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BRIEF REPORT

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Genetic Evidence for a Potential Environmental Pathway to Spillover Infection of Rat-Borne Leptospirosis

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In this study, we genotyped samples from environmental reservoirs (surface water and soil), colonized rat specimens, and cases of human severe leptospirosis from an endemic urban slum in Brazil, to determine the molecular epidemiology of pathogenic *Leptospira* and identify pathways of leptospirosis infection. We identified a well-established population of *Leptospira interrogans* serovar Copenhageni common to human leptospirosis cases, and animal and environmental reservoirs. This finding provides genetic evidence for a potential environmental spillover pathway for rat-borne leptospirosis through the environment in this urban community and highlights the importance of environmental and social interventions to reduce spillover infections.

Keywords. environmental reservoir; *Leptospira interrogans*; leptospirosis; pathogenic *Leptospira*; *Rattus norvegicus*; genotyping; soil; water.

Leptospirosis is an environmentally transmitted zoonotic disease that is emerging as an epidemic in urban slum communities in developing countries [1]. Spillover leptospirosis requires the continuous release of pathogenic *Leptospira* from their animal reservoirs and their survival and dispersal in the environment before human exposure and infection [2]. However, the pathways of leptospirosis transmission are not well established due to the diversity of animal reservoirs. Furthermore, the fastidious characteristics of leptospires, especially from environmental sources, impacts our ability to isolate and genetically characterize this pathogen.

Previously, we found that pathogenic *Leptospira* species 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 define the molecular epidemiology of pathogenic *Leptospira* by investigating the genetic diversity of the pathogen in environmental reservoirs, colonized rat carriers, and isolates from severe human leptospirosis cases.

METHODS

Samples

To characterize the circulating pathogenic *Leptospira* species, we randomly selected 152 surface water and soil samples collected from 2012 to 2014 in this urban slum with a positive quantitative polymerase chain reaction (qPCR) result for pathogenic *Leptospira* [4]. Samples were selected by random stratification to include different sampling seasons, types of samples, and locations within the urban slum. 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] (Supplementary Table 2). In addition, we included in the analysis 84 pathogenic *Leptospira* isolates from severe human leptospirosis cases in Salvador (Brazil) from 1996 to 2012, and for which we have obtained whole-genome sequences (Supplementary Table 3) [7]. Among these clinical isolates, 5 came from patients residing in the same slum where rats and environmental samples were collected. Of note, all human isolates obtained in our studies in the city of Salvador have been identified as *Leptospira interrogans*.

PCR Reactions

We amplified a 245-bp fragment of the *secY* gene suitable for the discrimination between pathogenic *Leptospira* species. Because of the low concentrations reported previously in water and soil (<100 cells/mL or g), we performed a nested PCR (nPCR) with primers SecYII and SecYIV in a first step, and internal primers G1 and G2 in a second step, modifying a protocol described elsewhere [8]. All human and rat samples and a selection of 31 environmental samples, were further characterized with a partial multilocus sequence typing (MLST) with genes *glmU, pfkB,* and *tpiA* adapting a nPCR protocol described elsewhere with minor modifications [9]. PCR reactions contained 10× PCR buffer, 200 µM of each deoxynucleotide triphosphate (dNTP), 400 µM of the first or second pair of primers (Supplementary Table 1), 0.2 µg/µL of bovine serum albumin (Ambion), 1.25 U of Illustra Taq polymerase (GE Life Sciences), and 5 µL of DNA extract or the product of the first reaction in a total volume of 25 µL. *SecY* gene amplifications were conducted in a MyCycler Thermal Cycler (BioRad) using the following program: 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C

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for 45 seconds, and 72°C for 60 seconds; and a final step at 72°C for 7 minutes. For *glmU, pfkB,* and *tpiA* reactions, the annealing temperature was set at 46°C for both rounds of amplification. We also amplified by nPCR a fragment of gene *lic12008* from 35 environmental samples and all rat specimens as described previously [7] and retrieved the *lic120008* gene sequence from all cluster human and rat isolates.

