Outbreak of Intermediate Species *Leptospira venezuelensis* Spread by Rodents to Cows and Humans in *L. interrogans*–Endemic Region, Venezuela

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Leptospirosis is a common but underdiagnosed zoonosis. We conducted a 1-year prospective study in La Guaira State, Venezuela, analyzing 71 hospitalized patients who had possible leptospirosis and sampling local rodents and dairy cows. *Leptospira rrs* gene PCR test results were positive in blood or urine samples from 37/71 patients. *Leptospira* spp. were isolated from cultured blood or urine samples of 36/71 patients; 29 had *L. interrogans,* 3 *L. noguchii*, and 4 *L. venezuelensis*. Conjunctival suffusion was the most distinguishing clinical sign, many patients had liver involvement, and 8/30 patients with *L. interrogans* infections died. The *Leptospira* spp. found in humans were also isolated from local rodents; *L. interrogans* and *L. venezuelensis* were isolated from cows on a nearby, rodent-infested farm. Phylogenetic clustering of *L. venezuelensis* isolates suggested a recently expanded outbreak strain spread by rodents. Increased awareness of leptospirosis prevalence and rapid diagnostic tests are needed to improve patient outcomes.

Leptospirosis, one of the most common zoonoses worldwide, (*1*,*2*) is caused by *Leptospira* spp. In humans, its most severe, multiorgan, potentially fatal form is known as Weil's disease (*3*). *Leptospira* can also infect animals, such as cattle, sheep, cats, and dogs. Rodents are the reservoir for most *Leptospira* spp.; rodent kidneys can become colonized with *Leptospira* and chronically shed the bacteria in urine. Except for occupational or recreational exposure, leptospirosis generally occurs in residents of marginal, rodent-infested areas, often in coastal regions of tropical countries (*3*).

According to their ability to cause human disease, *Leptospira* bacteria were originally divided into fully pathogenic (P1), intermediate pathogenic (P2), and saprophytic or nonpathogenic (S1 and S2) subclades; this phylogenetic separation is confirmed by genome sequencing (*4*,*5*). The pathogenic species, most commonly *L. interrogans*, can cause leptospirosis and Weil's disease, but the role of intermediate species in human illness is unclear (*5*). Intermediate *Leptospira* spp. have been discovered by environmental sampling of soil and water (*5*), but they have also been found in animals and humans, where they are thought to cause only mild, self-limited illness without liver, kidney, or pulmonary involvement (*5*).

Leptospira infections are classically diagnosed by using the microscopic agglutination test (MAT) to detect *Leptospira*-specific antibodies, but diagnosis often requires comparing titers of acute and convalescent serum samples. Culturing *Leptospira* for a definitive bacteriologic diagnosis is difficult and takes weeks

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to months. Therefore, *Leptospira* bacteria are usually detected by PCR of blood or urine samples and identified by sequencing the amplified genes and comparing those sequences to known *Leptospira* spp. (*6*).

Venezuela is considered a moderate-incidence country for leptospirosis (*7*), but the true incidence is unknown because of a lack of clinical recognition of the disease and difficulties in laboratory diagnosis. To determine the presence of *Leptospira* spp., identify local strains, and evaluate leptospirosis incidence in Venezuela, we performed a prospective study in La Guaira, a small state on Venezuela's Caribbean coast. Although the study was conducted in 2010–2011 and reporting delayed because of Venezuela's economic situation, we believe the clinical leptospirosis data and epigenomic study of an intermediate *Leptospira* sp. outbreak remain relevant.

Methods

Ethics Approval

The Bioethics Commission of the Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela, approved the human study. The National Office of Biologic Diversity within the Venezuela Ministry for the Environment (Document 0264) and the Instituto Venezolano de Investigaciones Científicas Commission on Animal Bioethics approved the capture of rodents.

Study Area

We included patients with possible leptospirosis in La Guaira State, located on the northern Caribbean coast of Venezuela. La Guaira contains a shipping port and the nation's principal airport and has a population of ≈353,000. It is a beach resort for residents of Caracas but also contains low socioeconomic urban and rural areas. In the 1999 Vargas tragedy, mudslides destroyed much of the infrastructure of La Guaira (formerly Vargas State), causing thousands of fatalities.

Patient Selection

We visited Dr. José María Vargas Hospital during March 2010–March 2011 and Dr. Rafael Medina Jiménez Hospital during March–July 2010 and February–March 2011; visits were ≥2 times per week each. We reviewed diagnoses of new patients at admission and questioned hospital staff about new patients who had clinical symptoms suggestive of leptospirosis. Inclusion criteria were residence or place of work in La Guaira and an initial evaluation that included ≥1 sign or symptom of leptospirosis as described by the World Health Organization (*8*): fever >38°C with unknown etiology for ≤21 days, fever with renal failure (anuria, oligouria, or elevated creatinine), abdominal or muscle pain, icterus, conjunctival suffusion, hypokalemia or hyponatremia, hemoptysis or pulmonary hemorrhage, or an initial diagnosis of hepatitis or dengue. After patients voluntarily signed an informed consent form, we interviewed those patients and collected their clinical histories and places of residence. We also consulted the physician's notes. We excluded patients who were unable to complete the interview or provide adequate data. We enrolled a total of 71 patients from whom blood and urine specimens were obtained. Of those 71 patients, 38 had serologic tests for dengue and 39 for hepatitis A or B. Frozen serum samples from some patients were subsequently tested for hepatitis viruses A and B by PCR (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/30/8/23-1562-App1.pdf).

Leptospira Cultures

Leptospira were cultured at 28–30°C in liquid or semisolid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium with 10% supplement and 50–100 mg/mL of 5-fluorouracil for initial cultures (Appendix). All solutions and media were prepared according to the World Health Organization technical manual (*8*).

Rodent Capture

We set up Sherman aluminum traps in urban areas close to the residences of patients who were PCR positive for *Leptospira* (Appendix). The species of captured rodents were determined by amplifying and sequencing a subunit of the cytochrome c oxidase gene (*9*).

Cow Samples

We collected blood with and without EDTA anticoagulant from the caudal vein of 16 crossbred *Bos taurus* × *Bos indicus* (predominantly *Bos taurus*) dairy cows. Cows were 3–10 years of age and located on a farm in Miranda State, Venezuela, ≈30 km from La Guaira State (Appendix). We collected urine samples from the same cows after intramuscular injection of the diuretic furosemide (1 mg/kg). We cultured blood and urine samples and performed PCR for the *Leptospira* genes *rrs* (16S rDNA) and *lipL32*.

Passaging of Isolates in Hamsters

We intraperitoneally injected *Leptospira* isolates from second to fourth passages of liquid culture into 4-week-old male Syrian golden hamsters (*Mesocricetus auratus*). Sixteen days after injection, we euthanized the hamsters and removed and macerated the kidneys. We placed the kidney tissue into EMJH medium to sediment and then inoculated culture medium with the supernatant.

Molecular Detection of *Leptospira*

We amplified *lipL32* (*10*) and regions V3–V6 of the *rrs* gene from isolated DNA by using PCR (*11*) (Appendix Table 1). We purified the *rrs* gene amplification products (QIAGEN, https://www.qiagen.com), which were then sequenced by Macrogen (https://www.macrogen.com). We also sequenced the *lig* gene from a few specimens (*12*). We used the *L. interrogans* genes *pntA*, *sucA*, *pfkB*, *tpiA*, *mreA*, *glmU*, and *caiB* (*13*,*14*) for multilocus sequence typing (MLST). We performed variable-number tandem-repeat (VNTR) analysis of *L. interrogans* isolates as previously described (*15*).

MAT of Bovine Serum Samples

MATs were performed in the bacteriology laboratory of the Instituto Nacional de Investigaciones Agricolas according to 2003 Pan American Health Organization standards (https://www.paho.org/es/documentos/leptospirosis-humana-guia-para-diagnostico-vigilancia-control). MATs were considered positive when ≥50% of *Leptospira* bacteria were agglutinated.

Phylogenetic Reconstruction of *L. venezuelensis* Isolates

We used Velvet (*16*) for de novo assembly of genome contigs from sequencing reads of *L. venezuelensis* isolates. We used cow isolate 201502610 (GenBank Biosample accession no. SAMEA5168082) as a reference to map reads from the other *L. venezuelensis* isolates (Appendix).

Statistics

We performed statistical analyses of patient signs and symptoms and clinical test values by using Stata 13 (StataCorp LLC, https://www.stata.com). We did not adjust p values for multiple statistical testing.

Results

PCR and Cultures of Patient Specimens

Through twice-weekly visits to the 2 hospitals in La Guaira state over a 1-year period, we identified 71 patients who met the inclusion criteria (Appendix Tables 2, 3). We PCR amplified the *Leptospira rrs* gene from blood samples of 17, urine samples of 22, and both blood and urine samples of 2 patients. We also cultured *Leptospira* bacteria from blood samples from 13, urine samples from 20, and both blood and urine samples from 3 patients (Appendix Table 4). Using PCR amplification of *Leptospira rrs* in either blood or urine samples as confirmation of leptospirosis, the sensitivity of the *rrs* gene for diagnosing leptospirosis was 46% for blood and 59% for urine specimens; both sample types had 100% specificity. For *lipL32* PCR amplification, sensitivity was 41% for blood, 35% for urine, and 70% when both blood and urine samples were tested; all samples had 100% specificity. For blood cultures, sensitivity was 43%, and specificity was 100%; for urine cultures, sensitivity was 59%, and specificity was 97%; for either positive blood or urine cultures, sensitivity was 95%, and specificity was 97% (Table 1; Appendix Tables 4–6). The *rrs* gene was amplified from 2 patients who had negative *Leptospira* cultures: from a blood specimen of a patient with jaundice and from the urine of a patient who died of severe pulmonary disease.

