THE PREVALENCE OF *LEPTOSPIRA* IN SMALL MAMMALS ON FIVE PUERTO RICAN CATTLE FARMS

by

Kathryn Michelle Benavidez, B.S.

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Committee Members:

Iván Castro-Arellano, Chair

Dittmar Hahn

David Rodriguez

Joseph Veech

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ABSTRACT

Leptospirosis is thought to be the most widespread zoonotic disease in the world. For this study 124 mice (*Mus musculus*), 99 rats (*Rattus rattus* and *R. norvegicus*), and 89 small Asian mongooses (*Herpestes auropunctatus*) from five farms in Puerto Rico were tested for renal carriage of *Leptospira* and approximately 38% of the sampled individuals were positive. I evinced a heterogeneous distribution of *Leptospira* prevalence among the sites with a farm in Lajas having the highest prevalence at 52%. Among tested species, mice had the highest prevalence of *Leptospira* at 59% and mongooses had the lowest at 13%. Comparative sequence analysis of the *LipL32* gene revealed the presence of two species of *Leptospira: Leptospira borgpetersenii* and *Leptospira interrogans*. These two *Leptospira* species were equally distributed at four farms, however, at the farm at San Sebastián 100% of the samples sequenced were of the species *L. borgpetersenii.* Significant associations of *Leptospira* prevalence with landscape features were observed at a farm in Naguabo, where the average distance of positive samples was closer to the tested landscape features than negative samples, and at the farm in Sabana Grande where the average distance of positive samples were closer to a human dwelling than the negative samples. These results show that rural areas of Puerto Rico are in need of management and longitudinal surveillance of *Leptospira* in order to prevent continued infection of Leptospirosis by focal susceptible species (i.e. humans and cattle)

1. INTRODUCTION

Along with increasing globalization, climate change, and urban expansion, the rising number of emerging infectious diseases is a major concern for humans. Among emerging infectious diseases, over 60% are multi-host zoonoses that over the past decade have cost the global economy over $20 billion. Many of these are considered as "neglected" according to the World Health Organization (WHO) due to a general lack of epidemiological knowledge about them, with the situation exacerbated in the tropics (WHO 2011; Webster et al. 2016). Perhaps the most widespread neglected zoonotic disease in the world is Leptospirosis, which has an estimated annual global incidence of 1.03 million human cases with a projected number of 60,000 as fatal cases (Jancoles et al. 2014). If left untreated, Leptospirosis can have a fatality rate in humans of around 29% (Jancoles et al. 2014). Another reason that the WHO considers Leptospirosis a neglected disease is because the clinical signs are nonspecific and serology testing is often misleading (WHO 2011). Leptospirosis is included into diseases addressed by the One Health initiative because the interactions between humans, wildlife, and ecosystems plays a vital role in persistent disease transmission (Jancloes et al. 2014). The One Health Initiative is a global movement joined by individual researchers, government agencies, NGO's and global organizations, such as the WHO, that seeks to improve the health of all humans and animals by integrating human and veterinary medicine along with environmental science.

Leptospirosis is a bacterial disease affecting a variety of mammals and is caused by pathogenic spirochetes of the genus *Leptospira* (Bourhy et al. 2011; Li et al., 2013). Within the genus *Leptospira* there are at least 12 known pathogenic species with over 250

serovars (Ki et al. 2009). Susceptible animal species contract *Leptospira* through direct or indirect contact with the urine of animal reservoirs. In known natural reservoirs, such as dogs, rodents, and cattle, *Leptospira* persists and multiplies within the renal tubules of these hosts. The bacteria are then dispersed via movement and urination of the hosts throughout the local landscape (Plank 2000; Bharti et al. 2003). Once in soil and water, this bacterium can remain viable for several months and can infect susceptible species through open-skin wounds and mucus membranes (Ko et al. 2009).

Humans are incidentally infected and can contract Leptospirosis following exposure to soils or water that is contaminated with animal urine containing *Leptospira* (Plank 2000). Specific Leptospirosis signs in humans vary but can include severe pulmonary hemorrhage (Vijayachari 2015). Other manifestations include nausea, diarrhea, jaundice, enlargement of the liver, renal damage, fever, and other severe flu-like symptoms (Alexander 1963; Levett 2001). A variety of environmental and behavioral factors are associated with contracting Leptospirosis. Oftentimes, transmission rates increase in association with water bodies and rainfall events (Boqvist et al. 2012). Outbreaks in economically developed areas are associated with water-related recreational activities, while outbreaks in developing and impoverished areas are more closely correlated with flooding, rainfall, and contact with animals such as cattle (Mwachui et al. 2015).

Previous research has shown that Leptospirosis can also negatively affect the livestock industry (Webster 1995; Lilenbaum and Santos 1996; Alonso-Andicoberry et al. 2001). *Leptospira* infects both dairy and beef cattle. Clinical signs of primary concern in cattle include abortions, birth complications, and reduced milk production. In

tropical areas, cattle are at higher risk for contracting Leptospirosis likely as a result of climatic conditions conducive for longer periods of pathogen persistence in the environment (Ellis 1984). Since humans rely on livestock for food, milk, and economic gain, Leptospirosis outbreaks are a threat to overall food security in affected areas.

South Pacific island farms have been shown to be particularly at risk of Leptospirosis outbreaks due to both the farmer's lack of knowledge and the shortage of trained veterinarians throughout the entire region. Because of these and prevailing environmental conditions in this region, Leptospirosis is ranked the number one endemic disease in South Pacific island countries (Brioudes et al. 2014). Leptospirosis is also widespread in the Caribbean islands where Leptospirosis outbreaks have occurred in Haiti, Jamaica, and Martinique among several others (Petreakovsky et al. 2014). Moreover, the islands of Trinidad and Tobago have the highest incidence of Leptospirosis in the Caribbean (Pulido-Villamarin et al. 2014). However, incidence and prevalence of Leptospirosis are largely underestimated throughout tropical environments especially since co-infections with dengue and malaria have been documented and can complicate the gathering of accurate epidemiological data (Cestero-Rivera 2006; CDC 2012; Sharma et al. 2014; Pérez Rodríguez et al. 2014; Durski 2014; Sharp et al. 2016). Since Leptospirosis is commonly misdiagnosed in tropical regions, the actual prevalence very likely is much higher than reported prevalence figures.

