DOI: 10.7589/2015-09-245

Journal of Wildlife Diseases, 52(3), 2016, pp. 688–693
© Wildlife Disease Association 2016

Characterization of *Leptospira santarosai* Serogroup Grippotyphosa Serovar Bananal Isolated from Capybara (*Hydrochaeris hydrochaeris*) in Brazil

Luisa Z. Moreno,[1] Fabiana Miraglia,[1] Maria F. V. Marvulo,[2] Jean C. R. Silva,[1] Catia D. Paula,[1] Barbara L. P. Costa,[1] Zenaide M. Morais,[1] Fernando Ferreira,[1] José S. Ferreira Neto,[1] Odir A. Dellagostin,[3] Rudy A. Hartskeerl,[4] Silvio A. Vasconcellos,[1] and Andrea M. Moreno[1,5] [1]Universidade de São Paulo, Faculdade de Medicina Veterinária e Zootecnia, Departamento de Medicina Veterinária Preventiva e Saúde Animal, Av. Prof. Dr. Orlando Marques de Paiva, 87, Cidade Universitária, 05508 270, São Paulo/São Paulo, Brazil; [2]Faculdade Max Planck, Curso de Medicina Veterinária, Rodovia João Ceccon, Km 4, Indaiatuba/São Paulo, Brazil; [3]Centro de Desenvolvimento Tecnológico, Núcleo de Biotecnologia, Universidade Federal de Pelotas, Campus Universitário Capão do Leão, CP 354, 96010-900, Pelotas/Rio Grande do Sul, Brazil; [4]WHO/FAO/OIE and National Leptospirosis Reference Centre, KIT Biomedical Research, Royal Tropical Institute, Mauritskade 63, 1092 AD, Amsterdam, Netherlands; [5]Corresponding author (email: morenoam@usp.br)

ABSTRACT: Leptospirosis is a widespread zoonosis caused by bacteria of the genus *Leptospira*. Rodents appear to be the most important reservoirs of infection. They contaminate the environment and food and can transmit the pathogen when they are consumed by carnivores. Capybara (*Hydrochaeris hydrochaeris*) are efficient reservoirs of *Leptospira*, and because they are in close contact with farm animals and are found in semiurban areas, they represent a risk to public health. We isolated five *Leptospira* strains from capybara kidneys in Sao Paulo State, Brazil, in 2001 and typed them using serologic and molecular techniques. These strains include the *Leptospira santarosai* serogroup Grippotyphosa serovar Bananal. Pulsed field gel electrophoresis resulted in a unique pattern distinct from the reference strains, and the isolates clustered with greater than 85% similarity. The isolates also presented higher growth rates than other *Leptospira* serovars, with high minimal inhibitory concentration values for most of the tested antibiotics, with the exception of penicillin and ampicillin. This isolation and characterization of the *L. santarosai* serogroup Grippotyphosa serovar Bananal from capybara, highlights the importance of wild and sinantropic rodents as carriers of pathogenic leptospires.

Key words: Capybara, *Leptospira santarosai*, microdilution, PFGE, serogroup Grippotyphosa.

Leptospirosis is a widespread zoonosis with a higher incidence in tropical climates. *Leptospira* is maintained in nature by the infection of a wide variety of wild and domestic animals (Faine et al. 1999). Rodents appear to be the most important reservoirs, contaminating the environment and food, and they can transmit the infection when consumed by carnivores (Suepaul et al. 2010).

Capybara (*Hydrochoerus hydrochaeris*) are the largest living rodents and are distributed throughout South America. These rodents are exclusive herbivores that live in flooded grasslands (Cueto et al. 2000). Capybara can be efficient reservoirs of *Leptospira* because they live in areas with abundant water, which is an important environmental factor for disease transmission. Antibody prevalence studies and the isolation of *Leptospira* from capybara kidneys have been reported in Brazil (Ito et al. 1998; C.D.P. pers. comm.), demonstrating that capybaras are susceptible to *Leptospira* infection but serve primarily as carriers (Marvullo et al. 2009).

Because capybara are in close contact with farm animals and live in semiurban areas, they represent a risk to public health and can result in economic losses in production animals (Milagres 2004). We report the isolation of *Leptospira* strains from capybara kidneys, their serologic and molecular characterizations as *Leptospira santarosai* serogroup Grippotyphosa serovar Bananal, and the evaluation of their antibiotic susceptibility profiles.

