Am. J. Trop. Med. Hyg., 94(3), 2016, pp. 519–521
doi:10.4269/ajtmh.15-0505

Infection with *Leptospira kirschneri* Serovar Mozdok: First Report from the Southern Hemisphere

Carlos Eduardo Pouey da Cunha, Samuel Rodrigues Felix, Amilton Clair Pinto Seixas Neto, Anelize Campello-Felix, Frederico Schmitt Kremer, Leonardo Garcia Monte, Marta Gonçalves Amaral, Márcia de Oliveira Nobre, Éverton Fagonde da Silva, Cláudia Pinho Hartleben, Alan John Alexander McBride, and Odir Antonio Dellagostin*

Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Pelotas, Brazil; Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, Brazil

Abstract. Leptospirosis is a global zoonosis caused by pathogenic *Leptospira* spp. In this study, we characterized two *Leptospira kirschneri* serogroup Pomona serovar Mozdok isolates, one obtained from a dog and the other from a patient with severe leptospirosis, 4 years later. Histopathological analysis showed that both isolates caused severe tissue damage when used to infect hamsters. While *L. kirschneri* serogroup Pomona serovar Mozdok is endemic in animals in Europe, there is only one report of human leptospirosis in the literature. Although strains belonging to *L. kirschneri* serogroup Pomona have been identified in cases of human leptospirosis in Europe, serovar Mozdok has not yet been implicated. The 4-year interval between isolations and the fact that this is the first report of serovar Mozdok as the causative agent of human leptospirosis in the southern hemisphere, demonstrates its epidemiological importance to public health. Moreover, the presence of serovar Mozdok in Brazil has the potential to affect vaccine and diagnostic test development.

Leptospirosis is a reemerging zoonotic disease, and the global burden is showing an upward trend. The original estimates in 1999[1] predicted some 500,000 annual cases compared with the latest prediction of 873,000 cases and 49,000 mortalities per year, a 74.6% increase over 15 years.[2] Accurate laboratory diagnosis continues to be a limiting factor, meaning that the true global burden of leptospirosis is likely to be much higher.[3] In Latin America, the prevalence of severe leptospirosis is high (10,000 cases a year) due to the tropical climate and lack of appropriate sanitation.[3] Although the city of Pelotas has a subtropical climate, > 50 cases of human leptospirosis per 100,000 inhabitants are reported each year, one of the highest rates in southern Brazil.[4] The infection rate in Pelotas is higher than the Brazilian average for the same period (3.5/100,000) and other regions with similar climatic conditions (> 10/100,000).[5]

At present, there are 10 pathogenic *Leptospira* spp. classified into > 260 serovars[6] and *Leptospira interrogans*, *Leptospira borgpetersenii*, and *Leptospira kirschneri* are most commonly associated with human leptospirosis.[7] In Brazil, *L. interrogans* serogroup Icterohaemorrhagiae serovars Icterohaemorrhagiae and Copenhageni are the main cause of urban leptospirosis and have been widely studied,[3] whereas rural leptospirosis and the associated serovars have been largely neglected. To the best of our knowledge, *L. kirschneri* serogroup Pomona serovar Mozdok has only been implicated in a case of human leptospirosis in Cuba.[8] Serovar Mozdok is endemic to Croatia where it is prevalent in wild rodents. Human leptospirosis caused by serogroup Pomona is common in that region and while serovar Mozdok has not been implicated in any human cases,[9] it is a causative agent of canine leptospirosis in Europe.[10]