Initial diagnoses were similar for patients in this study who had positive or negative *Leptospira* PCR and were most commonly dengue, hepatitis, icteric hemorrhagic syndrome, febrile syndrome, or unknown. Leptospirosis was listed as an initial diagnosis for 6 patients from whom *Leptospira* spp. were isolated and for 1 patient who had negative *Leptospira* cultures. Dengue

Table 1. Tests used for leptospirosis diagnoses in study of outbreak of intermediate species *Leptospira venezuelensis* spread by rodents to cows and humans in *L. interrogans*–endemic region, Venezuela*

Diagnostic test†	*rrs* PCR+, n = 37‡	*rrs* PCR–, n = 34‡	% Sensitivity (95% CI)	% Specificity (95% CI)	% PPV (95% CI)	% NPV (95% CI)	% Accuracy (95% CI)
rrs PCR							
Blood, +	17	0	46 (30–63)	100 (90–100)	100 (80–100)	63 (56–70)	72 (60–82)
Blood, –	20	34	NA	NA	NA	NA	NA
Urine, +	22	0	59 (42–75)	100 (90–100)	100 (85–100)	69 (61–77)	79 (68–88)
Urine, –	15	34	NA	NA	NA	NA	NA
Both, +	2	0	NA	NA	NA	NA	NA
Culture							
Blood, +	16	0	43 (27–61)	100 (90–100)	100 (80–100)	62 (55–68)	70 (58–81)
Urine, +	22	1	59 (42–75)	97 (85–100)	96 (76–99)	69 (60–77)	77 (66–87)
Either, +	35	1	95 (82–99)	97 (85–100)	97 (84–100)	94 (81–98)	96 (88–99)
lipL32 PCR							
Blood, +	15	0	41 (25–58)	100 (90–100)	100 (78–100)	61 (54–67)	69 (57–79)
Urine, +	13	0	35 (20–53)	100 (90–100)	100 (75–100)	59 (53–64)	66 (54–77)
Either, +	26	0	70 (53–84)	100 (90–100)	100 (87–100)	76 (65–84)	85 (74–92)

*Diagnostic values were obtained for *Leptospira* cultures and PCR of *Leptospira lipL32* and *Leptospira rrs* (16S rDNA) genes of blood and urine specimens from hospitalized patients. NA, not applicable; NPV, negative predictive value; PPV, positive predictive value; –, negative; +, positive.
†Test results are shown for patients who had *Leptospira* detected in blood, urine, or either blood or urine.
‡Total numbers of study patients who had the *Leptospira rrs* gene detected by PCR of either blood or urine samples.

was diagnosed in 3 patients and hepatitis in 4 patients who had positive *Leptospira* cultures; dengue was diagnosed in 5 patients and hepatitis in 4 patients who had negative cultures. *Leptospira* isolation was not correlated with seasonal variation in precipitation.

We compared PCR sequences of *rrs* with GenBank sequences by using BLAST (*17*). We identified 29 sequences as *L. interrogans*, 3 as *L. noguchii*, and 4 were 99% identical to the intermediate species *L. licerasiae* and *L. wolffi* (Appendix Tables 7, 8); genome sequencing showed those 4 isolates belonged to a novel intermediate species that we then named *L. venezuelensis* (*18*). The *lig* gene (*12*) was amplified by PCR from the urine of the culture-negative patient who died of pulmonary disease and was identified as belonging to *L. interrogans* by using BLAST. Patients who had positive tests for hepatitis or dengue and positive *Leptospira* blood or urine cultures all grew *L. interrogans* and were assumed to be co-infected. Serum samples from *L. venezuelensis*–positive patients were negative for hepatitis viruses A and B (Appendix Table 9).

Clinical Characteristics

Patients who had PCR-amplified *rrs* were more likely to have conjunctival suffusion, dyspnea, cough, hemoptysis, and myalgias (Figure 1; Appendix Tables 10, 11). Eight (27%) of the 30 patients who had *L. interrogans* infections died of their illness, whereas no deaths were recorded among the 34 patients who had no evidence of leptospirosis. Leptospirosis patients who died had more severe infections, with pulmonary and renal involvement, than did those who survived (Figure 2; Appendix Tables 12, 13). Patients who died of leptospirosis had more hemoptysis but less abdominal pain and myalgias and also had higher mean urea and creatinine levels, higher leukocyte counts, higher percentages of neutrophils, and lower percentages of lymphocytes than those who survived.

Cultures from Captured Rodents

To delineate reservoir hosts for *Leptospira*, we captured 45 rodents from 27 communities where patients who had positive cultures resided. We captured 30 *Mus musculus* mice and 11 *Rattus rattus* and 4 *R. norvegicus* rats. We amplified the *rrs* gene by PCR and cultured *Leptospira* from kidney tissue samples from all 45 rodents; 36 (80%) isolates were *L. interrogans*, 4 (9%) were *L. noguchii*, 3 (7%) were the intermediate species *L. fainei*, and 2 (4%) were *L. venezuelensis*. *L. interrogans* was isolated from all 3 rodent species, *L. noguchii* was isolated only from mice, and the intermediate species *L. fainei* and *L. venezuelensis* were only isolated from *R. rattus* rats.

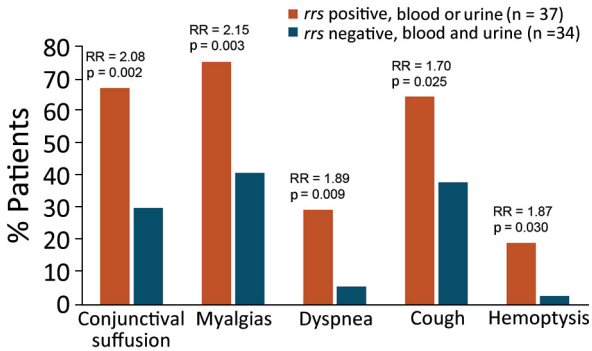

Figure 1. Distinguishing clinical features of hospitalized patients in study of outbreak of intermediate species *Leptospira venezuelensis* spread by rodents to cows and humans in *L. interrogans*–endemic region, Venezuela. The most statistically different clinical symptoms are shown for hospitalized patients considered to have leptospirosis according to positive PCR for the *Leptospria rrs* gene in either blood or urine specimens compared with those without leptospirosis according to negative *rrs* PCR in both blood and urine samples. PCR primers for *rrs* amplify a region of the gene encoding 16S rRNA that is highly conserved in *Leptospira* (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/30/8/23-1562-App1.pdf). One patient whose urine culture grew *L. venezuelensis* was *rrs* PCR negative, and leptospirosis was not diagnosed (Appendix Table 9). Comparisons of all clinical features with 95% CIs were also determined (Appendix Tables 10, 11). RRs and Pearson χ^2 test p values were calculated by using Stata 13 (StataCorp LLC, https://www.stata.com). RR, risk ratio.

Leptospira in Cows on Nearby Farm

Leptospira spp. are known to infect cattle. In a preliminary study, we performed MATs on serum samples from 48 cows on 8 small farms in adjacent Miranda State. We found *Leptospira*-specific antibodies against ≥1 *Leptospira* serovars in 2 animals from a single farm located ≈30 km from where the leptospirosis patients in this study resided. We then obtained blood and urine specimens from 16 cows randomly selected from that single farm and performed MATs against live antigens of 23 *Leptospira* reference strains; 9 samples agglutinated ≥1 serovar (Table 2; Appendix Table 14). Of those 9 cows, 8 had urine positive for *Leptospira rrs* by PCR; 2 urine samples had positive cultures of *L. interrogans*, and 7 had positive cultures of *L. venezuelensis*. The cows that had *L. venezuelensis*–positive urine had MAT titers of 1:400 to 1:800 against reference strain *L. interrogans* serovar Wolffi, serogroup Sejroe. The 2 *L. interrogans*–positive cows had high MAT titers for other serovars: 1:400 for *L. hebdomadis* (cow 5) and 1:400 for *L. mini* (cow 9). Cows 1 and 8 were negative according to MATs and *rrs* PCR of their urine, but their blood samples were *rrs* PCR–positive. Urine of cow 8 grew *L. interrogans*, whereas urine of cow 1 grew *L. venezuelensis* (Table 2; Appendix Table 14). Cow 11 had a MAT titer of 1:1,600 for *L. interrogans* serovar Bataviae and *rrs*-positive urine, but no *Leptospira*

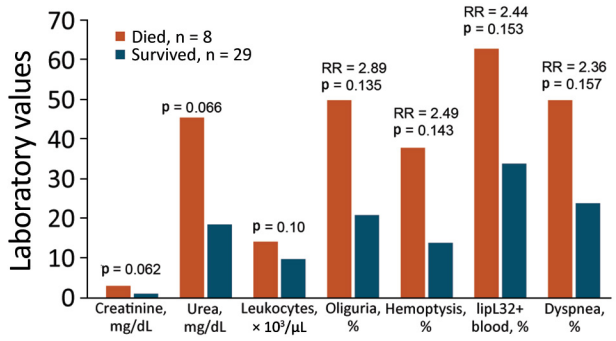

Figure 2. Clinical features most strongly associated with fatal outcomes in study of outbreak of intermediate species *Leptospira venezuelensis* spread by rodents to cows and humans in *L. interrogans*–endemic region, Venezuela. Clinical features are shown for hospitalized patients who had positive PCR tests for the *Leptospira rrs* (16S rDNA) gene in blood or urine and either survived or succumbed to their illness. Laboratory units of measure are indicated on the x axis for each bar. Comparisons of all clinical features with 95% CIs were also determined (Appendix Tables 12, 13, https://wwwnc.cdc.gov/EID/article/30/8/23-1562-App1.pdf). p values comparing creatinine, urea, and number of lymphocytes were obtained from Pearson χ^2 tests. p values comparing percentages of patients with oliguria, hemoptysis, *lipL32*, and dyspnea were obtained from 2-tailed *t*-tests. All statistical calculations were performed by using Stata 13 (StataCorp LLC, https://www.stata.com). RR, risk ratio.

spp. were isolated from either the blood or urine. *L. venezuelensis* isolates did not agglutinate with antiserum to common *L. interrogans* serovars, although antiserum to serovar Wolffi was not included.

