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Identification Of Leishmania Spp. And T. Cruzi Parasites In Stray Felines In El Paso, Texas Through Polymerase Chain Reaction

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IDENTIFICATION OF *LEISHMANIA SPP*. AND *T. CRUZI* PARASITES IN STRAY FELINES IN EL PASO, TEXAS THROUGH POLYMERASE CHAIN REACTION

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by

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ABSTRACT

Chagas disease and leishmaniasis are considered neglected parasitic diseases, and although dogs are considered the main domestic reservoirs, infected cats have recently been found in endemic areas in several countries and became a public health concern. These diseases can be transmissible to other animals and are known to infect humans as well. Natural infection of cats by *Leishmania spp*. and *Trypanosoma cruzi* has been demonstrated in several European, Latin American, and Asian countries. A recent field study found dogs and wild animals tested positive for both *Leishmania* and *Trypanosoma cruzi* in the El Paso, Texas Region. Cats, however, have not been tested in this region. In this study we aimed to detect *Leishmania* parasites and *T. cruzi* in tissue samples from 155 stray cats from the El Paso, Texas Region. Samples from the spleen, skin, and heart of each cat were collected and subjected to molecular analysis (PCR). Positive samples where then sequenced as an alternate method to detect the presence of DNA from *Leishmania* parasites and *T. cruzi*. Percentage of frequency of positive samples was calculated and their distribution was mapped throughout El Paso region and surrounding areas according to place of capture. PCR results show positive identification of *Leishmania spp*. in nearly twenty percent of skin tissues, and no cases of *T. cruzi* in any of the tissues tested. DNA sequencing proved infection of *Leishmania spp*. parasites in 19 of the 155 cats indicative of the presence of the parasites in the tissues of the studied stray cat population. No geographical pattern was observed among the positive samples.

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INTRODUCTION

Neglected diseases are responsible for causing disease in more than 1.4 billion people across the world (World Health Organization, 2015). Together leishmaniasis and Chagas affect more 300 million people (World Health Organization, 2015). They are considered neglected diseases due to the lack of funds allocated towards their research. Since they affect mainly the poor, little monetary profit is made from any advances made in the diagnosis and treating of these diseases, which is the reason why not enough research is invested in them. Currently no routine surveillance studies are conducted by the city of El Paso or the state for either of these diseases. Several studies have found the presence of the parasites causing these diseases in the El Paso Region and its surrounding areas in tissue from canines and wild animals (Mariscal & Armijos, 2013; (Tenney, Curtis-Robles, Snowden, & Hamer, 2014). For these reasons, it is the aim of this study to investigate if tissues from stray cats from the same region are also infected with these parasites.

BACKGROUND AND SIGNIFICANCE

Leishmaniasis

Leishmaniasis is caused by *Leishmania* parasites and is transmitted through the bite of a female phlebotomus sandfly. There are approximately 2.3 million cases a year, 300,000 of them being Visceral leishmaniasis (VL) (World Health Organization, 2013).

LEISHMANIASIS WORLDWIDE

Combined, the four different types of leishmaniases are prevalent in 98 countries with 90% of the visceral cases occurring in Bangladesh, Brazil, Ethiopia, India, Nepal, South Sudan and Sudan. Most of cutaneous cases are seen in Afghanistan, Algeria, Brazil, Colombia, the Islamic Republic of Iran, Pakistan, Peru, Saudi Arabia, the Syrian Arab Republic and Tunisia and the mucocutaneous in Brazil, Peru and the Plurinational State of Bolivia (World Health Organization, 2013). Since this disease tends to occur in remote and indigenous areas, only about 600,000 of the cases them are reported (Alvar et al., 2012).

LEISHMANIASIS IN TEXAS

In the United States, leishmaniasis has been identified in the southwest, concentrating mainly in Arizona and Texas (Raymond, McHugh, Witt, & Kerr, 2003). Wright et al. found 9 autochthonous cases in residents of North Texas (Wright, Davis, Aftergut, Parrish, & Cockerell, 2008) and Clarke et al. found 3 cases outside the endemic region of Texas, one in Oklahoma city, the second one in Lamar County in Texas, and the third one in McCurtain County in Oklahoma(Clarke, Bradley, Wright, & Glowicz, 2013). None of these patients had any travel history to areas known to be endemic for leishmaniasis. The spread on this disease has been linked to the woodrat, *Neotoma micropus* in Texas (Kerr, McHugh, & Dronen Jr, 1995)and

Neotoma albigula Arizona (Kerr, McHugh, & Merkelz, 1999). These woodrats were infected by the *Lutzomyia anthophora* and *Lutzomyia diabolica* species of sandflies (Kerr et al., 1995). The population of infected woodrats in both of states exceeds 20% (Kerr et al., 1995). From January 2004 through April 2008, there have eight domestic cats found with cutaneous leishmaniasis in Texas. Veterinarians who saw these cats at their clinics reported them. All of these cats were from different counties but none from El Paso County (Trainor, Porter, Logan, Hoffman, & Snowden, 2010).

LEISHMANIASIS IN EL PASO

Mariscal & Armijos (2013) did the only study specific to the El Paso region where they tested skin, spleen, and heart tissue from stray dogs and sylvatic animals through Polymerase Chain Reaction (PCR). They found two positive cases among 96 canines, and 12 positive cases among 20 sylvatic animals. Though more than half of the sylvatic animals came to be positive there have been no registered cases of leishmaniasis in humans in this region (Mariscal & Armijos, 2013).

LEISHMANIASIS IN CATS

Leishmaniasis has already been found in cats across the world. Infection of domestic cats *Leishmania infantum* has been demonstrated in several European, Latin American, and Asian countries (Chatzis et al., 2014). Since 2009, leishmaniasis in Madrid has been unusually high and a study found 11 out of 346 (3.2%) stray cats that tested positive for *L. infantum* (Miró et al., 2014). A different study in Greece tested clinically normal cats (n=50) and cats with several clinical manifestations (n=50) for *L. infantum* and found that 42% of the healthy cats were infected and 40% of the sick cats were infected (Chatzis et al., 2014). In Brazil 50 domesticated

cats were tested and 2(4%) female cats came to be positive (Braga et al., 2014). Infected cats have also been found in Mexico, which is important since El Paso is a border city with this country. Sera from 95 cats tested from the Yucatan Peninsula in Mexico for *L. mexicana, L. infantum, and L. braziliensis* showed 35(31%) samples tested positive for at least one form *Leishmania* and 11(12%) samples tested positive for more than one strain of *Leishmania* (Longoni et al., 2012).

VECTOR FOR TRANSMISSION OF *LEISHMANIA*

The parasite is transmitted to humans through the bite of infected female sandflies, *Phlebotomus* in the Old World and *Lutzomyia* in the New World. Only about 10% of the 600 known species of sandflies belonging to this genus are known vectors, and only 30 are known to spread *Leishmania* parasites (Sharma & Singh, 2008). They are found throughout the world's inter-tropical and temperate regions and are approximately 2 – 3 mm in length, hairy and soundlessly flying insects (Sharma & Singh, 2008). They breed in specific organic wastes such as feces, manure, rodent burrows, leaf litter, and in dark corners in the crevices of walls for humidity and temperature (Sharma & Singh, 2008). Most of the time, these tend to be around human habitats and they remain there since they are poor flyers and only have a flight range of a few kilometers (Sharma & Singh, 2008). In order to develop eggs and get the necessary proteins, female sandflies ingest blood meals. They especially find their meals between dusk and dawn from animals and humans (Sharma & Singh, 2008). It is during these meals when the infected female sandfly transmits the parasite to the human or the animals it is feeding on. As to vector density and development time, it all depends on the environmental conditions.

Figure 1 ***Lytzomyia tortura***

Figure 1 is a male sandfly of species *Lutzomyia tortura* found in Andean Jungle in the tropics of Ecuador. Photo taken by the author in Ecuador, 2014

LEISHMANIA LIFE CYCLE

The *Leishmania* life cycle consists of two stages. It starts when the sandfly takes a blood meal and injects the promastigotes into the skin (CDC, 2015). These promastigotes are phagocytized by the macrophages, where they transform from promatigotes to amastigotes (CDC, 2015). They then multiply and infect the host's cells and tissues (CDC, 2015). The sandlfly then takes another blood meal and ingests infected macrophages (CDC, 2015). In the midgut of the insect, the amastigotes transform into promastigotes, divide, and migrate to the proboscis of the sandfly to be ready to be injected into the mammal once again (CDC, 2015).

