A cross-sectional epidemiological study of domestic animals related to human leptospirosis cases in Nicaragua

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Abbreviations

Domestic animals (DA), Ministry of Health (MINSA, from its Spanish acronym), Pan American Health Organization (PAHO), Microscopic Agglutination Test (MAT), confidence intervals (CI) and Chi square test (X^2).

ABSTRACT

Leptospirosis is one of the most extended zoonosis worldwide and humans become infected most commonly through contact with the urine of carrier animals, either directly or via contaminated water or soil. The aim in this study was to analyse the epidemiological behaviour of *Leptospira* spp., from domestic animals around the sites of human leptospirosis cases in Nicaragua, from 2007 through 2013. We report the results of a cross-sectional epidemiological study with a non-probability sampling of blood (n=3050) and urine (n=299) from Domestic Animals (DA) around the sites of human leptospirosis cases in Nicaragua. We analysed data obtained through Microscopic Agglutination Test (MAT), *in-vitro* culture, real time PCR and sequencing of lfb1 *locus*. Frequencies of 30.31% (95% CI: 28.66-31.95) and 15.38% (95% CI: 11.12-19.64) were obtained from serological test and from *in-vitro* culture, respectively. Although similar frequencies from serology test ($P \geq 0.05$) were found in DA species, *in-vitro* culture frequencies were significantly higher from bovine, equine and sheep ($P<0.05$) in comparison with swine and canine species. Ten serogroups of pathogenic *Leptospira* spp. were encountered, with the highest presence of Icterohaemorrhagiae serogroup 34.65% (95% CI: 29.35-39.94). We identified 7 samples homologous to *L. interrogans* species Pyrogenes serovar and 3 samples as *L. noguchii* Louisiana or Panama serovars by analysis of lfb1 sequences. We were able to establish a temporal and spatial correlation from DA and cumulative incidence of human cases. Therefore an effective epidemiological surveillance should be implemented with a specific control program toward DA in order to reduce human leptospirosis incidence.

Keywords: leptospirosis, domestic animals, Nicaragua, human cases.

1. Introduction

Leptospirosis is a serious threat to public health and is considered the most extended zoonosis worldwide. Humans usually become infected with leptospires through direct or indirect exposure to the urine of infected wild or DA, which frequently occurs in developing countries with poor sanitation where humans and animals often live in close proximity (Reller et al., 2014;Thiermann, 1984; Wasiński and Dutkiewicz, 2013).

The *Leptospira* life cycle involves haematogenous dissemination to the kidneys, shedding in the urine, persistence in the environment and acquisition of a new host (Haake and Levett, 2015). DA with subclinical infections as well as those who recover from the clinical disease become an important source of infection for humans and other hosts, since they continue shedding leptospires for a long time (Faine, 1957; Valverde et al., 2008). It is worth noting that transient leptospire shedding does occur during human infection, but human-to-human transmission is extremely rare (Haake and Levett, 2015).

Leptospirosis is a good example of the interaction between humans and their environment, and particularly human interface with DA (Lau et al., 2010). The countries most affected are those located in tropical and subtropical areas, where conditions such as temperatures, relative humidity, rainfall, structure, soil composition and pH are optimal for the pathogen to survive and multiply (Wasiński and Dutkiewicz, 2013; Schneider et al., 2012).

Leptospirosis has acquired increased worldwide attention following epidemics characterized by severe pulmonary haemorrhage syndrome without jaundice or renal complications. This syndrome was originally diagnosed in China and Korea, then in Nicaragua following hurricane flooding in rural areas (Lehmann et al., 2014; Park et al., 1989; Trevejo et al., 1998; Zaki and Shieh, 1996). In Nicaragua, several human leptospirosis outbreaks with end-stage pulmonary haemorrhage have been observed since

1995, most notably in 1998 and 2007 (Ashford et al., 2000; Trevejo et al., 1998). Leptospirosis behaves as an endemic disease in urban and rural areas, with average notification of 2 cases per week in the rainy season (May to November). This average might rise to 8 cases per week in the West Region (Departments of León and Chinandega), where outbreaks occur more frequently (Schneider et al., 2012).