Growth in Hamsters

We purified all *Leptospira* isolates by injecting early passage cultures into the peritoneal cavities of Syrian golden hamsters and performing necropsies 16 days after inoculation; 3 hamsters infected with *L. interrogans* and 1 infected with *L. noguchii* died before 16 days. We cultured aliquots of macerated kidney extracts from all inoculated hamsters and performed PCR to detect *Leptospira rrs*. In each case, sequences of *rrs* from the hamster kidney extracts were identical to the sequences from the corresponding original specimens and also the *Leptospira* cultured from those hamster kidney extracts.

Molecular Epidemiology of *L. interrogans*

Among the *L. interrogans* strains isolated from humans or rodents, 3 clusters had 7/7 identical MLST alleles; in each cluster, 2 patients resided in the same residential zone (Table 3). Of the 27 different MLST profiles, only 4 were present in the *Leptospira* PubMLST database (https://pubmlst.org/organisms/leptospira-spp), 2 of which (sequence types 27 and 50) were in clusters that had 7/7 identical alleles. Sequence types 20 and 37 were clustered with strains that had 6/7 identical alleles. VNTR clustering was not concordant with MLST clustering (Appendix Table 15). Two of the 3 *L. interrogans* strains isolated from cows had identical alleles in 4 VNTR loci (Appendix Table 16) but were not analyzed by using MLST.

New Intermediate Species of *Leptospira*

L. venezuelensis, isolated from 4 patients (Appendix Table 9), 2 rodents, and 7 cows, is located on the phylogenetic tree within the *Leptospira* intermediate pathogen or P2 subclade (5). Three of the 4 patients infected with *L. venezuelensis* resided in the same municipality; the fourth patient resided in an adjacent

Table 2. Analysis of blood and urine specimens from cows in study of outbreak of intermediate species *Leptospira venezuelensis* spread by rodents to cows and humans in *L. interrogans*–endemic region, Venezuela*

	rrs PCR		*lipL32* PCR		Serology		Cultures		Sequenced
Cow no.	Blood	Urine	Blood	Urine	MAT titer	Serovar	Blood	Urine	species†
1	+	–	–	–	Negative	NA	+	–	*L. venezuelensis*
2	–	–	–	–	Negative	NA	–	–	NA
3	–	+	–	–	1:800	*L. wolffii*	–	+	*L. venezuelensis*
4	–	–	–	–	Negative	NA	–	–	NA
5	–	+	–	+	1:400	*L. hebdomadis*	–	+	*L. interrogans*
6	–	–	–	–	Negative	NA	–	–	NA
7	–	+	–	–	1:800	*L. wolffii*	–	+	*L. venezuelensis*
8	+	–	+	–	Negative	NA	+	–	*L. interrogans*
9	–	+	–	+	1:400	*L. mini*	–	+	*L. interrogans*
10	–	–	–	–	Negative	NA	–	–	NA
11	–	+	–	–	1:1,600	*L. bataviae*	–	–	NA
12	–	+	–	–	1:800	*L. wolffii*	–	+	*L. venezuelensis*
13	–	+	–	–	1:400	*L. wolffii*	–	+	*L. venezuelensis*
14	–	+	–	–	1:800	*L. wolffii*	–	+	*L. venezuelensis*
15	–	–	–	–	Negative	NA	–	–	NA
16	–	+	–	–	1:400	*L. wolffii*	–	+	*L. venezuelensis*

*Results for MAT serology, cultures, and PCR of the *Leptospira lipL32* and *rrs* (16S rDNA) genes from cultured isolates. MAT, microscopic agglutination test; NA, not applicable; –, negative; +, positive.
†*Leptospira* spp. were determined by sequencing the PCR-amplified *rrs* gene.

Table 3. MLST of *Leptospira interrogans* isolates in study of outbreak of intermediate species *L. venezuelensis* spread by rodents to cows and humans in *L. interrogans*–endemic region, Venezuela*

Isolates†	MLST allele nos.							ST‡
	glmU	*pntA*	*sucA*	*tpiA*	*pfkB*	*mreA*	*caiB*	
Human								
CAB-H41	1	1	2	1	7	7	8	NP*
CAY-U48	1	1	2	1	7	4	3	20
CAB-U03	1	1	2	2	7	4	3	NP
MAC-H04	1	1	2	2	7	4	5	NP
URI-U06	**1**	**1**	**3**	**2**	**7**	**4**	**3**	NP
URI-H01	**1**	**1**	**3**	**2**	**7**	**4**	**3**	NP
CLM-H09	1	1	3	2	4	7	5	NP
CLM-U30	1	3	2	2	4	4	19	NP
MAC-H63	1	3	2	2	7	7	19	NP
CAY-H65	1	3	3	1	4	5	5	NP
SOB-U13	1	12	3	3	10	4	5	NP
MAQ-U18	1	12	3	3	10	5	19	NP
CLM-U22	1	12	2	3	10	6	19	NP
CLM-U28	**1**	**12**	**3**	**3**	**10**	**6**	**19**	27
CLM-H08	**1**	**12**	**3**	**3**	**10**	**6**	**19**	27
GUA-H40	**1**	**12**	**3**	**3**	**10**	**6**	**19**	27
CLM-U45	**3**	**3**	**3**	**2**	**4**	**5**	**5**	NP
CLM-U47	**3**	**3**	**3**	**3**	**4**	**5**	**5**	37
NAG-U02	6	1	3	2	4	7	3	NP
CAY-U49	6	1	3	3	76	7	3	NP
CLM-U46	6	2	3	3	7	7	19	NP
CLM-U24	6	1	3	12	4	5	5	NP
GUA-H52	6	3	2	2	4	4	3	NP
GUA-H64	6	3	2	3	4	7	5	NP
CAB-U11	6	3	3	2	4	5	5	NP
GUA-H21	6	3	3	3	1	7	5	NP
CAO-U23	6	3	3	3	4	5	19	NP
MAQ-H53	**6**	**8**	**2**	**2**	**9**	**7**	**5**	50
MAQ-H60	**6**	**8**	**2**	**2**	**9**	**7**	**5**	50
Rat								
CLM-R09-A	1	1	2	2	7	4	8	NP
CLM-R11-A	1	1	3	3	4	6	19	NP
SOB-R13-B§	1	12	3	3	10	6	19	27

*Bold font indicates isolates that had identical profiles. MLST, multilocus sequence typing; NP, not present in database; ST, sequence type.
†The first 3 letters for each isolate indicate the area of the patient's residence or where the rodent was captured in the state of La Guaira, Venezuela: CAB, Caraballeda; CAO, Caruao; CAY, Carayaca; CLM, Catia La Mar; GUA, La Guaira; MAC, Macuto; MAQ, Maiquetia; NAG, Niguata; SOB, Soublette; or URI, Urimare. The fourth letter is H (isolated from human blood), U (isolated from human urine), or R (isolated from rat tissue).
‡STs found in the *Leptospira* PubMLST database (https://pubmlst.org/organisms/leptospira-spp) (*14*).
§Rat sequence shared an MLST profile for some alleles with human isolates CLM-U28, CLM-H08, and GUA-H40.

district. This municipality was the most frequent residence of leptospirosis patients, home to 10 of 32 patients with other *Leptospira* infections. Of the 2 rats infected with *L. venezuelensis*, 1 was trapped in the same municipality and the other in a nearby district.

Phylogenetic reconstruction of genomes from the 6 sequenced *L. venezuelensis* isolates uncovered limited genetic diversity (Figure 3). The isolates from human, rodent, and bovine hosts all differed by <12 single-nucleotide polymorphisms (SNPs), suggesting a recent outbreak of a *L. venezuelensis* strain that was spread, presumably by rodents, to different host populations. The *L. venezuelensis* genome sequence data have been deposited in GenBank (Biosample accession nos. SAMEA5168082, SAMEA5168083, SAMEA5168130, SAMEA5168133, SAMEA5168318, SAMN06855518, SAMN39993761, SAMN39993762, and SAMN39993763).

Discussion

The true incidence of leptospirosis in La Guaira state has been unknown, likely because it has been difficult or impossible to diagnose and has not been considered by clinicians, even in patients with characteristic signs and symptoms. Our prospective search for leptospirosis cases in La Guaira's 2 hospitals during a 1-year period found *rrs* PCR evidence of *Leptospira* spp. in blood or urine specimens from 37 hospitalized patients, including 8 patients who died. We also cultured *Leptospira* from 36 patient samples. Two patients with positive *rrs* PCR had negative cultures, but 1 of those patients had an *L. interrogans lig* gene fragment amplified from their urine. The population of La Guaira is ≈353,000, corresponding to a borderline high incidence of 10 leptospirosis cases/100,000 population. However, this figure is almost certainly an underestimate because the study only included

patients ill enough to require hospitalization and did not capture patients with less severe illness, who represent up to 90% of leptospirosis cases (*2*).