We report the isolation and characterization of two isolates of serovar Mozdok recovered from cases of canine and human leptospirosis in Pelotas, southern Brazil. The canine strain was isolated in 2009 during a municipal dog neutering campaign. Urine samples were aseptically collected from the bladder during ovarian hysterectomy, via aspiration using an insulin 30-G needle and syringe (BD Biosciences, Franklin Lakes, NJ). The urine was immediately inoculated into unsupplemented Ellinghausen-McCullough-Johnson-Harris (EMJH; Difco, Sparks, MD) medium (100 μL urine/5 mL EMJH), incubated for 1 hour and then subcultured into EMJH containing 10% of a commercial supplement (Difco). The dog from which the strain was isolated was asymptomatic and was released after the surgical procedure. The second isolate was obtained from the blood culture of a 56-year-old female patient from a rural area of the city. The patient presented with headache, myalgia, fever, vomiting, fatigue, sleepiness, and arthralgia and reported contact with dogs, rats, pigs, cattle, and flood water. The isolate was cultured in EMJH medium as described for the canine isolate. Both isolates were identified as *L. kirschneri* by means of *secY* gene sequencing.[11] Multilocus sequence typing (MLST)[7] further characterized the isolates as *L. kirschneri* serogroup Pomona serovar Mozdok (ST 117). All sequencing procedures were performed using the paired-end technology on an Illumina Solexa platform (Illumina, San Diego, CA; GenBank accession numbers for sequences are shown in Table 1). The canine isolate was named 3759, and the human isolate 61H.

To assess the taxonomic relationship of the 3759 and 61H strains with previous isolates from Pelotas, concatenated sequences based on the loci used for MLST were used to generate a phylogenetic tree by means of the maximum composite likelihood method using MEGA software.[12] The 3759 and 61H strains grouped in a separate branch of the tree, showing weak relatedness to the other isolates (Figure 1). Of note, Mozdok isolates grouped more closely to serogroup Icterohaemorrhagiae strains than to other strains.

Golden Syrian hamsters (*Mesocricetus auratus*) were infected with 10^8 leptospires to determine the virulence of the isolates via the intraperitoneal route. Three hamsters per isolate were used and the experiment was performed once for the canine isolate and twice for the human isolate. The Committee on the Ethics of Animal Experimentation of the Federal University of Pelotas approved the protocol used (permit number 6843). Both isolates caused lethal leptospirosis, and the infected hamsters met end-point criteria for euthanasia within 4–7 days postinfection. The average lethal dose (LD_{50})

*Address correspondence to Odir Antonio Dellagostin, Núcleo de Biotecnologia, Centro de Desenvolvimento Tecnológico, Campus Universitário s/n, Caixa Postal 354, 96010-900, Pelotas, Brazil. E-mail: odir@ufpel.edu.br

TABLE 1
GenBank accession numbers for nucleotide sequences generated in this work to characterize the isolates

Gene	Isolate 61H	Isolate 3759
mreA	KP114449	KP125524
glmU	KP114450	KP125525
caiB	KP114451	KP125526
pfkB	KP114452	KP125527
pntA	KP114453	KP125528
sucA	KP114454	KP125529
tpiA	KP114455	KP125530
secY	KP114457	KP125532

of the human isolate was 170 leptospires using a standard protocol[13]; while the virulence of the canine isolate was confirmed, the LD_{50} was not determined. Histopathological analysis revealed the presence of hemorrhagic lesions in the kidneys with infiltration by mononuclear cells and urinary casts. The liver exhibited leukocyte infiltration, hemorrhage, congestion, and atypical hepatocyte architecture; the lungs showed edema, congestion, hemorrhage, and hemosiderin within 7 days of infection. The imprint technique[14] confirmed the presence of leptospires in the kidneys, liver, and lungs of infected animals. The presence of putative virulence factors LigA and LigB, as well as LipL32[15] was confirmed by means of indirect immunofluorescence using rabbit polyclonal sera against each antigen conjugated with fluorescein isothiocyanate (excited at 450 nm). Leptospiral DNA was stained with Hoechst 33258 (Sigma-Aldrich, St. Louis, MO; excited at 350 nm). Supplemental Figures 1 and 2 are representative of the histopathological damage and indirect immunofluorescence results observed in infected hamsters, respectively.