In the present report, we analysed the epidemiological behaviour of *Leptospira* spp. from DA around the sites of human leptospirosis cases in Nicaragua from 2007 through 2013. This study constituted part of the national plan for the prevention and control of leptospirosis, supported and integrated by the MINSA, Ministry of Agriculture and Forestry (MAGFOR), National Autonomous University of Nicaragua-León (UNAN-León, from its Spanish acronym) and the PAHO.

2. Material and methods

2.1. Study setting

We conducted a cross-sectional epidemiological study from 2007 through 2013 in 16 out of 17 Departments of Nicaragua, in Central America, located between 10°45' and 15º 15' North latitude and 83°00' and 88º 00' West longitude. This region is frequently affected by natural disasters. The number of bovine reaches around 4 million heads reflecting the importance of cattle ranching in the country.

2.2. Sampling

A non-probability sampling was conducted upon MINSA notification of 406 human leptospirosis cases. Whole blood and urine samples were collected from DA found nearby the human patient's residence, which included bovine, canine, equine, sheep and swine species. We conducted sampling within a 30 meter diameter surrounding the patient's residence in urban areas, and up to 100 meters diameter in rural areas.

2.3. Serological testing by MAT

The MAT to detect specific leptospira's antibodies was performed using a standard microtiter method. We searched for antibodies against *L. interrogans* (*sensu lato*) using twenty-eight reference pathogenic strains along with Patoc 1 and ICF strains, which represented non-pathogenic species (Table S1).

2.4. Culture of *Leptospira* spp.

Approximately 2.5 ml of urine were filtered through a 0.45 μm nitrocellulose membrane, and 0.5 ml of the filtrate was inoculated into 3 ml of Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium (Difco, USA) supplemented with 5-fluorouracil (200 μg/ml) and enriched with 1% rabbit serum.

The cultured tubes were incubated between 28–30 °C for a 3-month period and at least once a week a 10 μl droplet from each culture was examined under dark-field microscopy for lepstospires presence.

2.5. Pathogenic leptospire identification

To determine whether these isolates were pathogenic or non-pathogenic leptospires, spirochetes from previously obtained cultures were reactivated in the EMJH medium, and after 7 days post-incubation, the media were centrifuged at 16,000 rpm for 10 minutes. The supernatant was discarded and 200 μl of re-suspended precipitate was used for DNA extraction, following the manufacturer´s instruction (UltraClean® bacterial® DNA Culture kit MO BIO, USA).

Molecular analyses were performed in the Molecular Diagnostic Laboratory of the Infectious Diseases and Epidemiology Unit, Faculty of Veterinary Sciences, University of Zaragoza (Spain). We performed the Real time PCR (qPCR) with primers previously described (Levett et al., 2005), LipL32 270F

120 (CGCTGAAATGGGAGTTCGTATGATT), LipL32 692R
121 (CCAACAGATGCAACGAAAGATCCTTT), which amplify a 423 bp fragment of
122 LipL32 gen, that is believed to be conserved in the pathogenic serovars. Samples were
123 run in triplicate, nuclease free water was included as a negative control and DNA from a
124 pure culture of Pomona strain was used as a positive control.

125 **2.6. Genotyping**

126 Isolates with results under 34 CT value were analyzed by sequencing of lfb1 *locus* (331
127 bp) (Perez and Goarant, 2010). DNA purifications were performed with the commercial
128 kit according to the manufacturer UltraClean® 15 DNA Purification MO BIO, USA.
129 The sequences of the *locus* of each strain were performed in the Laboratory Service
130 Sequencing and Functional Genomics, University of Zaragoza (Spain) and were aligned
131 using ClustalW 1.6 software. The history of evolution was inferred using the neighbor-
132 joining method (Saitou and Nei, 1987) and the analysis were conducted using the
133 MEGA6 software (Tamura et al., 2013).