Leptospira spp. were isolated from the kidneys of all 45 rodents captured in the region. *Leptospira* species distributions were similar in rodents and humans; most isolates were *L. interrogans,* which is globally the species most associated with severe human illness. *L. venezuelensis* was also isolated from 7 cows on a nearby farm, whereas *L. interrogans* was isolated from only 3 cows on the same farm (Table 2; Appendix Table 14).

Leptospirosis is difficult to diagnose in a clinically useful time frame, but *rrs* PCR of both blood and urine samples detected 37 cases. The most discriminative clinical finding in patients was conjunctival suffusion (*19*), but *Leptospira*-positive patients also had more myalgias, dyspnea, cough, and hemoptysis than did hospitalized *Leptospira*-negative patients. *L. interrogans* was recovered from patients with the most severe cases, and 27% (8/30) of *L. interrogans*–infected patients died. However, for patients with mild to moderate disease, the infecting species could not be distinguished by patient signs, symptoms, or laboratory values (Appendix Table 8).

The intermediate species *L. fainei* was isolated from the kidneys of 3/45 captured rodents. *L. fainei* has been reported to cause disease in humans (*20*) but was not isolated from any human patient or bovid in this study. *L. venezuelensis* is phylogenetically closer to other intermediate species reported to cause human illness, such as *L. liceraciae* (*21*) and *L. wolffi* (*22*) and is phylogenetically close to *Leptospira* spp. isolated from environmental samples in Malaysia, Mayotte, and New Caledonia (*5*).

Few studies have been conducted to determine the phylogenetic relatedness of different strains of *Leptospira* spp. isolated from a particular geographic region. *L. interrogans* isolates from this study had many MLST profiles, including clusters of profiles found in the *Leptospira* PubMLST database (Table 3). MATs showed that serum samples from 3 cows each reacted to a different *L. interrogans* serovar, including 2 whose isolates had the same VNTR pattern (Appendix Tables 14, 16). The heterogeneity of *L. interrogans* strains suggests a long-term endemic presence in the local rodent population. In contrast, the genomes of 6 *L. venezuelensis* isolates differed by a maximum of 11 SNPs (Figure 3), suggesting an outbreak strain. Although only 6 of the 13 *L. venezuelensis* isolates were sequenced, they were

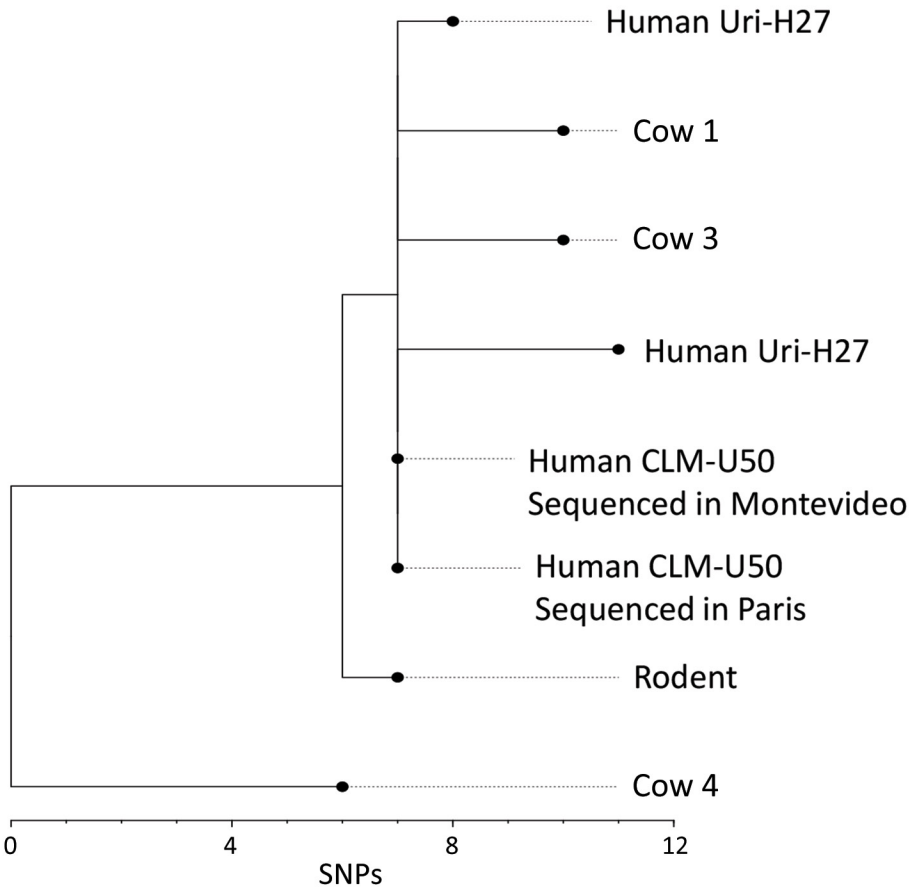

Figure 3. Phylogenetic analysis of *Leptospira venezuelensis* isolates in study of outbreak of intermediate species *L. venezuelensis* spread by rodents to cows and humans in *L. interrogans*–endemic region, Venezuela. Branch length indicates the number of SNPs separating *L. venezuelensis* strains. Phylogenetic tree was reconstructed according to comparisons of whole-genome sequences from 6 *L. venezuelensis* strains isolated from hospitalized leptospirosis patients in La Guaira State on the Caribbean coast of Venezuela, from rodents captured near the residences of hospitalized leptospirosis patients, and from dairy cows on a farm 30 km away from La Guaira State. Human isolate CLM-50 was sequenced at both the Institute Pasteur in Paris, France, and the Institute Pasteur in Montevideo, Uruguay. Human isolate Uri-H27 was sequenced twice at the Institute Pasteur in Paris; the genome of the isolate after many passages in culture contained 3 SNPs that were not present in the same isolate from an earlier passage. Scale bar indicates number of SNPs per site. SNP, single-nucleotide polymorphism.

obtained from a diverse sampling of hospitalized humans, rats captured in La Guaira, and cows on a farm 30 km away from patient residences. Unless *L. venezuelensis* has a mutation rate even slower than slow-mutating *Mycobacterium tuberculosis* (*23*), the low genetic diversity reflects a recently expanded bacteria population. Greater genomic heterogeneity would be expected if *L. venezuelensis* evolved from a local environmental *Leptospira* sp. Instead, the close genomic similarity between isolates suggests a recent introduction of *L. venezuelensis* into the region, perhaps arriving with rats on a ship that docked in the port of La Guaira and then spread within the local rodent population.

Infections with intermediate clade *Leptospira* spp. have only rarely been associated with icteric human illness (*6*), but 3 of 4 patients from whom *L. venezuelensis* was isolated were icteric, had liver aminotransferase values >250 (Appendix Table 9) and negative test results for hepatitis viruses A and B. Only 1 patient with *L. venezuelensis* infection was tested for dengue, but all 4 had platelet levels within reference ranges, which is uncharacteristic for acute dengue.

Although intermediate *Leptospira* spp. are thought to be incapable of surviving in an animal model, infection of rats has been reported for the intermediate species *L. licerasiae* (*24*). We recovered all 13 *L. venezuelensis* isolates from hamster kidneys 16 days after intraperitoneal inoculation of low passage isolates, although later passages of the same isolates could not be recovered from hamsters after high-dose intraperitoneal infections (data not shown). The acquisition of SNPs and loss of virulence during *in vitro* passages of *Leptospira* isolates has been previously described (*25*,*26*).

Leptospira intermediate species are often isolated from environmental samples (*5*), but it seems unlikely that *L. venezuelensis* was an environmental or laboratory contaminant. The *rrs* PCR of the original human, bovine, and rodent specimens; the isolate cultures; and hamster infection studies were all performed separately before sequencing results were available, and the samples containing *L. venezuelensis* were not temporally linked. The MAT titers of serum samples from *L. venezuelensis*–positive bovids all showed the same presumed cross-reaction with *L. interrogans* serovar Wolffi, consistent with genomic evidence of an outbreak strain. Human disease causality could be confirmed by high or rising MAT titers in patient serum samples, but acute serum samples from 2 *L. venezuelensis* and 4 *L. interrogans* patients did not have titers >1:50, and convalescent patient blood samples were not collected.

In Argentina (*27*), *L. wolffii* was isolated from a patient who died of a severe respiratory syndrome, but PCR results suggested an *L. interrogans* co-infection. Similarly, 2 of the 4 *L. venezuelensis*–positive patients in this study had positive *lipL32* PCR results (Appendix Table 9). The *lipL32* primers were designed to amplify *lipL32* from *L. interrogans* or other pathogenic *Leptospira* spp. but not from intermediate species, such as *L. venezuelensis*. Although the amplified *lipL32* fragments were not sequenced, the 2 *lipL32*-positive patients could have been co-infected with *L. venezuelensis* and *L. interrogans.* Another patient from whom *L. venezuelensis* was cultured had negative *rrs* PCR results in both blood and urine. The pathogenicity of this intermediate species could not be confidently evaluated from the 4 *L. venezuelensis*–positive patients in this study.

In conclusion, an *L. venezuelensis* outbreak circulating in rodents appears to have spread to cows in the region and also infected humans, in whom it might have caused febrile illness with hepatic involvement. Our findings indicate the need for increased awareness of leptospirosis prevalence and characteristics in Venezuela and other tropical, rodent infested coastal regions and also indicates an urgent need for rapid point-of-care tests to diagnose leptospirosis and improve patient treatment and outcomes.