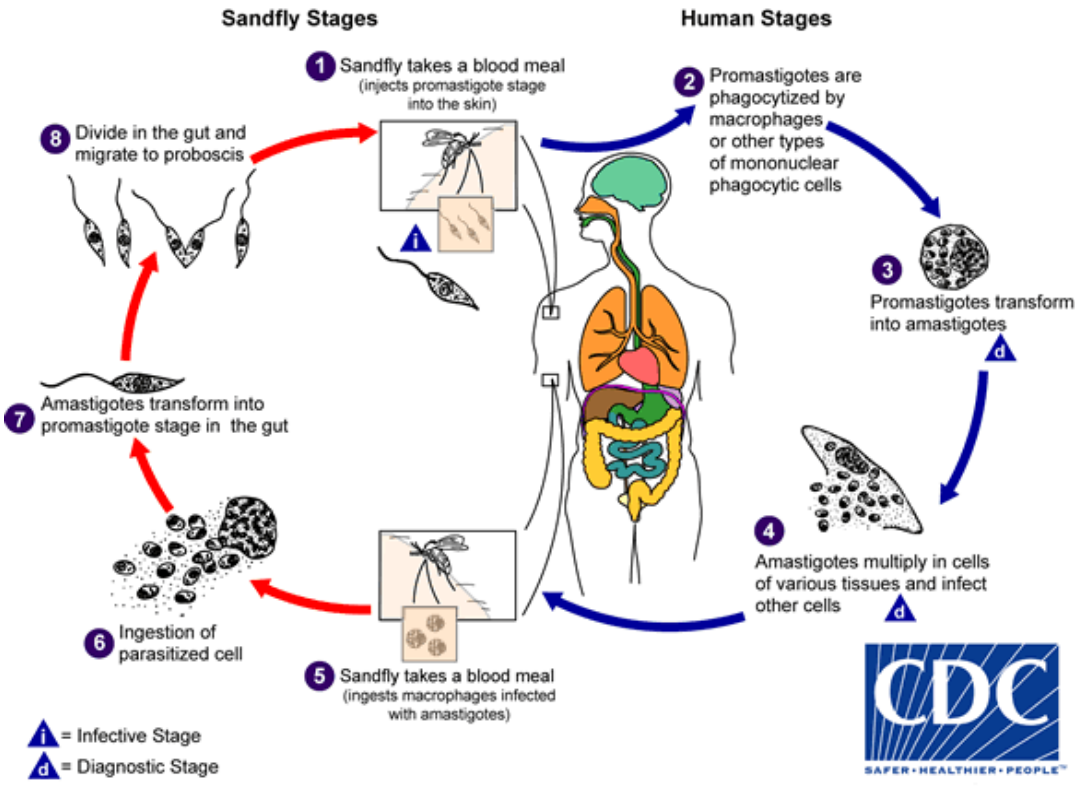

Figure 2 *Leishmania* Life Cycle

Figure 1.2 was retrieved from the Center for Disease Control and Prevention (CDC), (2013).

LEISHMANIASES CLINICAL MANIFESTATIONS IN RELATION TO THE SPECIES

Leishmania parasites exhibit a wide spectrum of clinical manifestations depending on the species that is causing the infection and its reaction with the human host. Kala-azar or visceral leishmaniasis (VL), mainly caused by *L. donovani, L. chagasi,* and *L. infantum* (Hartley, Ronet, Zangger, Beverley, & Fasel, 2012), tends to stereotypically manifest itself with fever, weight loss, hepastosplenomegaly, and pancytopenia (CDC, 2013). If untreated, VL has a mortality rate of almost 100% (Sharma & Singh, 2008). *L. braziliensis, L. guyanensis* and *L. peruviania* can start by showing cutaneous lesions and can later develop into mucocutaneous leishmaniasis (MCL) (Hartley et al., 2012), which is when the lesions spread all over the mucous membranes

of the nose, mouth and throat cavities and surrounding tissues (Sharma & Singh, 2008). This however, only happens in 10% of the cases (Hartley et al., 2012). Cutaneous leishmaniasis (CL), the mildest manifestation of the disease, is mainly caused by *L. major, L. tropica, L. amazonensis* and *L. mexicana* (Hartley et al., 2012). CL presents itself as red lesions at the site of bite from the sand fly sometimes weeks or even years after (Palumbo, 2010). Skin ulcers can range from 1 to 200 in some cases (Sharma & Singh, 2008).The last form is diffuse cutaneous leishmaniasis (DCL). *L. amazonesis, L. mexicana* and *L. aethiopica* have been known to sometimes develop into this type (Sharma & Singh, 2008). It is a variant of localized CL, where the lesions are disseminated throughout the body, resembling lepramatous leprosy (Sharma & Singh, 2008).

Chagas Disease

Caused by the protozoan *Trypanosoma cruzi* (*T. cruzi)*, American trypanosomiasis, also known as Chagas disease, is the cause of infection of about 7 to 8 million people worldwide.

CHAGAS DISEASE AND *TRYPANOSOMA CRUZI* WORLDWIDE

T. cruzi is mostly found in South and Central American countries such as Argentina, Belize, Bolivia, Brazil, Chile, Colombia, Costa Rica, Ecuador, El Salvador, French Guyana, Guatemala, Guyana, Honduras, Mexico, Nicaragua, Panama, Paraguay, Peru, Suriname, Uruguay, and Venezuela (World Health Organization, 2013). While these countries together have most of the total prevalence of Chagas disease, in 2007, control efforts in the South and Central America were joined by an initiative to recognize that presence of imported cases to Europe, North America, and Japan (Beard et al., 2003). The United States, however, did not take place in this initiative even when the CDC estimates that there are more than 300,000 cases of American trypanosomiasis (CDC, 2015), and the whole southern tier of states from Georgia to California

contains established enzootic cycles of *T. cruzi*, involving several triatomine vector species (Bern, Kjos, Yabsley, & Montgomery, 2011), and mammalian hosts such as raccoons, opossums, and domestic dogs (Beard et al., 2003).

T. CRUZI AND CHAGAS DISEASE IN TEXAS

Garcia et al. found 5 patients that were positive for *T. cruzi* around the Houston area (Garcia et al., 2014). Based on each patient's travel history it was determined that these cases were autochthonous and the infection was acquired from recreational exposure (Garcia et al., 2014). These patients however, showed no clinical manifestations. Instead they were found through routine bloods scans but were not registered. Since 1955, there have been seven confirmed and registered autochthonous vector-borne infections in the United States, four of them which where in Texas (Bern et al., 2011). Tenney et al. took serum samples from 205 stray dogs and dogs in shelters in seven major cities in Texas and 18 were seropositive for *T. cruzi* antibodies (Tenney et al., 2014). However, when tested with three different methods, PCR, Chagas STAT-PAK, which is a commercially available rapid immunochromatographic test, and indirect fluorescent antibody test, there were only three positive samples.

T. CRUZI IN EL PASO

Though no cases of Chagas disease have been registered in El Paso, the parasite has been seen in the region. Of the 18 seropositive samples that Tenney et al. (2014) found, two of them were from El Paso. Of the three that were tested by the three different methods one of them was from an animal shelter in El Paso (Tenney et al., 2014). Mariscal et al. (2013) found 10 PCR confirmed samples infected with *T. cruzi* from this same region in stray dogs as well. This team

also tested 20 sylvatic animals and found 13 PCR confirmed positive samples. Cats, however, were not tested in any of these studies.

T. CRUZI IN CATS

Cats are major domestic reservoir hosts of *Trypanosoma cruzi*. In Argentina, serum from 40 domestic cats were tested for detection of *T. cruzi* infection and 10 (25%) were found positive (Enriquez, Cardinal, Orozco, Schijman, & Gürtler, 2013). ELISA and PCR was performed in Merida, Yucatan in Mexico on serum samples and DNA from 220 cats and 19 (8.6%) of them tested positive for *T. cruzi* antibodies and 75 (34%) tested positive through PCR (Jiménez-Coello, Acosta-Viana, Guzman-Marin, Gomez-Rios, & Ortega-Pacheco, 2012). A different study from the Yucatan Peninsula tested sera from 95 cats and 7(7%) were positive(Longoni et al., 2012). In Brazil, PCR was performed on serum samples from 50 cats and 10 cats (20%) came out to be positive (Eloy & Lucheis, 2012).