134 **2.7. Data sources and Statistical analysis**

135 An epidemiological questionnaire was filled for each DA in the study. Relative
136 frequencies with 95% CI were calculated for statistical descriptive analyses. A X^2 or
137 Fisher exact test were applied to compare between variable categories. For the control
138 of confounding variables, stratifications and logistic regression models were applied.
139 Cohen's kappa index was calculated to compare the results of the MAT and direct
140 culture techniques (Agresti and Kateri, 2011). All data were recorded and analysed in
141 statistic software Package for Social Sciences Software (SPSS, version 19).

142 Our results were compared with human case cumulative incidence rate (per 10,000
143 inhabitants), as previously reported by other authors (Sánchez, 2012; Schneider et al.,

144 2012; Soto, 2012), and with epidemiological bulletins published on the MINSA official
145 website (http://www.minsa.gob.ni/).

146 **2.8. Ethics statement**

147 The procedures were performed by veterinary staff of the School of Veterinary
148 Medicine, UNAN-León. We requested in each case the acceptance and approval of the
149 patient through an informed written consent statement.

150 **3. Results**

151 A total of 3050 whole blood and 299 urine samples were collected from DA. Sampling
152 was conducted upon MINSA notification of 406 human leptospirosis cases from 2007
153 through 2013.

154 **3.1. Correlation of results obtained by different techniques**

155 Frequencies of seropositive samples by MAT and *in-vitro* culturing from DA were
156 30.31% (95% CI: 28.66-31.95) and 15.38% (95% CI: 11.12-19.64), respectively. A
157 Cohen's Kappa value of 0.176 (P=0.001) indicates a low correlation between these two
158 techniques. One third (31.10%) of cultured samples (95% CI: 25.8-36.51) resulted
159 positive in qPCR for LipL32. There were no statistical differences ($P \geq 0.05$) found
160 between species, sex, year, Departments as for serogroups or antibody titters in either
161 qPCR or MAT techniques.

162 León and Chinandega Departments showed the highest frequencies of positives in both
163 serology and culture tests. In serology (MAT) Chinandega Department showed the
164 highest frequency of positives with 33.40% (95% CI: 29.38-37.40), followed by León
165 with 33.30% (95% CI: 30.95-35.63), while in culture, samples from León had the
166 highest frequencies with 23.93% (95% CI: 18.58-29.32) followed by Chinandega with
167 23.40% (95% CI: 14.31-32.49).

Similar serology frequencies were found between DA species ($P \geq 0.05$). The frequencies of positives varied from 28.10% in goats and sheep to 35.10% in equine. However, the analysis of the culture results showed significant differences between species ($P < 0.05$), with lowest positive values (9.80%) in swine to highest values of 30.30% and 33.33% in equine and sheep, respectively.

The highest percentage of seropositive according to MAT were found in February and July, while the month of September had the lowest frequency ($P < 0.001$). The highest percentage of direct culture containing pathogenic leptospires corresponded to the month of April while July and December showed the lowest frequency of positives ($P < 0.001$).

3.2. Logistic regression model for seropositive DA

In the logistic regression model for seropositive DA, the spatial analysis revealed an OR=13.87 (95% CI: 1.08-178.29) for Jinotega Department versus Boaco Department. The OR values were even greater between Jinotega Department and those of Nueva Segovia and Managua, with values of 26.76 (95% CI: 1.98-3622.59) and 33.41 (95% CI: 1.08-1029.71), respectively. Results according to months factor, June has 87% less odds to be positive in MAT ($P < 0.05$) than February. The year 2013 showed significantly lower frequencies compared to 2007 ($P < 0.05$). Equine species has two times OR (95% CI: 1.11-4.66) compared to bovine species in being positive in culture, while the sex variable has no effect. In culture we found that positive animals sampled in the months of May and October had 96% and 97% less odds over the animals tested in April ($P < 0.05$). No such differences were observed ($P \geq 0.05$) between Departments, year, species and sex variables (Table 1).

3.3. *Leptospira* serogroups found in DA

Ten serogroups of pathogenic *Leptospira* spp., (*sensu lato*) were found using MAT. The Icterohaemorrhagiae serogroup was predominant with 34.65% (95% CI: 29.35-39.94), followed by Sejroe 25.23% (95% CI: 20.38-30.07), (Table 2).