Leptospirosis was first suspected to be present in Puerto Rico by the Department of Public Health in 1918, and later *Leptospira* presence was confirmed in 1939 (Alexander 1963). Although cases of Leptospirosis are now documented throughout Puerto Rico, current published data for this island are limited, but reported incidence has

increased over the past decade (CDC 2012). Livestock workers are among the most likely groups of people that could contract Leptospirosis in Puerto Rico (CDC 2012). Furthermore, the dairy industry in Puerto Rico is among the top grossing agricultural industries with up to 25% of the agriculture-related income; therefore the dairy industry is historically the most important agricultural commodity in this island (USDA 2014; Gruebele and Barahona 1974). For these reasons, assessing the risk of humans and cattle potentially contracting *Leptospira* on rural farm areas of Puerto Rico is important to assess measures to reduce transmission.

Puerto Rico does not possess any native terrestrial mammals that are known to act as a reservoir for *Leptospira*. However, by the 19th century four species had been introduced and are now ubiquitous throughout the island. These include two rat species (*Rattus rattus* and *R. norvegicus*), mice (*Mus musculus*), and the small Asian mongoose (*Herpestes auropunctatus*). All of these species are known *Leptospira* reservoirs in other locations (Desavars 2011; Webster et al. 2016). Animal surveys for *Leptospira* in Puerto Rico were last conducted in the late 1940s and early 1950s but little research has been conducted since this time. Positive specimens in a previous study in Puerto Rico identified a 37% prevalence (n = 63) in *R. rattus*, 40% prevalence (n = 10) in *R. norvegicus*, 48% prevalence (n = 27) in house mice, and 20% prevalence (n = 55) in small Asian mongooses (Alexander et al. 1963). In another study, Rust (1948) found a 39% prevalence (n = 59) in both rat species. Both of these studies were limited to the urban area of San Juan, and to date, there are no studies that address *Leptospira* prevalence in animal reservoirs in any rural area of Puerto Rico. In other regions, rural areas tend to have a higher infection risk than urban areas, likely due to the presence of

outdoor water bodies and the close proximity to animal reservoirs (Gheim et al. 2007; Gongora et al. 2008). Without general knowledge of what species are primarily responsible for maintaining *Leptospira* in both rural and urban environments, it is difficult to create management plans that will effectively reduce the risk of cattle and people contracting Leptospirosis.

Surveillance and management of *Leptospira* in animal reservoirs is a key element for implementing efficient protocols to control Leptospirosis outbreaks (Reperant 2010; CDC 2012; Jobbins et al. 2013; Mendes and Moraes 2014). One of the most effective methods of managing zoonotic disease outbreaks is managing the wildlife reservoirs responsible for spread. Invasive and pest (i.e. commensal rodents) species are of particular concern because they tend to readily adapt to human activity and urban settings, which places them in closer proximity to humans (Reperant 2010; McKinney 2008). A first step in preventing the continued spread of *Leptospira* is delineating the mechanisms by which the bacteria are dispersed and maintained.

The objective of the current study was to provide data on the prevalence of *Leptospira* in rural farms in Puerto Rico. Owing to the differences in the landscape and environmental conditions throughout Puerto Rico, I expected a heterogeneous distribution of *Leptopsira* species in mammal reservoirs across the island. Additionally, since *Leptospira* prevalence is influenced by water bodies and proximity to farm animals, there was a high likelihood that infected mammal reservoirs would be located closer to certain landscape features on the farms, such as stock ponds and milking areas, than non-infected individuals.

2. METHODS

Study Sites

Figure 1: Map of Puerto Rico. All farm locations sampled for this study are indicated with a black star.

I trapped mongooses and rodents on cattle farms from five municipalities in Puerto Rico during the summers of 2014 and 2015. Municipalities sampled for 2014 included dairy cow farms in Lajas (18.001189ºS, -67.042908ºW), Isabela (18.46116 ºS, -67.05652 ºW), San Sebastián (18.378265 ºS, -67.022423 ºW), and Naguabo (18.238525 ºS, -65.719208 ºW). During 2015 the same municipalities were sampled along with a beef cattle farm in Sabana Grande (18.036125 ºS, -66.931173 ºW) (Figure 1).

Lajas and Sabana Grande are both located in the southwestern Caribbean Sea side island and have a tropical savannah climate. Sampling sites in San Sebastián and Isabela are on the northeastern Atlantic Ocean side of the island and have a tropical rainforest

climate. Naguabo represents the coastal east side of the island and receives the most amount of rain of the five farms in part due to its close proximity to El Yunque rain forest. Owners of the sampled farms volunteered their farms to be included in this study.

Sample Collection

Two trap types were used in the field including small Sherman live traps (7.62 cm x 8.89 cm x 22.86 cm) (H.B. Sherman Traps, Inc., Tallahassee, FL, USA) baited with rolled oats, and medium Tomahawk Live Traps© (50.8 cm x 17.78 cm x 17.78 cm) (Tomahawk Live Traps, Hazelhurst, WI, USA) baited with tuna fish. Transects were established as either one line of 40 or two lines of 20 traps. Tomahawk traps were placed approximately 15 – 20 meters apart and Sherman traps were placed approximately 2 – 5 meters apart depending on the habitats sampled, that included ecotones, grasslands, cattle pastures, riparian zones, and around human dwellings present at each site. The positions of captured animals were recorded with a GPS unit (Garmin Montana 650, Garmin Corp., Kansas City, KS, USA). I actively checked the Tomahawk traps for captures throughout diurnal hours, three times a day (early morning, midday, and evening) to target mongooses and left the Tomahawk and Sherman traps open overnight to target rodents. Animal processing using sterile equipment occurred in the field immediately after capture. Animals were euthanized by cervical dislocation after first being rendered unconscious with isoflurane. Weight and measurements of individuals were taken along with tissue samples that included kidneys, a liver fragment, the GI tract, heart, and lungs. During summer 2014, tissue samples were stored in 70% ethanol (EtOH) and transferred to 95% EtOH at the end of the field season. During summer 2015, kidney samples were stored in 95% EtOH and kept cool at approximately 4°C throughout the field season. In

total, 312 animals were collected in summers 2014 and 2015. I tested renal tissues for the presence of pathogenic strains of *Leptospira*. Renal carriage is an integral part of persistent Leptospirosis incidence since carrier animals distribute *Leptospira* from the kidneys into the environment via urination (Adler et al. 2010). The Institutional Animal Care and Use Committee (Protocol #0514_0303_07) at Texas State University (San Marcos, Texas, USA) approved all field and sample collection methods.