VECTOR FOR TRANSMISSION OF *T. CRUZI*

There are several routes of in which *T. cruzi* can infect its host. Congenital transmission is possible. Anywhere between 1 and 10% of infants of *T. cruzi*-infected mothers are born with congenital Chagas disease (Azogue & Darras, 1990). Blood-borne transmission is another method, especially seen in less developed countries where resources for blood screening are not available. With technological advances the risk of transmission has decreased but has not been eliminated (Schmunis & Cruz, 2005). Though not very common, organ derived transmission and oral transmission have also been recorded (Schmunis & Cruz, 2005). Ingestion of the infected bug can also cause infection in an insectivorous host (Bern et al., 2011). The most common method of transmission is vector-borne transmission. *T. cruzi* is transmitted to humans and wild

mammals through sylvatic triatomine species, most commonly known as kissing bugs. Unlike sandflies and mosquitoes, kissing bugs of both sexes must take blood meals to develop through their nymphal stages to adults, and females require a blood meal to lay eggs (Bern et al., 2011). Adult kissing bugs are attracted to human dwellings by light and most of the time feed nocturnally without waking the host (Bern et al., 2011). The Americas are endemic to more than 130 triatomine species that are able to spread the parasite. However, only 11 species have been recorded in the United States, *Triatoma gerstaeckeri*, *T. incrassata*, *T. indictiva*, *T. lecticularia*, *T. neotomae*, *T. protracta*, *T. recurva*, *T. rubida*, *T. rubrofasciata*, *T. sanguisuga*, and *Paratriatoma hirsute* (Bern et al., 2011). Seven of these species have been seen in Texas, making it the state with the most diversity of triatomines (Bern et al., 2011).

Figure 3 *Triatoma sanquisuga*

Figure 1.3 is a picture of an adult female kissing bug of the species *Triatoma sanquisuga* found in Bandera County in South Texas. Picture was retrieved from the Region 8 Texas Department State Health Services.

T. CRUZI LIFE CYCLE

The life cycle of the *T. cruzi* parasite consists of three stages. The cycle starts when an infected triatomine bug intakes a blood meal from a human or animal and releases the parasite in the trypomastigote stage in its feces near the site of bite wound (CDC, 2015). The host then aids in the migration of the parasite into site of the bite wound by scratching. These then infect the host through the wound and into intact mucosal membranes and invade nearby cells where they turn into their second stage, which is intracellular amastigotes (CDC, 2015). These then replicate through binary fission and return to their trypomastigote stage. They can then infect other host tissues and differentiate into their amastigote form again, which is when the clinical manifestations are seen or they can burst out of the cell and into the bloodstream where they can be consumed by the triatomine bug in the blood meal and infect the vector. If the triatomine takes in the parasite in the trypomastigote stage, they travel to the midgut where turn into their third and final stage, which is the epimastigote stage and they multiply (CDC, 2015). From there they move into the hindgut and transform into trypomastigotes where they can be released in feces (CDC, 2015).

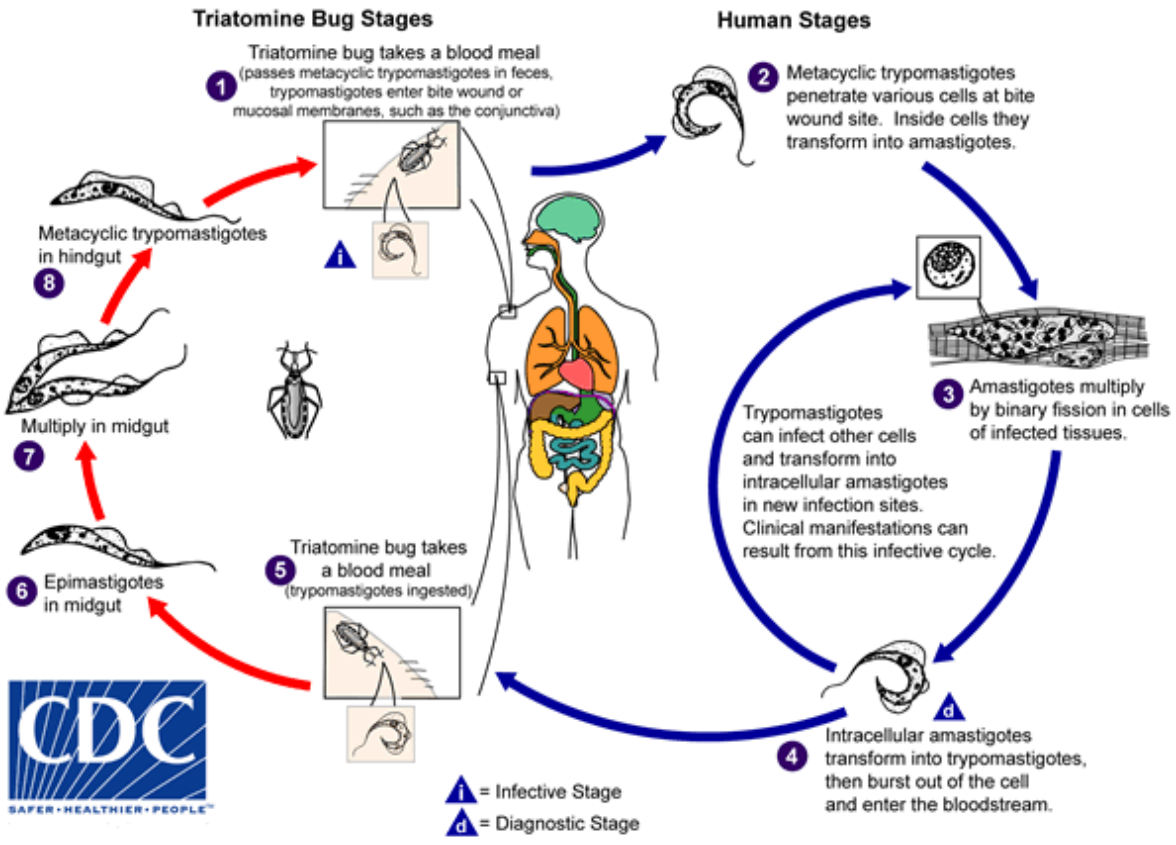

Figure 4 *T. cruzi* Life Cycle

Figure 1.2 shows the full life cycle of *T. cruzi* and was retrieved from the Center for Disease Control and Prevention (CDC), (2013).

CLINICAL MANIFESTATIONS OF CHAGAS DISEASE

The parasite is found in the blood and causes the acute phase of the disease immediately after infection. This consists of fever, or swelling around the site of inoculation (CDC, 2015). Following this, most people go into a prolonged asymptomatic period called the chronic indeterminate, which is where most of the infection remain for the rest of patient's life. In 20-30% of the cases however, the infection can cause more severe symptoms that include heart rhythm abnormalities that can cause sudden death, a dilated heart that prevents proper pumping

of the blood, and a dilated esophagus or colon that leads to difficulties in eating and bowel movement (CDC, 2015).

Environmental Factors

Impact of Climate Change in the Spread of the Disease

Climate change has been mentioned as a reason for the spread of these vector-borne diseases. Looking at vector and reservoir distribution in Mexico, Canada, and the United States for these diseases over the years has lead to conclusions like these. Through the construction of ecological models the distribution of certain vector and reservoirs and their expansion has been predicted. Scenarios for years 2020, 2050, and 2080 have been made where all vectors and reservoir species will see an expansion of their potential range northwards (González et al., 2010). By year 2080 the number of human individuals exposed to leishmaniasis will at least double its present value (González et al., 2010).

Cats are Reservoirs in the Environment and Serve as Pets for Humans

Cats are naturally susceptible to the infection by both of these protozoan parasites, normally without developing any clinical signs and when present they are generally cutaneous lesions (Maia & Campino, 2011) that can be confused with everyday lesions for stray cats. Since they are exposed to the environment at all times they are a constant blood source (Maia & Campino, 2011) for phlebotomines, and triatomines. in domestic cats has also been found. This especially poses a problem because they are among the most popular pet animals around the world (Maia & Campino, 2011). Infection was first described in Algeria in 1912 (Soares, Duarte, & Sousa, 2015). Since then it has been globally reported in countries but more frequently found in countries around the Mediterranean Sea and in Central America, Brazil, and Paraguay (Soares et

al., 2015). Though in this study domestic cats are not be tested, it is important to recognize if cats in this area are infected since domestic cats can also spend a great amount of their time outdoors.

Social and Behavioral Factors

Border Economic Changes in Relation to the Spread of the Disease

The economic change the world is going through has also had an impact on the expansion of these diseases (Esteve-Gassent et al., 2014). The United States and Mexico share a border spanning 3,100 km from the Gulf of Mexico to the Pacific Ocean. Approximately 14 million people reside within the area found roughly 100 km on either side of the international line between the two countries, with 7.3 million residing in the US and 6.8 million in Mexico (Environmental Protection Agency-SEMARNAT, 2013). More specifically the Texas-New Mexico-Chihuahua region stretches 500 miles from the Coronado National Border to the Big Bend National Park which includes the major sister cities of Columbus-Palomas, Las Cruces-El Paso-Ciudad Juarez, and Presidio-Ojinaga (EPA, 2013). This region is home to the second largest metropolitan area along the U.S. – Mexico border known as the Paso Del Norte region with more than two million residents (EPA, 2013). As the border activity in this area broadens, it opens for a constant flow of goods, products, and pathogen dissemination (Esteve-Gassent et al., 2014). At the same time human populations are in constant mobility, increasing the possibility that they come in contact with infectious agents. As the countries improve economically, so does travel and the world trade which in turn contributes to the spread of diseases.