Australis serogroup was the only one that did not show cross reactions with other serogroups. Icterohaemorrhagiae serogroup presented cross reactions with eight of the remaining nine serogroups, mainly with Sejroe serogroup (13/114), (Table 2).

We found differences ($P \leq 0.05$) in the distribution between serogroups in the Departments of Chinandega, Estelí, Granada, Jinotega and León. The Icterohaemorrhagiae serogroup was the most frequent in León, Sejroe serogroup in Chinandega, Canicola in Estelí and Pomona was the most prevailing one in Granada Department.

In the distribution of serogroups by year, significant differences were found between the years 2008, 2009, 2011 ($P<0.001$) and 2012 ($P<0.05$). In 2008, Pomona serogroup had the highest frequency, whereas in the years 2009, 2011 and 2012, Icterohaemorrhagiae serogroup was the predominant.

Icterohaemorrhagiae serogroup was present in high a percentage of 5 species analysed without specific association with any particular species ($P \geq 0.05$). The Canicola serogroup presented significant association with the canine species ($P<0.001$). Similarly, Louisiana serogroup was found to be associated with sheep, while the Pomona serogroup showed a significant association with swine ($P<0.001$). The Pyrogenes and Louisiana serogroups also showed a high frequency in canines ($P<0.05$). For Australis, Hebdomadis, Grippotyphosa, Sejroe and Panama serogroups, no significant associations were observed with any particular species.

The analysis of lfb1 sequences identified 7 samples homologous to *L. interrogans* species Pyrogenes serovar and 3 samples such *L. noguchii* Louisiana or Panama serovars (Figure supplementary 1). We found 3 of the 7 strains of *L. interrogans* in bovines and one was found in equine, ovine, canine and porcine species. Moreover, 3 strains of *L. noguchii* were found in equine, canine and bovine, respectively (Table 3).

4. Discussion

Cohen's Kappa value of 0.176 (P=0.001) indicates a poor correlation between (MAT) results and *Leptospira in-vitro* culturing from DA. This could be explained by the fact that most of the animals were asymptomatic, showing low or negative antibody titres but still secreting spirochetes in the urine. Hammond and Levett reported similar findings for the serological and urine *in-vitro* culture of *Leptospira* spp. from animals (Hamond et al., 2014; Levett, 2003).

The spatial analysis for the serology (MAT) and culture results from DA show that the highest frequencies corresponded to Chinandega and León Departments. Results from both techniques are consistent with the results reported in previously published studies by several authors (“From the Centers for Disease Control and Prevention. Outbreak of acute febrile illness and pulmonary hemorrhage--Nicaragua, 1995,” 1995; Zaki and Shieh, 1996) , regarding the second epidemic of haemorrhagic pulmonary syndrome affecting 2259 inhabitants in the Department of León (referred to as Achuapa fever).

The first study of leptospirosis in wild animals in Nicaragua was conducted by Clark, which reported the highest frequency of culture in the West Region and identified for the first time the strain 1011, known as Nicaragua serovar, from a weasel (*Mustela nivalis*) (Clark et al., 1966). The data surveillance of the MINSA, presented by Soto, showed that in 2011, 71% of cases of human leptospirosis in Nicaragua were found in the Departments of León and Chinandega. Therefore, our study is confirming that both

Departments are highly endemic areas for leptospires with persistent chronic infections in animals (Soto, 2012).

In the years 2007 and 2010 we observed high frequencies of seropositive animals as well as high frequencies of positives *in-vitro* cultures. A marked decrease in frequencies from both tests was observed for the year 2013. Our results are in agreement with those documented by PAHO ("Impacto del huracán Mitch en Centro América," 1998), showing the impact of floods in Central America on leptospirosis incidence. In fact, we found high frequencies of positive animals in both MAT and culture techniques through the years 2008 and 2010 when major floods happened in Nicaragua: hurricane Felix in 2007 and Tropical Storm Matthew in 2010 ("NASA - Hurricane Season 2010," 2015). Schneider documented an increase in the occurrence of human cases of leptospirosis in Nicaragua, associated with floods in 2007 and 2010 in endemic areas of the country (Schneider et al., 2012). Naranjos , also published outbreaks of leptospirosis in Honduras after flooding from Hurricane Mitch in 1998 (Naranjo et al., 2008).