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Outbreak of Intermediate Species *Leptospira venezuelensis* Spread by Rodents to Cows and Humans in *L. interrogans*–Endemic Region, Venezuela

Appendix

Additional Methods

Leptospira Cultures

Leptospira were cultured at 28–30°C in liquid or semisolid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium containing 10% supplement (bovine serum albumin, Tween 80, chlorides, sulfides, and vitamins), and 50–100 µg/mL of 5-fluorouracil (5-FU) for initial cultures. All solutions and culture media were prepared according to the World Health Organization technical manual (*1*) using freshly made copper and iron solutions. All culture media, antimicrobial drugs, and solutions were prepared by using analytical or molecular biology grade reagents from commercial sources.

Four or five drops of whole patient blood were inoculated into 5 mL of EMJH 5-FU broth in duplicate. For urine cultures, 500 µL of unconcentrated urine were inoculated into 5 mL of EMJH 5-FU broth. In addition, 5 mL of urine was centrifuged at 3220 × g for 10 minutes, the supernatant was discarded, the pellet was resuspended in 500 µL EMJH 5-FU, and then 500 µL was inoculated into 5 mL EMJH 5-FU broth. When *Leptospira* growth was noted, primary cultures were passaged by using serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) into semisolid EMJH-5-FU medium, which was then monitored for growth by dark field microscopy weekly for several months.

After the exclusive presence of spirochetes typical of *Leptospira* was observed, the culture was passaged into fresh sterile EMJH 5-FU (semisolid medium at 1:10 vol/vol and 2× liquid media at 1:1 vol/vol, both in duplicate) and then monitored weekly for growth by dark field microscopy. After good growth was noted, the strains were passed weekly to fresh semisolid EMJH medium at 1:10 vol/vol until 1,000 bacteria cells/mL was obtained or until the characteristic Dinger's ring was observed, indicating growth of *Leptospira*.

Rodent Capture

The National Office of Biologic Diversity of the Venezuela Ministry for the Environment (Document 0264) and the Instituto Venezolano de Investigaciones Científicas Commission on Animal Bioethics approved the capture of rodents in Sherman aluminum traps set in urban areas close to residences of patients who were PCR positive for *Leptospira*. The species of captured rodents were determined by isolating DNA from liver samples by using a DNeasy Blood and Tissue kit (QIAGEN, https://www.qiagen.com) and then sequencing a PCR-amplified subunit of the cytochrome c oxidase gene (*2*).

The rodents were euthanized according to the 2010 Guidelines of the Canadian Council on Animal Care (https://ccac.ca/en/guidelines-and-policies/the-guidelines). The kidneys were removed by using sterile technique, macerated, placed in a 15 mL tube containing 5 mL of EMJH 5-FU broth, and left to sediment at room temperature for 25 minutes. The supernatants were examined by dark field microscopy for the presence of *Leptospira* and 500 μL of supernatant was inoculated into 2 tubes of EMJH 5-FU broth.

Bovine Samples

Blood from the caudal vein of cows on farms in Miranda State, ≈30 km from La Guaira State, was collected with and without EDTA as an anticoagulant. Two or 3 drops of uncoagulated blood were inoculated onto 5 mL of semisolid EMJH medium in duplicate, incubated at 30°C, and examined weekly by using darkfield microscopy. After spirochetes were detected, the cultures were inoculated into fresh semisolid media and incubated at 30°C.

Urine samples from cows were collected in sterile 50 mL tubes after intramuscular injection of the diuretic furosemide (1 mg/kg). Ten mL of urine samples were centrifuged for 30 minutes at 3,220 × g and then 0.5 mL of the pellet was mixed with 4.5 mL liquid EMJH medium (dilution 1:10) supplemented with albumin and 5-FU and incubated at 30°C. In addition, 10 mL

of urine samples were passed through a 0.45 micron syringe filter and 500 μL of the filtrate was inoculated in duplicate onto semisolid EMJH medium and incubated at 30 C. Two or 3 drops of uncoagulated blood were inoculated onto 5 mL semisolid EMJH medium in duplicate, incubated at 30°C, and examined weekly by using darkfield microscopy. After spirochetes were detected, the cultures were inoculated onto fresh semisolid EMJH medium and incubated at 30°C.

Molecular Detection of Pathogenic *Leptospira* in Blood and Urine

Patient blood samples (2.5 mL) were collected in EDTA tubes and DNA was isolated by using the AxyPrep Blood Genomic DNA Miniprep kit (Corning, https://www.corning.com). Midstream urine samples (15 mL) were collected and adjusted to pH 7.5 with 1–2 mL 8.5% sodium bicarbonate supplemented with 1% 5-FU; DNA was isolated as previously described (*3*). DNA from 300 μL blood collected from cow caudal veins was extracted by using the Geneaid DNA Isolation Kit (Geneaid, https://www.geneaid.com) and stored at −20°C until PCR analysis. Immediately after urine samples were collected from cows, ≈30 mL urine per cow was spun at 1,600 × g for 30 minutes. Then, 200 μL of the pellet was processed by using the Geneaid kit, and the isolated DNA was used in standard PCR with primers for *lipL32* and regions V3–V6 of the *rrs* gene (*4*) (Appendix Table 1). Detection of *lipL32* was conducted as previously described (*5*). The *rrs* gene PCR products were purified and sequenced by Macrogen (https://www.macrogen.com).

Molecular Analysis of Cultured *Leptospira* Species

To identify *Leptospira* spp. in cultured material, the V2–V9 regions of the *rrs* gene (16S rRNA) were PCR amplified by using the primers rrsfull-D and rrsfull-R (Appendix Table 1). The resulting 1,500 bp product was then subjected to a second round of PCR of *rrs* regions V3–V6 by using the nested primers 16S-D(int) and 16S-R(int). The primers and nested-PCR strategy used to detect *lipL32* have been previously described (*5*).

The variable-number tandem-repeats VNTR4, VNTR7, VNTR10, and VNTR-Lb5 loci were amplified by PCR as previously described (*6*); products were analyzed on 2% agarose gels and the sizes of the DNA bands were compared with a 100 bp DNA ladder. The genes amplified and sequenced for multilocus sequence typing were *pntA*, *sucA*, *pfkB*, tpiA, *mreA*, *glmU*, and *caiB* (Appendix Table 1) (*6*). When the yield of genomic DNA was poor, a nested PCR protocol and additional primers were used as previously described (*7*); new primers were designed to

amplify *caiB* from *L. interrogans* and *L. noguchii.* The amplified DNA fragments were purified and sequenced (Macrogen) and aligned with GenBank sequences by using BLAST (https://blast.ncbi.nlm.nih.gov); sequences were compared with *Leptospira* reference species obtained from Genbank by using the ClustalW algorithm in MacVector (https://www.macvector.com).

MATs of Cow Serum Samples

Blood without anticoagulant was centrifuged for 10 minutes at room temperature at 3,220 × g. The serum samples were then transferred to new tubes and stored at −20°C until processed at the Bacteriological Animal Health Laboratory at the Instituto Nacional de Investigaciones Agrícolas in Maracay, Aragua, Venezuela. The microscopic agglutination test (MAT) was performed in the Animal Health Laboratory according to the 2001 Pan American Health Organization standards. The serum samples were inactivated in a water bath at 56°C for 30 minutes and, during the screening phase, diluted 1:25 by adding 0.25 mL serum sample to 6 mL phosphate-buffered saline (PBS).

For live antigens, 23 serovars of *Leptospira* were grown in liquid medium, examined by microscopy to rule out contamination, then diluted in PBS to a 0.5 McFarland turbidity standard. MAT assays were performed in 96-well plates with 100 µL of the diluted serum sample and 100 µL of live antigen. A human serum sample known to be negative for *Leptospira* antibodies was used as a negative control, and a well containing equal parts antigen and PBS without serum sample was used as a blank. The plates were incubated at 37°C for 1 hour and then a drop from each well was analyzed for agglutination by using a darkfield microscope with a 16× objective. MAT tests were considered positive when ≥50% of the *Leptospira* were agglutinated. Titering was also performed in 96-well plates by using 2-fold serial dilutions of serum samples in PBS. The titer was the maximum serum sample dilution in which agglutination was observed.

Phylogenetic Reconstruction of *L. venezuelensis* Isolates

Velvet (*8*) was used for de novo assembly of genome contigs obtained from sequencing reads of *L. venezuelensis* isolates. Isolate 201502610 (GenBank Biosample no. SAMEA5168082) was used as a reference to map the sequencing reads from the other *L. venezuelensis* isolates. The 201502610 reference genome contains 29 assembled contigs with a total length of 4,250,319 bp. Sickle (*9*) was used to trim the reads from genome sequencing data

and sequencing reads with a Phred base quality score >20 and read lengths >30 bp were kept for analysis. Sequencing reads were mapped to the reference genome by using Bowtie (*10*), and then SAMtools version 1.3.1 (*11*) was used for calling single-nucleotide polymorphisms that had a mapping quality score >30. Fixed mutations having a frequency of ≥95% and ≥20 supporting reads were identified by using VarScan version 2.3.9 (*12*); small insertions or deletions identified by VarScan were excluded in the analysis. The alignments of polymorphic positions from all *Leptospira* strains were used for phylogenic reconstruction by using MEGA 7.0 (*13*). The neighbor-joining method was used for initial inference of the phylogenic structure under the number of differences model.

Tests for Dengue and Hepatitis

Tests for dengue were performed at the Instituto Nacional de Higiene in Caracas by using a solid phase immunochromatographic assay that detects IgG and IgM against dengue virus serotypes 1–4, together with a rapid chromatographic immunoassay against dengue virus nonstructural protein 1 antigen. Frozen serum samples from *L. venezuelensis*–culture positive patients were tested for hepatitis A virus by using an ELISA to detect virus-specific IgM and tested for hepatitis B by using an ELISA to detect hepatitis B surface antigen and hepatitis B core antibody.

