**Genetic evidence for a potential environmental pathway to spillover infection of rat-borne leptospirosis.**

**Running head: Environmental pathway to leptospirosis spillover**

Arnau Casanovas-Massana\*, Daiana de Oliveira, Andrew G. Schneider, Mike Begon, James E. Childs, Federico Costa, Elsio A. Wunder Jr, Mitermayer G. Reis, and Albert I. Ko\*

Yale School of Public Health, New Haven, Connecticut, USA (A. Casanovas-Massana, A.G. Schneider, J.E. Childs, F. Costa, E.A. Wunder, A.I. Ko)

Institute of Integrative Biology, University of Liverpool, Liverpool, UK (Mike Begon, F. Costa)

Oswaldo Cruz Foundation, Salvador, Bahia, Brazil (D. de Oliveira, F. Costa, E.A. Wunder, Mitermayer G. Reis, A.I. Ko)

Universidade Federal da Bahia, Salvador, Bahia, Brazil (F. Costa)

\*Corresponding authors

Yale School of Public Health – Department of Epidemiology of Microbial Diseases

60 College Street, LEPH Room 319B, P.O. Box 208034

New Haven, CT, 06520-8034 USA

Phone: +1 203 785 6292

Fax: +1 203 785 6193.

E-mail: arnau.casanovas@yale.edu; albert.ko@yale.edu

**Abstract** (47 words)

We identified by molecular methods a population of *L. interrogans* Copenhageni common to the environmental reservoirs (surface water and soil), colonized rat specimens and cases of human severe leptospirosis in an endemic urban slum. This finding provides evidence for a potential environmental spillover pathway for rat-borne leptospirosis.

**Keywords**

Pathogenic *Leptospira*; *Leptospira interrogans*; leptospirosis; environmental reservoir; soil; water; *Rattus norvegicus*.

**DISPATCH (1,127 words)**

 Leptospirosis is an environmentally-transmitted zoonotic disease that is emerging as an epidemic in urban slum communities in developing countries (1). Spillover leptospirosis infections require the continuous release of pathogenic *Leptospira* from their animal reservoirs and their survival and dispersal in the environment (2). However, the pathways of leptospirosis transmission are not well established due to the diversity of animal reservoirs and the difficulties of isolation from environmental sources. Previously, we found that pathogenic *Leptospira* spp*.* were widely distributed in surface waters (3) and soils (4) from an urban slum in Salvador (Brazil) at high risk for leptospirosis infection (5) where Norway rats (*Rattus norvegicus*) are the main reservoir of the pathogen (6). In this well characterized urban setting, we aimed to identify potential pathways to *Leptospira* spillover by investigating the genetic diversity of the pathogen in environmental reservoirs, colonized rat carriers and isolates from severe human leptospirosis cases.

To characterize the circulating pathogenic *Leptospira spp.*, we randomly selected 152 surface water and soil samples collected from 2012 to 2014 in this urban slum with a positive qPCR result for pathogenic *Leptospira* (3,4). We also included 6 *Leptospira* isolates from Norway rats captured in the same area in 1998 (7) and 28 urine specimens from rats captured from 2010 to 2013 (6). Using a nPCR approach with primers SecYII and SecYIV and internal primers G1 and G2 (8), we amplified and sequenced a 245-bp fragment of the *secY* gene suitable for the discrimination between species. Finally, we retrieved the *secY* sequences of 84 human clinical isolates of *L. interrogans* from severe leptospirosis cases in Salvador (Brazil) from 1996 to 2012 (7). Among these clinical isolates, 5 came from patients residing in the same slum where rats and environmental samples were collected. We then inferred a Maximum Likelihood phylogenetic tree using GTR substitution model and 1000 bootstraps (Technical Appendix).

We obtained suitable sequences for analysis from 84 of 152 (55%) environmental samples and from all 34 rats. The 245-bp *secY* gene sequences exhibited 80 ̶ 100% nucleotide identity to pathogenic *Leptospira* species (Technical Appendix for accession numbers). The phylogenetic tree showed that the samples formed 5 clusters within the pathogenic group (A to E). Cluster A comprised 135 samples including all 84 human isolates, all 34 rat samples, 41 environmental samples (38 surface water and 3 soil) and the reference strain *L. interrogans* Copenhageni L1-130. The sequences exhibited a 100% nucleotide identity among them, apart from one soil sample (98.8% identity) (Fig. 1).

The remaining 43 environmental samples grouped in 4 clusters (B to E) and 5 sequences remained ungrouped (Fig. 1). These phylogroups had nucleotide identities ranging from 89% to 95% to other pathogenic *Leptospira* spp., but only 69 ̶ 71% and 77 ̶ 78% to intermediate and saprophytic species, respectively, which suggests that some may represent novel species within the pathogenic cluster. This is consistent with recent observations that the diversity of *Leptospira* in environmental reservoirs is still largely underexplored (9,10). The role that these potential pathogens play in the epidemiology of urban leptospirosis seems limited, since active surveillance programs have never identified them, but their contribution to subclinical infections and animal disease should be investigated.

