

Risk factors for contracting CL throughout the world can differ widely depending on numerous epidemiologic variables across different endemic regions. Some common risk factors for contracting the disease include sex (males typically have jobs or social roles that put them in increased contact with phlebotomine vectors), age (older people in endemic areas often have developed some degree of immunity), household construction, and the presence of domestic or peri-domestic animals living in close association with people (Reithinger et al, 2007). Other important risk factors for the development CL, especially in the New World, are the degree of deforestation, agricultural development, and resource exploitation in a given area. The degradation of a landscape can often cause phlebotomine vectors to adopt a more anthrophilic lifestyle whereby they bite humans and domestic animals more regularly than they would if the

landscape around them was intact and other sylvatic mammal species were more abundant (Desjeux, 2001). CL is also a disease closely associated with poverty and the lack of adequate medical infrastructure. Poverty-associated factors such as poor housing conditions, poor nutrition, lack of bed net usage, sleeping outside, lack of regular garbage collection, and inadequate vector control efforts all are greatly associated with increased risk for contracting CL (Alvar et al, 2006).

1.1.6 Emergence of Cutaneous Leishmaniasis in the United States

Between 1903 and 1996, approximately 29 reported cases of autochthonous human CL were reported in the United States, all from the state of Texas (Clarke et al, 2013). In 2008, a cluster of 9 autochthonous cases of CL was reported in north Texas in an area that had previously never had any evidence of *Leishmania* transmission, possibly indicative of a recent northward expansion of the disease (Wright et al, 2008). In all recent cases of human CL in Texas, the pathogen responsible has been *L. mexicana*, a species that most often causes CL but is also known to cause diffuse cutaneous leishmaniasis in a small subset of infected individuals. Recent research has also indicated that the primary reservoir of CL in south, central and north Texas is likely the Southern Plains woodrat (*Neotoma micropus*). Infected woodrats have been discovered in the vicinity of all recent documented human exposures and also in areas of the state where human cases have not yet been reported (McHugh et al, 1990; Raymond et al, 2003; González et al, 2010).

Reports of CL in domestic dogs in Texas have also been reported, though these have not been backed up by molecular testing and to date no comprehensive seroprevalence or PCR-prevalence study testing for *L. mexicana* has been conducted in the state (Petersen, 2009). Outbreaks of canine leishmaniasis caused by *L. infantum* among hunting dogs imported from the Mediterranean basin have also occurred throughout the U.S. since 1999. Although no autochthonous human cases of VL caused by *L. infantum* have been reported, it has been shown

that dogs can act as capable reservoirs of this parasite and evidence for local transmission between dogs in the U.S. has been well-documented (Enserink, 2000; Petersen, 2009).

Evidence also suggests that in future years, the distribution of CL across the state of Texas and across the entire southern United States will expand significantly, in large part due to climate change-mediated factors. Predicted reservoir distributions created using ecological niche modeling under various climate change scenarios suggest a widening and northwardly expansion of the reservoir species *Neotoma micropus, Neotoma albigula, Neotoma floridana,* and *Neotoma mexicana*. Also the same predictive modeling applied to the endemic phlebotomine vector species, *Lutzomyia anthophora* and *Lutzomyia diabolica*, also predicts a widening and northwardly expansion (González et al, 2010). In addition to climate change, other factors associated with the emergence of CL into new regions of the United States and into areas where humans will be at greater risk of infection might include habitat destruction, increased tourism and immigration, environmental pollution, insecticide resistance, and increasing human encroachment into uninhabited and rural areas (Harrus and Baneth, 2005).

1.2 American Trypanosomiasis (Chagas Disease)

The genus *Trypanosoma* (Kinetoplastida: Trypanosomatidae) contains two notable human pathogens, *Trypanosoma cruzi* and *Trypanosoma brucei*, the cause of Chagas disease (American trypanosomiasis) and African sleeping sickness (African trypanosomiasis), respectively. In addition to these two important human pathogens, the genus *Trypanosoma* also contains numerous other protozoa that are known to parasitize animals of all vertebrate classes (Haag et al, 1998). Trypanosome parasites also differ widely in their pathobiology and can be transmitted by a variety of vectors including numerous species of insect, arachnid, and even some leeches (Haag et al, 1998). Even between the two major human trypanosomiases, there are significant differences in transmission and pathobiology with *T. cruzi* being transmitted by the triatomine (i.e., kissing bug) and being an intracellular infection in its mammalian host, and *T. brucei* being transmitted by the dipteran Tse-tse fly and being an extracellular infection in its host. Indeed, these two trypanosome species have likely been geographically isolated for millions of years and have had ample evolutionary time to diverge and develop their strikingly different life cycles (Machado et al, 2006).

Human infection with *T. cruzi*, the causative agent of Chagas disease, can lead to a broad spectrum of clinical symptoms depending on the length and burden of infection, the type of tissue most affected by the parasite, and by numerous other host and parasite factors (Despommier et al, 2000). Clinical Chagas disease can, however, usually be divided into three phases: an initial acute phase, a prolonged indeterminate phase, and a final symptomatic chronic phase. Acute infection with *T. cruzi* can be asymptomatic or is sometimes characterized by fever, malaise, and lymphadenopathy. These symptoms of acute Chagas disease are often transient and can easily be misdiagnosed. Acute Chagas disease is most common in young children and can even be fatal if serious complications develop, such as meningitis, encephalitis, myocarditis or pneumonitis (Rassi Jr et al, 2010). After an acute incubation period of between 4 and 12 weeks, an infected individual will enter an asymptomatic or indeterminate phase of that can last many years or decades. Finally, an individual may progress into the chronic phase of Chagas disease

that is responsible for the vast majority of the disease's observed morbidity and mortality. Progression to this symptomatic stage occurs in an estimated 30% to 40% of those asymptomatically infected and is most often characterized by the progressive destruction of the myocardium, the esophagus, or the colon (World Health Organization, 2015; Rassi Jr et al, 2010). Chagas disease-associated morbidity and mortality can be due to a number of causes including myocarditis, congestive heart failure, arrhythmia, thromboembolism, megaoesophagus, and megacolon (Rassi Jr et al, 2010; Tanowitz et al, 1992).

In addition to the traditional vector-borne route of transmission for *T. cruzi*, infection can also occur when the parasite comes in contact with a host's ocular or conjunctival membrane or is directly ingested. In cases of vector-borne transmission, a large swollen and erythematous area may develop around the site of the triatomine bite wound and known as a chagoma. If the parasite is introduced into the body through contact with the ocular membrane, a unilateral swelling may develop around the eye, referred to as Romaña's sign (Despommier et al, 2000). Transmission of *T. cruzi* via blood transfusion, organ transplant, from mother to offspring, and through the ingestion of uncooked food contaminated with infected triatomines has also been documented, though with far less frequency and epidemiologic relevancy than traditional vector-borne transmission. (World Health Organization, 2015).

1.2.1 Pathobiology of Chagas Disease

Infection with *T. cruzi* begins when a human is bitten by an infected triatomine. Rather than inoculating the infective parasites directly into the bite wound, however, the triatomine will actually defecate in the area surrounding the bite wound and release a flagellated *T. cruzi* trypomastigote, the infective stage of the parasite, in its feces (Figure 4). The bitten human or other mammalian host will often then proceed to rub or scratch at the bite wound, driving these infective metacyclic trypomastigotes into the subcutaneous tissue. In some cases, the bitten individual will rub or scratch the bite wound and proceed to touch their eyes, allowing the

parasite to gain entry to the body through the conjunctiva or ocular membrane. Once inside the host, these metacyclic trypomastigotes can directly penetrate a number of different host cell types, and once inside a host cell will lose their flagellum and transform into the amastigote stage (Figure 4). These intracellular amastigotes will proceed to divide within the host cell, eventually transforming into trypomastigotes that burst from the host cell and enter the host bloodstream. Once in the bloodstream trypomastigotes can travel to and infect distant tissues, often showing a proclivity for myocardiocytes and cells of the hollow digestive organs. In these tissues the parasite will transform back into the amastigote where it can then replicate or lay dormant for many years, and may eventually lead to symptomatic chronic Chagas disease (Despommier et al, 2000) (Figure 5).

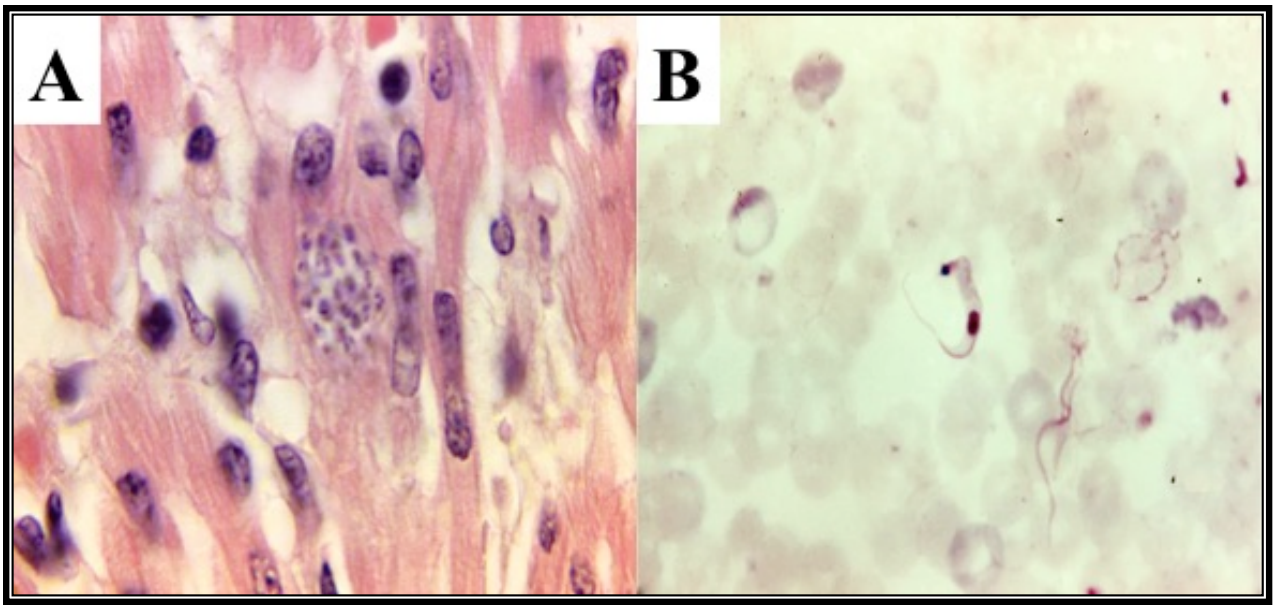

Figure 4: *Trypanosoma cruzi* parasites under light microscopy. (A) Cluster of intracellular amastigotes visible in central myocardiocyte. (B) Trypomastigote in peripheral blood smear. Images adopted from CDC Public Health Image Library

It has been observed that different strains of *T. cruzi* vary in their tropism for different tissues. Studies in mice have identified some *T. cruzi* strains with a proclivity for spleen, liver, or

bone marrow, some with a proclivity for myocardiocytes, and others that preferentially infect the smooth muscle of hollow digestive organs (Melo and Brener, 1978). The various strains or discrete typing units of *T. cruzi* that have been identified are also known to have somewhat varying degrees of tissue tropism that ultimately may affect the clinical manifestations of chronic Chagas disease in those infected. For example, TcI (a discrete typing unit found throughout the Americas and into Mexico and the United States) has been shown to be more prone to cause cardiac manifestations than some of the other *T. cruzi* discrete typing units, however gastrointestinal complications caused by this parasite can still be observed (Espinoza et al, 2011).