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Appendix Table 1. PCR primers used in the study

Primer name	Primer sequence, 5′–3′	Annealing temperature, °C	Reference
glmU-D(Ext)	AGGATAAGGTCGCTGTGGTA	52	(*14*)
glmU-R(Ext)	AGTTTTTTTCCGGAGTTTCT		
glmU-D(Int)	GGAAGGGCACCCGTATGAA	52	(*15*)
glmU-R(Int)	TCCCTGAGCGTTTTGATTT		
pntA-D(Ext)	TGCCGATCCTACAACATTA	52	(*15*)
pntA-D(Int)	TAGGAAARATGAAACCRGGAAC		
pntA- R	AAGAAGCAAGATCCACAAYTAC		(*14*)
sucA-D(Ext)	TCATTCCACTTYTAGATACGAT	58	(*14*)
sucA-R(Ext)	TCTTTTTTGAATTTTTGACG		
sucA-D(int)	AGAAGAGGCCGGTTATCATCAG	52	(*15*)
sucA-R(int)	TTCCGGGTCGTCTCCATTTA		
tpiA-D(Ext)	TTGCAGGAAACTGGAAAATGAAT	52	(*14*)
tpiA- R(Ext)	GTTTTACRGAACCHCCGTAGAGAAT		
tpiA-D(int)	AAGCCGTTTTCCTAGCACATTC	52	(*15*)
tpiA-R(int)	AGGCGCCTACAAAAAGACCAGA		
*pfkB*13-D(Ext)	CGGAGAGTTTTATAARAAGGACAT	52	(*14*)
*pfkB*13-R(Ext)	AGAACACCCGCCGCAAAACAAT		
pfkB-D(int)	CCGAAGATAAGGGGCATACC	52	(*15*)
pfkB-R(int)	CAAGCTAAAACCGTGAGTGATT		
*mreA*13-D(Ext)	GGCTCGCTCTYGACGGAAA	58	(*14*)
*mreA*13-R(Ext)	TCCRTAACTCATAAAMGACAAAGG		
mreA-D(int)	GTAAAAGCGGCCAACCTAACAC	52	(*15*)
mreA-R(int)	ACGATCCCAGACGCAAGTAA		
caiB-D(Ext)	TAGAAATTTTGCRGGACACG	54	This study
caiB-R(Ext)	TAAAGTTCGGTAGATAGACT		
caiB(int)	ACACCTCAGATTCCAGGAT	55	
caiB(int)	GGAATACCGGRTCCTTAAT		
*rrs*full-D	GCTCAGAACTAACGCTGGCG	60	This study
*rrs*full-R	TATTCACCGCGGCATGCTGA		
16s-D(int)	CATGCAAGTCAAGCGGAGTA	58	(*16*)
16s-R(int)	AGTTGAGCCCGCAGTTTTC		
lipL32F	ATCTCCGTTGCACTCTTTGC	58	(*16*)
lipL32R	ACCATCATCATCATCGTCCA		
lipl32-P662	CTAAGTTCATACCGTGATTT	58	(*17*)
lipl32 P663	TTCTGACGCGACTAAGTAAT		
lipl32 Internal 1	GACGGTTTAGTCGATGGAAAC	58	(*17*)
lipl32Internal 2	GGGAAAAGCAGACCAACAGA		
209-5′NCR HCV	ATACTCGAGGTGCACGGTCTACGAGACCT	50	(*18*)
211-5′NCR HCV	CACTCTCGAGCACCCTATCAGGCAGT		
939-5′NCR HCV	CTGTGAGGAACTACTGTCTT	53	(*19*)
940-5′NCR HCV	TTCACGCAGAAAGCGTCTAG		
58-Surface HBV	CCTGCTGGTGGCTCCAGTTC	58	(*20*)
1101n-Surface HBV	GAAAGGCCTTGTAAGTTGGCGAG		
S3s-Surface HBV	TGCCTCATCTTCTTRTTGGTTCT	53	(*21*)
S3as-Surface HBV	CCCCAAWACCAVATCATCCAT		
HAV1-VP1 HAV	GTTTTGCTCCTCTTTATCATGCTATG	52	(*22*)
HAV7-VP1 HAV	CTGGAGTGAACCAGGCCATGCCATC		
HAV6-VP1 HAV	AGGAAATGTCTCAGGTACTTTCTTTGCTAAAACTG		
Lig1	TCAATCAAAACAAGGGGCT	48	(*23*)
Lig2	ACTTGCATTGGAAATTGAGAG		

Appendix Table 2. Age of patients in the study according to sex and PCR results for *Leptospira rrs* gene

	rrs-positive blood or urine		*rrs*-negative blood and urine	
Patient sex	No. patients	Average age, y (range)	No. patients	Average age, y (range)
M	21	34.7 (13–81)	22	30.3 (2 mo–72 y)
F	15	31.6 (4–71)	13	36.6 (16–60)
Total	36	33.4	35	32.7

Appendix Table 3. Source hospitals of patients in the study according to sex and PCR results for *Leptospira rrs* gene

	rrs-positive blood or urine			*rrs*-negative blood and urine		
Hospital where patient was contacted	M	F	Total	M	F	Total
Dr. Jose Maria Vargas	20	11	31	18	12	31
Dr. Rafael Medina Jiménez (Pariata)	2	4	6	3	1	4
Total no. patients	22	15	37	21	13	34

Appendix Table 4. PCR results for *Leptospira* spp. compared with cultures of blood and urine samples from hospitalized patients*

					lipL32 PCR						*rrs* PCR					
	Positive culture				Blood		Urine		Both		Blood		Urine		Both	
Leptospira spp.	Blood	Urine	Both	Total	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
L. interrogans	10	16	3	29	11	18	12	17	2	8	12	17	19	10	2	0
L. noguchii	2	1	0	3	2	1	0	3		1	3		0	3	0	0
L. venezuelensis	1	3	0	4	1	3	1	3		2	1	3	2	2		1
Total	13	20	3	36	14	22	13	23	2	11	16	20	21	15	2	1

*PCR was conducted to detect *lipL32* and *rrs Leptospira* genes. Neg, negative; Pos, positive.

Appendix Table 5. Average number of days from onset of symptoms in hospitalized patients to collection of blood or urine positive for *Leptospira* by PCR or culture*

	Blood		Urine		
Detection method	No. samples	No. days (range)	No. samples	No. days (range)	p value†
lipL32 PCR	15	9.0 (1–21)	13	10.38 (1–21)	0.52
rrs PCR	17	9.18 (1–21)	20	10.55 (1–22)	0.48
Culture	16	9.69 (1–21)	20	10.43 (1–21)	0.35

*PCR was conducted to detect *lipL32* and *rrs Leptospira* genes.
†*t*-test was used to compare duration of symptoms before a positive test for *Leptospira* was obtained in blood verses urine.

Appendix Table 6. Comparison of positive PCR results for *Leptospira* in blood, urine, or both samples from hospitalized patients whose cultured blood or urine specimens grew *Leptospira**

	lipL32-positive PCR			*rrs*-positive PCR		
Leptospira-positive cultures	Blood	Urine	Blood and urine	Blood	Urine	Blood and urine
Blood, n = 13	11	0	0	13	0	0
Urine, n = 20	2	10	1	1	17	1
Blood and urine, n = 3	0	1	1	1	2	0

*PCR was conducted to detect *lipL32* and *rrs Leptospira* genes.

Appendix Table 7. Average number of days of symptoms in hospitalized patients before specimens were obtained for diagnostic tests according to *Leptospira* spp. isolated and positive or negative test results

	lipL32 PCR, blood				*lipL32* PCR, urine				*rrs* PCR, blood				*rrs* PCR, urine				Blood culture				Urine culture			
	Pos		Neg		Pos		Neg		Pos		Neg		Pos		Neg		Pos		Neg		Pos		Neg	
Leptospira spp.	Pts	Days	Pts	Days	Pts	Days	Pts	Days	Pts	Days	Pts	Days	Pts	Days	Pts	Days	Pts	Days	Pts	Days	Pts	Days	Pts	Days
L. interrogans	11	7.82	18	10.44	12	10.58	17	8.65	12	8.75	17	9.94	12	11.42	16	8.06	13	9.85	16	9.13	19	10.37	10	7.7
L. noguchii	2	10.5	1	2	0	NA	3	7.67	3	7.67	0	NA	1	2	2	10.5	2	6.5	1	10	1	10	2	6.5
L. venezuelensis	1	14	3	11	1	8	3	13	1	14	3	11	3	11	1	14	1	14	3	11	3	11	1	14

*PCR was conducted to detect *Leptospira lipL32* and *rrs* genes. Data show number of patients in each group and average number of days. NA, not applicable; Neg, negative; Pos, positive; Pts, patients.