Dogs are also a likely contributor to *Leptospira* maintenance in Puerto Rico (Ferrington and Sulzer 1982). Even though sampling domestic animals was beyond the scope of this study I conducted observations at each farm in regard to the presence of dogs at these premises. To qualitatively assess the potential effect of the presence of dogs on *Leptospira* prevalence, I used opportunistic sampling methods to approximate the number of dogs present at each farm. For this sampling, I used personal observation and anecdotal accounts from the farm owners.

Genetic analyses

DNA extractions from kidneys were performed using Qiagen DNeasy® Blood and Tissue Extraction Kits following the manufacturer's instructions (Qiagen Inc., Valencia, CA, USA). All extractions were performed following BSL II safety protocol for infectious disease. DNA extracts were stored frozen at -18°C until *q*PCR assays were performed. I used an Applied Biosystems StepOne Plus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) to test for the presence of pathogenic *Leptospira* bacteria in the tissue samples using *q*PCR TaqMan assays and targeting the *LipL32* gene. *LipL32* codes for a lipoprotein located on the outer membrane of pathogenic species of *Leptospira*. TaqMan probes utilize a fluorescent reporter dye on

the 5' end of the sequence that allows for higher detection compared to end-point PCR. I used StepOne Software v2.3 to analyze the results collected from the *q*PCR assay. As a positive control I used DNA of the *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130, provided by Dr. Albert Ko from the Yale School of Public Health. I used the UltraClean® DNA Purification Kit Cat. #12100-300 (MO BIO Laboratories, Carlsbad, CA) to extract the positive control DNA and a Peltier Thermal Cycler 200 (MJ Research, Cambridge, MA) to validate the positive control with end-point PCR amplification.

The volume of each reaction totaled 25µl (Appendix A). I used a 96-well reaction plate with all samples run in duplicate with detection in one sample constituting positive identification (Appendix A). Cycle holding stages were at 95°C for 20 seconds, then 40 cycles at 95°C for three seconds and 60°C for 30 seconds. Assays were only considered valid if the negative control did not show an amplification signal. Samples with cycle threshold (Ct) values less than or equal to 38 were considered positive and samples with Ct values greater than 38 were considered negative (Stoddard et al. 2009). I used an Applied Biosystems Genetic Analyzer 3500xL (Applies Biosystems) to determine the *Leptospira* species of a subset of individual animal specimens chosen from the positive samples. The sequence amplicon from a partial *LipL32* gene was used to compare the samples from this study to known reference sequences in GenBank® (National Center for Biotechnology Information, U.S. National Library of Medicine). I chose samples that showed low Ct values (i.e. high pathogen loads) to increase the likelihood of successfully generating DNA sequence data. Since *Herpestes* and *Rattus* spp. samples contained low

loads of *Leptospira*, I could only sequence *Mus* samples (Appendix A). In total I was able to sequence 45 *Mus* specimens, which included individuals from all five farms.

Statistical Analyses

I estimated crude prevalence among and within species and farms and calculated Jeffreys confidence intervals using R with the "prevalence" package v.0.4.0. Jeffreys confidence intervals are recommended for proportional data (bounded between 0 and 1) and are appropriate for both small and large samples sizes. I used an alpha value of 0.05 for significance assessments (Brown et al. 2001). I conducted a chi-squared test to determine if there were differences in prevalence among the farms. I analyzed the distribution of *Leptospira* across the landscape with SaTScan v9.4.2 software to assess whether positive samples were significantly clustered in any location.

Since both rainfall and anthropogenic factors, such as housing type and waste management, can influence the epidemiology of Leptospirosis, I also tested for a relationship between prevalence and landscape features on the farms (WHO 2011). I measured the distance of positive and negative samples against specific features on each farm to determine if prevalence was associated with these landscape features. I examined ponds, slurry ponds, milking areas of the farm, and human buildings. I identified these features while fieldwork was in progress then approximated location coordinates with Google Earth. Due to GPS system errors for some individuals, the sample size for this analysis totaled 293, so 6% (n = 19) of the total dataset was excluded. I used the GPS points of captured animals collected in the field and the haversine formula to calculate the distance of each individual to the chosen landscape features. I used Welch's t-test (unequal variances) to determine if the mean distances of positive and negative samples

to the landscape feature were significantly different from one another in terms of distance from individual landscape features.

3. RESULTS

Prevalence

Over the two trapping seasons 312 individuals were sampled. *Leptopsira* prevalence was approximately 38% (Jeffreys CI: 0.33 to 0.44) among all species. Positives for pathogenic *Leptospira* bacteria were found in all mammal species tested although the distribution of positive specimens among species and sites was highly heterogeneous (Table 1 and 2). Ct values for positive samples ranged from 19.93 to 37.55 (Appendix B).

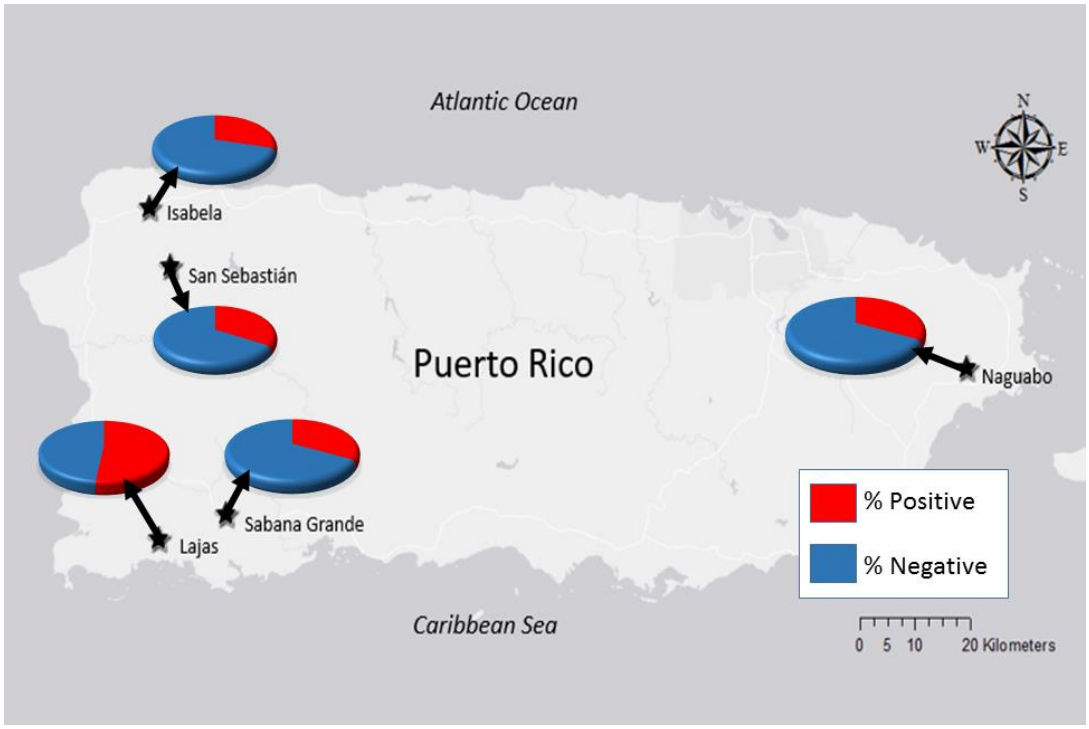


Figure 2: Geographic distribution. Distribution of *Leptospira* prevalence at five rural farms in Puerto Rico.