PCR products were visualized with 2% agarose gels, amplicons were purified using the Monarch DNA Extraction kit (NEB) prior to Sanger sequencing, and obtained sequences were deposited in GenBank.

Phylogenetic Analyses

Pairwise comparisons were carried out using the maximum composite likelihood with uniform rate of change in MEGA 7.0.18. For the phylogenetic analysis, sequences were aligned with MUSCLE 3.7 and curated with Gblocks 0.91b. Then, maximum likelihood trees were inferred by PhyML 3.0 using GTR substitution model and 1000 bootstrap replicates to assess confidence in nodes. The *secY* gene tree included sequences from all pathogenic, intermediate, and saprophytic leptospires retrieved from GenBank (Supplementary Table 4). The MLST tree included sequences from *L. interrogans* isolates belonging to different serovars retrieved from the MLST website (https://pubmlst.org/leptospira/) (Supplementary Table 5).

RESULTS

Using the nPCR procedure for *secY*, we obtained sequences suitable 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 (Supplementary Table 2). 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 1 soil sample (98.8% identity; Figure 1).

The remaining 43 environmental samples grouped in 4 clusters (B to E) and 5 sequences remained ungrouped (Figure 1). These phylogroups had nucleotide identities ranging from 89% to 95% to other pathogenic *Leptospira* species, 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.

To further characterize the *L. interrogans*-like cluster A, we performed a partial MLST with genes *glmU, pfkB,* and *tpiA* adapting a nPCR [9] to a random selection of 32 of 41 (78%) environmental samples from cluster A, and all rat samples (34) and human clinical isolates (84). The sequences from the 3 genes were concatenated to obtain a 1302-bp fragment and maximum