Comparison of monthly serology and culture results showed little agreement. This mismatch could be explained, as proposed by Zuerner, by the presence of several molecular patterns of leptospires that are recognized by a variety of receptors in the hosts (Zuerner, 2015). Another plausible explanation could be that bacterial colonization is limited to specific organs, mainly the kidney, causing a poor immune response with low antibody titters while the animal is still shedding leptospires in its urine.

Culture frequencies in our study are the highest in heavy rainfall months. These results are in agreement with the results reported by Schneider where a significant increase of leptospirosis in humans is observed between weeks 37 to 45 in Nicaragua (Schneider et

al., 2012). Both studies highlight the importance of animal surveillance for the prevention of new human cases of leptospirosis.

The seropositive frequencies obtained in the present study are similar between the different animal species sampled. However a significant association was observed between bovine, equine and sheep species for *in-vitro* culture positive results. These higher frequencies may be due to the pH of urine in these species. Carnivore urine has lower pH that might affect the leptospires survival in the sample. Furthermore, the MAT results show that all species sampled had similar susceptibility to infection. This higher percentage of bovine shedding of cultivable leptospires justifies that 81% of the preventive treatments applied by health authorities in Nicaragua focused on bovine species, in order to minimize shedding spirochetes to the environment (Sánchez, 2012).

The logistic regression analysis for the *in-vitro* culture results suggested that the time variable ¨month¨ was an associated factor. Logistic regression analysis for seropositive frequencies revealed that not only time, but also variables such as Departments, years, and species were associated risk factors as well. These data reinforce the policy that animal sampling should best be performed in the months from June to August, as proposed by the Nicaraguan health authorities and the PAHO/WHO in Nicaragua in their preventive program. This timing would help to predict the likelihood of human leptospirosis case increases in the following months of September and October (Soto, 2012).

The reported high number of cross-reactions in our MAT technique results agree with other published studies (Katz et al., 2003; Levett, 2003), where the possibility of co-infection with multiple serovars is a factor that alters the results of the MAT technique in highly endemic areas. Cross-reactions by MAT technique could also be due to the presence of several common antigens in different leptospiras (Katz et al., 2003).

We found that Canicola, Louisiana and Pyrogenes serogroups are associated with canine species. This finding indicates that even though a given *Leptospira* serovar usually has preference for a given species, a particular animal species can be similarly infected by different ones, as described Vijayachari (Vijayachari et al., 2008). For instance, the results obtained for Icterohaemorrhagiae serogroup is a good example of a given serovar being capable of infecting several species with quite similar affinities. Our findings for Icterohaemorrhagiae serogroup are similar to those reported by Romero-Vivas where Icterohaemorrhagiae serogroup had the highest frequency in canines (Romero-Vivas et al., 2013). However, Calderon found that Grippotyphosa, Pomona and Tarassovi serogroups were the most common in canine species in Colombia (Calderón et al., 2014). Trevejo, in another study conducted in the municipality of Achuapa in Nicaragua, reported that 60% of positive samples from dogs had Canicola serogroup in areas close to human leptospirosis cases (Trevejo et al., 1998).

In 10 samples *locus* lfb1 was sequenced. The classification obtained by phylogenetic analysis identified two groups, one consisting of 7 samples with similarity to the species *L. interrogans* serovar Pyrogenes, while the other group was formed by 3 samples identified as *L. noguchii* Louisiana or Panama serovars. Our results in the phylogenetic method coincides with that described in other studies (Perez and Goarant, 2010). In this study the polymorphism found at this *locus* allowed rapid and proper classification of serovars.