Methodology of Preference

POLYMERASE CHAIN REACTION

The PCR approach for the detection of *Leishmania* and *T. cruzi* DNA has been only recently been used due to the scarcity of these parasites where this method has been used. Because it is considered to be an expensive technique in most cases only the more developed countries have access to the machinery required (Weiss, 1995). However, it has been proven to be fast, sensitive and specific, and most importantly, since this study involves dead animal tissue, the parasite do not need to be viable (Weiss, 1995). A review done in 2014 on detection of *Leishmania* parasite in cats found that out of the 24 studies reviewed, 17 used the PCR approach (Pennisi et al., 2015). A different study used the ELISA approach to detect *T. cruzi* in dogs a found 18 seropositive cases but when PCR was used to verify these positive cases only 3 came out positive (Tenney et al., 2014) proving PCR is a better approach.

SEQUENCING

After PCR is conducted the product can then be sequenced. By getting the whole genome sequence of a sample provided, one can simply put in a database available online and the genus and species of the sample can be known. Sequencing also a highly sensitive test available for detection of microorganisms (Kraaijeveld et al., 2015) and it will be able to classify species as well as genus.

Organs of Preference

Organs used in this study were chosen based on the location of parasites in the body. *T. cruzi* is found in the blood (CDC, 2015) and the *Leishmania* species endemic to areas around El Paso typically manifest themselves as CL (Hartley et al., 2012) therefore they are found in macrophages around the skin ulcer therefore a sample of skin tissue will be collected. The spleen filters the blood as part of the immune system and recognizes and attacks pathogens foreign to

the body and the heart circulates blood throughout the whole body therefore samples from these organs will be collected as well.

STUDY RATIONALE

Both leishmaniasis and Chagas disease, caused by the protozoan *Leishmania spp*. parasites and *T. cruzi* parasites respectively, have been considered neglected diseases, meaning more research should be invested in them worldwide. Climate and economical changes that the world is experiencing has contributed to the northward spread of the disease. Studies tested both wild animals and canines and found that *T. cruzi* and *Leishmania* parasites are already present in El Paso, Texas. Though no human cases have yet been recorded in the area, it might not be long until they are seen since their presence has already been confirmed. Multiple studies have proven that *T. cruzi* and *Leishmania* parasites are capable of using cats as reservoirs. Though studies to search for these diseases in the El Paso area have been done, none have attempted to look for them in felines or with a large sample size like the one attempted in this study. This study aims to identify the frequency of captured cats infected with *T. cruzi* and *Leishmania spp*. parasites by testing tissue from a convenience sample in the El Paso region and if enough positive samples are found to find a geographic pattern in distribution of positive samples.

GOAL AND OBJECTIVES

Problem Statement

There are no routine surveillance studies being conducted in the city of El Paso to monitor either Chagas disease or leishmaniasis. With proof of the presence of the *T. cruzi* and *Leishmania spp*. parasites in this region, veterinarians and health professionals could be actively seeking for these parasites and place more emphasis on prevention methods that can be used to break the transmission cycle and in-turn prevent any future human case manifestations.

Research Question

What is the frequency of stray felines infected with *Leishmania spp*. and *T. cruzi* parasites in the El Paso Region?

Overall Goal of the Study

The goal of this study was to determine if stray cats are infected with *T. cruzi* and *Leishmania spp*. parasites in the El Paso, TX region.

Objectives

1. To determine the frequency of stray cats infected with *T. cruzi* and/or *Leishmania spp*. parasites in the El Paso region by testing spleen, skin, and heart tissue from a convenience sample.
2. To find a pattern in distribution of positive cases in the El Paso region by GIS mapping all positive samples from the tissues tested.

METHODOLOGY

Experimental Design

An observational, cross sectional/transversal study was performed to quantitatively describe the frequency of stray cats testing positive for *T. cruzi* and *Leishmania spp*. found in a convenience sample. Tissue from five cats a week for a period of 10 months was collected and the data was used to summarize the sample in an attempt to represent the stray cat population from the city of El Paso and across the greater El Paso area.

Sample Population

Tissue samples were collected from stray cats that were picked up by trained animal control officers at the City of El Paso and complied with all local and state regulations to be put down for state surveillance purposes. All cats collected by the City of El Paso Animal Services were found in the city of El Paso or across the greater El Paso area. Samples were collected once a week, three weeks per month for a period of 10 months. Tissues from five different cats were collected per visit. Each cat picked up by Animal Services the day of the visit was visually examined for the presence of ulcerative or non-ulcerative skin lesions prior to skin biopsy collection. If a lesion was identified, a biopsy was collected around the afflicted site. Cats with lesions were given preference for collection. Each animal'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 near the apex. Information on each animal's approximate age, sex, and fur color was recorded, in addition to the GPS coordinates of the location the animal was picked up by the City of El Paso Animal Services. A regional public health veterinarian working for the Texas Department of State Health Services granted permission and oversaw the progress of the sample

collection. Approval from the University of Texas at El Paso's Institutional Animal Care and Use Committee was not required as none of the cats surveyed were euthanized for the purpose of this study, but rather were euthanized for routine animal control surveillance programs conducted by the city of El Paso Animal Services. Approval from the Institutional Biosafety/Recombinant DNA Committee from the University of Texas at El Paso was required and obtained in order to comply with university policies to work with tissue possibly infected with *Leishmania spp*. parasites and *T. cruzi,* which are considered Risk Group 2 Organisms.

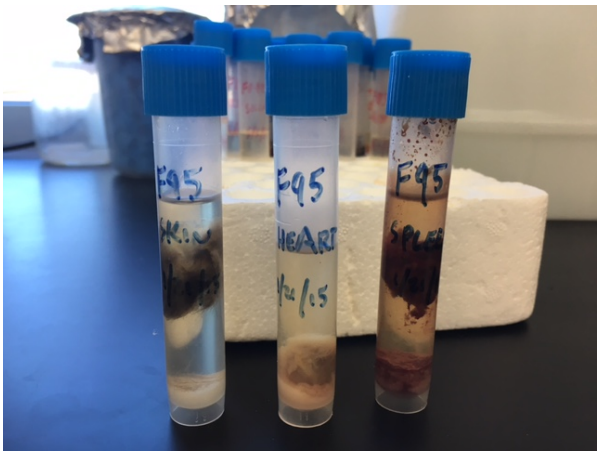

Figure 5 Samples Preserved in DMSO/EDTA/Salt Solution

Figure 4.1 shows the tissues from the cats preserved in DMSO/EDTA/Salt Solution at room temperature in the laboratory until the time of DNA extraction. Each cat was given a code, which was written in each tube along with the date of collection and the organ the tissue belongs to, either skin, spleen, or heart.

Instruments and Equipment

DATA COLLECTION SHEET

A data collection sheet was designed to record each cat's code which was the sample number, date of collection, GPS coordinates of the location where the cat was picked up, its age, sex, color, and whether or not the sample presented any lesions or organ discoloration. The information taken from these forms was inputted into Microsoft Excel and coordinates of positive samples were mapped using ArcGIS.

EQUIPMENT

Once samples were collected, they were immediately taken back to the lab and preserved in a DMSO/EDTA/Salt solution. A lysis buffer (SNET) composed of 400 mM NaCl, 1% (w/v) SDS, 20 mM Tris-Cl (pH 8.0), and 5 mM EDTA (pH 8.0) was prepared and Proteinase K was added to a final concentration of 400μg/mL in order to denature the proteins of each sample. Tissues were left over night in the SNET/Proteinase K solution inside an Amarex Instruments, Inc.® SteadyShake 757 Benchtop Incubator Shaker and once taken out they were vortexed in a Vortex-Genie® 1 and an equal amount of Phenol/Chloroform/Isoamyl Alcohol as SNET was also added under a fume hood to finish disintegrating the tissue samples. Samples were then centrifuged in a Beckman Coulter® Allegra X-15R Refrigerated Benchtop Centrifuge. The aqueous layer was placed in 1.5 ml Eppedorf® tubes with ice-cold isopropanol and centrifuged in an Eppedorf® Centrifuge 5415D to separate the DNA from the rest of the solution. DNA concentration, purity, and quality was determined using a NanoDrop® ND-1000. All DNA amplifications were made on a Bio-Rad® Thermal Cycler, run on a Bio-Rad® Sub-Cell GT Cell, and bands were analyzed under UV light.