We isolated *Leptospira* strains from a group of 40 apparently healthy capybaras in 2001 in Sao Paulo State, Brazil. For isolation, 5 g of kidney samples were collected and homogenized in 50 mL of Sorensen saline, and 100-

Downloaded from http://meridian.allenpress.com/jwd/article-pdf/52/3/688/2248084/2015-09-245.pdf by guest on 07 September 2024

TABLE 1. Susceptibility of *Leptospira santarosai* isolates to Sensititre Standard Susceptibility minimal inhibitory concentration (MIC) Plate BOPO6F.

	MIC (μg/mL)									
Isolate	Ceftiofur	Tiamulin	Gentamicin	Florfenicol	Chlortetracycline	Oxytetracycline	Penicillin	Ampicillin	Danofloxacin	Neomycin
2ACAP	1	>64	>32	>16	>16	>16	≤0.125	≤0.25	>2	>64
7ACAP	1	>64	>32	>16	>16	>16	≤0.125	≤0.25	>2	>64
10ACAP	2	>64	>32	>16	>16	>16	≤0.125	≤0.25	>2	>64
6BCAP	0.5	>64	>32	>16	>16	>16	≤0.125	≤0.25	>2	>64
21CAP	1	>64	>32	>16	>16	>16	≤0.125	≤0.25	>2	>64
L1.130[a]	≤0.25	≤0.5	≤1	0.25	0.5	≤0.5	≤0.125	≤0.25	1	8
Pomona[a]	≤0.25	2	8	2	>16	>16	≤0.125	≤0.25	>2	>64
MIC_{90}	1	>64	>32	>16	>16	>16	≤0.125	≤0.25	>2	>64

[a] Reference strains *Leptospira interrogans* serogroup Pomona serovar Pomona (1937, Australia) and *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni strain L1.130 (1996, Brazil).

TABLE 1. Extended.

	MIC (μg/mL)							
Isolate	Trimethoprim/ sulfamethoxazole	Spectinomycin	Tylosin tartrate	Tulathromycin	Tilmicosin	Clindamycin	Sulphadimethoxine	Enrofloxacin
2ACAP	>4/72	>128	>64	>128	>128	>32	>512	>4
7ACAP	>4/72	>128	>64	>128	>128	>32	>512	>4
10ACAP	>4/72	>128	>64	>128	>128	>32	>512	>4
6BCAP	>4/72	>128	>64	>128	>128	>32	>512	>4
21CAP	>4/72	>128	>64	>128	>128	>32	>512	>4
L1.130[a]	>4/72	≤8	≤0.5	≤1	≤4.0	≤0.25	>512	0.5
Pomona[a]	>4/72	≤8	≤0.5	≤1	>128	0.5	>512	2
MIC_{90}	>4/72	>128	>64	>128	>128	>32	>512	>4

Downloaded from http://meridian.allenpress.com/jwd/article-pdf/52/3/688/2248084/2015-09-245.pdf by guest on 07 September 2024

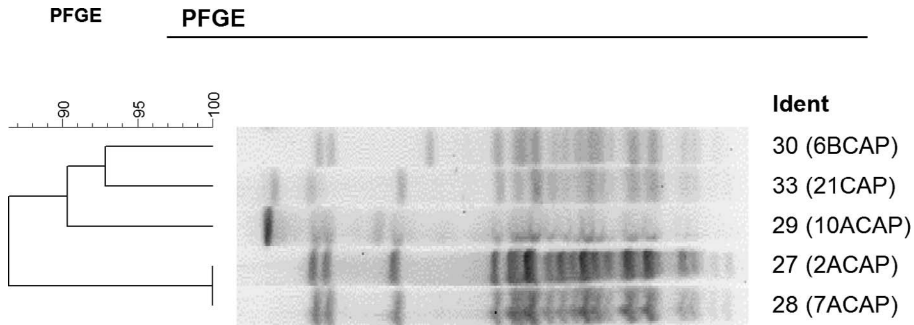

FIGURE 1. Dendrogram, generated by BioNumerics v7.5, showing the relationships among the pulsed field gel electrophoresis (PFGE) patterns from the *Leptospira santarosai* serogroup Grippotyphosa serovar Bananal isolated from capybara (*Hydrochaeris hydrochaeris*) in Sao Paulo State, Brazil, in 2001 (Ident=identification of strains).