The "kissing bug" vector becomes infected when it feeds on an acutely infected mammalian host and ingests circulating trypomastigotes from the host's bloodstream. Within the triatomine vector, the newly ingested trypomastigotes will transform into epimastigotes in the vector's midgut and replicate via binary fission. Eventually, the epimastigotes will migrate to the vector's hindgut and rectum where they transform back into the infectious stage, the metacyclic trypomastigote that can now be transmitted to an uninfected mammalian host following a blood meal (Rassi Jr et al, 2010; Despommier et al, 2000) (Figure 5).

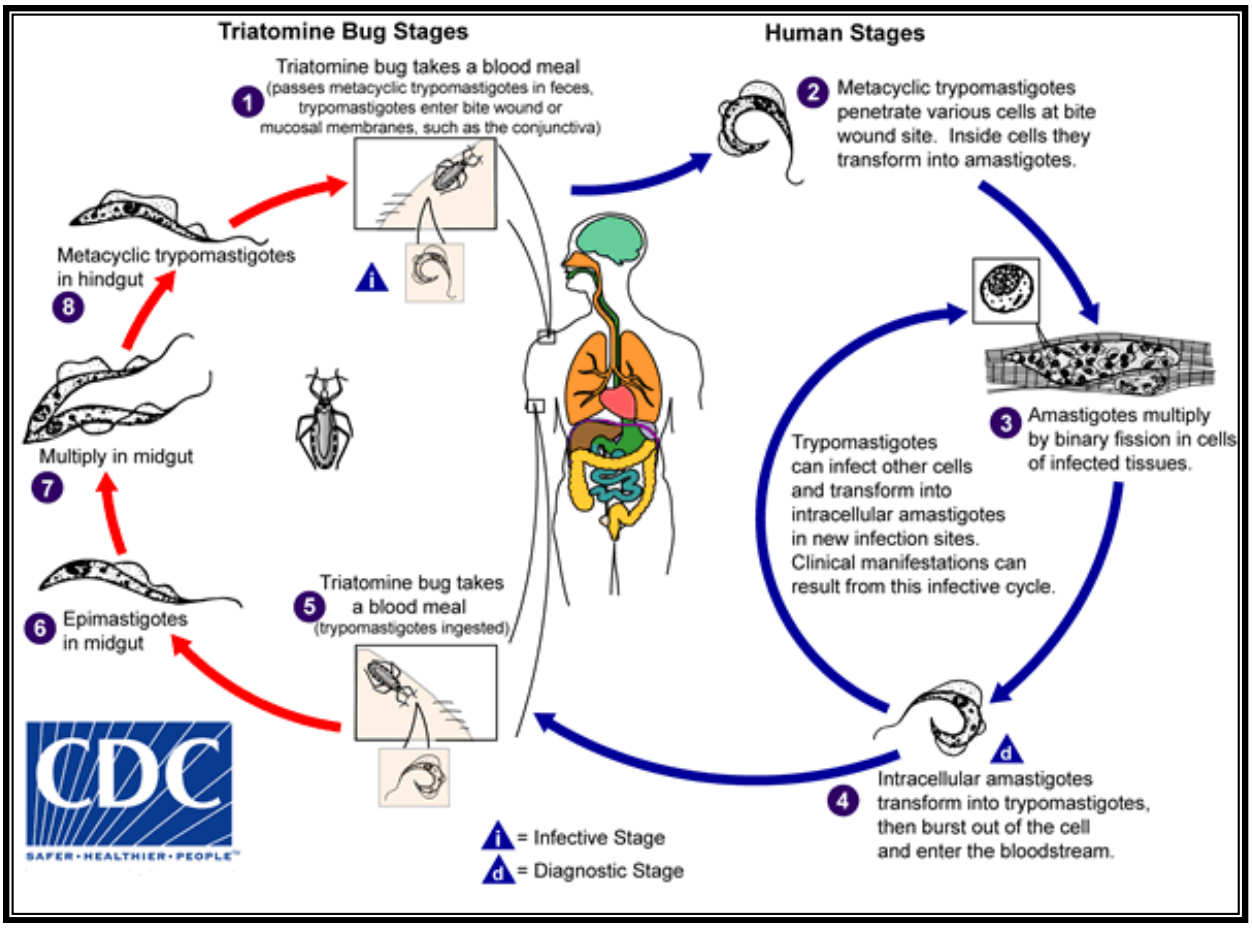

Figure 5: Lifecycle of *Trypanosoma cruzi* within the human host. Figure adopted from the U.S. Centers for Disease Control and Prevention

1.2.2 Biology of Triatomine Vectors

Trypanosoma cruzi is most often transmitted to human and other mammalian species thorough the bite of an infected triatomine "kissing bug" (Hemiptera: Reduvidae: Triatominae). Currently, there are 140 different species of triatomine that have been formally described and although all are likely competent carriers of *T. cruzi*, many species may have natural histories that do not make them epidemiologically relevant vectors of human Chagas disease (Schofield and Galvão, 2009). Indeed, many triatomine species differ greatly in their anthropophily, biting preferences, and time between feeding and defecation, all of which can affect their competency as Chagas disease vectors (Stevens et al, 2011). Nearly all triatomines are found in the New

World in tropical, subtropical and temperate regions extending in latitude from approximately 46º N to 46º S. Common names for triatomines differ greatly across the Americas and include the "kissing bug", the "chinche", the "chinchoro", and the "vinchuca", among numerous others (Schofield and Galvão, 2009).

Triatomines are considered true bugs, and all are six-legged and winged as adults with narrow heads and elongate bodies. Many also have an elaborate orange or yellow banding pattern surrounding their abdomen that can often serve as a distinguishing feature. Species in three genera, the *Triatoma*, the *Rhodnius*, and the *Panstrongylus*, have been implicated as important *T. cruzi* vectors. All of these vector species (both male and female) are secretive and nocturnal, and feed on the blood of a variety of vertebrate species (Stevens et al, 2011).

About 40 *Triatoma spp.* are found in North America, with eight species being shared between the United States and Mexico, 28 being found only in Mexico, and four being found exclusively in the United States (Stevens et al, 2011). In the United States, triatomines can be found from California to Florida, and as far north as Pennsylvania and New Jersey, though much of the species diversity is contained in the southern United States and within the state of Texas (Klotz et al, 2014). *Triatoma gerstaeckeri, Triatoma protracta, Triatoma indictiva, Triatoma rubida, Triatoma lecticularia, Triatoma neotomae, Triatoma recura,* and *Triatoma sanguisuga* are all species that have been identified in different regions throughout the state (Klotz et al, 2014). The overall *T. cruzi* infection rate among triatomines is also higher in Texas than in any other state, with infection rates of greater than 50% being reported by some studies (McPhatter et al, 2012; Kjos et al 2009).

1.2.3 Mammalian Reservoirs for Trypanosoma cruzi and American Trypanosomiasis in Canines

More than 150 species of domestic and sylvatic mammals have been found with evidence of *T. cruzi* infection worldwide, including domestic dogs, cats, guinea pigs, many different rodents, armadillos, and some marsupials (Rassi Jr et al, 2010). In many remote and uninhabited

settings, enzootic transmission cycles of the disease are restricted to rodent species, primates, opossums, armadillos, and other sylvatic wild mammals with only incidental infection of domestic animals or humans reported. In peri-domestic and domestic settings, however, infection is much more common in domestic dogs, cats, some synanthropic rodent species, and as a consequence, humans (Gürtler and Cardinal, 2015).

In the United States, *T. cruzi* has been detected in raccoons, skunks, opossums, and a wide variety of rodent species (Charles et al, 2013; Bern et al, 2011). A recent seroprevalence study conducted across Texas found an 8.8% seropositivity among stray shelter dogs, with a 6.9% seropositivity being found in shelter dogs in the El Paso region (Tenney et al, 2014).

Clinical infection in domestic dogs is somewhat similar to that in humans, with an initial acute phase and long indeterminate or asymptomatic phase. Mortality can be especially high in dogs less than one year old, often due to various cardiac complications. Many dogs exhibit a high parasitemia a few days following initial infection and lasting up to a month, making them potentially effective reservoirs of the disease in some settings and capable of transmitting the infection back to triatomines. Differences in the clinical outcome and potential infectivity of infected dogs vary greatly by age, breed, nutritional status, and the *T. cruzi* strain or discrete typing unit causing infection (Gürtler and Cardinal, 2015; Kjos et al, 2008; Bern et al, 2011).

1.2.4 Diagnosis and Treatment of Chagas Disease

Definitive diagnosis of acute Chagas disease often requires microscopic observation of circulating bloodstream trypomastigotes. Detection of circulating bloodstream parasites becomes much more difficult during the indeterminate or chronic phases of the disease, as most parasites exist in the host in the form of intracellular amastigotes (Despommier et al, 2000). Diagnosis of Chagas disease in the chronic phase usually requires the detection of IgG antibodies against *T. cruzi* (detected through either ELISA, immunofluorescence, or indirect haemagglutination). PCR diagnosis of chronic Chagas disease is not widely employed as a diagnostic tool due to recurring

problems with sensitivity, standardization, cross-contamination, and the need for more sophisticated laboratory infrastructure that is not available in many resource-limited settings (Rassi Jr et al, 2010; Bern et al, 2011).

Chagas disease is traditionally treated with the antiprotozoal drugs benznidazole and nifurtimox. If given to an individual soon after infection these drugs can be highly efficacious with cure rates approaching 100% (World Health Organazation, 2015). However, cure rates are significantly diminished in cases of chronic infection. Despite this, treatment of infected individual in the indeterminate or chronic phase of the disease is often recommended as it can halt the progression of the disease and limit development of more severe symptoms (World Health Organization, 2015; Despommier et al, 2000). No human vaccine against *T. cruzi* is currently available despite a great need for one. Modern vaccination techniques such as DNA vaccination, recombinant protein vaccination, and carbohydrate vaccination have shown some experimental success, raising the prospects that a vaccine will be available in future years (Camargo, 2009).