To further characterize the *L. interrogans*-like cluster A, we performed a partial multilocus sequence typing (MLST) with genes *glmU,* *pfkB* and *tpiA* adapting a nPCR (11) (Technical Appendix) to a random selection of 32 of 41 (78%) environmental samples from cluster A, and all rat samples and human clinical isolates (Technical Appendix). The sequences from the 3 genes were concatenated to obtain a 1,302-bp fragment and Maximum Likelihood phylogenetic trees were constructed as described above. All human and rat samples, and 17 of 24 environmental samples had suitable sequences for analysis for all the selected genes (*glmU*, *pfkB* and *tpiA*). Environmental and rat samples shared a 100% nucleotide identity among them and grouped with *L. interrogans* strains belonging to serogroup Icterohaemorrhagiae (serovars Copenhageni and Icterohaemorrhagiae) (Fig. 2). In addition, 83 of 84 human isolates had also identical sequences to those of the environmental and rat samples. Notably, the 83 identical human isolates and the 6 rat isolates had been previously serotyped as serovar Copenhageni (data not published). The different human isolate was identified as serovar Canicola. Overall, the partial MLST confirmed the clonality of the samples from cluster Aalready observed for *secY* (Fig. 1).

To determine if the sequences that grouped with *L. interrogans* serogroup Icterohaemorrhagiae strains were serovar Copenhageni or Icterohaemorrhagiae, we amplified by nPCR a sequenced a fragment of gene *lic12008* from 35 environmental samples and all rat specimens (Technical Appendix). Additionally, we retrieved the *lic120008* gene sequence from all cluster A human and rat isolates. This gene is related to LPS biosynthesis and contains an indel that can genetically distinguish *L. interrogans* serovars Copenhageni and Icterohaemorrhagiae (7). Of the 136 samples with suitable sequence results (83 human, 34 rat and 19 environmental), all had identical sequences with *L. interrogans* Copenhageni reference strains (Technical Appendix). This indicates that all *L. interrogans* detected in cluster A likely belonged to serovar Copenhageni, with the exception from one serogroup Canicola human isolate.

This study was limited in that we did not characterize isolates or specimens from alternative animal reservoirs present in this slum. Although a few pigs, horses and stray dogs exist, their potential contribution to the environmental load is likely outweighed by that of *R. norvegicus*, which are present in high numbers and are chronic shedders or the pathogen (6,12). In addition, our sequencing approach may have not completely captured all the variability within *L. interrogans* and consequently, some clustering between or within human, rats and the environment may still exist. Future studies should aim at isolating *L. interrogans* from water and soil to allow for a finer characterization of the pathogen in its environmental reservoirs using whole genome sequencing.

Despite these limitations, we identified by molecular methods a common population of *L. interrogans* Copenhageni in human clinical cases, rat reservoirs and the environment (surface water and soil) in a Brazilian urban slum, confirming that both rats and the environment are sources for human infection. Previous cross-sectional and longitudinal epidemiological studies showed that contact with mud and water were strongly associated with *Leptospira* infection among slum dwellers (5,13,14). Conversely, direct contact with rats is virtually non-existent. Therefore, although an infection route through direct exposure to rat urine cannot be ruled out, our results support the existence of spillover pathway for rat-borne leptospirosis through the environment in this urban community. Given the poor success of rodent control strategies due to regrowth after extermination [(15)](https://paperpile.com/c/V0q8hO/4i9z%2Bo3a6), public health interventions to prevent or reduce exposures to environmental sources of the pathogen are critical to weaken the spillover pathway to the human population in urban slums and prevent *Leptospira* infection and outbreaks of severe leptospirosis.

**Acknowledgements**

We thank the joint collaborative effort of the resident associations, community leaders and residents, which constitute the Urban Health Council of Pau da Lima, as well as the members of the Leptospirosis Group from Gonçalo Moniz Institute, Salvador, Brazil. This research was supported by the National Institutes of Health research grants [R01 AI052473], [U01 AI088752], [R25 TW009338], [R01 TW009504] and [R01 AI121207], and the Wellcome Trust grant [102330/Z/13/Z].

**Biographical sketch**

Dr. Casanovas-Massana is a Postdoctoral Associate at Yale School of Public Health. His research interests include the ecology of microbial pathogens in environmental reservoirs.

**REFERENCES**

1. Costa F, Hagan JE, Calcagno J, Kane M, Torgerson P, Martinez-Silveira MS, et al. Global Morbidity and Mortality of Leptospirosis: A Systematic Review. PLoS Negl Trop Dis. 2015;9(9):e0003898.

2. Plowright RK, Parrish CR, McCallum H, Hudson PJ, Ko AI, Graham AL, et al. Pathways to zoonotic spillover. Nat Rev Microbiol. 2017;15(8):502–10.

3. Casanovas-Massana A, Costa F, Riediger IN, Cunha M, de Oliveira D, Mota DC, et al. Spatial and temporal dynamics of pathogenic Leptospira in surface waters from the urban slum environment. Water Res. 2018 Mar;130:176–84.