The results of the molecular classification showed no serovar associated to years, animal species or Departments, suggesting that in Nicaragua leptospirosis is related to multiple serovars or serogroups. These results differ from a study conducted in 2010 in New Caledonia, where they found that the group of isolates based on the sequences of

313 the locus lfb1 were monophyletic and probably corresponded to a single serovar (Perez
314 and Goarant, 2010).

315 ## 5. Conclusions

316 This work confirms that leptospirosis is endemic in Nicaragua and its epidemiological
317 behaviour in DA is an associated risk factor related to human cases. Implementing
318 appropriate measures of control in DA must be considered when developing preventive
319 measures for human leptospirosis control.

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TABLES

Table 1: Logistic regression model of the results in MAT and culture of *Leptospira* spp., in DA considering the variables; Departments, months, years, species and sex

Variables	Value	MAT				Culture			
		Significance	OR	95% CI		Significance	OR	95% CI	
				Lower	Higher			Lower	Higher
Departments	Boaco*	0.010				0.948			
	Chinandega	0.656	1.68	0.17	16.40	0.840	1.22	0.17	8.74
	Chontales	0.377	3.69	0.20	66.89	0.998	0.00	0.00	.
	Estelí	0.138	5.87	0.57	60.87	0.251	4.35	0.35	53.47
	Jinotega	0.044[a]	13.87	1.08	178.29	1.000	1.53	0.00	.
	León	0.085	7.29	0.76	69.73	0.299	2.59	0.43	15.66
	Madriz	0.999	0.00	0.00	.				
	Managua	0.045[a]	33.41	1.08	1029.71				
	Masaya	0.318	3.27	0.31	33.63				
	Matagalpa	0.361	3.42	0.24	48.12	0.142	10.16	0.46	223.90
	Nueva Segovia	0.013[a]	26.76	1.98	362.59				
	RAAS	0.599	2.20	0.12	41.11	1.000	6.68	0.00	.
	Rivas	0.999	0.00	0.00	.	1.000	7.10	0.00	.
Months	February *	0.002							
	March	0.168	0.13	0.01	2.36				
	April	0.234	0.28	0.03	2.30	0.384			
	May	0.058	0.10	0.01	1.09	0.016[a]	0.04	0.00	0.54
	June	0.035[a]	0.13	0.02	0.87	0.999	0.00	0.00	.
	July	0.746	0.74	0.12	4.55	0.998	0.00	0.00	.
	August	0.103	0.21	0.03	1.37	0.052	0.04	0.00	1.02
	September	0.998	0.00	0.00	.	0.070	0.10	0.01	1.21
	October	0.056	0.11	0.01	1.06	0.025[a]	0.03	0.00	0.65
	November	0.542	0.53	0.07	4.10	0.147	0.10	0.00	2.26
	December	0.142	0.22	0.03	1.65				
Years	2009*	0.001							
	2010	0.142	4.49	0.60	33.34				
	2011	0.999	0.00	0.00	.	1.000			
	2012	0.234	0.63	0.30	1.34	0.999	0.00	0.00	.
	2013	0.000[b]	0.10	0.03	0.30	0.998	0.00	0.00	.
Species	Bovine*	0.124				0.986			
	Canine	0.164	1.48	0.85	2.59	0.557	1.44	0.43	4.80
	Equine	0.025[a]	2.28	1.11	4.66	0.999	0.00	0.00	.
	Ovine	0.647	2.16	0.08	57.64	1.000	1.62	0.00	.
	Swine	0.999	1.00	0.53	1.90	0.808	1.22	0.24	6.20
Sex	Female*								
	Male	0.204	0.75	0.48	1.17	0.362	1.65	0.56	4.88

433 **a**: Significant at 0.05 (95% CI) difference from the contrast value, **b**: Significant at 0.01

434 (99% CI) difference from the contrast value.

435 *: contrast value.