1.2.5 Epidemiology of Chagas Disease

Transmission of *T. cruzi* occurs almost exclusively within Latin America and the southern United States, however numerous infections have been reported in returning travelers or migrants in the northern United States, Canada, throughout Europe, and in nations of the western Pacific (Rassi Jr et al, 2010). An estimated 25 million people are at risk for infection with *T. cruzi* and an estimated 6 to 7 million individuals are believed to be actively infected worldwide, with the majority of infections being in Central America, South America, or Mexico (World Health Organization, 2015). Other estimates propose these figures to be much higher, with some studies estimating the overall prevalence to be as high as 17 million, the estimated incidence to be as high as 700,000-800,000 new cases annually, and the annual number of Chagas disease-related deaths to be as high as 45,000 (Moncayo and Silveira, 2009).

Across the Americas, *T. cruzi* parasites can be subdivided into six different discrete typing units referred to as TcI through TcVI. Each different discrete typing unit varies in its geographic distribution across the Americas, in its preferred mammalian hosts and triatomine vectors, in the ecological environment in which it is found, and in the clinical Chagas disease symptoms it causes in humans (Zingales et al, 2012) (Figure 6). Two discrete typing units are known to be endemic to the southern United States, TcI and TcIV, where both appear to be primarily infecting sylvatic mammal species with only incidental infection of domestic animals or humans being reported (Zingales et al, 2010; Charles et al, 2013).

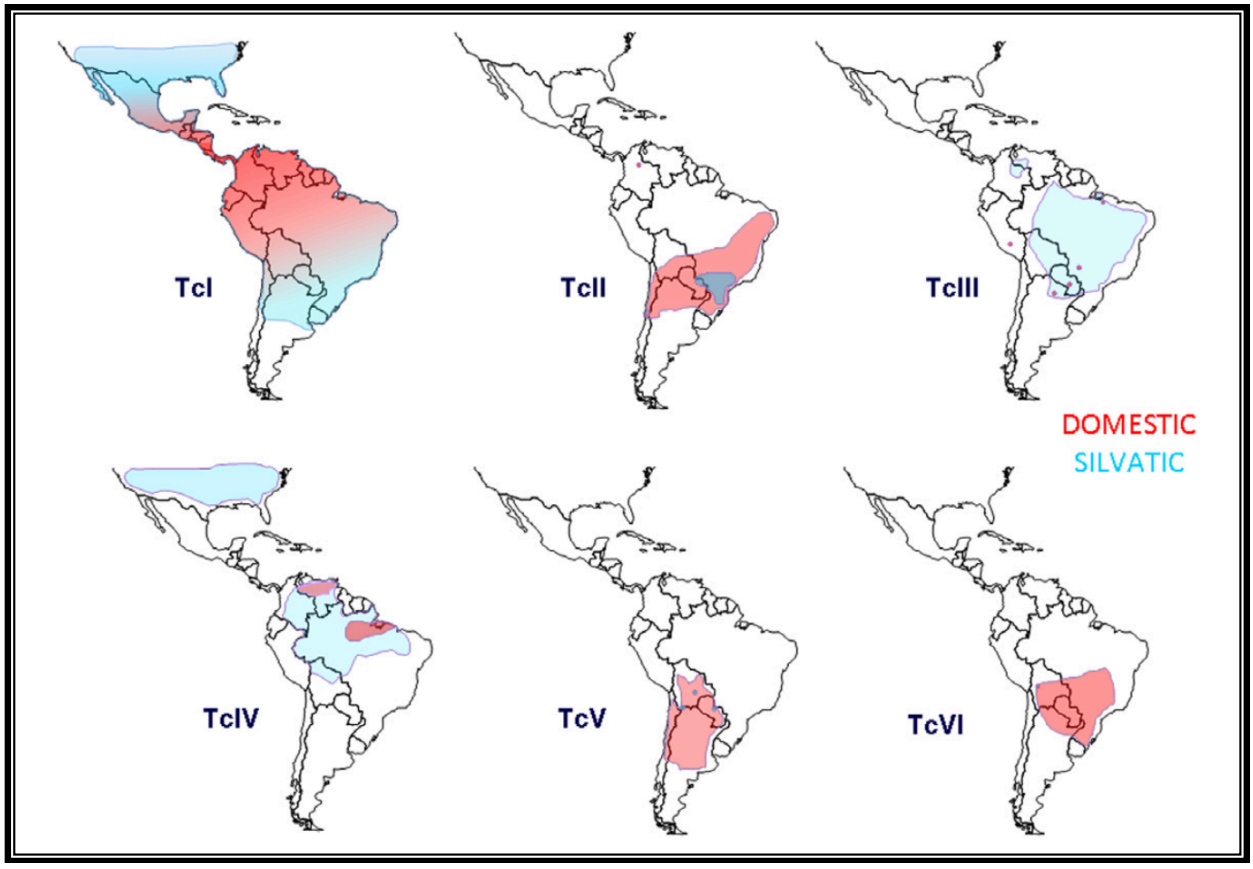

Figure 6: Geographic distribution of *Trypanosoma cruzi* by discrete typing unit. Figure adopted from Zingales et al, 2012

In South and Central America, the Chagas disease burden is heaviest in the nations of Brazil, Argentina, Bolivia, Colombia, Venezuela and Ecuador. In Brazil and Argentina alone, an infection prevalence of 1.9 million and 1.6 million has been estimated, respectively (Bern et al, 2011). In Mexico, Chagas disease has an estimated annual incidence of approximately 70,000 cases and seroprevalance studies have proposed an estimated overall infection prevalence of about 1 or 2 million people (Carabarin-Lima et al, 2013; Hotez et al, 2013). Estimates of *T. cruzi* infection prevalence in the United States vary significantly and to date no estimates have been able to distinguish between imported and autochthonous cases. The highest estimates of Chagas disease prevalence in the United States report an estimated 1 million infections, with Texas alone having an estimated 267,000 infections (Hanford et al, 2007). More conservative estimates place the overall number of infected individuals in the United States closer to 300,000 (Bern and Montgomery, 2009; Sarkar et al, 2010). Again, these estimates have the limitation in that they do not distinguish between travel-associated and autochthonous cases, and an estimate of the number of autochthonous Chagas disease cases in the United States or in Texas has not yet been made available.

1.2.6 Emergence of Chagas Disease in the United States

The first case of autochthonous Chagas disease in the United States was described along the Texas-Mexico border in 1955. However, the disease has likely been present along the Rio Grande Valley for many thousands of years (Bern et al, 2011; Hotez et al, 2013). Despite conservative estimates of approximately 300,000 cases of Chagas disease currently present in the United States, the number of confirmed autochthonous cases has remained quite low. By 2011, only seven confirmed vector-borne autochthonous cases had been described, with four from the state of Texas, and the remaining three from California, Tennessee, and Louisiana (Bern et al, 2011). In 2014, a cluster of five new autochthonous and likely vector-borne cases was reported from southeast Texas. Most of the cases were likely infected near their residencies, although two

may have been infected during recreational outdoor activities (Garcia et al, 2014). Even though there have not been large numbers of autochthonous case reports of Chagas disease in the United States, there are likely a very large number of cases going unrecognized and undiagnosed. Much of the reason for this underdiagnosis is due to the obscure nature of many of the symptoms of chronic Chagas disease. Many cases of idiopathic heart disease and congestive heart failure in the United States may in fact be due to chronic Chagas disease, and increasing awareness among the American physicians community to consider Chagas disease in their differential diagnosis will be an important public health challenge in upcoming years (Hanford et al, 2007).

Ecological niche modeling examining future habitat suitability for *T. cruzi* vectors and reservoirs under climate change predictive models has identified areas of Texas below 30º N latitude of being at significant risk for local transmission (Sarkar et al, 2010). Other climate change risk models for Chagas disease predict a widening of current triatomine species distributions and an increased risk for Chagas disease across the southern and central United States (Lambert et al, 2008). In any case, it is likely that the number of autochthonous cases in the United States will continue to go underreported, even if transmission of the parasite expands and increases in the next years and decades.

Chapter 2: Study Rationale, Aims, and Research Questions

2.1 Study Rationale and Aims

Stray canines have been implicated as potential reservoirs of both *Leishmania* and *T. cruzi* across South America, Central America, and into Mexico. Numerous details regarding the enzootic transmission of these pathogens in the southern United States remain either uninvestigated or unresolved, including the role that domestic dogs may be playing in enzootic transmission cycles. In Texas, all human cases of cutaneous leishmaniasis have been linked to the rodent reservoir *Neotoma micropus*, however, *Leishmania* has also been identified in numerous other mammal species (including domestic dogs) in south and central Texas and other parts of the southern United States. Although no human cases have been reported in west Texas or the El Paso area, it is acknowledged that phlebotomine and triatomine vectors and competent reservoirs are likely to inhabit the region but whether they are actively transmitting these protozoal pathogens is still unknown. Given the behaviors of many stray canines, dogs may have the potential to move these pathogens from sylvatic cycles in rural or undeveloped areas into more domestic and peri-urban settings where the potential for transmission to humans is greater. The discovery of either *Leishmania* or *T. cruzi* in stray canines in El Paso County would represent an emerging public health concern to people living in the region and could be evidence of novel transmission of these pathogens in the area.

A pilot study conducted by Mariscal in 2013 suggested that enzootic transmission of *T. cruzi* and *L. mexicana* is occurring in the El Paso region. This study examined 96 randomly selected stray canines from El Paso County and found a 10% overall *T. cruzi* infection rate. This same pilot study also found *Leishmania* present in two stray canines from this sample as well as in numerous rodent species collected in Mason County, Texas (Mariscal, 2013). It was unclear as to whether these infection were due to *Leishmania mexicana* or another *Leishmania spp*. These results, however, suggest that there is a significant likelihood of finding further evidence for transmission of pathogens among stray dogs in the El Paso region.

The overall aim of this study is to determine whether evidence of *Leishmania* or *T. cruzi* infection can be found among a group of stray dogs collected from across El Paso County, Texas. PCR-amplification and direct sequencing or PCR-positive amplicons will allow for the determination of infecting *Leishmania* species and *T. cruzi* discrete typing unit among infected dogs, providing important epidemiologic information on these pathogens in the region that is currently unknown. Ultimately these results will allow for a better understanding of the enzootic transmission of *Leishmania* and *T. cruzi* in the El Paso area, the Chihuahuan desert, and along the entire Rio Grande Valley and U.S.-Mexico border. This study may also provide valuable information as to whether canines in the region have the potential to act as competent reservoirs for these pathogens and whether there is significant risk posed to people living in urban, peri-urban, or rural areas of the region.