Although serovar Mozdok has been implicated in canine leptospirosis, mainly in Europe,[10] to our knowledge, this is the first report of its isolation. Similarly, this is the first isolate from a patient in the southern hemisphere and only the second worldwide.[8] The 4-year interval between isolations suggests that the serovar has adapted to at least one reservoir host and is circulating in the city, with the potential to cause infection. Furthermore, asymptomatic dogs carrying a virulent *Leptospira* strain are of particular public health concern, especially in a city with > 20,000 stray animals.[16]

The most effective way to ensure protection from leptospirosis is vaccination. The current vaccines, however, are heat-killed whole cells of *Leptospira* spp., which provide only serovar-specific protection, and only a few countries permit their use.[15] This type of vaccine could be effective in urban areas such as Salvador and São Paulo, where a single serovar is responsible for the majority of human and animal cases[3]; however, in mixed urban/rural areas such as Pelotas, where leptospirosis is caused by several serovars,[17–20] novel vaccines capable of inducing a cross-protective response are a necessity. Therefore, characterization of clinical isolates to the serovar level is of crucial importance, not only to understand the epizootiology of the disease but also for the development of novel vaccines and diagnostic tests. Thus, there is a risk that previously unreported serovars will appear in urban or rural settings without apparent epidemiological cues, affecting both diagnosis accuracy and vaccine efficacy.

In conclusion, we believe this to be the first report of human and animal leptospirosis caused by *L. kirschneri* serogroup Pomona serovar Mozdok in the southern hemisphere, making it one of the prevalent serovars causing disease in humans and animals in southern Brazil, and possibly in other parts of the world with similar environmental conditions. Furthermore, the epidemiological data presented here will be important for the development of both animal and human leptospirosis