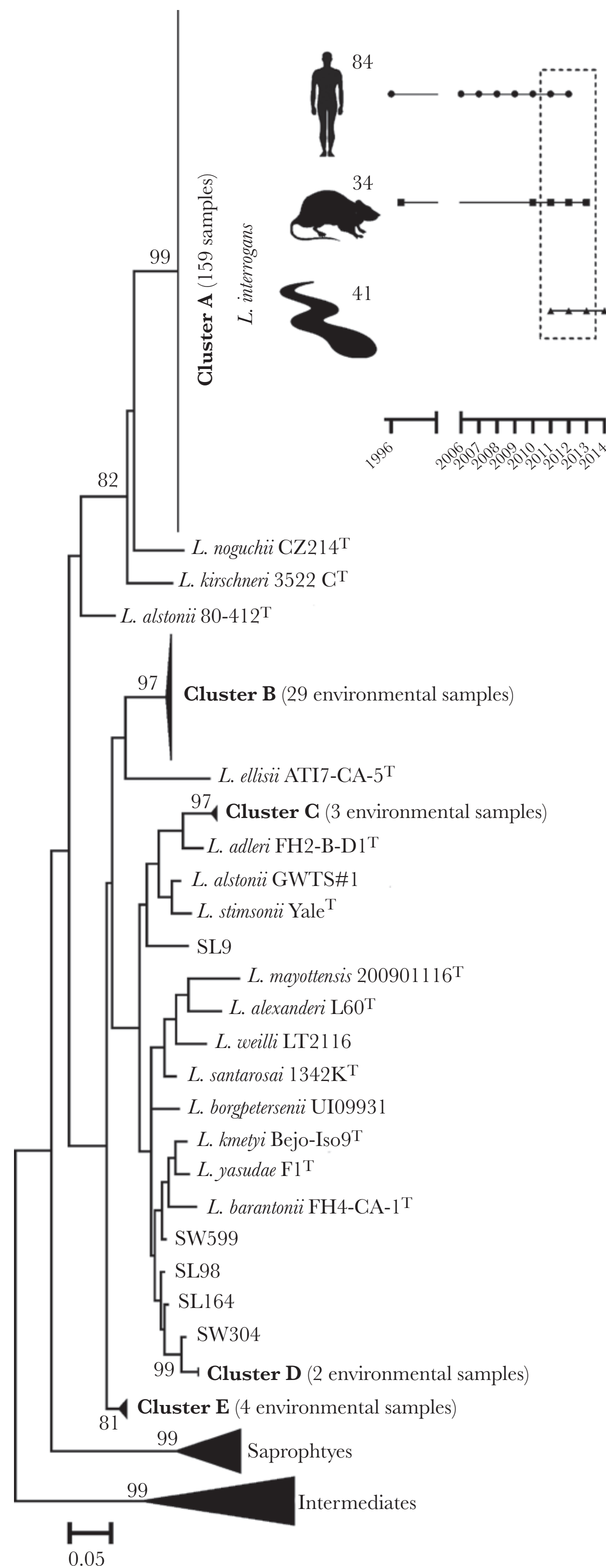

Figure 1. Phylogenetic analysis of 245-bp fragments of the *secY* gene sequences from environmental samples, rodent specimens, and human leptospirosis isolates. Reference *Leptospira* species sequences were retrieved from GenBank (Supplementary Material). A bootstrap of 1000 replicates was performed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

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likelihood phylogenetic trees were constructed as described above using 32 *L. interrogans* isolates from different serogroups as a reference. All human (84) and rat (34) samples, and 17 of 32 environmental samples had sequences suitable for analysis for all the selected genes (*glmU*, *pfkB,* and *tpiA*). Environmental and rat samples shared a 100% nucleotide identity among them (Supplementary Table 2) and grouped with *L. interrogans* strains belonging to serogroup Icterohaemorrhagiae (serovars Copenhageni and Icterohaemorrhagiae) (Figure 2). In addition, 83 of 84 human isolates also had 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 high clonality of the samples from cluster A already observed for *secY*.

To determine if the rat, human and environmental sequences that grouped with *L. interrogans* serogroup Icterohaemorrhagiae