2.2 Research Questions

This study is an initial attempt to determine whether enzootic transmission of *Leishmania spp*. and *Trypanosoma cruzi* is occurring among stray canines within El Paso County, Texas. The frequency of stray dogs infected with either *Leishmania* or *T. cruzi* in this sample will be determined through the PCR-amplification of one *Leishmania*-specific and two *T. cruzi*-specific gene segments and followed by direct sequencing. It is assumed that a relatively low infection prevalence of *L. mexicana* among canine skin biopsies will be detected. There is a small possibility that *L. infantum* will be detected in spleen biopsies from this group of canines. The discovery of any other *Leishmania spp*. is not expected. A very low infection prevalence of *L. mexicana* might suggest that dogs are only being incidentally infected with *L. mexicana* and are playing a relatively minor role in the overall transmission dynamics of the parasite, however, further studies will be needed to assess their overall competency as potential reservoirs of this disease. It is assumed that a higher infection prevalence will be detected in heart or skin biopsies of these stray canines with respect to *T. cruzi* relative to *Leishmania*. This finding would be consistent with other seroprevalance studies conducted across Texas and throughout the southern United States. Sequencing of positively identified PCR samples will allow for characterization of *T. cruzi* discrete typing unit. Zingales et al. 2012 identified discrete typing unit TcI and TcIV as being potentially present in sylvatic cycles in the area surrounding El Paso County and it is possible that either could be identified in this study. Again, further studies in this area will be necessary to determine whether these canines are only being incidentally infected in this region or whether they are acting as significant reservoirs in the transmission of *T. cruzi*.

In addition to the reporting of PCR-positive infection frequencies for these pathogens, all PCR-positive samples for both *Leishmania spp*. and for *T. cruzi* will be mapped based on the GPS coordinates of where each stray dog was initially found. Mapping of any PCR-positive samples will allow for an initial determination of whether infection in these dogs is occurring in a particular region of the county or within a particular ecological setting. These results could potentially help direct any future studies attempting to study the transmission of these pathogens

in the region. Furthermore, as canines were sampled regularly for an nearly an entire year, this study could also assess if there is any temporal pattern of infectivity among these dogs. A cluster of positive samples (especially if identified in any juvenile canines) being discovered in adjacent months could indicate that infection is occurring primarily within a particular season and will elucidate an important temporal aspect of the transmission and epidemiology of these zoonotic infections.

Direct sequencing of any PCR-positive amplicons for *Leishmania* or *T. cruzi* will also allow for the determination of epidemiologically valuable information on the species of *Leishmania* and the discrete typing unit of *T. cruzi* causing infection in these stray dogs. Alignment of these sequences with known sequences from reference strains of *Leishmania* and *T. cruzi* will allow for a much more detailed characterization of any infecting protozoa in these biopsies and can be taken as more definitive evidence of infection.

Chapter 3: Methods

3.1 Study Design and Stray Canine Tissue Sampling

This study attempted to identify *Leishmania spp*. and *Trypanosoma cruzi* among a sample of stray canines (*Canis familiaris*) from El Paso County, Texas. All canines in the sample were captured within El Paso County by trained Animal Control Officers at the City of El Paso Animal Services. Only animals slated by the City of El Paso Animal Services for euthanasia were included in this study. No preference was given to dogs of particular age, sex, or breed. Sample collection was conducted regularly beginning in July 2014 and continued until May 2015. Dogs surrendered by their owners for euthanasia were excluded from study. All euthanasia of dogs in this study was conducted by a trained animal control officer at the City of El Paso Animal Services and according to all local and state regulations. Institutional Animal Care and Use Committee (IACUC) approval was not required, as none of the animals surveyed were euthanized solely for the purpose of this study but rather were euthanized as part of routine animal control activities conducted by the City of El Paso Animal Services. IBC approval for this project was granted by The University of Texas at El Paso Institutional Biosafety Committee (IRBNet Identification Number: 807121-1).

In all dogs included in the study, information on their approximate age, sex, and breed was recorded. All dogs over the age of one year were labeled as adults and dogs less than one year of age had their age estimated in months based on dentition. The breed of each dog was recorded as it was listed by the Animal Control Officer who initially collected the animal. The approximate Geographical Positioning System (GPS) coordinates of where each animal was collected by the City of El Paso Animal Services was also calculated in decimal degrees using the program GPS Visualizer (http://www.gpsvisualizer.com/geocode).

Biopsies of skin, spleen, and heart tissue were collected from each animal following euthanasia. Each dog was visually examined for the presence of ulcerative or non-ulcerative skin lesions prior to skin biopsy collection and if a lesion was identified, the biopsy was collected

around the afflicted site. Each canine's spleen and heart was also visually examined for lesions or discoloration and, if present, biopsies were collected around the afflicted site. Spleen biopsies were collected around the margin of the organ and heart biopsies were collected at or near the apex.

Immediately following biopsy collection, tissue samples were stored in a cooler on ice and were transported to the laboratory for preservation for downstream analysis. To preserve skin, spleen, and heart tissue samples, each sample was placed in a 10 mL tube and covered with a DMSO/NaCl/EDTA solution (pH 8.0). This solution contained 20% dimethyl sulfoxide (DMSO) and 250 mM ethylenediaminetetraacetic acid (EDTA) and was saturated with sodium chloride (NaCl). Preservation of the tissue samples in this manner allowed for their storage at room temperature for extended periods of time and without sacrificing the quality or yield of extracted DNA (Sambrook and Russell, 2001).

3.2 Data Collection

For each sample, approximately 100 mg of tissue (preserved in DMSO/NaCl/EDTA solution) was excised from the preserved biopsy sample for genomic DNA extraction. Excised tissues were placed in a 15 mL tube containing 2 mL SNET (1% w/v sodium dodecyl sulfate; 400 mM NaCl; 5 mM EDTA; 20 mM Tris-Cl) and proteinase K at a concentration of 0.5 mg/mL (Sambrook and Russell, 2001). Each tube was incubated at 55º C and shaken at approximately 300 rpm overnight in order to break apart the tissue and suspend genomic DNA in the solution. After overnight incubation, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 mixture) was added to each tube. Tubes were then placed on a rocking platform at room temperature for 30 minutes and centrifuged for 15 minutes at approximately 2600 rpm to separate aqueous and organic phases. Next, 700 µl of the upper aqueous phase was removed and placed in a 1.5 mL tube and an equal volume of cold isopropanol was added to the tube and briefly mixed. Each tube was then centrifuged for 20 minutes at 4 ºC in a tabletop centrifuge at 13,000 rpm to allow for DNA precipitation. The upper layer of isopropanol was removed and the pellet containing extracted genomic DNA was rinsed with cold 70% ethanol and allowed to dry for approximately 30 minutes. The pellet was then resuspended by adding 30 µl of nuclease free water to the tube and briefly vortexed to ensure suspension of genomic DNA. The concentration of the extracted genomic DNA was determined using a NanoDrop® spectrophotometer.

A working solution of genomic DNA from each tissue sample was made by diluting the extracted DNA in nuclease free water to a final concentration of approximately100 ng/µl. All subsequent PCR reactions contained 100 ng of extracted genomic DNA (or parasite DNA for positive controls), 22 µl 1x PCR Master Mix, and 1 µl of both forward and reverse primers for a final reaction volume of 25 µl.

Assessment of the quality of extracted genomic DNA from each tissue sample was done by PCR-amplifying a 227 base pair segment of the mammalian gene encoding interphotoreceptor retinoid-binding protein (IRBP) (Table3.1). Thermocycler conditions for IRBP amplification involved an initial denaturation step at 94º C for 4 minutes, followed by 35 cycles at 94º C for 30

seconds, 57º C for 30 seconds, and 72º C for 1 minute and concluded with a final extension step at 72º C for 5 minutes (Ferreira et al, 2010) (Table3.2).

Detection of *Leishmania spp*. was done through the PCR-amplification of a 320 base pair non-coding segment of *Leishmania*-specific DNA known as the internal transcribed spacer (ITS1) and using the primer pair LITSR and L5.8S (Table 3.1). Thermocycler conditions for ITS1 amplification involved an initial denaturation step at 95º C for 2 minutes, 35 cycles at 95º C for 20 seconds, 53º C for 30 seconds, and 72º C for 1 minute, and concluded with a final extension step at 72º C for 6 minutes (El Tai et al, 2000) (Table 3.2).

Trypanosoma cruzi was detected through the amplification of two gene segments, the first of which was a 188 base pair repetitive nuclear sequence that used the primers Tcz1 and Tcz2 (Table 3.1). Thermocycler conditions for the amplification of this sequence included an initial denaturation step of 94º C for 5 minutes, followed by 35 cycles at 94º C for 20 seconds, 57º C for 10 seconds, and 72º C for 30 seconds, and concluded with a final extension step at 72º C for 7 minutes (Herwaldt et al, 2000; McPhatter et al, 2012) (Table 3.2). The other gene segment targeting *T. cruzi* was a 330 base pair segment of kinetoplast minicircle DNA that used the primers 121 and 122 (Table 3.1). Amplification of this segment included an initial denaturation step of 94ºC for 3 minutes, followed by 35 cycles at 94ºC for 30 seconds, 57ºC for 30 seconds, and 72ºC for 30 seconds, and concluded with a final elongation step at 72ºC for 7 minutes (Fitzwater et al, 2008) (Table 3.2).

Table 1: PCR primer pairs and gene segments used for the detection of *Leishmania spp*. and *Trypanosoma cruzi* in stray canine tissue biopsies

Gene Segment	Primer Name	Fragment Size	Purpose	5' → 3' Sequence
IRBP	IRBP - FW	227 bp	Internal control	TCCAACACCACCACTGAGATCTGGAC
	IRBP - RV			GTGAGGAAGAAATCGGACTGGCC
ITS 1	LITSR	320 bp	*Leishmania spp*.	CTGGATCATTTTCCGATG
	L5.8S			TGATACCACTTATCGCACTT
Tcz1/Tcz2	Tcz1	188 bp	*T. cruzi*	CGAGCTCTTGCCCACACGGGTGCT
	Tcz2			CCTCCAAGCAGCGGATAGTTCAGG
121/122	121	330 bp	*T. cruzi*	AAATAATGTACGGGGGAGATGCATGA
	122			GGTTCGATTGGGGTTGGTGTAATATA

Positive controls were run for ITS1, Tcz1/Tcz2, and 121/122 PCRs using genomic DNA template from *Leishmania mexicana* (strain M379) and *T. cruzi* (γ strain). Negative controls in which no template DNA is included were also run for all ITS1, Tcz1/Tcz2, and 121/122 PCRs.