Procedures

DNA Extraction

Genomic DNA was extracted from the tissue samples collected from each cat to make the DNA templates required for the PCR protocols. Each spleen, heart, and skin tissue sample was placed in the appropriate amount of SNET/proteinase K. Samples were incubated at 55°C overnight with agitation (300 rpm). After incubation, tubes were vortexed accordingly to dissolve tissue and an equal amount as SNET was added of phenol/chloroform/isoamyl alcohol and placed on a rocking platform for 30 minutes at room temperature. Samples were then centrifuged for 5 minutes at maximum speed. Phases were separated and 750 µL of the aqueous phase was combined with an equal amount of ice-cold isopropanol to precipitate DNA. Microcentrifuge tubes were then centrifuged for 15 minutes at maximum speed at 4°C. When taken out, a pellet was seen. Isopropanol was removed and pellet was washed with 100 µL of 70% ethanol. Ethanol was removed and pellets were allowed to air dry for 15 to 20 minutes at room temperature. Once dry, pellets were suspended in 100 µL of nuclease free water and vortexed until the pellet was completely dissolved. DNA concentrations, quality, and purity were measured and the samples were diluted with nuclease free water to end up with a concentration of 100 ng/µL. Samples were then ready to be used as templates for the PCR.

Primers and PCR Protocols

PCR content included 12.5 µL of PCR master mix, 1 µL of reverse primer, 1 µL of forward primer, 1 µL of template, and 9.5 µL of nuclease free water to have a total volume or 25 µL per tube.

Primers TCZ1 and TCZ2 were used to amplify 144 bp of the TCZ region (Braz et al., 2008) from *T. cruzi*. PCR conditions were the following: an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of 94°C for 20 seconds, 57°C for 10 seconds, 72° C for 1 minute and a final extension of 72° C for 10 minutes (McPhatter et al., 2011). *T. cruzi* DNA was used as template for the positive controls and 1 µL of nuclease free water was added in replacement of the template for the negative controls. Controls were included with every run.

Figure 6 BioRad® Thermal Cycler and PCR Tubes with Content

Figure 4.2 shows the Bio-Rad® Thermal Cycler (left) which is where all the PCR amplification were performed and the some of the PCR tubes with their contents (right).

IRBP-CF-FWD and IRBP-CF-REV which amplify 227 bp of the interphotoreceptor retinoid-binding protein (IRBP) of any mammalian species, were also be used to test the quality of the DNA extracted (Ferreira, Gontijo, Cruz, Melo, & Silva, 2010). PCR conditions for this primer were the following: an initial denaturation step of 94° C for 3 minutes, followed by 35 cycles at 94° C for 30 seconds, 57° C for 30 seconds, 72° C for 1 minutes and then a final extension step at 72° C for 10 minutes (Ferreira et al., 2010).

Primers LITSR and LITSV were used to amplify the 192 bp ITS region of any *Leishmania spp*. DNA. PCR protocol for this primer were as follows: an initial denaturation step of 95°C for 2 minutes, followed by 35 cycles of 95°C for 20 seconds, 53°C for 30 seconds, 72° C for 1 minute and a final extension of 72° C for 15 minutes (El Tai, Osman, El Fari, Presber, & Schönian, 2000). *Leishmania* DNA was used as template for the positive controls and 1 µL of nuclease free water was added in replacement of the template for the negative controls. Controls were included with every run.

Once PCR was completed, a 1.8% agarose gel was run at 100 volts for 20 minutes and bands were observed under UV light.

Table 1: Sequence of Primers Used

Gene	**Parasite ID**	**Primer Name**	**5'----------3'**	**Product Size**
TCZ region	*T. cruzi*	TCZ1 TCZ2	CGAGCTCTTGCCCACACGGGTGCT CCTCCAAGCAGCGGATAGTTCAGG	144 bp
ITS region	*Leishmania*	LITSR LITSV	CTGGATCATTTTCCGATG ACACTCAGG TCTGTAAAC	192 bp
IRBP	Control	IRBP-CF-FWD IRBP-CF-REV	TCCAACACCACCACTGAGATCTGGAC GTGAGGAAGAAATCGGACTGGCC	227 bp

Table 2: PCR Protocols

Gene segment	Starting temperature (x 1 cycle)		Denaturing Temperature (x 35 cycles)		Annealing Temperature (x 35 cycles)		Extending Temperature (x 35 cycles)		Ending temperature (x 1 cycle)	
TCZ	94°C	5min	94°C	20sec	57°C	10sec	72°C	30sec	72°C	10min
ITS	95°C	2min	95°C	20sec	53°C	30sec	72°C	1 min	72°C	10min
IRBP	94°C	3min	94°C	30sec	57°C	30sec	72°C	1 min	72°C	10min

Samples that tested positive through PCR were subjected to sequencing using the University of Texas at El Paso Molecular Core Facility (BBRC).

DATA ANALYSIS

A descriptive analysis was performed to describe the distribution of possible cases of infected stray cats in the El Paso region. Location of positive cases was mapped based on their GPS location. The maps depict the geographic distribution of *Leishmania spp*. and *T. cruzi* infections in stray cats.

RESULTS

COLLECTED SAMPLES

Collection was only possible for 32 weeks from the first week of July, 2014 to the last week of May, 2015. Spleen, heart, and skin tissues from a total of 155 cats were collected.

T. CRUZI PCR RESULTS

Tissues collected from the 155 cats were all tested for *T. cruzi*. No samples produced evident bands resulting in negative results for every cat. Since no samples gave positive results through PCR, none of the samples were sent to sequencing.

LIESHMANIA PCR AND SEQUENCING RESULTS

Tissues collected from the 155 cats were all tested for *Leishmania spp*. parasites. Out of those tested, 19 (12%) cases were found to be infected with *Leishmania* in the El Paso region and its surrounding areas (Figure 7). Skin samples of all of these 19 cases were sent to sequencing and all of these cases were confirmed to be infected with *Leishmania mexicana*.

LOCATION OF IDENTIFIED CASES

No obvious pattern was seen among the location were the infected cats were picked up (Figure 7). There were two cases found in each other following zip codes, 79849 (F5 and F8), 79903 (F49 and F65), 79922 (F7 and F57) and 79924 (F21 and F52). There were four identified cases (F1, F53, F61, and F67) in the 79905 zip code, which is in the central part of town.

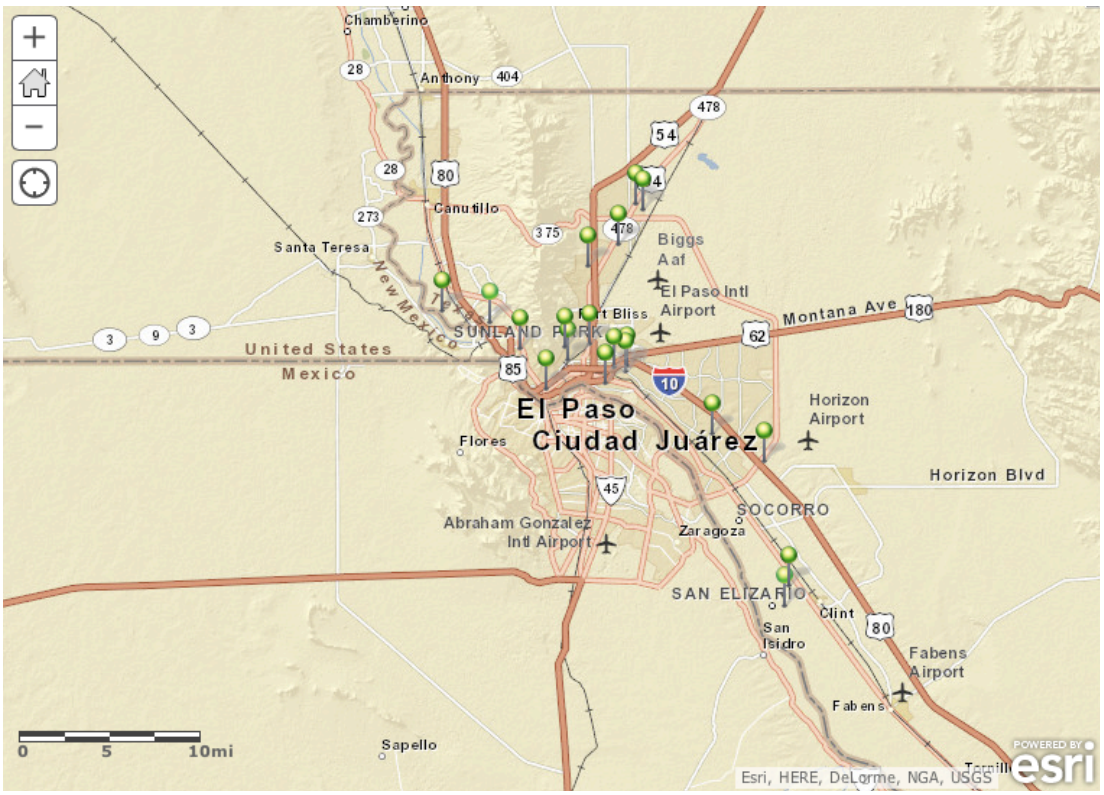

Figure 7 Positive cases of *Leishmania* infected Cats

DATES OF IDENTIFIED CASES

All of the cases identified were in the latter part of 2014 (Table 3). Five of the 19 positives were found in July 2015, three in August, one in September, six in October, three in November, and the last one was identified in December. Collection went on for five more months but no cases were found.