μL aliquots of 10^{-1} to 10^{-3} dilutions were inoculated into duplicate tubes containing Ellinghausen–McCullough–Johnson–Harris bacterial culturing medium (EMJH; DIFCO, Detroit, Michigan, USA) enriched with 15% rabbit serum, 5-fluorouracil, and nalidixic acid. Once isolated, the cultures were stored in EMJH semisolid medium at 30 C.

Serotyping was performed at the World Health Organization and National Collaborating Centre for Reference and Research on Leptospirosis (Kit Biomedical Research, Amsterdam, the Netherlands). For the determination of presumptive serogroup and serovar, the isolates were subjected to microscopic agglutination test using the following panel of monoclonal antibodies: F71C9, F71C17, F165C1, F165C2, F165C3, and F165C7.

The pulsed field gel electrophoresis (PFGE) requirements for the culture conditions, plugs preparation, DNA extraction and digestion with *Not*I (New England Biolabs, Ipswich, Massachusetts, USA) were performed as described by Miraglia et al. (2013). The PFGE run was performed according to the Galloway and Levett (2010) protocol. Gels were stained with 1× SYBR® Safe (Invitrogen Corporation, Carlsbad, California, USA) for 40 min and photographed under ultraviolet transillumination. For the PFGE analysis, the isolates were grouped into different pulsotypes when they differed by four or more bands (Van Belkum et al. 2007). Fingerprint patterns were analyzed by comprehensive pairwise comparisons of the restriction fragment sizes using the Dice coefficient. The coefficient mean values were employed in unweighted pair group method with arithmetic mean using BioNumerics 7.5 (Applied Maths, Sint-Martens-Latem, Belgium) to generate a dendrogram.

Purified DNA was recovered according to Boom et al. (1990). The 16S ribosomal (r)RNA gene amplification was performed as described by Morey et al. (2006) with primer modifications (D1mod, GTTTGATCCTGGCTCAG; P2mod, GGCTACC TTGTTACGACTT). The 1,467–base pair–amplified fragments were purified using Illustra GFXTM PCR DNA and Gel Band Purification kit (GE Healthcare, São Paulo, Brazil) and sequenced at the Human Genome Research Center (University of São Paulo, São Paulo, Brazil).

Sequence analysis (trimming, consensus generation, and alignment) was performed with MEGA 5.10 (Tamura et al. 2011), and a phylogenetic tree was constructed using the maximum likelihood method with the Tamura three-parameter model. A total of 500 bootstrap replicates were used for branch support statistical inference. The DNA sequences were deposited in GenBank under accessions KJ946433–KJ946437.

The broth microdilution method was adapted from the Murray and Hospenthal (2004) protocol for the Sensititre® Standard Susceptibility MIC Plate BOPO6F (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The inoculum preparation, incubation, and addition of alamarBlue® (Thermo Fisher

Downloaded from http://meridian.allenpress.com/jwd/article-pdf/52/3/688/2248084/2015-09-245.pdf by guest on 07 September 2024