Table 2: Thermocycler specifications for the amplification of gene segments used for the detection of *Leishmania spp*. and *Trypanosoma cruzi* (*Ferreria et al, 2010; †El Tai et al, 2000; **McPhatter et al, 2012; ††Fitzwater et al, 2008)

Gene Segment	Initial Denaturation		Denaturation (x35 cycles)		Annealing (x35 cycles)		Elongation (x35 cycles)		Final Elongation	
IRBP*	94°C	4 min	94°C	30 sec	57°C	30 sec	72°C	1 min	72°C	5 min
ITS 1†	95°C	2 min	95°C	20 sec	53°C	30 sec	72°C	1 min	72°C	6 min
Tcz1/Tcz2**	94°C	5 min	94°C	20 sec	57°C	10 sec	72°C	30 sec	72°C	7 min
121/122††	94°C	3 min	94°C	30 sec	57°C	30 sec	72°C	30 sec	72°C	7 min

3.3 Analysis of PCR Results and Sequencing Data

All PCR products were visualized under ultraviolet light on ethidium bromide-stained agarose gels (containing 1.8% agarose and run in TAE buffer at 100 volts for 20 minutes). Each gel was made with two combs containing 15 wells each, allowing for the simultaneous analysis of two canine samples per gel. Gels for each canine sample contained a well with 100 bp or 1 kb DNA ladder (New England Biolabs®) in order to estimate the size in base pairs of any DNA fragments obtained through PCR. Gels for each canine sample also contained IRBP, ITS1, and Tcz1/Tcz2 PCR products for each spleen, heart, and skin biopsy from the given canine. Positive and negative controls were also included from both the ITS1 and Tcz1/Tcz2 PCR. 121/122 PCR products were run on separate gels including the corresponding positive and negative controls. Each canine's skin, spleen, or heart samples were considered PCR-positive if a DNA fragment is visually observed with the same approximate size and migration pattern as that of the respective positive control.

The frequencies of PCR-positive biopsies and PCR-positivity percentages were calculated. Positive samples were also examined according to canine sex, age, breed group, and lesion status in order to make a preliminary determination of potential trends among infected dogs. The date in which any positive sample is identified was also examined in an attempt to determine whether dogs are being infected during a particular time of year. Finally, PCR-positive samples were mapped based on the GPS coordinates of where each dog was initially collected in order to determine whether any spatial or ecological pattern exists with respect to any PCR-positive samples. Maps were created using the computer program Epi Info™ (Version 7.1.4) developed by the U.S. Centers for Disease Control and Prevention.

Samples identified as PCR-positive after visualization through agarose gels electrophoresis were randomly selected for bidirectional sequencing. Sequencing services were provided by the Genomic Analysis Core Facility at the Department of Biological Sciences at The University of Texas at El Paso. Sequenced PCR products were analyzed using the Basic Local Alignment Search Tool (BLAST®) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the program

MView (http://www.ebi.ac.uk/Tools/msa/mview/) was used to create a multiple sequence alignment comparing sequenced PCR products.

Chapter 4: Results

4.1 Stray Canine Sample Description

Tissue biopsies were collected from stray canines at City of El Paso Animal Services between July 2, 2014 and May 27, 2015. During this period, biopsies were collected on 33 separate days with approximately five canines being sampled on each collection date. In total, skin, spleen, and heart biopsies from 159 stray dogs were obtained. Data from all dogs were recorded into a spreadsheet, and dogs were cataloged according to the order in which their biopsies were taken. For convenience, all dogs surveyed were given the moniker "K" followed by a number, with the first dog surveyed being cataloged as "K1", the second dog as "K2", and so forth for all 159 dogs for which tissues were collected.

A nearly even distribution of male and female dogs was found with 81 being female and 78 being male, representing 50.9% and 49.1% of the sample, respectively (Table 3). Based on dentition patterns, the majority of dogs in the sample were classified as adults (i.e., greater than one year of age) with only 14.5% of dogs in the sample being less than one year of age. An age range of 2 months to 10 months was found among the 23 dogs in the group considered to be less than one year of age. Skin lesions were noted on 41 of the dogs surveyed with lesions being distributed across the animals' bodies and often on more exposed areas. Biopsies of both ulcerative and non-ulcerative lesions were collected if present. Lesions, discoloration or other abnormalities of the spleen were noted in 43 dogs and visible lesions of the myocardium were noted in 30 dogs (Table 3).

Each stray dog was also categorized into one of seven different breed groups based on the standards of the American Kennel Club (AKC), a determination that was made based solely on each animal's external appearance or by the breed noted by the Animal Control Officer that initially collected it. Any dog that was clearly of mixed breed was included in the breed group that was considered most dominant based on external appearance. In all, 31 dogs, representing 19.5% of the sample, were included in the Herding group, which included the breeds of German

shepherd, Australian shepherd, and border collie, among others. Two dogs were included in the Hound group, both of which were recorded as beagles. A total of 17 dogs were included in the Non-sporting group, the majority of which were poodles. 11 dogs were included in the Sporting group, which included the breeds Labrador retriever, cocker spaniel, and golden retriever. The largest breed group in the sample was the Terrier group, which included 57 dogs and represented 35.8% of the sample. Breeds included in the Terrier group included pit-bull terriers and Russell terriers, among other terrier mixes. 33 dogs were included in the Toy group, representing 20.8% of the sample, the majority of which were considered Chihuahuas or Chihuahua mixes. Finally, 8 dogs were included in the Working group, which represented 5.0% of the sample and included the breeds St. Bernard, boxer, and husky (Table 3).

Table 3: El Paso County stray canine sample characteristics (*Age was estimated based on dentition characteristics; †American Kennel Club breed group determination made based on external appearance)

Sample Characteristic	**No. (%) (n=159)**
Sex (Female)	81 (50.9)
Age (< 1 year old)*	23 (14.5)
Skin lesions	41 (25.8)
Spleen lesions and/or abnormalities	43 (27.0)
Myocardial lesions	30 (18.9)
AKC Breed Group† (Herding)	31 (19.5)
AKC Breed Group† (Hound)	2 (1.3)
AKC Breed Group† (Non-sporting)	17 (10.7)
AKC Breed Group† (Sporting)	11 (6.9)
AKC Breed Group† (Terrier)	57 (35.8)
AKC Breed Group† (Toy)	33 (20.8)
AKC Breed Group† (Working)	8 (5.0)

Stray canines were collected from across El Paso County with the large majority coming from suburban, peri-urban, and agricultural areas from the western portion of the county near the city of El Paso and its surrounding communities. The GPS coordinates at which each stray dog was captured were recorded, and a map of all 159 capture locations was created using Epi Info™ 7.1.4 (Figure 7). Clusters of capture locations with large numbers of stray canines are visible in the northeastern portion of the city of El Paso and along the agricultural regions to the southeast of El Paso and following the Rio Grande. Fewer dogs comparatively came from the area west of the Franklin Mountains and from urban, more densely populated areas of the city.

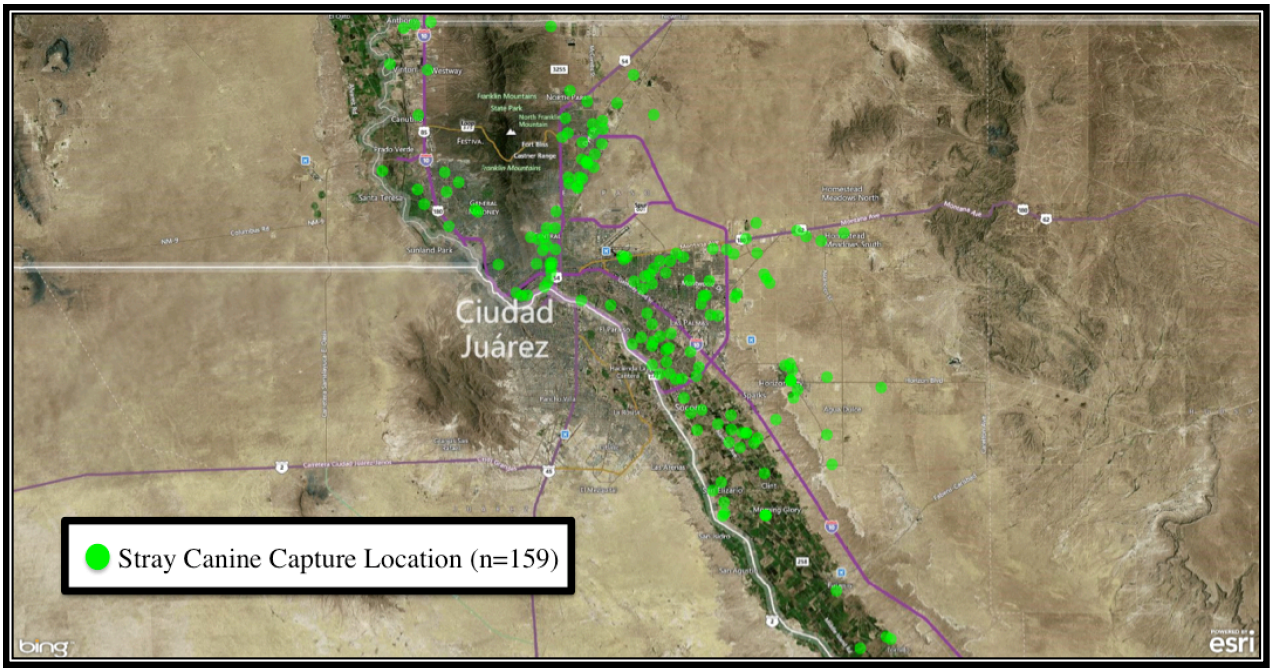

Figure 7: Distribution of stray canine capture locations across El Paso County (Map prepared with Epi Info™ 7.1.4)

4.2 PCR Detection of Leishmania spp. and Trypanosoma cruzi

PCR amplification of the IRBP segment was positive for the majority of skin, spleen and heart biopsies when viewed under agarose gel electrophoresis and indicated that preceding extraction of genomic DNA from tissue biopsies was largely successful. IRBP amplification of skin, spleen, or heart biopsies was only unsuccessful in three of the 159 stray dogs. These three samples were excluded from subsequent analyses and a final sample size of 156 was thus used for all remaining frequency calculations.

Agarose gel electrophoresis was conducted for all samples with each gel including one canine's skin, spleen, and heart biopsy PCR products for the gene segments IRBP, ITS 1 and Tcz. In addition to PCR products for each sample, positive and negative controls were also included for ITS 1 and Tcz as well as a DNA ladder with fragments of known sizes. A representative example of an agarose gel that was considered negative for both *Leishmania* (ITS 1) and *T. cruzi* (Tcz) is shown in Figure 8.