Table 1: Prevalence of *Leptospira* among sampled mammals. Table includes total sample size (N) as well as Jeffreys Confidence Intervals.

Species	N	# Positive	Prevalence	Jeffreys Confidence Interval
Mus musculus	124	73	0.59	0.50 to 0.67
Rattus rattus	94	33	0.34	0.25 to 0.45
Herpestes auropunctatus	89	12	0.13	0.08 to 0.22
Rattus norvegicus	5	1	0.20	0.02 to 0.63
Total	312	119	0.38	0.33 to 0.43

Table 2: Prevalence of *Leptospira* among sites and host species. Table includes total sample size (N) and Jeffreys Confidence Intervals for each site and species within each site.

Location/Host species	N	# positive	Prevalence	Jeffreys Confidence Interval
Lajas	89	46	0.52	0.41 to 0.62
Herpestes auropunctatus	29	6	0.21	0.09 to 0.38
Rattus rattus	23	14	0.61	0.41 to 0.79
Rattus norvegicus	0	--	--	--
Mus musculus	37	26	0.70	0.54 to 0.83
San Sebastián	97	33	0.34	0.25 to 0.44
Herpestes auropunctatus	21	3	0.14	0.04 to 0.33
Rattus rattus	39	10	0.26	0.14 to 0.41
Rattus norvegicus	3	0	0.00	0.00 to 0.44
Mus musculus	34	20	0.59	0.42 to 0.74
Naguabo	33	11	0.33	0.19 to 0.50
Herpestes auropunctatus	8	1	0.13	0.01 to 0.45
Rattus rattus	7	0	0.00	0.00 to 0.23
Rattus norvegicus	0	--	--	--
Mus musculus	18	10	0.56	0.33 to 0.76
Sabana Grande	36	12	0.33	0.20 to 0.50
Herpestes auropunctatus	7	0	0.00	0.00 to 0.23
Rattus rattus	5	2	0.40	0.09 to 0.79
Rattus norvegicus	0	--	--	--
Mus musculus	24	10	0.42	0.24 to 0.61
Isabela	57	17	0.30	0.19 to 0.42
Herpestes auropunctatus	24	2	0.08	0.02 to 0.24
Rattus rattus	20	7	0.36	0.17 to 0.57
Rattus norvegicus	2	1	0.50	0.06 to 0.94
Mus musculus	11	7	0.64	0.35 to 0.86

I successfully sequenced 45 *Mus* samples across all five sampling sites. I detected both *L. borgpetersenii* and *L. interrogans* which had an unequal distribution across sites (Table 3). All sequences showed 100% sequence similarity at 242 bp to known *Leptospira* sequences (U89709, KF922037) in GenBank®.

Table 3: Sequencing Results. *Leptospira* species identified in Puerto Rico using *LipL32* sequence data. All sequenced individuals were from the host species *Mus musculus*.

Site	**Number of Samples**	***L. borgpetersenii* (KF928037)**	***L. interrogans* (U89708)**
Sabana Grande	11	45.45 %	54.55 %
San Sebastián	10	00.00 %	100.0 %
Lajas	10	60.00 %	40 .00%
Naguabo	9	44.44 %	55.56 %
Isabela	5	40.00 %	60.00 %

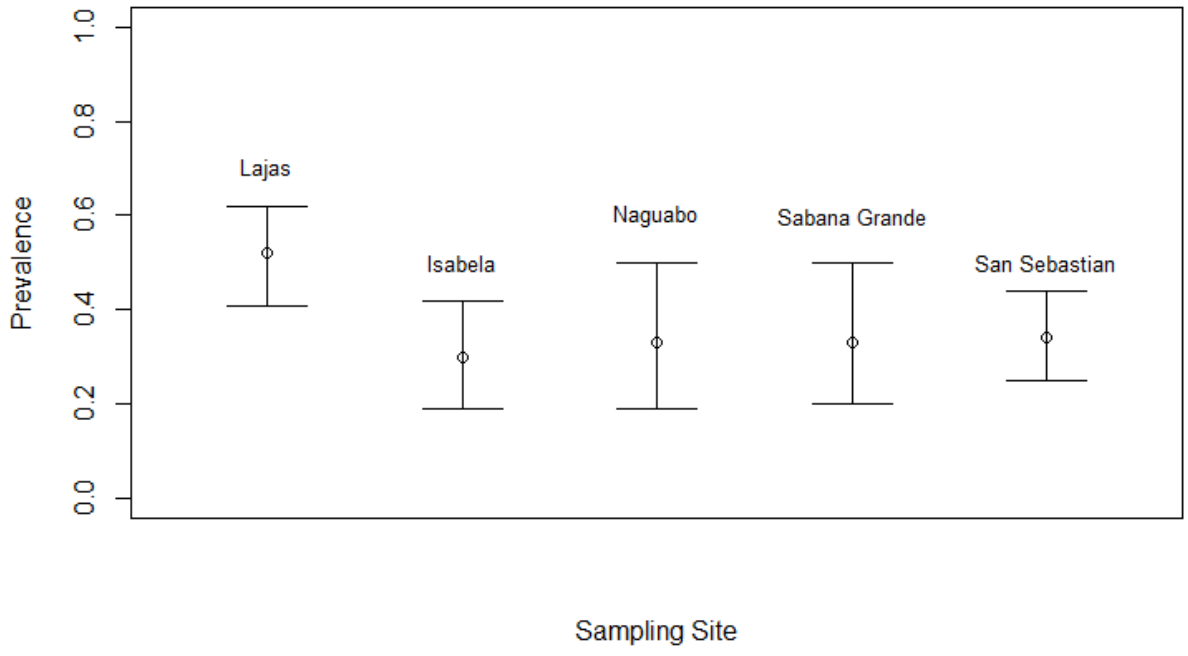


Figure 3: Prevalence comparison between farms. Prevalence of *Leptospira* for five farms in Puerto Rico. Error bars are Jeffreys Confidence Intervals.