436

437 **Table 2:** Leptospires serogroups found in DA with positive reactions and cross-reactions

438 MAT

Serogrup/Serogrups		1	2	3	4	5	6	7	8	9	10	Frequency (%)	95% CI	
													Lower	higher
1	**Australis**	6	0	0	0	0	0	0	0	0	0	1.82	0.23	3.42
2	**Canicola**	0	25	0	0	8	0	0	1	4	2	12.16	8.47	15.84
3	**Grippotyphosa**	0	0	16	2	3	0	0	4	0	1	7.90	4.83	10.97
4	**Hebdomadis**	0	0	2	15	3	0	1	5	3	3	9.73	6.37	13.08
5	**Icterohaemorrhagiae**	0	8	3	3	69	1	2	8	7	13	34.65	29.35	39.94
6	**Louisiana**	0	0	0	0	1	2	0	0	0	0	0.91	0.19	2.64
7	**Panama**	0	0	0	1	2	0	0	0	0	0	0.91	0.19	2.64
8	**Pomona**	0	1	4	5	8	0	0	52	2	1	22.19	17.54	26.83
9	**Pyrogenes**	0	4	0	3	7	0	0	2	26	4	13.98	13.98	17.88
10	**Sejroe**	0	2	1	3	13	0	0	1	4	59	25.23	20.38	30.07

439

440 **Table 3:** Sequences analyzed by lfb1 *locus*

Samples	Serovars	Animal species	Departments	Years
0024	Pyrogenes	Bovine	León	2011
0045	Pyrogenes	Equine	León	2011
0087	Louisiana, Panama	Equine	León	2011
0202	Pyrogenes	Ovine	León	2011
0205	Pyrogenes	Bovine	León	2011
0212	Louisiana, Panama	Canine	León	2011
0273	Louisiana, Panama	Bovine	León	2011
0465	Pyrogenes	Bovine	León	2010
0485	Pyrogenes	Porcine	Chinandega	2010
0639	Pyrogenes	Canine	Matagalpa	2012

441

Table S1

Reference strains used with the MAT for diagnosis of leptospirosis in DA

Id	Species	Serogrups	Serovars	Strains
1	*L. interrogans*	Australis	Australis	Ballico
2	*L.noguchii*	Australis	Nicaragua	1011
3	*L. interrogans*	Autumnalis	Autumnalis	Akiyami A
4	*L. borgpetersenii*	Ballum	Castellonis	Castellon 3
5	*L. interrogans*	Bataviae	Bataviae	Swart
6	*L. interrogans*	Canicola	Canicola	Hond Utrecht IV
7	*L. weilii*	Celledoni	Celledoni	Celledoni
8	*L. kirschneri*	Cynopteri	Cynopteri	3522 C
9	*L. interrogans*	Djasiman	Djasiman	Djasiman
10	*L. kirschneri*	Grippotyphosa	Grippotyphosa	Moskva V
11	*L. interrogans*	Hebdomadis	Hebdomadis	Hebdomadis
12	*L. interrogans*	Icterohaemorrhagia	Icterohaemorrhagia	RGA
13	*L. interrogans*	Icterohaemorrhagia	Copenhageni	M20
14	*L. interrogans*	Icterohaemorrhagia	Copenhageni	Wijnberg
15	*L. interrogans*	Icterohaemorrhagia	Icterohaemorrhagia	Kantorowic
16	*L. borgpetersenii*	Javanica	Javanica	Veldrat Batavia
17	*L. noguchii*	Louisiana	Louisiana	LSU 1945
18	*L. weilii*	Manhao	Qingshui	L 105
19	*L.borgpetersenii*	Mini	Mini	Sari
20	*L. noguchii*	Panama	Panama	CZ 214
21	*L. interrogans*	Pomona	Pomona	Pomona
22	*L. interrogans*	Pyrogenes	Pyrogenes	Salinem
23	*L. meyeri*	Ranarum	Ranarum	ICF
24	*L. weilii*	Sarmin	Sarm111	Sarmin
25	*L.borgpetersenii*	Sejroe	Sejroe	M84

26	*L. interrogans*	Sejroe	Hardjo	Hardjoprajitno
27	*L. interrogans*	Sejroe	Wolffi	3705
28	*L. biflexa*	Semaranga	Patoc	Patoc 1
29	*L. santarosai*	Shermani	Shermani	1342 K
30	*L. borgpetersenii*	Tarassovi	Tarassovi	Perepelitsin