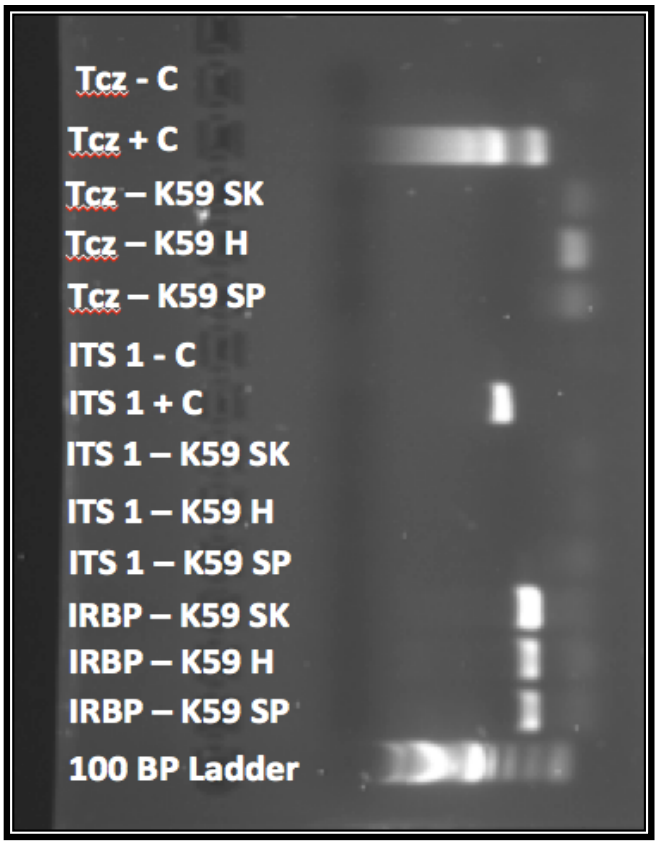

Figure 8: Representative agarose gel with negative results for both ITS 1 and Tcz PCRs (K59 represents the canine sample catalog number) (SP: Spleen; H: Heart; SK: Skin; + C: Positive Control; - C: Negative Control)

Of the 156 stray dogs for which IRBP amplification was successful, the ITS 1 region was amplified in 41, representing 26.3% of the total sample. Samples were considered positive if a clear fragment was observed that resembled the positive control in both size (320 bp) and migration pattern. Positive samples were identified in spleen, heart and skin biopsies, with skin and spleen biopsies being those that had the largest proportion of PCR-positive fragments. Figure 9 shows an agarose gel that was PCR-positive in the ITS 1 region in all three biopsy tissue types.

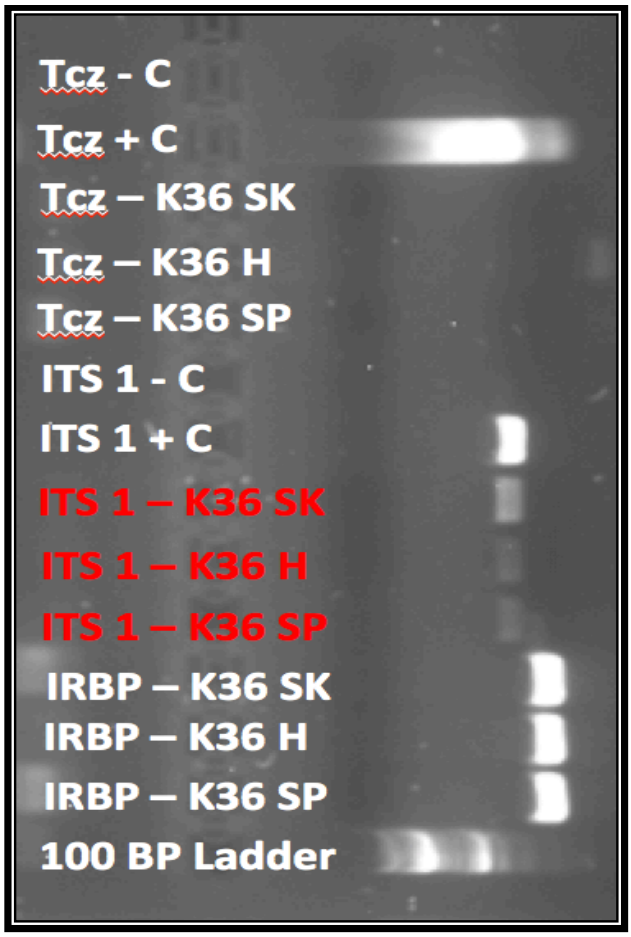

Figure 9: Agarose gel with positive results for *Leishmania spp*. in skin, spleen, and heart biopsy PCRs (PCR-positive biopsies indicated in red) (SP: Spleen; H: Heart; SK: Skin; + C: Positive Control; - C: Negative Control)

Amplification of 121/122 and Tcz gene segments was successful in a total of 21 stray canines for a final PCR-positivity frequency of 13.5%. Kinetoplast minicircle DNA from *T. cruzi* using the 121/122 primers was detected in heart biopsies from seven canine samples (Agarose gel not shown). Amplification of Tcz was primarily successful using genomic DNA from heart biopsies but was also detected in a smaller number of spleen biopsies. Figure 10 shows an agarose gel that was negative for the ITS 1 region but positive for the Tcz gene segment with a visible fragment of the expected size (188 bp) seen in the heart biopsy PCR product.

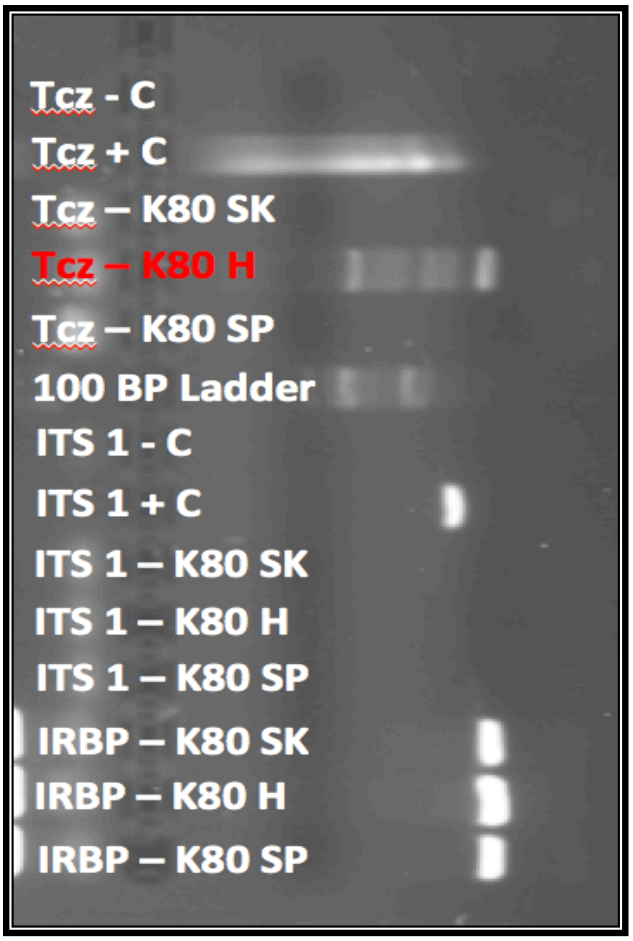

Figure 10: Agarose gel with positive result for *Trypanosoma cruzi* in heart biopsy PCR (PCR-positive biopsy indicated in red) (SP: Spleen; H: Heart; SK: Skin; + C: Positive Control; - C: Negative Control)

The demographic characteristics of the 41 stray canines that were found to be positive for *Leishmania spp*. closely resembled those of the overall sample. No clear sex difference existed among the positive samples with 53.7% being male dogs and 46.3% being female (Table 4). 29.3% of positive samples were taken from stray dogs that showed visible skin lesions at the time of biopsy collection, which was slightly higher than the 25.6% skin lesion prevalence in the total sample (Table 4). The prevalence of spleen lesions or abnormalities in the positive samples was also slightly higher than that of the overall sample with 29.3% of dogs showing evidence of such lesions in the positive group compared to 26.3% in the overall sample (Table 4). Positive

samples did not appear to cluster within dogs of a particular breed group, with the distribution in *Leishmania spp*. PCR-positive dogs closely resembling that of the overall sample (Table 4.) Of the 41 dogs testing positive for *Leishmania spp*., 28 (68%) were taken from dogs captured during the late summer and early fall months, from July to September 2014. Most of the remaining samples were detected in the winter months from November 2014 to February 2015.

Table 4: Comparison of demographic characteristics between stray canines identified as PCR-positive for *Leishmania spp*. and the overall sample tested

Demographic Characteristic	***Leishmania spp.* PCR-Positive No. (%) (n=41)**	**Total Sample No. (%) (n=156)**
Sex (Female)	19 (46.3)	79 (50.6)
Age (< 1 year old)	6 (14.6)	23 (14.7)
Skin lesions	12 (29.3)	40 (25.6)
Spleen lesions and/or abnormalities	12 (29.3)	41 (26.3)
Myocardial lesions	7 (17.0)	28 (17.9)
Herding AKC Breed Group	10 (24.5)	31 (19.8)
Hound AKC Breed Group	1 (2.4)	2 (1.3)
Non-sporting AKC Breed Group	5 (12.2)	16 (10.3)
Sporting AKC Breed Group	2 (4.9)	10 (6.4)
Terrier AKC Breed Group	13 (31.7)	56 (35.9)
Toy AKC Breed Group	7 (17.0)	33 (21.2)
Working AKC Breed Group	3 (7.3)	8 (5.1)

Dogs that tested positive for *Leishmania spp*. were distributed across El Paso County. The majority of positive samples were found in the western and northeast portions of the city of El Paso and along the Rio Grande in agricultural areas to the southeast of the city (Figure 11). Of note is that of the few stray canines that were captured west of the Franklin Mountains, about half tested positive for *Leishmania*, indicating that this may be a potential area of greater transmission.