4. Schneider AG, Casanovas-Massana A, Hacker KP, Wunder EA, Begon M, Reis MG, et al. Quantification of pathogenic Leptospira in the soils of a Brazilian urban slum. PLoS Negl Trop Dis. 2018 Apr;12(4):e0006415.

5. Hagan JE, Moraga P, Costa F, Capian N, Ribeiro GS, Wunder EA, et al. Spatiotemporal Determinants of Urban Leptospirosis Transmission: Four-Year Prospective Cohort Study of Slum Residents in Brazil. PLoS Negl Trop Dis. 2016;10(1):e0004275.

6. Costa F, Wunder EA, De Oliveira D, Bisht V, Rodrigues G, Reis MG, et al. Patterns in Leptospira Shedding in Norway Rats (Rattus norvegicus) from Brazilian Slum Communities at High Risk of Disease Transmission. PLoS Negl Trop Dis. 2015 Jun 5;9(6):e0003819.

7. Santos LA, Adhikarla H, Yan X, Wang Z, Fouts DE, Vinetz JM, et al. Genomic Comparison Among Global Isolates of L. interrogans Serovars Copenhageni and Icterohaemorrhagiae Identified Natural Genetic Variation Caused by an Indel. Front Cell Infect Microbiol [Internet]. 2018;8:193. Available from: https://www.frontiersin.org/article/10.3389/fcimb.2018.00193/full

8. Victoria B, Ahmed A, Zuerner RL, Ahmed N, Bulach DM, Quinteiro J, et al. Conservation of the S10-spc-alpha locus within otherwise highly plastic genomes provides phylogenetic insight into the genus Leptospira. PLoS One. 2008;3(7):e2752.

9. Thibeaux R, Iraola G, Ferrés I, Bierque E, Girault D, Soupé-Gilbert M-E, et al. Deciphering the unexplored Leptospira diversity from soils uncovers genomic evolution to virulence. Microb Genomics. 2018 Jan 1;4(1).

10. Vincent AT, Schiettekatte O, Goarant C, Neela VK, Bernet E, Thibeaux R, et al. Revisiting the taxonomy and evolution of pathogenicity of the genus Leptospira through the prism of genomics. Martins EAL, editor. PLoS Negl Trop Dis [Internet]. 2019 May 23 [cited 2019 Jul 24];13(5):e0007270. Available from: http://dx.plos.org/10.1371/journal.pntd.0007270

11. Weiss S, Menezes A, Woods K, Chanthongthip A, Dittrich S, Opoku-Boateng A, et al. An Extended Multilocus Sequence Typing (MLST) Scheme for Rapid Direct Typing of Leptospira from Clinical Samples. PLoS Negl Trop Dis. 2016;10(9):e0004996.

12. Faria MT de, Calderwood MS, Athanazio DA, McBride AJA, Hartskeerl RA, Pereira MM, et al. Carriage of Leptospira interrogans among domestic rats from an urban setting highly endemic for leptospirosis in Brazil. Acta Trop. 2008;108(1):1–5.

13. Felzemburgh RDM, Ribeiro GS, Costa F, Reis RB, Hagan JE, Melendez AXTO, et al. Prospective study of leptospirosis transmission in an urban slum community: role of poor environment in repeated exposures to the leptospira agent. PLoS Negl Trop Dis. 2014 May;8(5):e2927.

14. Reis RB, Ribeiro GS, Felzemburgh RDM, Santana FS, Mohr S, Melendez AXTO, et al. Impact of environment and social gradient on Leptospira infection in urban slums. PLoS Negl Trop Dis. 2008;2(4):e228.

15. Minter A, Diggle PJ, Costa F, Childs J, Ko AI, Begon M. A model for leptospire dynamics and control in the Norway rat (Rattus norvegicus) the reservoir host in urban slum environments. Epidemics. 2018;25:26–34.

**FIGURE CAPTIONS**

**Fig 1.** Phylogenetic analysis of 245-bp fragments of the *secY* gene sequences from environmental samples, rodent specimens and human leptospirosis isolates. Reference *Leptospira* spp. sequences were retrieved from GenBank (Technical Appendix). A bootstrap of 1000 replicates was performed and values above 75% are shown. SW and SL indicate samples obtained from sewage and soil, respectively. The top right panel shows the number of human, rat and environmental samples in cluster A, the year of collection (1996-2014), and the period of sample overlap.

**Fig 2.** Phylogenetic analysis of 1,302-bp fragments from the concatenated sequences obtained from genes *glmU*, *pfkB* and *tpiA* from environmental samples, rodent specimens and human leptospirosis isolates. Reference *L. interrogans* sequences were retrieved from the Leptospira MLST website (Technical Appendix). A bootstrap of 1000 replicates was performed and values above 75% are shown. SW and SL indicate samples obtained from sewage and soil, respectively.

**Figure 1**



**Figure 2**