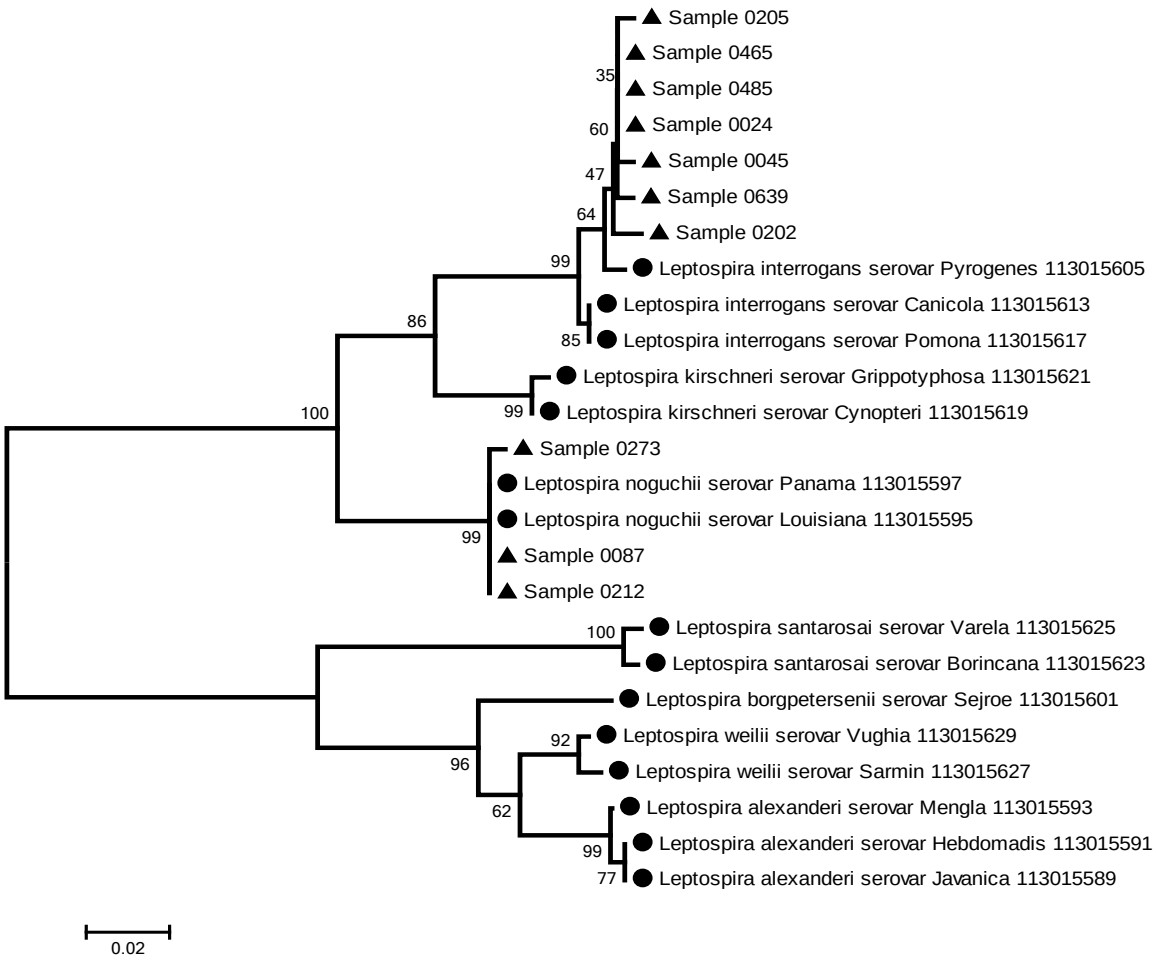

Figure Supplementary 1.
Phylogenetic tree for locus sequences lfb1

▲ Sample sequences; ● GenBank sequences

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.52232019 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 25 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 261 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

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