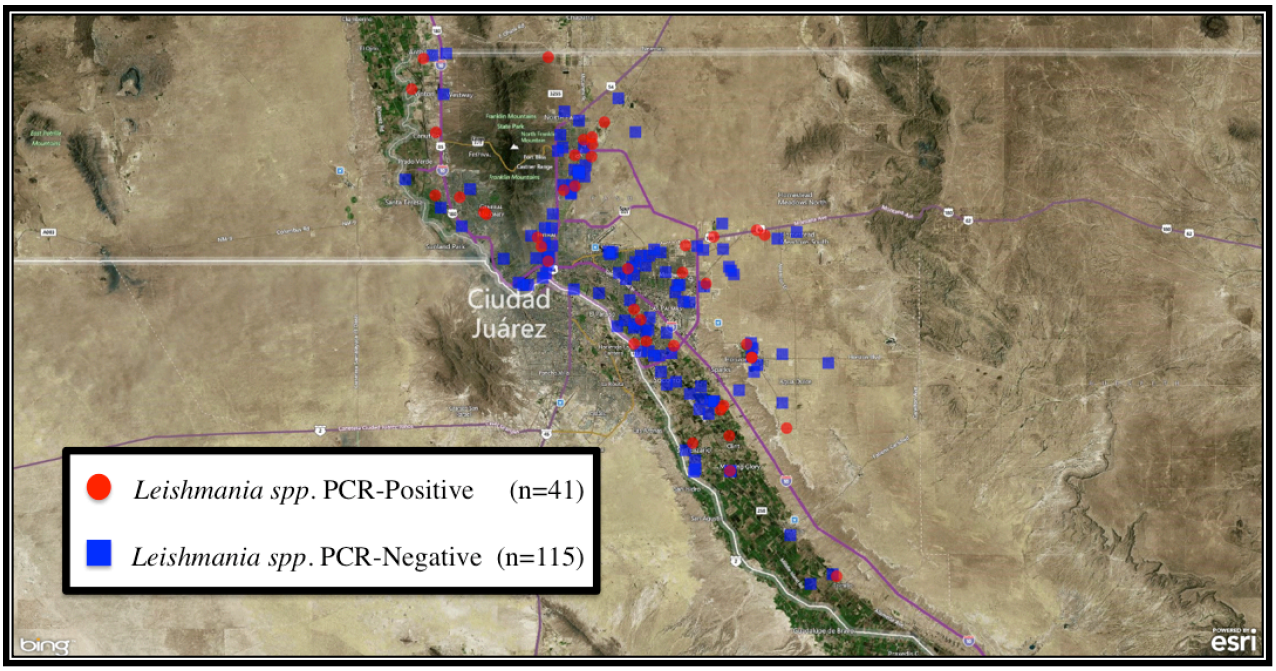

Figure 11: Geographic distribution of stray canines identified as PCR-positive and PCR-negative for *Leishmania spp*. (Map prepared with Epi Info™ 7.1.4)

Demographic characteristics of the 21 stray canine samples found to be PCR-positive for *T.cruzi* largely resembled those of the overall sample with some notable exceptions. No clear sex difference existed in the positive sample with 52.4% of positive dogs being female as compared to 50.6% of the overall sample (Table 5). No clear difference was also seen between the positive group and overall sample with respect to age with 14.3% of the positive dogs and 14.7% of the overall sample being less than one year of age (Table 5). An interesting difference was noted with respect to the proportion of myocardial lesions between the dogs with evidence of *T. cruzi* infection and the overall sample. In 42.9% of dogs testing positive for *T. cruzi,* visible lesions of the myocardium were observed, while only 17.9% of dogs in the overall sample showed such lesions (Table 5). In terms of breed groups, no obvious differences were observed between the positive group and overall sample, however there were slightly more dogs considered to be in the Terrier group and slightly fewer in the Toy group in the positive sample as compared to the overall sample (Table 5). The majority of PCR-positive *T. cruzi* samples were detected in stray

canines captured during the winter months between November 2014 and February 2015. 15 stray canines, representing 71% of the positive sample, were captured during these months.

Table 5: Comparison of demographic characteristics between stray canines identified as PCR-positive for *Trypanosoma cruzi* and the overall sample tested

Demographic Characteristic	***Trypanosoma cruzi* PCR-Positive No. (%) (n=21)**	**Total Sample No. (%) (n=156)**
Sex (Female)	11 (52.4)	79 (50.6)
Age (< 1 year old)	3 (14.3)	23 (14.7)
Skin lesions	2 (9.5)	40 (25.6)
Spleen lesions and/or abnormalities	5 (23.8)	41 (26.3)
Myocardial lesions	9 (42.9)	28 (17.9)
Herding AKC Breed Group	5 (23.7)	31 (19.8)
Hound AKC Breed Group	0 (0.0)	2 (1.3)
Non-sporting AKC Breed Group	2 (9.5)	16 (10.3)
Sporting AKC Breed Group	1 (4.8)	10 (6.4)
Terrier AKC Breed Group	9 (42.9)	56 (35.9)
Toy AKC Breed Group	3 (14.3)	33 (21.2)
Working AKC Breed Group	1 (4.8)	8 (5.1)

The distribution of stray dogs found to be PCR-positive for *T. cruzi* also indicated that positive dogs were captured in areas throughout El Paso County and were not isolated to a particular region or locale within the county. A small cluster of six positive cases was observed in the northeast portion of the city of El Paso that can be seen in Figure 12. The remaining cases were distributed in the western and eastern portions of the city of El Paso and in the region along the Rio Grande to the city's southeast (Figure 12).

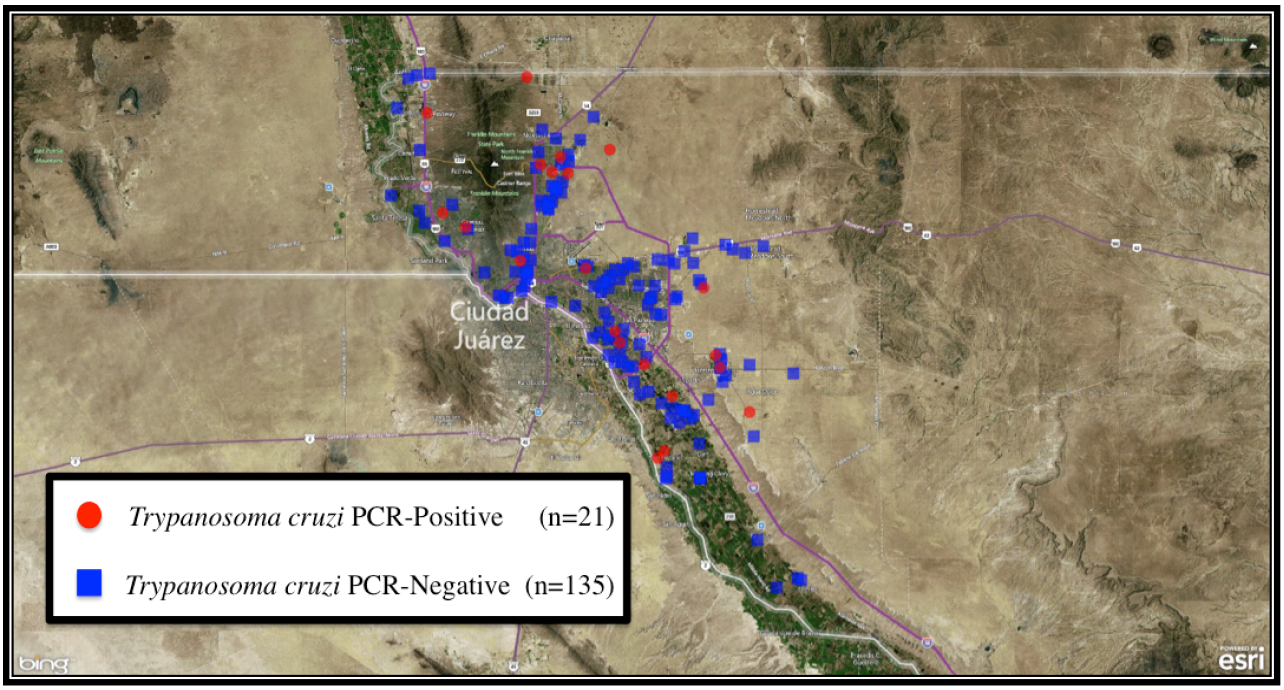

Figure 12: Geographic distribution of stray canines identified as PCR-positive and PCR-negative for *Trypanosoma cruzi* (Map prepared with Epi Info™ 7.1.4)

4.3 Sequencing Results

Of the 41 samples determined to be PCR-positive for *Leishmania spp*. following amplification of the ITS 1 region, 18 were randomly chosen for sequencing. BLAST® analysis of sequences from these samples indicated that all 18 clearly fit into the *Leishmania mexicana* clade with a large degree of sequence homology to the sequences of known *L. mexicana* reference strains. A multiple sequence alignment was created using the program MView in which the amount of ITS 1 sequence homology to one reference strain of *L. mexicana* (M379) can be seen (Figure 13). The ITS 1 sequence of *L. major* (Friedlin strain) was included as an outgroup to highlight sequence differences between these distantly related *Leishmania* species.

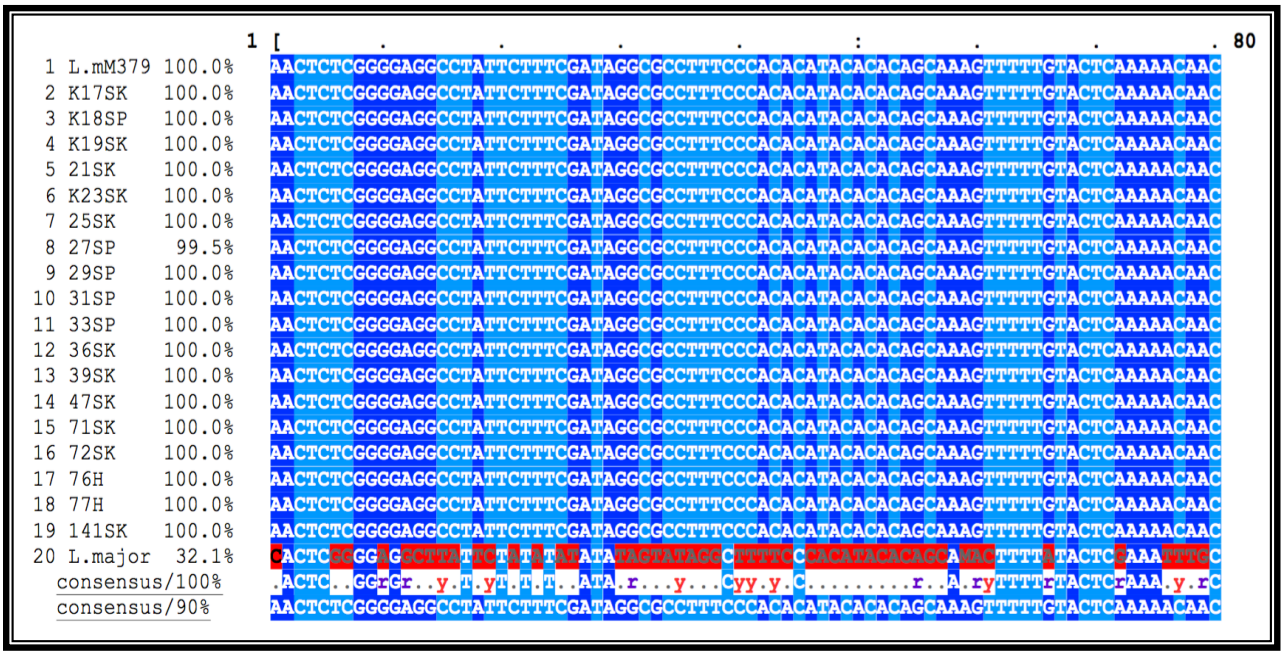

Figure 13: Abbreviated multiple sequence alignment showing *L. mexicana* (M379) reference strain (Row 1) and 18 samples identified as PCR-positive for *Leishmania spp*. (*L. major* Friedlin strain included as outgroup in Row 20)

Amplification of *T. cruzi* PCR products was attempted in six tissue biopsies that tested positive for the Tcz primer pair and in three biopsies that tested positive for the 121/122 primer pair. The sequenced PCR products that were obtained from these biopsies as well as those from

the *T. cruzi* γ strain control could not confirm the presence of *T. cruzi*. BLAST® analysis of all sequenced PCR products, including controls, was inconclusive and thus multiple sequence alignments were not created for these samples.