PHYSICAL CHARACTERISTIC OF IDENTIFIED CASES

Out of the 19 positively identified cases, 15 (79%) were adult cats. Ages of the others were of six months, five months, four months, and 5 weeks. Twelve of the cats were male (63%) and there was no specific color of fur that had more positive cases (Table 3).

LESIONS OR ORGAN DISCOLORATION

All of the cats surveyed were visually examined and 53% of the total 155 cats tested presented a skin lesion or organ discoloration. However, of the 19 cats that tested positive, eight (42%) of the cats showed some kind of organ or skin lesion (Table 3).

Table 3: Description of Identified *Leishmania* Cases

Sample	**Date Collected**	**Age**	**Sex**	**Color/Breed**	Lesions or **Organ Discoloration**	Zip code Picked Up
F2	July 2 - 2014	6 months	M	Black-White	negative	79904
F5	July 2 - 2014	Adult	F	Tortoiseshell	Negative	79849
F7	July 16 - 2014	Adult	F	Gray-Tan	Negative	79922
F8	July 16 - 2014	Adult	M	White-Gray	Negative	79849
F11	July 30 - 2014	Adult	M	Gray-White	Negative	79928
F16	Aug 6 -2014	Adult	F	Tortoiseshell	Negative	79905
F19	Aug 6 -2014	Adult	M	White-Gray	Lesion on Left side	79924
F21	Aug 13 -2014	5 months	M	Orange Tabby	Skin Lesion on Left paw	79924
F49	Sept 24 - 2014	Adult	M	Black	Negative	79903
F51	Oct 1 - 2014	4 months	F	Black	Negative	79901
F52	Oct 1 - 2014	Adult	M	Orange Tabby/White	Negative	79924
F53	Oct 1 - 2014	Adult	F	Calico	Skin Lesion on nose	79905
F55	Oct 15 - 2014	Adult	M	Brown Tabby	Skin lesion on base of tail	79902
F57	Oct 15 - 2014	Adult	M	Gray Tabby	Lesion on nose	79922
F61	Oct 22 - 2014	Adult	M	Orange/White	Lesion on nose Spleen lesions	79905
F65	Nov 12 - 2014	Adult	M	Black-White	Lesion on nose Spleen lesions	79903
F67	Nov 12 - 2014	Adult	F	Calico	Negative	79905
F73	Nov 19 - 2014	Adult	M	Brown Tabby	Myocardial Lesion	79930
F76	Dec 3 - 2014	5 weeks	F	Gray Tabby	Negative	79907

DISCUSSION

A study performed in canines in this region in search of *Leishmania spp*. and *T. cruzi* parasites found that Chagas disease was more common than leishmaniasis in dogs in El Paso, TX. This study showed somewhat different results in felines. No cases of *T. cruzi* were found in any of the cats tested, however 12% of the 155 cats sampled tested positive for *Leishmania mexicana* parasites. Though there was no obvious pattern in distribution for these cases the central part of El Paso did show a higher number of infections than all other parts of El Paso which is contrary to Mariscal et. al's (2013) findings which only reported two cases of canine leishmaniasis, none being in the central part of the city. Similar to what Mariscal et al. (2013) had reported in canines regarding the sex and breed of positive cases in both leishmaniasis and Chagas disease, sex and fur color of the cats did not seem influence vulnerability to infection. Mariscal et al. (2013) found that nine out ten positive cases of *T. cruzi* in fall months concluding temporal range of infection for *T. cruzi* to be in the summer. Similarly, all of the positive cases in this study were found in the summer and winter meaning that temporal range of infection for *Leishmania mexicana* could potentially be in the summer or late spring as well. However, further research is required to make conclusion regarding seasonality. This study had several limitations, the most important being lack of proper funding. Had the required funds been available, sequencing would have been possible in every samples collected, a larger research team could have been hired in order to increase samples size to every feline collected by City of El Paso Animal Services on the day of the visit rather than just five felines per visit. Another limitation was that visits for sample collection depended on the stray feline collection by the City of El Paso Animal Services. Therefore, sample collection was only possible from ten months and visits were scheduled based on City of El Paso Animal Services work schedule. Future research in this subject should concentrate on establishing seasonality of infection in felines since two studies have already mentioned this possibility based on their results. Further research in distribution of

leishmaniasis feline infection should also prove useful in order for the City of El Paso Department of Public Health to be prepared should there be a human case manifestation.

CONCLUSION

The data collected in this study shows that cats in El Paso, Texas and its surrounding areas have not yet been infected with *Trypanosoma cruzi* but *Leishmania mexicana* has infected nearly 20% of the stray cat sample population in the city. Data collected from this study should serve to increase awareness regarding the prevalence of leishmaniasis in stray cats among veterinarians and public health practitioners, leading to a greater emphasis placed on prevention methods that can be used to break the transmission cycle and in-turn prevent any future human case manifestations. The presence of *Leishmania mexicana* in nearly one-fifth of the sampled El Paso stray cat population warrants further research in the seasonality of infection of the parasite, and evaluation of felines in El Paso as possible reservoirs.

RESOURCES

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APPENDIX

Table 4: *T. cruzi* Stray Cats Sample Population Characteristics

Table 5: *Leishmania* Stray Cats Sample Population Characteristics

Electrophoresis gels of positive PCR samples

Table 4: *T. cruzi* Stray Cats Sample Population Characteristics

Demographic Characteristics	Positive Cases	Negative Cases
	0	155 (100%)
Age: 5 weeks		1 (1%)
Age: 4 months		4 (3%)
Age: 5 months		3 (3%)
Age: 6 months		2 (1%)
Age: 10 months		1 (1%)
Age: One year		4 (3%)
Age: Adult		135 (87%)
Age Range		5 weeks - Adult
Sex: Male		91 (59%)
Color: Black		22 (14%)
Color: Black and White		26 (17%)
Color: Brown Tabby		6 (4%)
Color: Calico		8 (5%)
Color: Gray		28 (18%)
Color: Gray and Red		1 (1%)
Color: Gray and White		17 (11%)
Color: Gray and Black		2 (1%)
Color: Gray and Tan		2 (1%)
Color: Orange Tabby		14 (9%)
Color: Orange and White		9 (6%)
Color: Siamese		6 (4%)
Tan and White		4 (3%)
Tortoiseshell		7 (5%)
Tricolor		1 (1%)
White		1 (1%)
White and Brown		1 (1%)
Lesions/Organ Discoloration		82 (53%)

Table 5: *Leishmania* Stray Cats Sample Population Characteristics

Demographic Characteristics	Positive Cases	Negative Cases
	19 (12%)	136 (88%)
Age: 5 weeks	1 (5%)	0
Age: 4 months	1 (5%)	3 (2%)
Age: 5 months	1(5%)	2 (1%)
Age: 6 months	1(5%)	1 (1%)
Age: 10 months	0	1 (1%)
Age: One year	0	4 (3%)
Age: Adult	15 (79%)	125 (92%)
Age Range	5 weeks - Adult	4 months - Adult
Sex: Male	12 (63%)	79 (58%)
Color: Black	2 (11%)	20 (15%)
Color: Black and White	2 (11%)	24 (18%)
Color: Brown Tabby	2 (11%)	4 (3%)
Color: Calico	2 (11%)	6 (4%)
Color: Gray	2 (11%)	26 (19%)
Color: Gray and Red	0	1 (1%)
Color: Gray and White	3 (16%)	14 (10%)
Color: Gray and Black	0	2 (1%)
Color: Gray and Tan	1 (5%)	1 (1%)
Color: Orange Tabby	1 (5%)	13 (10%)
Color: Orange and White	2 (11%)	7 (5%)
Color: Siamese	0	6 (4%)
Tan and White	0	4 (3%)
Tortoiseshell	2 (11%)	5 (4%)
Tricolor	0	1 (1%)
White	0	1 (1%)
White and Brown	0	1 (1%)
Lesions/Organ Discoloration	8 (42%)	74 (54%)