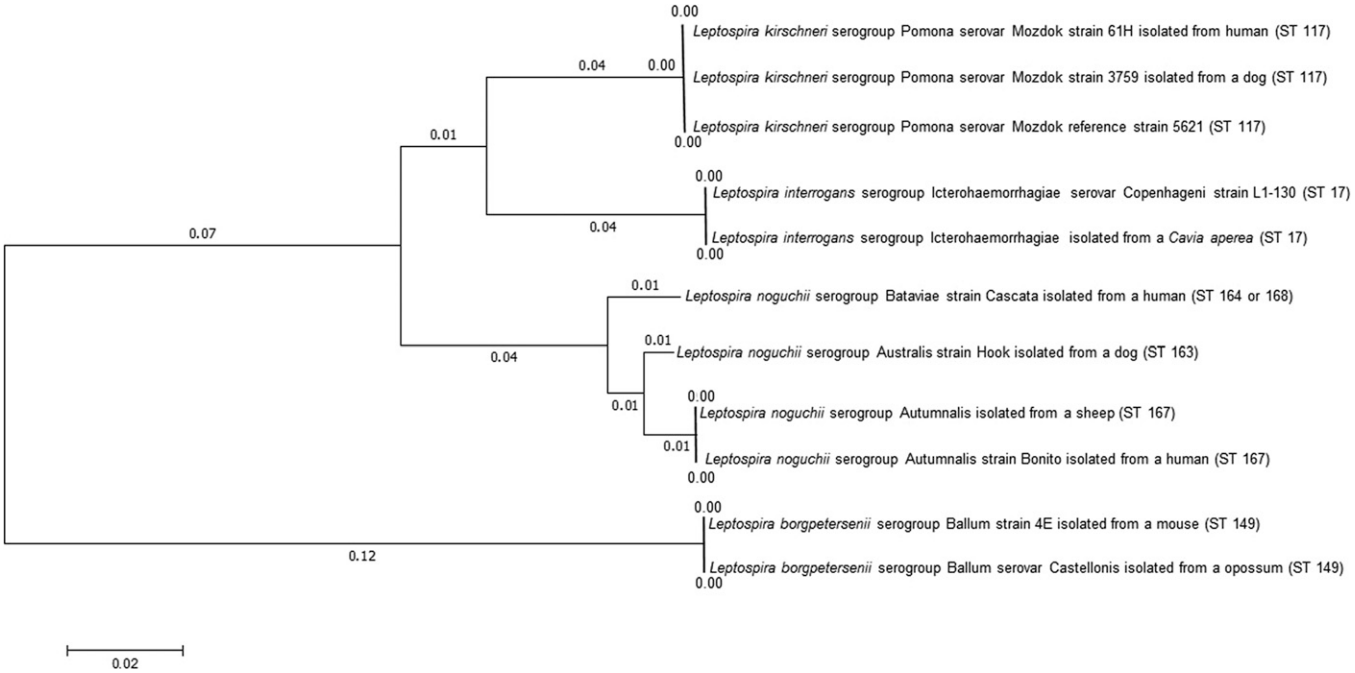

FIGURE 1. Dendrogram constructed from the concatenated sequences of 7 multilocus sequence typing (MLST) loci. Patient and animal isolates from the city of Pelotas are presented as well as two reference strains (L1-130 and 5621). Branch length is displayed next to each branch. The evolutionary distances were computed using the maximum composite likelihood method and are shown as the number of base substitutions per site. The sequences used to construct this dendrogram were retrieved from http://leptospira.mlst.net/portable/portable.xls and concatenated on http://leptospira.mlst.net/sql/concatenate/default.asp.

vaccines and/or rapid diagnostic tests. Moreover, we recommend that serovar Mozdok be included in the microscopic agglutination test batteries used by the State reference laboratories (Laboratório Central do Estado [State's central lab - Portuguese]) for the diagnosis of leptospirosis.

Received July 7, 2015. Accepted for publication October 12, 2015.

Published online January 11, 2016.

Note: Supplemental figures appear at www.ajtmh.org.

Acknowledgments: We would like to thank Ana Iara Noronha for helping with patient screening.

Financial support: The funding and scholarships were provided by CNPq and CAPES.

Disclaimer: The funding agencies played no role in publication.

Authors' addresses: Carlos Eduardo Pouey da Cunha, Centro de Desenvolvimento Tecnológico, Núcleo de Biotecnologia, Universidade Federal de Pelotas, Pelotas, Brazil, E-mail: cpouey@gmail.com. Samuel Rodrigues Felix, Amilton Clair Pinto Seixas Neto, Anelize Campello-Felix, Márcia de Oliveira Nobre, and Éverton Fagonde da Silva, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, Brazil, E-mails: samuelrf@gmail.com, amiltonseixas@gmail.com, anelizecampellofelix@gmail.com, marciaonobre@gmail.com, and fagondee@gmail.com. Frederico Schmitt Kremer, Leonardo Garcia Monte, Marta Gonçalves Amaral, Cláudia Pinho Hartleben, Alan John Alexander McBride, and Odir Antonio Dellagostin, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Pelotas, Brazil, E-mails: fred.s.kremer@gmail.com, leonardogmonte@hotmail.com, martagamaral@gmail.com, hartlebenclaudia@gmail.com, alanmcb@gmail.com, and odirad@terra.com.br.