Site comparison and landscape features

Results from the chi-squared test indicated that at least one site had a significantly different prevalence estimate when compared to other sites ($x^2 = 9.97$, df = 4, $p < 0.04$). Based on confidence interval overlap, I detected a higher prevalence of *Leptospira* at Lajas. Sabana Grande, San Sebastián, Naguabo, and Isabela all had a similar prevalence of *Leptospira* (Figure 3). Spatial associations of infection to landscape features were significant in Naguabo for all chosen landscape features **(**including a human dwelling, dairy cow milking area, and a pond) (Table 4). At Sabana Grande, mean distance of positive animals were closer to a human dwelling than mean distance of negative samples (Table 4).

SaTScan using a Bernoulli model identified four clusters within the studied farms but none were statistically significant (i.e., spatial grouping of positive animals could not be distinguished from a random clustering). However, a cluster identified at the Lajas farm approached significance (Figure 4 and Table 5). This cluster was located in a field in close proximity to the milking area (0.14 km SE) and a building (0.15 km SW). Dog presence was recorded at all five farm locations and Lajas also possessed the largest number of stray dogs present in close proximity to the farm (Table 6).

Table 4: Landscape Features. Results from Welch's t-test assays to determine if *Leptospira* prevalence in wild mammal reservoirs was associated with particular landscape features of individual farms in Puerto Rico. Bold figures denote significant results.

Location	Feature	Avg. Negative (m)	Avg. Positive (m)	Df	T	P
Isabela	Horse Stables	565.04	571.06	29.23	-0.05	0.96
Isabela	Milking area	520.26	534.54	29.45	-0.13	0.90
Isabela	Slurry Pond	524.93	559.57	29.94	-0.33	0.74
Isabela	Stock Pond	601.45	620.19	29.23	-0.13	0.89
Lajas	Human dwelling	2043.12	2237.99	80.95	0.70	0.48
Lajas	Human dwelling	1339.47	1038.90	80.81	-0.97	0.34
Lajas	Milking area	1390.05	1076.59	80.84	-0.97	0.34
Lajas	Pond	1646.12	1466.84	79.87	-0.68	0.50
Lajas	Wetland	1452.45	1174.42	80.48	-0.85	0.39
Naguabo	**Human dwelling**	**278.93**	**126.35**	**23.94**	**2.60**	**< 0.02**
Naguabo	**Milking area**	**234.42**	**100.71**	**24.00**	**2.53**	**< 0.02**
Naguabo	**Slurry Pond**	**254.38**	**128.19**	**23.95**	**2.44**	**< 0.05**
Sabana Grande	**Human dwelling**	**337.88**	**133.45**	**28.98**	**-2.28**	**< 0.05**
Sabana Grande	Stock Pond	450.78	314.50	28.90	-1.37	0.18
San Sebastian	Milking area	392.33	346.09	61.70	-0.85	0.40
San Sebastian	Wetland Pond	454.29	537.13	69.53	1.10	0.27
San Sebastian	Slurry Pond	414.85	380.96	60.44	-0.67	0.50

Table 5: SaTScan Results. Cluster discovered using a Bernoulli model.

	Site	Radius	Expected	Observed	p
Cluster 1	Lajas	0.029	5.49	12	0.07
Cluster 2	Sabana Grande	0.024	1.57	4	0.95
Cluster 3	Naguabo	0.086	1.57	4	0.95
Cluster 4	San Sebastián	0.029	2.75	6	0.97

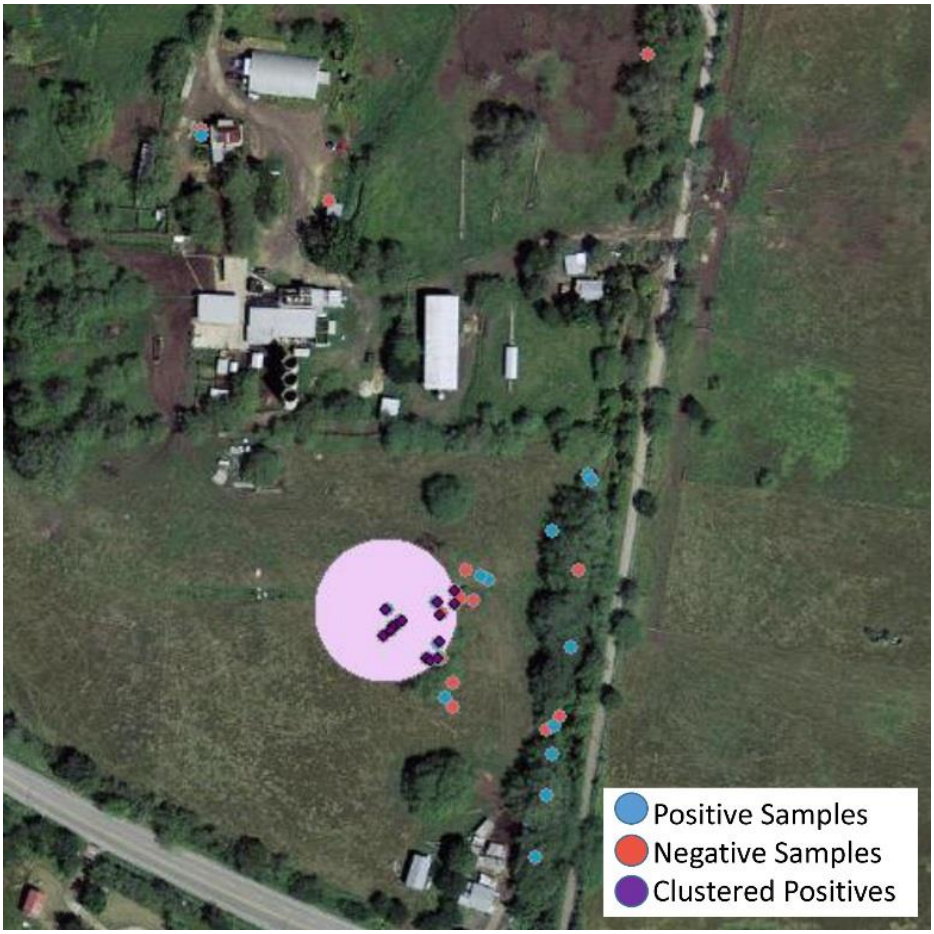


Figure 4: Results from SaTScan. Potential cluster of samples positive for *Leptospira* identified at Lajas. Structures in view include the milking area and a human dwelling.