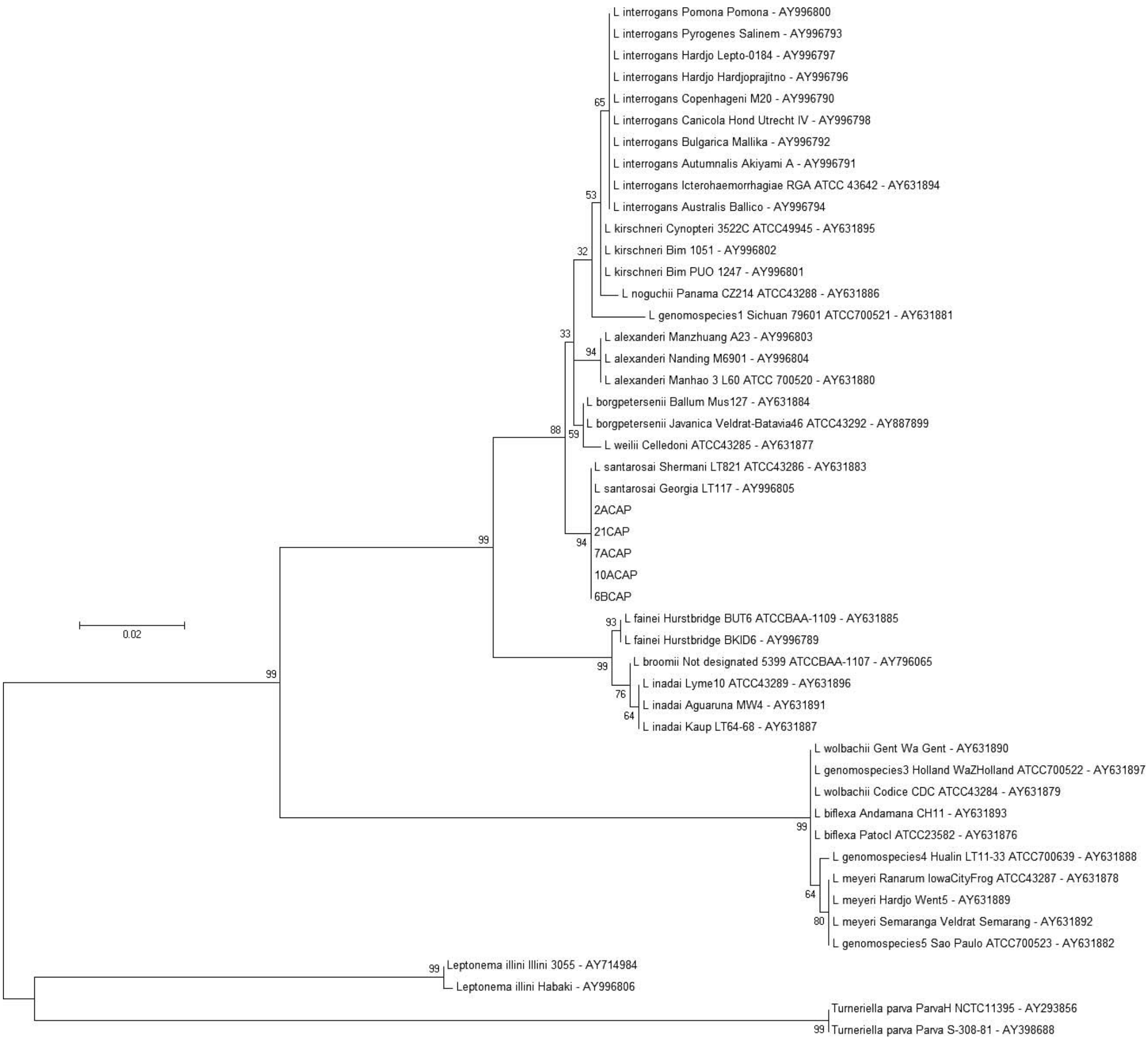

FIGURE 2. Dendrogram showing the evolutionary relationships among the *Leptospira santarosai* isolated from capybara (*Hydrochaeris hydrochaeris*) in Sao Paulo State, Brazil, in 2001 based on 16S ribosomal (r)RNA nucleotide sequences. The dendrogram was constructed using the maximum likelihood method with MEGA 5.10 software. The bootstrap values presented at the corresponding branches were evaluated using 500 replicates.

Downloaded from http://meridian.allenpress.com/jwd/article-pdf/52/3/688/2248084/2015-09-245.pdf by guest on 07 September 2024

Scientific) were performed as described by Miraglia et al. (2013). The minimal inhibitory concentrations (MICs) were assessed visually as the lowest concentrations of antibiotics in the wells without an alamarBlue color change on the third day of incubation. For internal and quality control, the *Leptospira interrogans* serogroup Pomona serovar Pomona (1937, Australia) and *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni strain L1.130 (1996, Brazil) were used.

Five strains were obtained from the capybara samples. The capybara strains had a higher growth rate than the reference strains and other collection isolates. Therefore, we adapted the incubation periods for all procedures and techniques. The isolates were serotyped as belonging to serogroup Grippotyphosa serovar Bananal. Genotyping using PFGE resulted in unmatched band patterns compared with the *Leptospira* reference strains. The capybara isolates clustered in one profile (P1) presenting high genetic similarity (Fig. 1). The phylogenetic analysis enabled us to identify the isolates as *L. santarosai* (Fig. 2); however, as expected, this analysis did not allow further study of the isolates at the serogroup and serovar levels.