REFERENCES

1. WHO, 1999. Leptospirosis worldwide. *Wkly Epidemiol Rec 74:* 237–242.
2. Picardeau M, Bertherat E, Jancloes M, Skouloudis AN, Durski K, Hartskeerl RA, 2014. Rapid tests for diagnosis of leptospirosis: current tools and emerging technologies. *Diagn Microbiol Infect Dis 78:* 1–8.
3. McBride AJA, Athanazio DA, Reis MG, Ko AI, 2005. Leptospirosis. *Curr Opin Infect Dis 18:* 376–386.
4. Barcellos C, Lammerhirt CB, Almeida MA, Santos E, 2003. Spatial distribution of leptospirosis in Rio Grande do Sul, Brazil: recovering the ecology of ecological studies [in Portuguese]. *Cad Saude Publica 19:* 1283–1292.
5. WHO, 2003. *Human Leptospirosis: Guidance for Diagnosis, Surveillance and Control*. Geneva, Switzerland: World Health Organization.
6. Bourhy P, Collet L, Brisse S, Picardeau M, 2014. *Leptospira mayottensis* sp. nov., a pathogenic *Leptospira* species isolated from humans. *Int J Syst Evol Microbiol 64:* 4061–4067.
7. Boonsilp S, Thaipadungpanit J, Amornchai P, Wuthiekanun V, Bailey MS, Holden MTG, Zhang C, Jiang X, Koizumi N, Taylor K, Galloway R, Hoffmaster AR, Craig S, Smythe LD, Hartskeerl RA, Day NP, Chantratita N, Feil EJ, Aanensen DM, Spratt BG, Peacock SJ, 2013. A single multilocus sequence typing (MLST) scheme for seven pathogenic *Leptospira* species. *PLoS Negl Trop. Dis 7:* e1954.
8. Obregón AM, Fernández C, Rodríguez I, Rodríguez J, 2007. The application of monoclonal antibody methodology as a tool for serotyping *Leptospira* isolates in Cuba. *Rev Cubana Med Trop 59:* 68–70.
9. Majetic ZS, Galloway R, Sabljic ER, Milas Z, Perko VM, Habus J, Margaletic J, Pernar R, Turk N, 2014. Epizootiological survey of small mammals as *Leptospira* spp. reservoirs in eastern Croatia. *Acta Trop 131:* 111–116.
10. Renaud C, Andrews S, Djelouadji Z, Lecheval S, Corrao-revol N, Buff S, Demont P, Kodjo A, 2013. Prevalence of the *Leptospira* serovars Bratislava, Grippotyphosa, Mozdok and Pomona in French dogs. *Vet J 196:* 126–127.
11. Victoria B, Ahmed A, Zuerner RL, Ahmed N, Bulach DM, Quinteiro J, Hartskeerl RA, 2008. Conservation of the S10-spc-alpha locus within otherwise highly plastic genomes provides phylogenetic insight into the genus *Leptospira*. *PLoS One 3:* e2752.
12. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S, 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol 30:* 2725–2729.
13. Reed LJ, Muench H, 1938. A simple method of estimating fifty percent endpoints. *Am J Hyg 27:* 493–497.
14. Chagas-Junior AD, McBride AJA, Athanazio DA, Figueira CP, Medeiros MA, Reis MG, Ko AI, McBride FWC, 2009. An imprint method for detecting leptospires in the hamster model of vaccine-mediated immunity for leptospirosis. *J Med Microbiol 58:* 1632–1637.
15. Dellagostin OA, Grassmann AA, Hartwig DD, Félix SR, da Silva ÉF, McBride AJA, 2011. Recombinant vaccines against leptospirosis. *Hum Vaccin 7:* 1215–1224.
16. Reis RB, Ribeiro GS, Felzemburgh RDM, Santana FS, Mohr S, Melendez AXTO, Queiroz A, Santos AC, Ravines RR, Tassinari WS, Carvalho MS, Reis MG, Ko AI, 2008. Impact of environment and social gradient on *Leptospira* infection in urban slums. *PLoS Negl Trop Dis 2:* e228.
17. Silva ÉF, Cerqueira GM, Seyffert N, Seixas FK, Hartwig DD, Athanazio DA, Pinto LS, Queiroz A, Ko AI, Brod CS, Dellagostin OA, 2009. *Leptospira noguchii* and human and animal leptospirosis, southern Brazil. *Emerg Infect Dis 15:* 4–7.
18. Silva EF, Félix SR, Cerqueira GM, Fagundes MQ, Neto ACPS, Grassmann AA, Amaral MG, Gallina T, Dellagostin OA, 2010. Preliminary characterization of Mus musculus-derived pathogenic strains of *Leptospira borgpetersenii* serogroup Ballum in a hamster model. *Am J Trop Med Hyg 83:* 336–337.
19. Silva EF, Santos CS, Athanazio DA, Seyffert N, Seixas FK, Cerqueira GM, Fagundes MQ, Brod CS, Reis MG, Dellagostin OA, Ko AI, 2008. Characterization of virulence of *Leptospira* isolates in a hamster model. *Vaccine 26:* 3892–3896.
20. Jorge S, Monte LG, De Oliveira NR, Collares TF, Roloff BC, Gomes CK, Hartwig DD, Dellagostin OA, Hartleben CP, 2015. Phenotypic and molecular characterization of *Leptospira interrogans* isolated from *Canis familiaris* in southern Brazil. *Curr Microbiol 71:* 496–500.