Table 6: Dog presence on sampled farms. Number of stray dogs observed at each farm over the course of the field season. Pet dogs included personal pets and working dogs present at the farms while stray dogs included those running at-large within the farm perimeter.

Location	Pet	Stray	Total
Lajas	16	9	25
Isabela	4	0	4
Sabana Grande	2	2	4
Naguabo	1	2	3
San Sebastian	2	0	2

4. DISCUSSION

Leptospirosis is a threat to the health of people and cattle in Puerto Rico. All four mammal species caught on the farms tested positive for pathogenic *Leptospira*. However, sample size for *Rattus norvegicus* was very low (n = 5). The low sample size is likely due to *R. norvegicus* preferring urban areas over rural areas (Feng et al. 2014), and hence not being well-targeted by my trapping design. Nevertheless, this species is likely a relevant reservoir for *Leptospira* in Puerto Rico as other studies have found relatively high prevalence of *Leptospira* in *R. norvegicus* from other locations (Desvars et al. 2013; Strand et al. 2015). For example, a longitudinal survey in France reported approximately 44% prevalence of *Leptospira* in *R. norvegicus* (Ayral et al. 2015). Although previous research has concluded that rats in general are the most important reservoir for *Leptospira*, results from this study suggests a high likelihood that *Mus musculus* plays a more important role as reservoir in Puerto Rico since prevalence was higher in this species than in both rat species (Table 1 and 2) (Sumanta et al. 2015). This was true of overall prevalence of *Leptospira* as well as prevalence at each individual farm. However, since sequencing data currently includes only *Mus musculus* samples, there is still a possibility that prevalence was over reported in this study. Research is currently in progress to confirm the presence of *Leptospira* species in *Herpestes* and *Rattus.*

Two species of *Leptospira* were discovered throughout sampled locations in Puerto Rico. These included *Leptospira interrogans* and *Leptospira borgpetersenii*. Interestingly, this is the same combination of *Leptospira* species that is known to persist in urban rat populations in Malaysia (Benacer et al. 2016). *L. interrogans* possesses over

200 serovars and it has a global distribution. Transmission of *L. interrogans* readily occurs through contact with surface waters since this species is capable of quickly adapting to changes in the environment (Cosson et al. 2014). *L. borgpetersenii* serovar Hardjo is often reported in cattle at other locations since cattle are a maintenance host for it (Cortese et al. 2014; Salgado et al. 2014). *L. borgpetersenii* is most frequently thought to infect through direct transmission, because it is not genetically well suited to survive in surface waters (Cosson et al. 2014). The identification of specific serovars for each *Leptospira* species in both cattle and humans was beyond the scope of this study so more research is needed in this area.

At almost all sites, there was nearly a 50% split for *Leptospira* species presence except for San Sebastián. This site was unique in terms of *Leptospira* species prevalence as *L. interrogans* constituted 100% of the samples that were sequenced and *L. borgpetersenii* was absent (Table 3). One possible explanation for this is that *L. borgpetersenii* only occurs in small numbers in this area; therefore, a larger sample size may be needed to detect its presence. Upon examining the landscape, there were no apparent features that would ecologically isolate this location from the other study sites. For example there were not large rivers, mountain ranges, or forested areas that would prevent the distribution of other *Leptospira* species to the farm in San Sebastián. Since *L. borgpetersenii* is well suited for direct transmission, perhaps the management strategies on this farm discourages transmission of *L. borgpetersenii* between individual cows. Climate may also influence *Leptospira* prevalence. In Colombia the prevalence of *Leptospira* is correlated with a climate gradient (Astudillo et al. 2012). For future

research, it will be interesting to sample a gradient across the island of Puerto Rico to determine if there is a spatial pattern to the distribution of *Leptospira* species.

Lajas was found to have the highest prevalence of *Leptospira* among all sampled farms. This highlights the finding of the potential disease spatial cluster at the Lajas farm, which was located only 145 meters away from the milking area. Over half of the animal reservoirs tested were positive for renal carriage of *Leptospira* therefore it is more likely that cattle and farm workers are coming into contact with the bacteria when compared to the other farms included in this study. Compared to the other farms surveyed in this study, Lajas had the greatest need for rodent and mongoose to help curb the risk for Leptospirosis transmission among susceptible species. Furthermore, if the prevalence of *Leptospira* continues to increase Lajas could serve as a source for surrounding areas, making it more difficult to control Leptospirosis on broader scale.

There are also several other factors that may contribute to the prevalence of *Leptospira* that are not included in this study. One important aspect not fully covered in this study is the presence of dogs, which play a major role in Leptospirosis transmission (Ayral et al. 2014; Suepaul et al. 2014; Aswal et al. 2015; Siuce et al 2015). Throughout the island, stray dogs are commonplace and oftentimes packs of wild dogs were seen roaming the sampled farms. Compared to all the other farms, Lajas had the greatest number of dogs (n = 25) on or adjacent to the areas surveyed for *Leptospira* as well as the highest prevalence rate among animal reservoirs. On the Lajas farm, some stray dogs (n = 3) were even seen roaming into the milking areas and were within close proximity to the cattle themselves. This interaction lends itself to a higher likelihood of transmission of *Leptospira* between the two animal species, especially since dogs act as reservoirs for

serovars that infect cattle (Bahari et al. 2011). A previous study in Población de Villavicencio, Columbia found that three associated elements increased the risk factor for *Leptopsira* infection, these included owning a pet dog, living/working in a rural environment, and coming into contact with rodents while working (Góngora et al. 2008). This potentially indicates that the dogs might be a more competent reservoir for *L. borgpetersenii* than rodents and mongooses. This also highlights the finding that *L. borgpetersenii* was absent in San Sebastián as there was also an absence of stray dogs and a low number of pet dogs. However surveys are needed on both cattle and dogs before this potential interaction can be assessed in Puerto Rico.

Naguabo was the only site from this study for which the landscape-feature hypotheses were supported. This relationship could likely be due to the geographical features of this farm. The farm is located in the valley of a mountainous area in close proximity to the El Yunque National Rainforest. Naguabo experiences an average rainfall of approximately 2134 mm, which is higher than all of the other sampling sites (Appendix B) (NOAA 2010). With heavy rainfall the bacteria and other pathogens present in the landscape are likely carried by runoff to structures located in areas with the lowest elevation of this farm. This is comparable to an urban slum area in Salvador, Brazil where houses that were in small valleys had higher *Leptospira* prevalence at the valley bottoms (Reis et al. 2008).