Appendix Table 8. Symptoms or treatments of hospitalized patients from whom the indicated *Leptospira* species was isolated

	No. patients infected with *Leptospira* spp.		
Symptoms/treatments	*L. interrogans*	*L. noguchii*	*L. venezuelensis*
Fever			
Yes	28	3	4
No	1	0	0
Antimicrobial drug treatment			
Yes	16	1	2
No	8	2	1
Icterus			
Yes	20	3	4
No	9	0	0
Conjuntival suffusion			
Yes	19	3	3
No	10	0	1
Dyspnea			
Yes	10	0	1
No	19	3	3
Hemoptysis			
Yes	5	0	1
No	23	3	3
Cough			
Yes	20	0	3
No	9	3	1
Oliguria			
Yes	8	0	2
No	19	3	2
Vomiting			
Yes	12	0	2
No	17	3	2
Diarrhea			
Yes	2	0	0
No	27	3	4
Rash			
Yes	6	0	1
No	23	3	3
Myalgias			
Yes	19	3	4
No	10	0	0
Arthralgias			
Yes	15	2	4
No	12	1	0
Abdominal pain			
Yes	17	3	2
No	12	0	2

Appendix Table 9. Laboratory results for the 4 hospitalized patients from whom *L. venezuelensis* was isolated*

Patient no.	*lipL32* +	*rrs* +	Culture +	AST, IU/L	ALT, IU/L	Indirect bilirubin, mg/dL	Direct bilirubin, mg/dL	Hepatitis A, B	Dengue	Platelets, $\times 10^3/\mu L$
1	Blood	Blood	Blood	412	1,483	0.7	2.7	Negative	NR	199
2	Urine	Urine	Urine	22	11	4.2	0.7	Negative	NR	220
3	Neg	Urine	Urine	1,160	1,260	0.6	0.5	Negative	Negative	228
4	Neg	Neg	Urine	280	670	1.9	7.6	Negative	NR	209

*PCR was used to determine the presence of *Leptospira* genes *lipL32* and *rrs*. NR, no results available: +, positive.

Appendix Table 10. Risk ratios for positive *Leptospira rrs* (16S rDNA) gene PCR according to symptoms in hospitalized patients*

Symptom/treatment	*rrs*-positive PCR, blood or urine	*rrs*-negative PCR, blood and urine	Risk ratio (95% CI)	p value†
Culture positive	35/37	1/34	17.04 (4.4–65.5)	**<0.001**
Conjunctival suffusion	25/37	10/33	2.08 (1.26–3.45)	**0.002**
Myalgias	28/37	14/34	2.15 (1.20–3.85)	**0.003**
Dyspnea	11/37	2/34	1.89 (1.31–2.73)	**0.009**
Cough	24/37	13/34	1.70 (1.04–2.76)	**0.025**
Hemoptysis	7/36	1/34	1.87 (1.29–2.72)	**0.030**
Antimicrobial drugs	21/31	11/24	1.51 (0.89–2.56)	0.102
Oliguria	9/35	4/33	1.46 (0.93–2.31)	0.15
Diarrhea	2/37	5/34	0.52 (0.16–1.72)	0.19
Vomiting	14/37	8/34	1.35 (0.88–2.09)	0.19
Death	8/36	0/6	1.23 (1.04–1.42)	0.20
Arthralgias	20/35	14/33	1.33 (0.83–2.14)	0.22
Dengue	3/21	5/17	0.63 (0.24–1.60)	0.25
Fever	36/37	31/34	2.15 (0.39–11.9)	0.26
Icterus	28/37	22/34	1.31 (0.75–2.27)	0.31
Rash	7/37	4/30	1.19 (0.71–1.98)	0.54
Hepatitis	4/23	4/16	0.82 (0.39–1.72)	0.56

*The *rrs*-positive and *rrs*-negative columns indicate the number of patients who had each feature over the total number of patients in each group for whom data were available.
†p values from Pearson's χ^2 test. Bold numbers indicate significant differences ($p<0.05$).

Appendix Table 11. Comparison of mean laboratory values from hospitalized patients who had positive versus negative PCR tests for the *Leptospira rrs* gene*

Laboratory test	*rrs-positive,* blood or urine Mean (95% CI)	*rrs-negative,* blood and urine Mean (95% CI)	p value	No. patients,† *rrs+/rrs–*
Potassium, mmol/L	3.8 (3.5–4.2)	4.1 (3.8–4.4)	0.17	34/34
Direct bilirubin, mg/dL	2.5 (1.6–3.3)	3.6 (2.0–5.2)	0.21	37/34
Creatinine, mg/dL	1.6 (0.7–2.4)	1.0 (0.8–1.2)	0.23	36/33
Indirect bilirubin, mg/dL	3.6 (1.3–3.4)	3.60 (1.6–5.6)	0.25	37/34
Hematocrit, %	35.7 (31.7–39.5)	38.2 (35.5–40.8)	0.29	36/34
AST, IU/L	394 (213–323)	503 (329–678)	0.38	37/34
Lymphocytes, %	22.7 (17.5–27.9)	20.2 (17.0–23.5)	0.41	27/28
Neutrophils, %	64.5 (57.5–71.4)	67.5 (60.4–74.5)	0.53	27/28
Urea, mg/dL	25 (17–33)	22 (13.3–30.4)	0.59	34/34
No. leukocytes, $\times 10^3/\mu L$	10.8 (8.6–13.0)	10.3 (8.3–12.2)	0.72	37/34
ALT, IU/L	585 (270–899)	561 (326–796)	0.90	37/34
No. platelets, $\times 10^3/\mu L$	243 (188–298)	243 192–293	0.99	37/34

*p values were calculated from 2-tailed *t*-tests. ALT, alanine aminotransferase; AST, aspartate aminotransferase; –, negative; +, positive.
†Number of patients (for whom data were available) who had a positive PCR test for the *Leptospira rrs* (16s rDNA) gene over the number of patients with a negative PCR test for *Leptospira rrs*.

Appendix Table 12. Risk ratios for fatal outcomes according to clinical features of hospitalized patients who had positive PCR tests for *Leptospira rrs* (16S rDNA) in either blood or urine*

Clinical feature	Survived, n = 29	Deceased, n = 8	Risk ratio (95% CI)	p value†
Oliguria	6/29	3/6	2.89 (0.71–11.83)	0.13
Hemoptysis	4/28	3/8	2.49 (0.77–8.00)	0.14
lipL32+ blood, PCR	10/29	5/8	2.44 (0.68–8.72)	0.15
Dyspnea	7/29	4/8	2.36 (0.72–7.79)	0.16
Icterus	21/29	7/8	2.25 (0.32–15.9)	0.38
rrs+ blood, PCR	12/29	5/8	1.96 (0.54–7.03)	0.29
Antimicrobial drug treatment	17/26	4/5	1.90 (0.24–14.9)	0.52
lipL32+ urine, PCR	10/29	3/8	1.11 (0.31–3.91)	0.87
Cough	19/29	5/8	0.90 (0.25–3.19)	0.87
Conjunctival suffusion	20/29	5/8	0.80 (0.23–2.81)	0.73
Blood culture positive	13/29	3/8	0.79 (0.22–2.82)	0.71
Arthralgias	17/29	3/6	0.75 (0.17–3.21)	0.70
Urine culture positive	18/29	4/8	0.68 (0.20–2.31)	0.54
rrs+ urine, PCR	18/29	4/8	0.68 (0.20–2.31)	0.54
Rash	6/29	1/8	0.61 (0.89–4.21)	0.60
Vomiting	12/29	2/8	0.55 (0.13–2.35)	0.40
Culture positive	28/29	7/8	0.40 (0.09–1.86)	0.32
Myalgias	24/29	4/8	0.32 (0.10–1.03)	**0.06**
Fever	29/29	7/8	0.19 (0.10–0.38)	**0.05**
Abdominal pain	21/29	1/8	0.10 (0.13 - 0.71)	**0.002**
Diarrhea	2/29	0/8	0	0.44

*Survived and deceased columns show the number of patients with each feature over the total number of patients in each group for whom data were available. PCR detected *Leptospira lipL32* and *rrs* genes. +, positive.
†p values were calculated from Pearson's χ^2 tests. Bolded p values indicate significant differences (p<0.05).

Appendix Table 13. Comparison of mean laboratory values in surviving versus deceased hospitalized patients who had positive PCR tests for *Leptospira rrs* (16S rDNA) in either blood or urine*

Laboratory test	Survived, n = 29 Mean (95% CI)	Deceased, n = 8 Mean (95% CI)	p value	No. deceased/ no. survived†	Cohen's d (95% CI)
Creatinine, mg/dL	1.1 (0.8–1.4)	3.1 (–1 to 7.4)	0.06	8/28	–0.77 (–1.57 to 0.04)
Urea, mg/dL	18.6 (13.4–23.8)	45.6 (16.4–74.8)	0.07	8/26	–1.36 (–2.21 to –0.49
No. leukocytes, $\times 10^3/\mu L$	9.9 (7.6–12)	14.3 (7.2–21.3)	0.10	8/29	–0.68 (–1.47 to 0.12)
ALT, IU/L	705 (311–1,097)	152 (5–299)	0.14	8/29	0.60 (–0.20 to 1.39)
Neutrophils, %	61.8 (55–69)	73.5 (49–98)	0.15	6/21	–0.68 (–1.60 to 0.25)
Lymphocytes, %	24.3 (19.3–29)	17.1 (–3 to 37)	0.25	6/21	–0.55 (–0.38 to 1.46)
AST, IU/L	448 (221–674)	202 (15–389)	0.26	8/29	0.45 (–0.34 to 1.24)
Potassium, mmol/L	3.7 (3.4–4.1)	4.1 (2.9–5.3)	0.33	8/26	–0.40 (–1.20 to 0.40)
No. platelets, $\times 10^3/\mu L$	257 (190–325)	193 (106–280)	0.33	8/29	0.39 (–0.40 to 1.18)
Hematocrit, %	36.4 (31.8–41.0)	32.7 (25–41)	0.45	7/29	0.32 (–0.51 to 1.15)
Direct bilirubin, mg/dL	2.6 (1.5–3.6)	2.17 (0.3–4.0)	0.72	8/29	0.14 (–0.64 to 0.92)
Indirect bilirubin, mg/dL	2.3 (1.1–3.5)	2.5 (0.1–4.9)	0.87	8/29	–0.07 (–0.85 to 0.72)

*p values were calculated from 2-tailed *t*-tests. ALT, alanine aminotransferase; AST, aspartate aminotransferase.
†No. deceased/no. survived indicates the number of patients in each group for whom data were available.