Chapter 5: Discussion

The results from this study suggest that transmission of both *L. mexicana* and *T. cruzi* is occurring across a widespread portion of El Paso County and in areas with significant human populations (i.e., the city of El Paso). This study found that a relatively large proportion of stray dogs in El Paso County show evidence of infection with *L. mexicana* and *T. cruzi*. More research will be needed to determine whether this represents a new emergence of these parasites and to fully assess the degree of risk for autochthonous transmission to people in the region.

This study found evidence of *Leishmania* infection in 41 dogs out of an overall sample of 156, for an overall positivity frequency of 26.3%. No major differences were found with respect to the sex, age, or breed group of dog infected, indicating that these variables are likely not major factors influencing a dog's susceptibility to *Leishmania*. Slightly more dogs that were positive for *Leishmania spp*. were found to have skin lesions as compared to the overall group, indicating that some dogs may be developing cutaneous lesions in response to infection. However, numerous PCR-positive dogs were found that lacked any cutaneous lesions, suggesting that lesion development is not a universal sign of infection. The largest cluster of PCR-positive canines was detected within the late summer and early fall months, between July and September. Taking into account incubation times before *Leishmania* parasites are detectable in tissue, this finding indicates that transmission to canines is likely occurring during the middle of the summer and into the early fall months. Interestingly, of the six PCR-positive dogs that were less than one year of age, five were detected during these few months, giving further support to the idea that peak transmission is likely occurring during this period. Furthermore, infections with *Leishmania* detected during this period were often found in all three tissue types (i.e., skin, spleen, and heart), a finding that indicates wide circulation of parasites through the circulatory system at the time of detection. There was no clear spatial pattern among infected dogs, as positive dogs were found throughout El Paso County. Positive cases can be seen in the northeast and far east portions of El Paso and along the Rio Grande east of the city. A possible cluster of positive cases is also

evident to the west of the Franklin Mountains where about half of all canines screened were infected suggesting that this area may be a focus of transmission.

Sequencing analysis from the 18 PCR-positive stray canines samples indicated that all infections were clearly caused by *Leishmania mexicana* with no other *Leishmania spp*. being detected. There was a remote possibility that *L. infantum* would be found in this group, as it has been reported in hunting dogs in parts of the eastern United States and Oklahoma, however this study found no evidence of its presence in the El Paso area.

The proportion of positive samples found in this study is markedly higher than that found by Mariscal in 2013 (2%) that also surveyed stray canines in El Paso County. There are numerous possible reasons for this discrepancy with a major reason being the primers used for PCR analysis. It is possible that the ITS 1 gene segment that was amplified in this study to detect *Leishmania spp*. has a greater sensitivity and is able to detect parasites in tissues at significantly lower levels. Another possible reason for this discrepancy is climatic. The summer, fall, and winter of 2014 and spring of 2015 had noticeably more precipitation and also had significantly warmer temperatures that previous years. Both these environmental factors could favor phlebotomine vector proliferation and lead to a greater number of canine infections. Interestingly, the Mariscal study in 2013 also found a temporal pattern to infectivity similar to that found in this study, with the majority of *Leishmania* infections being reported in the later summer and early fall months.

This study also found evidence of *T. cruzi* infection in 21 dogs out of the overall sample of 156, for a positivity frequency of 13.5%. As with *Leishmania,* no obvious differences were found with respect to sex, age, or breed group between the PCR-positive and PCR-negative stray canines. Stray canines testing positive for *T. cruzi*, however, were much more likely to have visible lesions of the myocardium when compared to the overall sample, with 42.9% of positive dogs having myocardial lesions compared to only 17.9% in the total sample. This finding and the fact that the majority of *T. cruzi* infections were found from heart biopsies is understandable given the pathobiology of the *T. cruzi* parasite and its proclivity to infect heart muscle. The

largest cluster of PCR-positive stray canines for *T. cruzi* was found between the months of November and February, indicating that this period might be a time during which increased transmission is occurring. However due to the chronic nature of many *T. cruzi* infections and the ability of the parasite to reside in tissue for many years, it is also possible that this apparent cluster of cases during these months does not reflect a time of greater transmission. No apparent spatial pattern to *T. cruzi* PCR-positive stray canines was found, suggesting that transmission of *T. cruzi* is occurring throughout El Paso County.

Sequencing of *T. cruzi* PCR products for the Tcz and 121/122 gene segments was inconclusive and unable to confirm the findings from PCR analyses. This precluded determination of discrete typing unit in any samples identified as PCR-positive. Despite the failure to confirm the presence of *T. cruzi* through sequencing, the samples determined to be PCR-positive were most likely infected with *T. cruzi*, but in levels that were low enough as to not provide an adequate amount of PCR product for sequencing. It is unlikely that DNA from other protozoal parasites was being amplified in these tissue biopsies. Attempts to amplify *Leishmania* DNA using either Tcz1/Tcz2 or 121/122 primer pairs did not lead to the visualization of any PCR product, making it unlikely that the results obtained were the result of any crossreactivity.

The results from the Mariscal 2013 study found a similar proportion of canines infected with *T. cruzi* (10%) as were found in this study (13.5%). The two studies also identified the late fall and winter months as those in which most positive canines were found, giving further support to the idea that this may be a time when greater transmission is occurring. Both studies also did not find a clear spatial pattern among positive samples and support the assumption that transmission is occurring over a widespread portion of El Paso County.

Overall, this study provides strong evidence that transmission of both *L. mexicana* and *T. cruzi* is occurring over a widespread portion of El Paso County. One major question that remains to be answered about the transmission of these pathogens concerns the biology of their respective vectors in the region. *L. mexicana* is potentially being transmitted to stray dogs by the sand fly

vectors *Lutzomyia anthophora* and *Lutzomyia diabolica*. These two vector species have been found throughout central and south Texas, where they have been linked to enzootic and zoonotic transmission of *L. mexicana,* but no study to date has identified either vector in the El Paso region. Similarly, it is unclear how dogs are being infected with *T. cruzi*. The triatomine vector species *Triatoma rubida, Triatoma protracta, Triatoma sanguisuga, Triatoma indictiva,* and *Triatoma gerstaeckeri* have all been collected from areas of west and south Texas with an ecology roughly similar to that of El Paso, and it is likely that some combination of these vector species are inhabiting the El Paso area. The question of which triatomine species are present in the area and how they may be infecting stray canines still remains to be answered. It is possible that canines, rather than being physically bitten by infected triatomines, are acquiring their infections by ingesting whole triatomine bugs. Further research will be needed to determine which route of infection is more likely to be occurring.

Another important concern that remains to be studied is the estimated risk that these pathogens pose to people living in the El Paso region. Small numbers of autochthonous cases of both cutaneous leishmaniasis caused by *L. mexicana* and Chagas disease have been reported from central, south, and north Texas. The presence of these pathogens in stray canines from El Paso County suggests that there is at least some potential for autochthonous human infection. People living in rural and agricultural areas of the county are likely to be at greatest risk for infection and increased efforts on behalf of the the public health community of El Paso are needed to increase awareness of these parasites and to provide efficacious methods to prevent potential infection. The data and results from this study will hopefully be used in conjunction with other studies that examine the presence of these parasites over extended periods of time, ultimately leading to a more comprehensive understanding of their epidemiology and the risk they pose to people. The results from this study may also be valuable in future years in order to assess whether transmission of these pathogens is increasing in response to global climate change.

Chapter 6: Conclusion

Collectively, the results from this study add to the understanding that enzootic transmission of *L. mexicana* and *T. cruzi* is occurring across a large portion of the southern United States and in areas where these pathogens were recently not believed to have been present. The relatively high proportion of stray canines with evidence of *L. mexicana* and *T. cruzi* infection found in this study suggest that transmission of these two parasites is occurring frequently and likely is infecting other domestic and sylvatic mammals in the region. Increased awareness of the presence of these pathogens in the El Paso area is greatly needed, especially among physicians, veterinarians, and those working in public health. Physicians in El Paso and across the southern United States should begin to consider cutaneous leishmaniasis and Chagas disease in their differential diagnoses in cases of idiopathic cutaneous lesions or cardiomyopathies, respectively. More research is needed in order to better understand the complex transmission dynamics of these pathogens between sylvatic and domestic reservoirs and their respective vectors.

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Vita

Evan James Kipp was born and raised in El Paso, Texas and is the only child of John Kipp and Deborah Caskey. In 2011 he graduated with honors from Texas A&M University with a Bachelor of Science in Biology. In the fall of 2013 he began his studies at The University of Texas at El Paso toward the completion of a Master in Public Health degree with a concentration in Hispanic and Border Health. While at The University of Texas at El Paso, Evan has been involved in numerous research projects. He has conducted research toward the development of a DNA vaccine against cutaneous leishmaniasis, has studied the emergence of enzootic leishmaniasis and trypanosomiasis in domestic and sylvatic mammalian reservoirs, has conducted research aimed at rapidly detecting leishmaniasis in inhabitants and travellers in rural Ecuador, and has participated in mosquito vector and arbovirus surveillance studies in the El Paso region. Evan has had the opportunity to present findings from these research projects at conferences in El Paso and Dallas, Texas and in Quito, Ecuador. Evan travelled to Ecuador in the summer of 2014 and participated in an epidemiologic training program where he observed cases of cutaneous leishmaniasis, studied diagnostic tools for leishmaniasis, and captured and identified phlebotomine sand fly vectors. During the summer of 2015, Evan served as a public health practicum intern at the City of El Paso Department of Public Health where he worked on the surveillance of emerging mosquito-borne diseases and created various educational materials aimed at preventing mosquito-borne disease transmission. In the future, Evan hopes to further develop skills as a researcher and epidemiologist of emerging zoonotic and vector-borne infectious diseases and ultimately hopes to obtain a doctoral degree in a related field.

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