Future research should focus on surveying cattle from farms in Puerto Rico. Knowing the rate at which cattle are contracting the bacteria, and what serovars are primarily affecting them, will help to direct future serology studies. This will be necessary to create effective vaccine regimes that are specific for rural areas in Puerto Rico. This

type of approach was successful in Italy where a combination of vaccinations and antibiotics were effective in reducing the prevalence of *Leptospira* among cattle herds (Mughini-Gras et al. 2014). Unless it is known what serovars the cattle are actually contracting, it will be more difficult to understand how the ecology of *Leptospira* reservoirs are interacting with the space cattle utilize.

Along with additional and continued surveillance of *Leptospira*, it is also important that other abiotic factors are surveyed to test for a relationship with the presence of *Leptospira.* Neither rainfall nor temperature alone were correlated with prevalence (Appendix B). Testing the soils and waters at the farm locations will demonstrate the capacity for the bacteria to persist in the environmental conditions of Puerto Rico (Wójcik-Fatla et al. 2014; Saito et al. 2013). For example Isabela has a stock pond that is used to water a nearby plantain crop. If *Leptospira* is persisting in great quantities in the soils and waters then using the potentially contaminated water for irrigation potentially places an increased risk on the agricultural workers that work in the field for harvest, planting, and maintenance. Testing of the environment should not only include soils and the nearby ponds, but also the moist areas within the milking structures.

Based on the results from this research, all farms in the study should implement rodent control to reduce the risk of susceptible focal species (i.e. humans and cattle) contracting Leptospirosis. At Lajas mice, rats, and mongooses were captured in relatively large numbers compared to the other farms. Lajas likely needs increased proactive management of all animal reservoirs in order to avoid an increase in *Leptospira* activity. Since the prevalence of *Leptospira* at Lajas was higher than at the other sites and a potential cluster of positive individuals was identified, there is a high likelihood for

Leptospirosis outbreaks at this area in the near future. Management plans in Naguabo should include rodent and mongoose control during periods of heavy rainfall across the landscape as opposed to only around the farm structures. At Sabana Grande, more effective rodent control near the human dwelling is also needed since more positive individuals were located near this area. Fewer mongooses and rats were captured compared to the number of mice captured at Sabana Grande, so rodent control should primarily target mice while continually monitoring the presence of rats and mongooses to assess changes in the population. *R. norvegicus* was captured only in San Sebastián and Isabela which are both located on the northwestern Atlantic Ocean side of Puerto Rico so rodent control on these farms should include methods to target both rat species.

Overall this research stresses the importance of constant surveillance in order to improve the health of rural areas in Puerto Rico. Leptospirosis is increasingly problematic in Puerto Rico so the urgency of managing the spread of *Leptospira* in the environment is becoming a pressing issue (CDC 2012). The results of this research will be useful for guiding future studies since it establishes baseline data for the general prevalence of *Leptospira* species on both the rural east and west coasts of Puerto Rico.

APPENDIX SECTION

Appendix A

*q*PCR setup and results.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10^7	10^7	Sam 1	Sam 1	Sam 8	Sam 8	Sam 16	Sam 16	Sam 24	Sam 24	Sam 32	Sam 32
B	10^6	10^6	Sam 2	Sam 2	Sam 9	Sam 9	Sam 17	Sam 17	Sam 25	Sam 25	Sam 33	Sam 33
C	10^5	10^5	Sam 3	Sam 3	Sam 10	Sam 10	Sam 18	Sam 18	Sam 26	Sam 26	Sam 34	Sam 34
D	10^4	10^4	Sam 4	Sam 4	Sam 11	Sam 11	Sam 19	Sam 19	Sam 27	Sam 27	Sam 35	Sam 35
E	10^3	10^3	Sam 5	Sam 5	Sam 12	Sam 12	Sam 20	Sam 20	Sam 28	Sam 28	Sam 36	Sam 36
F	10^2	10^2	Sam 6	Sam 6	Sam 13	Sam 13	Sam 21	Sam 21	Sam 29	Sam 29	Sam 37	Sam 37
G	10	10	Sam 7	Sam 7	Sam 14	Sam 14	Sam 22	Sam 22	Sam 30	Sam 30	Sam 38	Sam 38
H	01	01	Neg.	Neg.	Sam 15	Sam 15	Sam 23	Sam 23	Sam 31	Sam 31	Sam 39	Sam 39

Appendix A Figure 1: Plate setup for *q*PCR reactions.

Appendix A Table 1: Master Mix ratios for *q*PCR reactions.

Reagent	**Amount per Reaction (µl)**
TaqMan	12.5
Nuclease-free Water	3.75
Forward Primer	1.25
Reverse Primer	1.25
Probe	1.25

Appendix A Table 2: Mean Ct values for all positive samples used in this study.

Sample	Mean Ct	Species
MBT44	34.18	*Herpestes*
MBT31	35.58	*Herpestes*
MBT75	35.62	*Herpestes*
MBT64	35.99	*Herpestes*
MBT84	36.16	*Herpestes*
MBT85	36.20	*Herpestes*
MBT74	36.21	*Herpestes*
MBT46	36.34	*Herpestes*
MBT35	36.58	*Herpestes*
MBT59	37.02	*Herpestes*
MBT40	37.35	*Herpestes*
MBT83	37.55	*Herpestes*
KMB65	19.93	*Mus*
KMB39	19.96	*Mus*
KMB26	20.39	*Mus*
KMB145	20.85	*Mus*
KMB21	20.85	*Mus*
KMB24	20.94	*Mus*
KMB76	21.39	*Mus*
KMB20	21.53	*Mus*
KMB85	21.57	*Mus*
KMB44	21.57	*Mus*
KMB176	21.83	*Mus*
KMB67	22.26	*Mus*
CMR3	22.58	*Mus*
KMB170	22.71	*Mus*
KMB177	22.86	*Mus*
KMB51	23.08	*Mus*
KMB169	23.10	*Mus*
KMB151	23.13	*Mus*
KMB79	23.13	*Mus*
KMB48	23.34	*Mus*
KMB40	23.46	*Mus*
KMB174	23.52	*Mus*
KMB18	23.57	*Mus*
KMB75	23.61	*Mus*
KMB31	23.66	*Mus*
KMB68	23.88	*Mus*
KMB50	24.04	*Mus*