Appendix Table 14. Microscopic agglutination test antibody titers in serum samples from dairy cows at 1 farm*

	Serovars‡																						
Cow no., culture†	a	b	c	d	e	f	g	h	i	j	k	l	m	n	ñ	o	p	q	r	s	t	u	v
1, *L. venezuelensis*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3, *L. venezuelensis*	–	–	1:100	–	–	1:100	–	–	–	–	–	–	–	–	–	–	1:800	–	1:100	–	–	1:200	–
4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5, *L. interrogans*	–	–	–	–	–	–	–	–	–	1:400	–	–	–	1:200	-	–	–	–	1:400	–	–	–	–
6	–	–	1:50	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7, *L. venezuelensis*	–	–	–	–	–	1:200	1:400	–	–	–	–	–	–	–	–	–	1:800	-	1:400	–	–	1:100	–
8, *L. interrogans*	–	–	1:50	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
9, *L. interrogans*	–	–	–	–	–	1:200	–	–	–	–	1:200	–	–	–	1:50	-	1:100	1:100	1:200	–	–	1:400	–
10	–	–	–	–	–	–	–	–	–	–	-	–	–	–	–	–	–	–	–	–	–	–	–
11	–	–	1:50	1:1,600	–	–	–	–	–	–	1:100	–	–	–	–	–	1:200	1:50	1:200	–	–	1:400	–
12, *L. venezuelensis*	–	–	–	–	–	1:100	–	–	–	–	1:100	–	–	–	1:100	-	1:800	–	–	–	–	–	–
13, *L. venezuelensis*	–	–	1:100	–	–	1:100	–	–	–	–	1:100	–	–	–	–	–	1:400	-	1:200	–	–	–	–
14, *L. venezuelensis*	1:50	–	1:50	–	–	1:200	1:200	–	–	–	1:50	–	–	–	–	–	1:800	1:50	1:200	–	–	1:400	–
15	1:50	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
16, *L. venezuelensis*	–	–	1:50	–	–	–	–	–	–	1:100	–	1:200	–	–	–	–	1:400	–	1:400	–	–	–	–

*Antibody titers were determined by using live antigens of 23 reference *Leptospira* serovars. –, negative.

†*Leptospira* species isolated from urine cultures. Sixteen cows were tested, only 10 had *Leptospira*-positive cultures.

‡*Leptospira* serovars were: a, *L. javanica*; b, *L. grippothyphosa*; c, *L. hurstibridge*; d, *L. bataviae*; e, *L. autumnalis*; f, *L. hardjo*; g, *L. tarassovi*; h, *L. pomona*; i, *L.* celledoni; j, *L. canicola*; k, *L. sejroe;* l, *L. pyrogenes*; m, *L. icterohaemorrhagiae* RGA; n, *L. icterohaemorrhagiae* 3294; ñ, *L. copenhageni*; o, *L. shermani*; p, *L. wolffi*; q, *L. castelonis*; r, *L. hebdomadis*; s, *L. muenchen*; t, *L. cynoptery*; u, *L. mini*; v, *L. panama*.

Appendix Table 15. Comparison of VNTR loci and MLST alleles in *L. interrogans* strains isolated from hospitalized patients or rodents in La Guaira, Venezuela

	VNTR loci†				MLST alleles†						
Isolates*	VNTR4	VNTR7	VNTR10	VNTR-Lb5	*glmU*	*pntA*	*sucA*	*tpiA*	*pfkB*	*mreA*	*caiB*
CAB-H41	3	9	16	6	1	1	2	1	7	7	8
CAY-U48	1	9	3	NR	1	1	2	1	7	4	3
CAB-U03	3	NR	2	5	1	1	2	2	7	4	3
MAC-H04	2	NR	15	NR	1	1	2	2	7	4	5
CLM-R09-A	1	1	3	NR	1	1	2	2	7	4	8
CLM-H09	1	1	3	NR	1	1	3	2	4	7	5
URI-U06	3	2	NR	3	1	1	3	2	7	4	3
URI-H01	3	9	13	4	1	1	3	2	7	4	3
CLM-R11-A	2	1	7	5	1	1	3	3	4	6	19
CLM-U30	2	9	2	6	1	3	2	2	4	4	19
MAC-H63	2	9	NR	NR	1	3	2	2	7	7	19
CAY-H65	2	0	16	5	1	3	3	1	4	5	5
SOB-U13	3	9	14	NR	1	12	3	3	10	4	5
CLM-U22	3	12	7	NR	1	12	2	3	10	6	19
MAQ-U18	2	1	7	5	1	12	3	3	10	5	19
CLM-U28	1	11	3	4	1	12	3	3	10	6	19
GUA-H40	2	8	9	4	1	12	3	3	10	6	19
CLM-H08	3	12	9	NR	1	12	3	3	10	6	19
SOB-R13-B	3	9	12	NR	1	12	3	3	10	6	19
CLM-U45	2	10	3	NR	3	3	3	2	4	5	5
CLM-U47	2	15	NR	NR	3	3	3	3	4	5	5
NAG-U02	3	1	12	16	6	1	3	2	4	7	3
CAY-U49	2	4	16	6	6	1	3	3	76	7	3
CLM-U24	1	9	4	6	6	1	3	12	4	5	5
CLM-U46	2	NR	NR	4	6	2	3	3	7	7	19
GUA-H52	2	5	6	4	6	3	2	2	4	4	3
GUA-H64	1	9	15	5	6	3	2	3	4	7	5
CAB-U11	2	1	7	5	6	3	3	2	4	5	5
GUA-H21	2	9	3	5	6	3	3	3	1	7	5
CAO-U23	2	1	NR	NR	6	3	3	3	4	5	19
MAQ-H53	1	9	6	4	6	8	2	2	9	7	5
MAQ-H60	2	NR	2	8	6	8	2	2	9	7	5
GUA-R52	1	9	10	3	6	NA	NA	NA	NA	4	NA
URI-R01-B	0	3	NR	NR	NA	NA	NA	NA	NA	NA	NA
URI-R01-A	1	3	NR	NR	NA	NA	NA	NA	NA	NA	NA
GUA-R21	1	8	11	3	NA	NA	NA	NA	NA	NA	NA
CLM-R48-B	2	NR	1	NR	NA	NA	NA	NA	NA	NA	NA
CLM-R48-A	2	NR	3	7	NA	NA	NA	NA	NA	NA	NA
URI-R06-B	2	1	10	NR	NA	NA	NA	NA	NA	NA	NA
CLM-R46	2	1	10	2	NA	NA	NA	NA	NA	NA	NA
CAB-R03	2	10	NR	NR	NA	NA	NA	NA	NA	NA	NA
CLM-R11-B	2	10	17	6	NA	NA	NA	NA	NA	NA	NA
CLM-R22	2	12	10	3	NA	NA	NA	NA	NA	NA	NA
MAQ-R53	2	2	11	4	NA	NA	NA	NA	NA	NA	NA
CLM-R09-B	2	3	3	NR	NA	NA	NA	NA	NA	NA	NA
CLM-R09-C	2	3	3	NR	NA	NA	NA	NA	NA	NA	NA
URI-R01-D	2	9	16	6	NA	NA	NA	NA	NA	NA	NA
CLM-R08	2	9	3	5	NA	NA	NA	NA	NA	NA	NA
CAY-R49	3	NR	11	NR	NA	NA	NA	NA	NA	NA	NA
SOB-R13-C	3	0	NR	NR	NA	NA	NA	NA	NA	NA	NA
URI-R06-A	3	1	NR	4	NA	NA	NA	NA	NA	NA	NA
SOB-R13-A	3	2	12	5	NA	NA	NA	NA	NA	NA	NA
CLM-R45	3	3	NR	7	NA	NA	NA	NA	NA	NA	NA
CAY-R65	3	4	NR	NR	NA	NA	NA	NA	NA	NA	NA
MAC-R63	4	5	8	NR	NA	NA	NA	NA	NA	NA	NA
GUA-R64	8	3	10	NR	NA	NA	NA	NA	NA	NA	NA
URI-R01-C	8	3	3	5	NA	NA	NA	NA	NA	NA	NA

*The first 3 letters describing each isolate indicate the area of the patient's residence or where the rodent was captured in the state of La Guaira: CAB, Caraballeda; CAO, Caruao; CAY, Carayaca; CLM, Catia La Mar; GUA, La Guaira; MAC, Macuto; MAQ, Maiquetia; NAG, Niguata; SOB, Soublette; or URI, Urimare. The last 3 letters begin with H (isolated from human blood), R (isolated from rat tissue), or U (isolated from human urine). Rodent isolates are underlined. MLST, multilocus sequence typing; NA, not applicable; NR, no results for this locus;.VNTR, variable number of tandem repeats.

†Shading indicates clustered profiles that are identical, differ from each other by 1 allele. MLST profiles were only obtained for those rodent isolates with VNTR patterns similar to ≥1 patient isolate.

Appendix Table 16. VNTR typing of 3 *L. interrogans* strains isolated from dairy cows at 1 farm*

	No. copies of 4 VNTR loci			
Cow no.	VNTR4	VNTR7	VNTR10	VNTR-Lb5
5	2	9	13	3
8	2	9	13	3
9	3	11	9	4

*Shading indicates identical VNTR profiles. VNTR, variable number of tandem repeat.