Electrophoresis Gels of Positive Samples

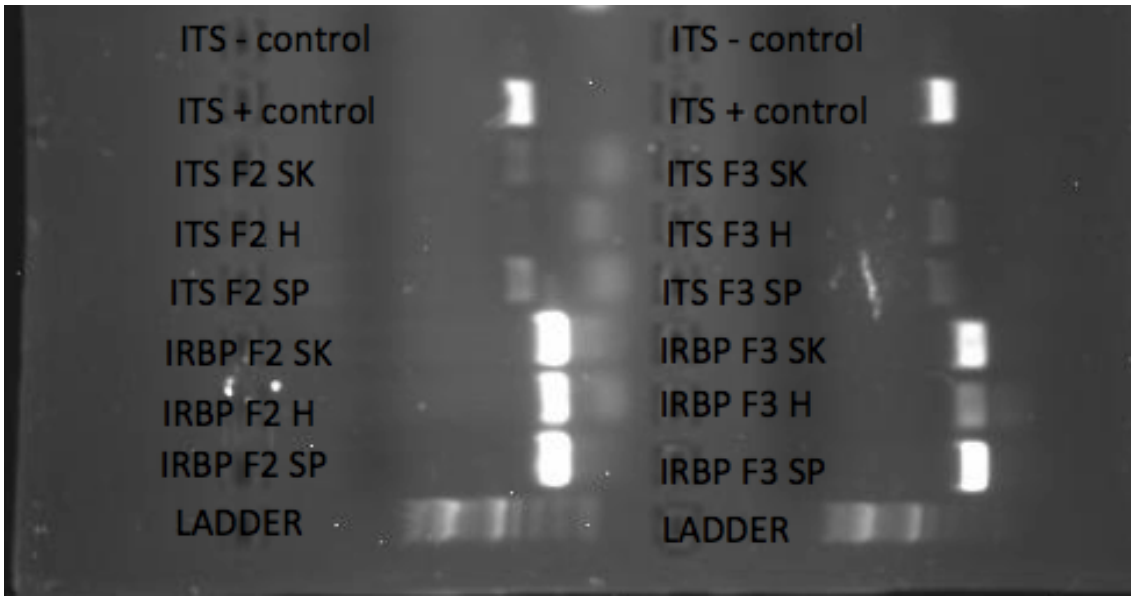

Figure 8: Electrophoresis Gel of Sample F2 and Sample F3

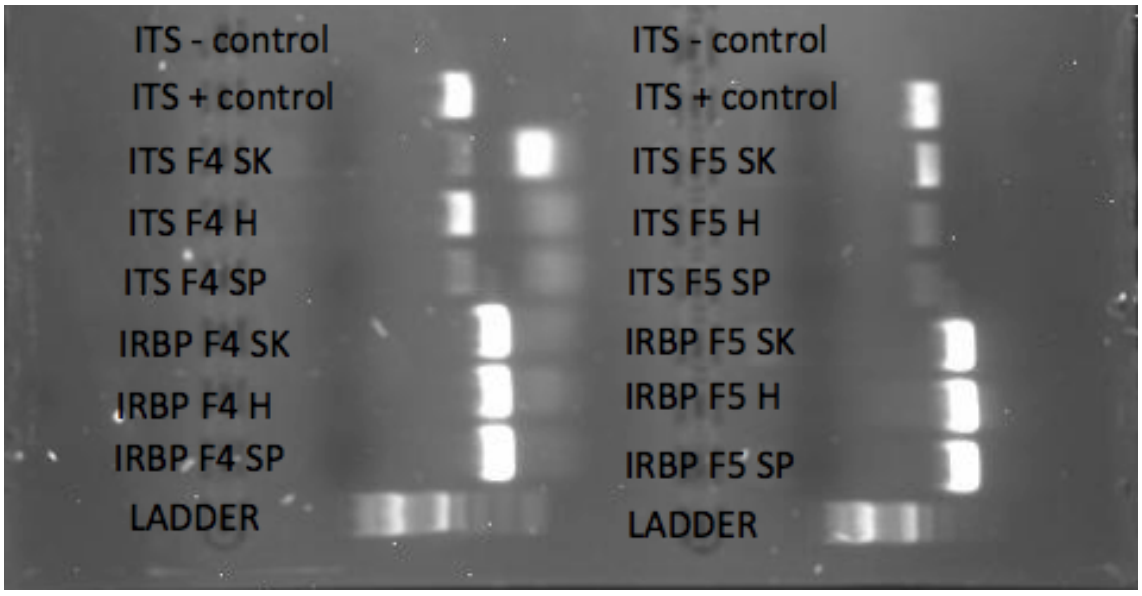

Figure 9: Electrophoresis Gel of Sample F4 and Sample F5

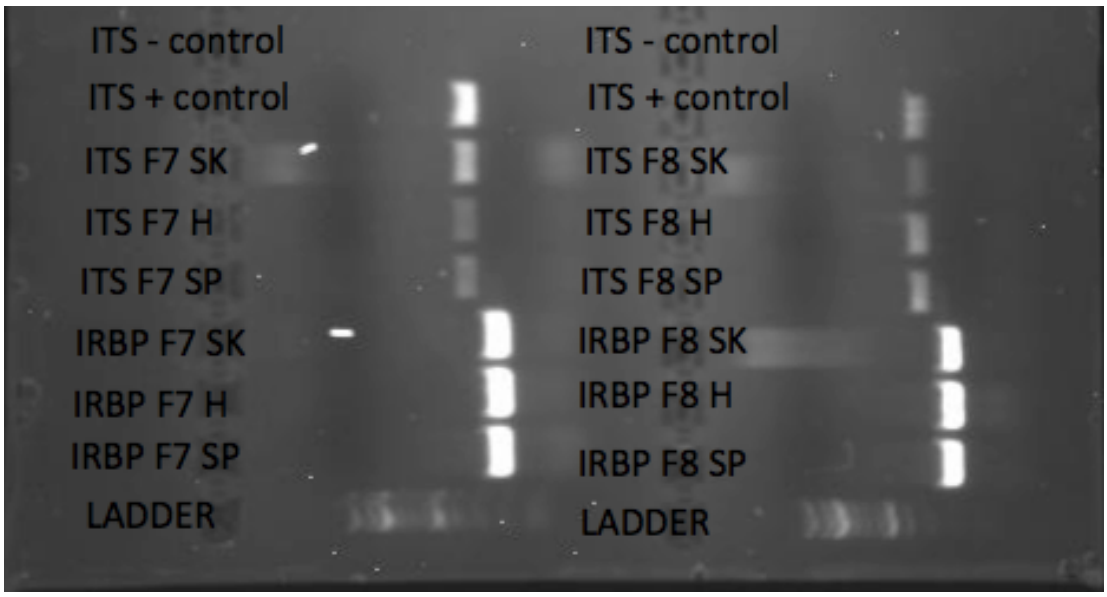

Figure 10: Electrophoresis Gel of Sample F7 and Sample F8

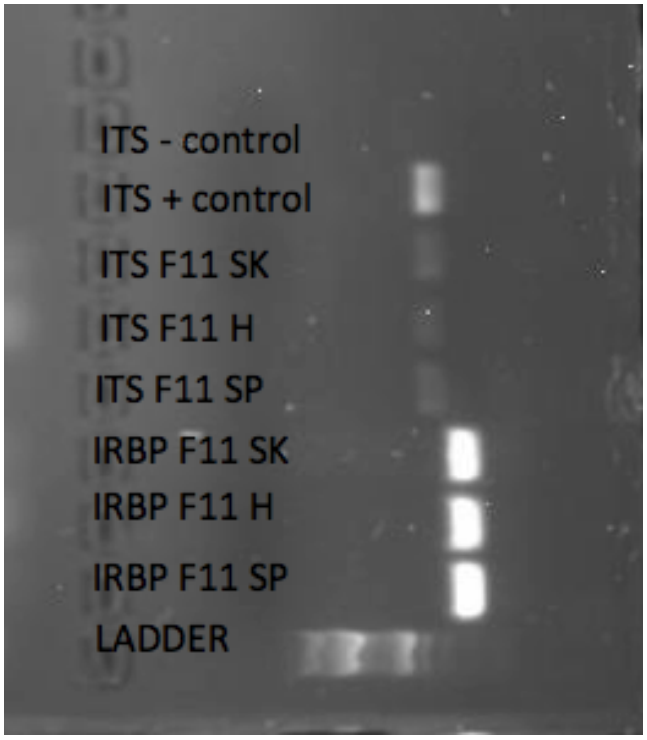

Figure 11: Electrophoresis Gel of Sample F11

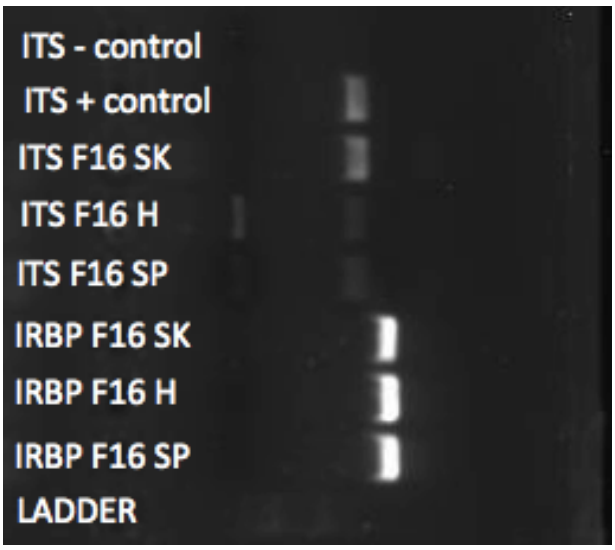

Figure 12: Electrophoresis Gel of Sample F16

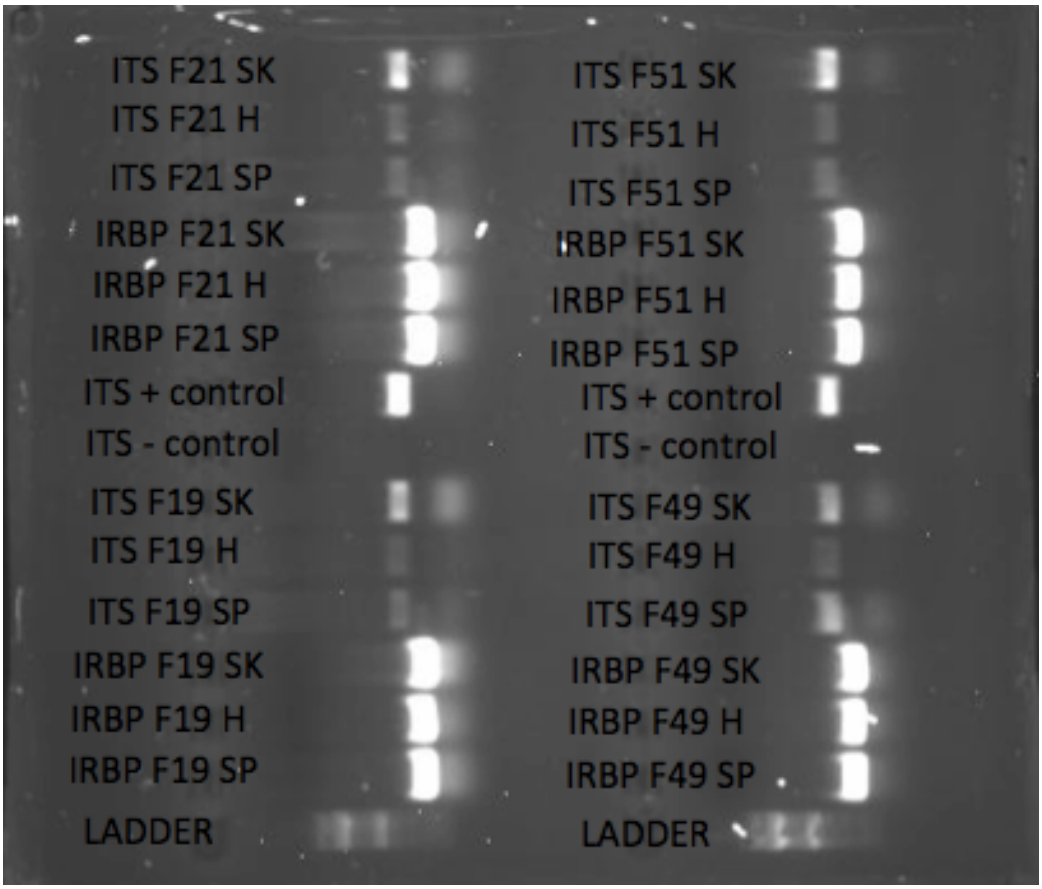

Figure 13: Electrophoresis Gel of Sample F19, F21, F49, and F51

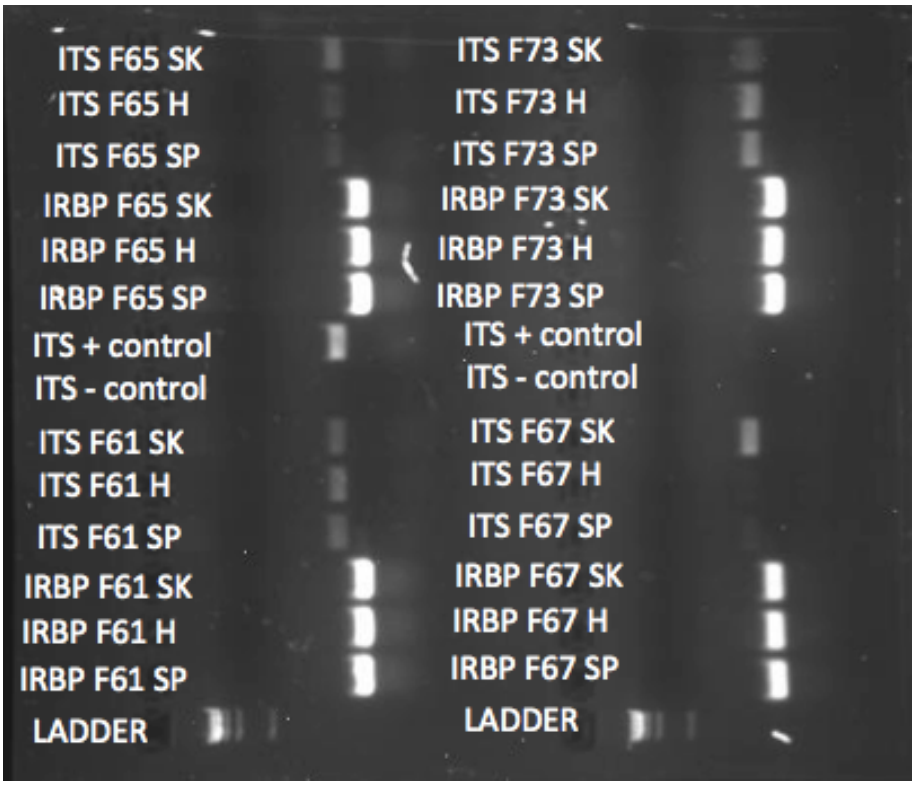

Figure 14: Electrophoresis Gel of Sample F61, F65, F67, and F73

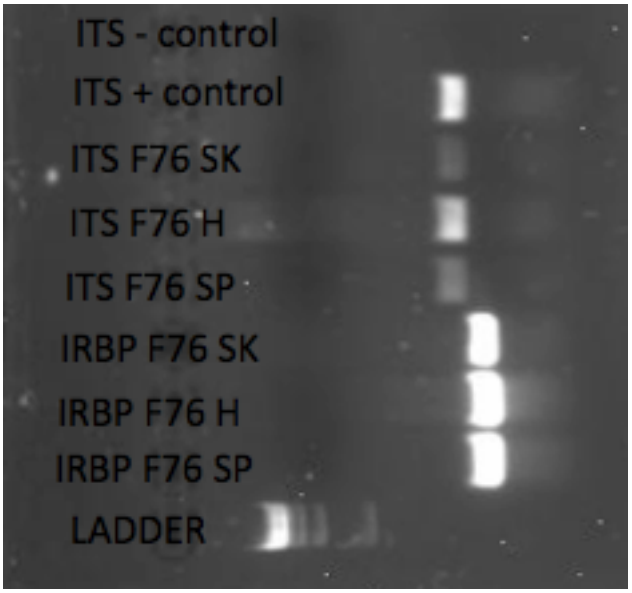

Figure 15: Electrophoresis Gel of Sample F76

VITA

Patricia Isabela Gonzalez, author of this thesis, was born in Guadalajara, Mexico. Soon after birth she was brought to El Paso, Texas and has lived there ever since. She is the daughter of Juan and Patricia Gonzalez and first of her family to acquire a college education. She graduated Cum Laude with a Bachelor of Science from the University of Texas at El Paso in 2012 where majored in Microbiology with a minor in Chemistry. Soon after graduation she pursued a Master of Public Health from the same university and graduated in 2015. She has participated in molecular biology research involving Chagas disease and leishmaniasis since 2013. Her research has involved attempting to design a vaccine *Leishmania mexicana* and identifying these parasites in El Paso, Texas where she currently resides. She traveled to Ecuador where she spent time in the Andean Jungle understanding the behavior and identification of *lutzomyia* sandflies and learning how to diagnose leishmaniasis. Patricia Isabela and her group of researchers were the only group from Texas invited to participate in the Second International Workshop of the Latin American Network of Molecular Epidemiology and Evolutionary Genetics (LAN MEEGID) and the III National Meeting for Infectious Disease Research and Tropical Medicine in Quito, Ecuador where Patricia Isabela and her team's research was awarded first place. Her future aspirations include working with infectious diseases specifically with vector control in her hometown of El Paso.

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This thesis was typed by Patricia Isabela Gonzalez.