Sample	Mean Ct	Species
KMB171	24.22	*Mus*
KMB27	24.22	*Mus*
KMB84	24.25	*Mus*
KMB28	24.51	*Mus*
KMB9	24.72	*Mus*
KMB116	25.03	*Mus*
KMB29	25.04	*Mus*
KMB105	25.15	*Mus*
KMB22	25.17	*Mus*
KMB159	25.22	*Mus*
KMB47	25.23	*Mus*
KMB46	25.49	*Mus*
KMB132	25.59	*Mus*
KMB30	25.85	*Mus*
KMB61	25.90	*Mus*
KMB8	25.94	*Mus*
KMB149	25.98	*Mus*
KMB178	26.15	*Mus*
KMB142	26.17	*Mus*
KMB120	26.19	*Mus*
KMB117	26.20	*Mus*
KMB104	26.40	*Mus*
KMB12	26.81	*Mus*
KMB45	26.84	*Mus*
KMB158	27.12	*Mus*
KMB140	27.13	*Mus*
KMB111	27.53	*Mus*
CMR7	27.54	*Mus*
KMB119	27.56	*Mus*
KMB32	28.12	*Mus*
KMB87	28.69	*Mus*
KMB98	28.81	*Mus*
KMB125	28.81	*Mus*
KMB43	28.92	*Mus*
KMB99	29.08	*Mus*
KMB150	29.12	*Mus*
KMB123	29.21	*Mus*
KMB25	29.58	*Mus*
KMB106	30.21	*Mus*

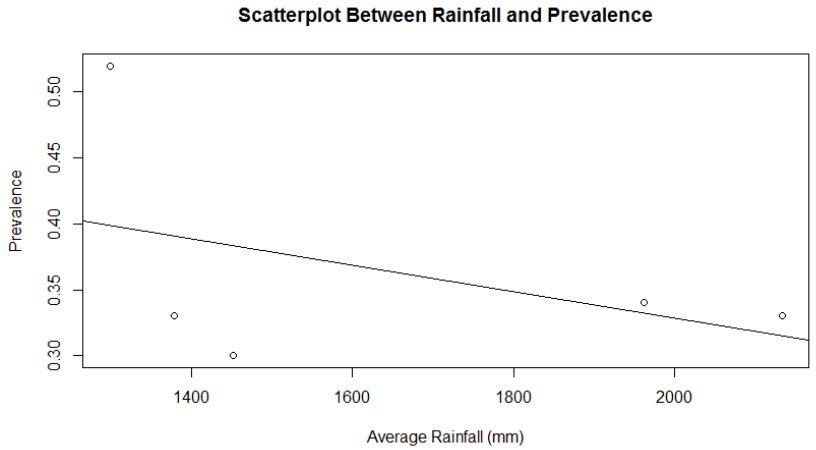
Sample	Mean Ct	Species
KMB92	30.51	*Mus*
KMB173	30.65	*Mus*
KMB131	31.25	*Mus*
KMB96	31.44	*Mus*
CMR2	34.17	*Mus*
CMR1	35.13	*Mus*
KMB154	37.05	*Mus*
KMB55	30.26	*Rattus*
KMB70	30.55	*Rattus*
CMR29	31.10	*Rattus*
CMR47b	31.64	*Rattus*
CMR38	31.65	*Rattus*
KMB3	31.66	*Rattus*
CMR46	31.76	*Rattus*
KMB37	31.94	*Rattus*
CMR36	32.41	*Rattus*
CMR37	32.50	*Rattus*
CMR44	32.79	*Rattus*
KMB139	32.84	*Rattus*
KMB10	32.96	*Rattus*
KMB133	33.89	*Rattus*

Sample	Mean Ct	Species
KMB6	34.76	*Rattus*
KMB6	34.76	*Rattus*
CMR26	35.22	*Rattus*
KMB57	35.29	*Rattus*
KMB59	35.42	*Rattus*
CMR21	35.63	*Rattus*
CMR30	35.72	*Rattus*
CMR28	35.76	*Rattus*
KMB16	35.88	*Rattus*
KMB58	35.91	*Rattus*
CMR27	36.07	*Rattus*
KMB7	36.24	*Rattus*
KMB2	36.29	*Rattus*
KMB138	36.33	*Rattus*
CMR20	36.40	*Rattus*
CMR13	36.48	*Rattus*
KMB17	36.54	*Rattus*
CMR34	36.55	*Rattus*
KMB38	36.79	*Rattus*
CMR41	37.49	*Rattus*

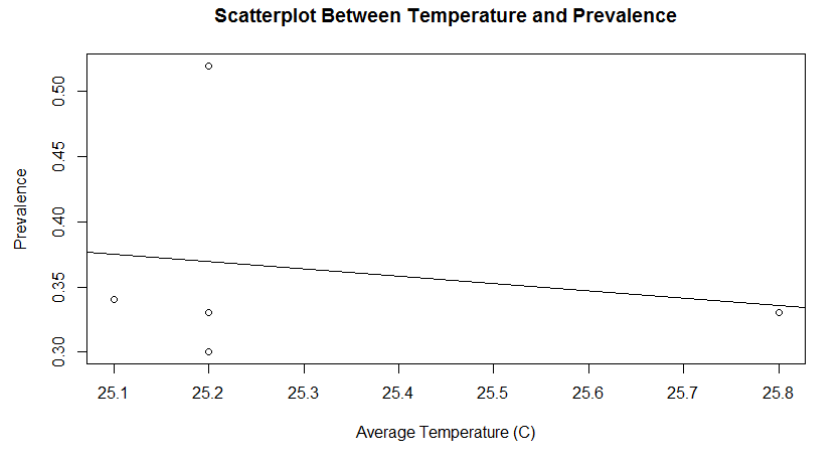
Appendix B

Rainfall and temperature compared with prevalence at each sampled farm. Prevalence was correlated with neither temperature (S = 26.88, p = 0.57) nor rainfall (S = 27.18, p = 0.55).

Location	Precipitation (mm)	Temperature (°C)	Altitude (m)	Prevalence
Naguabo	2134	25.8	17	0.33
San Sebastián	1962	25.1	73	0.34
Isabela	1451	25.2	60	0.3
Sabana Grande	1378	25.2	100	0.33
Lajas	1298	25.2	60	0.52



Appendix B Figure 1: Scatterplot of the average annual rainfall (mm) and prevalence of Leptospira in animal reservoirs from Puerto Rico.



Appendix B Figure 2: Scatterplot of average annual temperature (°C) and prevalence of Leptospira in animal reservoirs from Puerto Rico.

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