All isolates presented high MICs to trimethoprim/sulfamethoxazole, sulphadimethoxine, and neomycin (Table 1). The isolates appeared to be sensitive to penicillin and ampicillin, whereas they presented higher MICs for the other tested antibiotics, including gentamicin, florfenicol, tetracyclines, tiamulin, and clindamycin. The isolates also had higher than expected MIC values for fluoroquinolones.

Marvullo et al. (2009) reported that capybara are susceptible to *Leptospira*. Antibody responses, as well as the leptospiremic and leptospiruric phases in this species, appear to be very similar to those reported for other domestic and wild animals. Therefore, capybara can serve as a source of infection for other animals (Marvullo et al. 2009). Our results corroborated these findings and implicated capybara as asymptomatic carriers of the pathogenic *L. santarosai*.

The isolation of pathogenic leptospires from capybara kidneys has already been reported in Brazil (S. Jorge unpubl.), and they were classified by variable number tandem repeat as *L. interrogans* serogroup Icterohaemorrhagiae. Our five isolates were serotyped as the serogroup Grippotyphosa serovar Bananal, and *L. santarosai* identification was confirmed only via 16S rRNA sequencing. Although this species is not commonly associated with disease in Brazil, *L. santarosai* is important in human and animal leptospirosis in other countries (Carmona-Gasca et al. 2011; Valverde et al. 2013), especially in Asia (Chou et al. 2012).

Identification of our isolates as *L. santarosai* by 16S rRNA sequencing corroborated results of Ahmed et al. (2006). These authors had previously typed our isolate 2ACAP using their multilocus sequence typing scheme. With this scheme, it was possible to cluster our isolate with other *L. santarosai* strains; however, the scheme did not allow further serogroup or serovar distinctions within the species.

The PFGE analysis, in turn, generated a unique profile (P1) clustering isolates with greater than 85% similarity, even though they originated from different animals of the same population. This suggests a single infection source and the possibility of intrapopulation dissemination. Our results also corroborated the findings of Galloway and Levett (2010), who reported PFGE patterns of unidentified and potentially new serovars distinct from *Leptospira* reference strains.

Genotypic distinction was also observed for the antibiotic susceptibility profiles. The capybara isolates had high MICs for all antibiotics except penicillin and ampicillin. Although this was the first study of antibiotic susceptibility of the *L. santarosai* serogroup Grippotyphosa serovar Bananal, our results differed from reports of *L. interrogans* serogroup Grippotyphosa and *L. santarosai* susceptibilities (Hospenthal and Murray 2003; Ressner et al. 2008). We are also aware of the need to determine the kinetics of the studied strains and to adapt *L. interrogans* standardized protocols.

Downloaded from http://meridian.allenpress.com/jwd/article-pdf/52/3/688/2248084/2015-09-245.pdf by guest on 07 September 2024

Based on our results, greater attention should also be given to fluoroquinolone susceptibility. Despite previous reports of leptospiral sensitivity to most fluoroquinolones, including the report by Chakraborty et al. (2010), who supported the use of norfloxacin as an alternative empirical leptospirosis treatment, we obtained MIC_{90} values of >2.0 μg/mL and >4.0 μg/mL for danofloxacin and enrofloxacin, respectively.

In addition to highlighting the importance of this wild and synanthropic rodent as a carrier of pathogenic leptospires, we emphasize the importance of the molecular methods used for the identification of pathogenic species other than *L. interrogans* and atypical serovars and of the necessity of establishing *Leptospira* antibiotic susceptibility guidelines.

This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (projects 2010/51365-0, 2011/18290-0, and 2013/17136-2) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

LITERATURE CITED

Ahmed N, Manjulata DS, Valverde M, Vijayachari P, Machangu RS, Ellis WA, Hartskeerl RA. 2006. Multilocus sequence typing method for identification and genetic classification of pathogenic *Leptospira* species. *Ann Clin Microbiol Antimicrob* 5:1–10.

Boom R, Sol CJA, Salimans MMM. 1990. Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* 28:459–453.

Carmona-Gasca CA, Lara LL, Castillo-Sánchez LO, Ramírez-Ortega JM, Ko A, Palomera CL, de la Peña-Moctezuma A. 2011. Detection of *Leptospira santarosai* and *L. kirschneri* in cattle: New isolates with potential impact in bovine production and public health. *Vet Mex* 42:277–288.