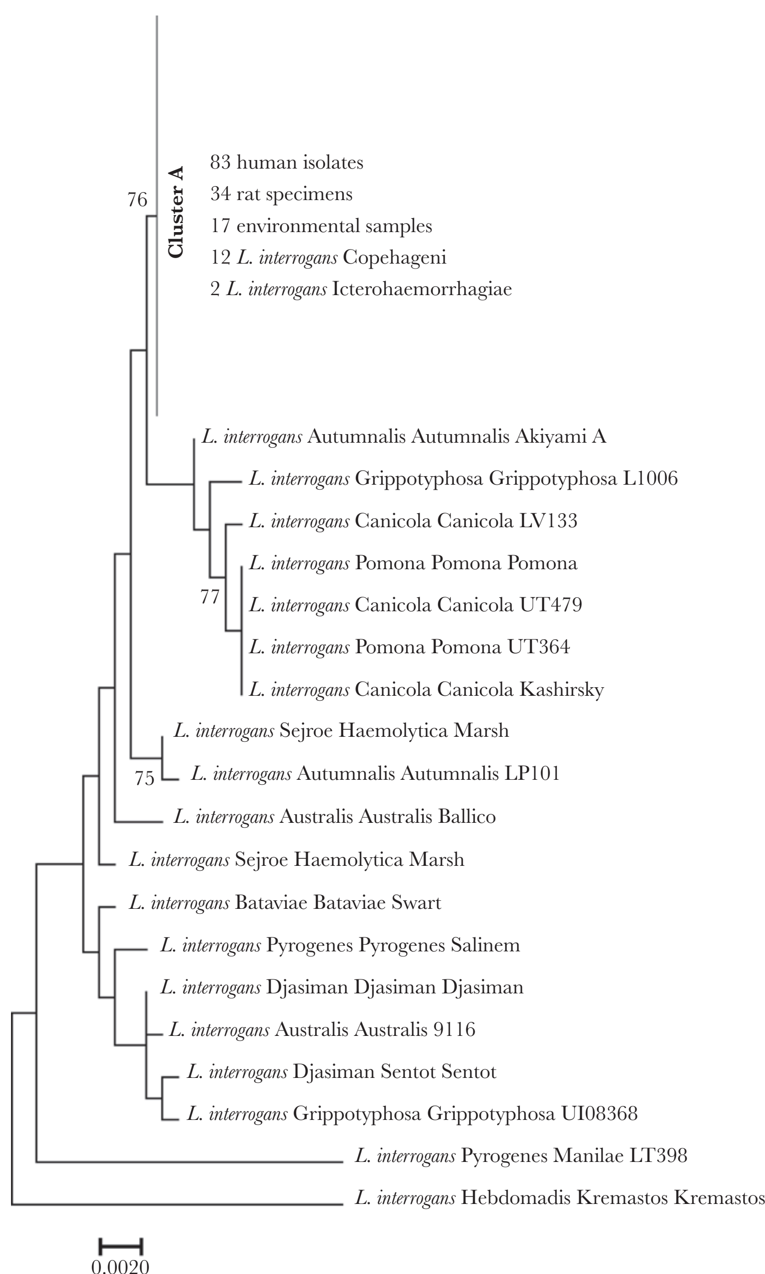

Figure 2. Phylogenetic analysis of 1302-bp fragments from the concatenated sequences obtained from genes *glmU*, *pfkB,* and *tpiA* from environmental samples, rodent specimens, and human leptospirosis isolates. Reference *Leptospira interrogans* sequences were retrieved from the *Leptospira* multilocus sequence typing website (Supplementary Material). A bootstrap of 1000 replicates was performed and values above 75% are shown. SW and SL indicate samples obtained from sewage and soil, respectively.

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strains were serovar Copenhageni or Icterohaemorrhagiae, we amplified by nPCR a sequenced of a fragment of gene *lic12008* from 35 of the 41 environmental samples belonging to cluster A, and all rat specimens. 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 (Supplementary Table 2).

DISCUSSION

The analysis of nucleotide identities is consistent with recent observations that the diversity of *Leptospira* in environmental reservoirs is still largely underexplored [10, 11]. The role that these potential pathogens play in the epidemiology of urban leptospirosis seems limited because active surveillance programs have never identified them, but their contribution to subclinical infections and animal disease should be investigated.

Our results indicate that all *L. interrogans* detected in cluster A likely belonged to serovar Copenhageni, with the exception of 1 serogroup Canicola human isolate. Overall, this indicates a high degree of clonality suggestive of an epidemiologically important population that is persistent over time and connects the environmental and rat reservoirs to human disease. These results add to previous studies that have used molecular approaches to identify links between human leptospirosis and environmental reservoirs [12] or animal reservoirs [13].

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 in this community, their potential contribution to the *Leptospira* environmental load is likely outweighed by that of *R. norvegicus*, which are present in high numbers and are chronic shedders of the pathogen [6]. Furthermore, only a small percentage (6%) of human isolates came from the same area where environmental and rat samples were selected. Our work in this community is focused on disease determinants, whereas our surveillance study that comprises isolation of the agent enrolls individuals from the entire city of Salvador, Brazil [1]. Nevertheless, all those isolates came from inhabitants of slum areas with similar epidemiological and living conditions across the city [1, 6, 14]. 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. Despite our compelling evidence of clonality, future studies should continue to aim at attempts to isolate *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 waters and soils) in a Brazilian urban slum. Our genetic-based results are consistent with previous epidemiological observations in case-control, cross-sectional and longitudinal studies of contact with mud and water as major risk factors for *Leptospira* infection among slum dwellers [5, 15]. Given that direct contact with rats is virtually nonexistent and the strong association of severe leptospirosis cases and seasonal rainfall [1], our findings support the existence of a spillover pathway for rat-borne leptospirosis through the environment in this urban community. Furthermore, our data suggest that a clonal population of *L. interrogans* is well established in this community and is persistent over time, being the cause of virtually all human leptospirosis cases. In view of the time-limited success of rodent control strategies due to regrowth after extermination [14] and the scarce resources available for public health in urban slums, interventions to reduce human exposures to the environmental sources of the pathogen (eg, construction of sanitary sewers, storm drains, or barriers to open sewers) should be explored. If successful, those actions can weaken the spillover pathway and prevent outbreaks of severe leptospirosis in similar settings around the world.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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