Chakraborty A, Miyahara S, Villanueva SYAM, Gloriani NG, Yoshida S. 2010. In vitro sensitivity and resistance of 46 *Leptospira* strains isolated from rats in the Philippines to 14 antimicrobial agents. *Antimicrob Agents Chemother* 54:5403–5405.

Chou LF, Chen YT, Lu CW, Ko YC, Tang CY, Pan MJ, Tian YC, Chiu CH, Hung CC, Yang CW. 2012. Sequence of *Leptospira santarosai* serovar Shermani genome and prediction of virulence-associated genes. *Gene* 511:364–370.

Cueto GR, Allekotte R, Kravetz FO. 2000. Scurvy in capybaras bred in captivity in Argentine. *J Zoo Wildl Med* 36:97–101.

Faine S, Adler B, Bolin C, Perolat P. 1999. Leptospira *and leptospirosis*. MediSci, Melbourne, Australia, 272 pp.

Galloway RL, Levett PN. 2010. Application and validation of PFGE for serovar identification of *Leptospira* clinical isolates. *PLoS Neg Trop Dis* 4:1–7.

Hospenthal D, Murray CK. 2003. In vitro susceptibilities of seven *Leptospira* species to traditional and newer antibiotics. *Antimicrob Agents Chemother* 47:2646–2648.

Ito FH, Vasconcellos SA, Bernardi F, Nascimento AA, Labruna MB, Arantes IG. 1998. Evidência sorológica de brucelose e leptospirose e parasitismo por ixodídeos em animais silvestres do Pantanal Sul-Mato-Grossense. *Ars Vet* 14:302–310.

Marvullo MFV, Silva JCR, Ferreira PM, Morais ZM, Moreno AM, Doto DS, Paixão R, Baccaro R, Vasconcellos SA, Ferreira Neto JS. 2009. Experimental leptospirosis in capybaras (*Hydrochaeris hydrochaeris*) infected with *Leptospira interrogans* serovar Pomona. *J Zoo Wildl Med* 40:726–730.

Milagres BS. 2004. *Epidemiological survey of diseases related to capybaras (*Hydrochaeris hydrochaeris*) in Brazil*. Masters Thesis, Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil, 65 pp.

Miraglia F, Matsuo M, Morais ZM, Dellagostin OA, Seixas FK, Freitas JC, Hartskeerl RA, Moreno LZ, Costa BL, Souza GO, et al. 2013. Molecular characterization, serotyping, and antibiotic susceptibility profile of *Leptospira interrogans* serovar Copenhageni isolates from Brazil. *Diagn Microbiol Infect Dis* 77:195–199.

Morey RE, Gallowey RL, Bragg SL, Steigerwalt AG, Mayer LW, Levett P. 2006. Species-specific identification of *Leptospiraceae* by 16S rRNA gene sequencing. *J Clin Microbiol* 44:3510–3516.

Murray CK, Hospenthal DR. 2004. Broth microdilution susceptibility testing for *Leptospira* spp. *Antimicrob Agents Chemother* 48:1548–1552.

Ressner RA, Griffith ME, Beckius ML, Pimentel G, Miller RS, Mende K, Fraser SL, Galloway RL, Hospenthal DR, Murray CK. 2008. Antimicrobial susceptibilities of geographically diverse clinical human isolates of *Leptospira*. *Antimicrob Agents Chemother* 52:2750–2754.

Suepaul SM, Carrington CVF, Campbell M, Borde G, Adesiyun AA. 2010. Serovars of *Leptospira* isolated from dogs and rodents. *Epidemiol Infect* 138:1059–1070.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony method. *Mol Biol Evol* 28:2731–2739.

Valverde MA, Goris M, González V, Anchía ME, Díaz P, Ahmed A, Hartskeerl RA. 2013. New serovars of *Leptospira* isolated from patients in Costa Rica: Implications for the public health. *J Med Microbiol* 62:1263–1271.

Van Belkum A, Tassios PT, Dijkshoorn L, Haeggman S, Cookson B, Fry NK, Fussing V, Green J, Feil E, Gerner-Smidt P, et al. 2007. Guidelines for the validation and application of typing methods for use in bacterial epidemiology. *Clin Microbiol Infect* 13:1–46.

Submitted for publication 17 September 2015.
Accepted 12 February 2016.

Downloaded from http://meridian.allenpress.com/jwd/article-pdf/52/3/688/2248084/2015-09-245.pdf by guest on 07 September 2024