Am. J. Trop. Med. Hyg., 81(5), 2009, pp. 906–910
doi:10.4269/ajtmh.2009.09-0195
Copyright © 2009 by The American Society of Tropical Medicine and Hygiene

Prevalence of *Leptospira* spp. in Urban Rodents from a Groceries Trade Center of Medellín, Colombia

Piedad Agudelo-Flórez, Andrés F. Londoño, Víctor H. Quiroz, Juan C. Ángel, Natalí Moreno, Erica T. Loaiza, Luis F. Muñoz, and Juan D. Rodas*

Grupo de Investigación Medicina Tropical, Instituto Colombiano de Medicina Tropical–Universidad CES, Colombia, Grupo de Investigación en Ciencias Veterinarias, "Centauro," Facultad de Ciencias Agrarias, Universidad de Antioquia, Medellín, Colombia

Abstract. Leptospirosis is a widely distributed zoonosis, and rats are its most common source of infection. Our goal was to determine the frequency for *Leptospira* infection in rodents in a farmers market in the city of Medellin. We performed a descriptive transversal study sampling 254 rodents. Rodents were bled and killed, and kidneys samples were taken. Supernatants of macerated kidneys were cultured on Fletcher medium. Microagglutination tests (MATs) with 11 serovars were also carried out in rat serum, and a polymerase chain reaction (PCR) specific for pathogenic species was used to test each bacterial culture. All animals were identified as *Rattus norvegicus*; 25% and 20% were positive by MAT and culture, respectively. PCR tests of 12 isolates were positive for pathogenic serovars, and 4 of them were confirmed as *L. interrogans* by sequencing. These data show the role of this natural carrier and shedder of pathogenic leptospires in the epidemiology of urban leptospirosis in Colombia.

INTRODUCTION

Leptospirosis is caused by spirochetes belonging to the genus *Leptospira*, of which 17 species are currently recognized and at least another 4 species are in the process of being described.[1] The infection, acquired through contact with several animal reservoirs such as rodents or an environment contaminated by their urine, produces a wide spectrum of clinical manifestations in humans. Fever can be the only identifiable symptom in many cases. At other times, in mild forms of the disease, chills, headache, and severe myalgias can be observed, and in a small percentage of individuals, when the disease progresses, it can cause severe multisystem complications such as jaundice, renal insufficiency, bleeding diatheses, and hemorrhagic pneumonitis. Severe forms of the disease are associated with case-fatality rates of 5–40%.[2,3] Leptospirosis has a worldwide distribution, and although the disease is most common in tropical and rural settings, leptospirosis is considered an under-recognized urban problem, mainly in cities where the lack of basic sanitation favors its rodent-borne transmission as in the Latin-American large urban settlements.

In Colombia, little is known about the real situation of leptospirosis, particularly regarding the prevalence data of human leptospirosis and the risk factors of infection in urban zones. The only documented epidemic in the country was in the Atlantic region in 1995,[4] with a total of 47 confirmed cases (17% mortality within this group), 284 suspect cases, and isolation of serotypes Icterohaemorrhagiae, Pomona, and Canicola of *L. interrogans*. Other Colombian studies have found general antibody prevalence in humans of 18%,[5] 23%,[6] and 13%,[7] directed mainly to serovars Icterohaemorrhagiae and Grippotyphosa, both associated with rodent transmission.

These findings show that *Leptospira* is widespread in Colombia, but the real role that is played by rodents in the urban epidemiology of this disease is still unknown. The purpose of this study was to determine the prevalence of leptospirosis in the urban rodent population and to assess some of the probable risk factors associated with the human infection.

*Address correspondence to Juan D. Rodas, Sede de Investigación Universitaria (SIU) T2–233, Universidad de Antioquia, Calle 62 52-59, Medellín, Colombia. E-mail: juandavid.rodas@gmail.com

MATERIALS AND METHODS

Study site description. Rodent trapping was performed in the urban farmers market called Plaza Minorista "Jose Maria Villa" of Medellín city, Colombia (Figure 1). This market is located close to the central urban area, and the Medellin River runs along its back from south to north of the city, separated only by the highway. It has a total surface of 26,623 m^2 and is visited daily by nearly 15,000 customers and also by homeless dogs and horses carrying groceries. Within the facilities, it is possible to find anything from vegetables to live animals (chickens and ornamental birds) and pig rearing. This type of market is not uncommon and very popular in cities in Colombia because it offers very competitive prices and is under lower regulatory conditions.

Capture protocol. We used 36 Tomahawk traps (15 × 15 × 45 cm), which were placed in sites that were strategically chosen, according to visual criteria or indications of rodent presence such as feces or dark and hidden corners with an abundance of crumbs of food and garbage (Figure 1). The bait used to attract rodents was based on oat flakes, peanut butter, and vanilla essence and was rolled in little balls that were placed inside the traps. Traps were set up during the evening, left open during the night, and checked very early the next morning, before the market was opened to the public. Trapping was conducted for 28 sessions during 7 months. The study was carried out with the consent of the Animal Ethics Committee of the University of Antioquia, Colombia.

Sample collection. Captured rodents were anesthetized with a mixture of ketamine and xylazine (9:2) using 0.1 mL/50 g. Rats were bled from the heart (2–3 mL depending of their size) in dry sterile red cap Vacutainer tubes (without anticoagulant), and the animals were killed with an overdose of sodium pentobarbital (Eutanex, INVET, Bogotá, Colombia; 0.1 mL of a solution containing 350 mg/mL). Finally, we measured, weighed, and sexed the rodents, classified them according to their approximate age, and examined them externally for any signs of disease, and the carcasses were labeled for further identification and packed in plastic bags to be transported

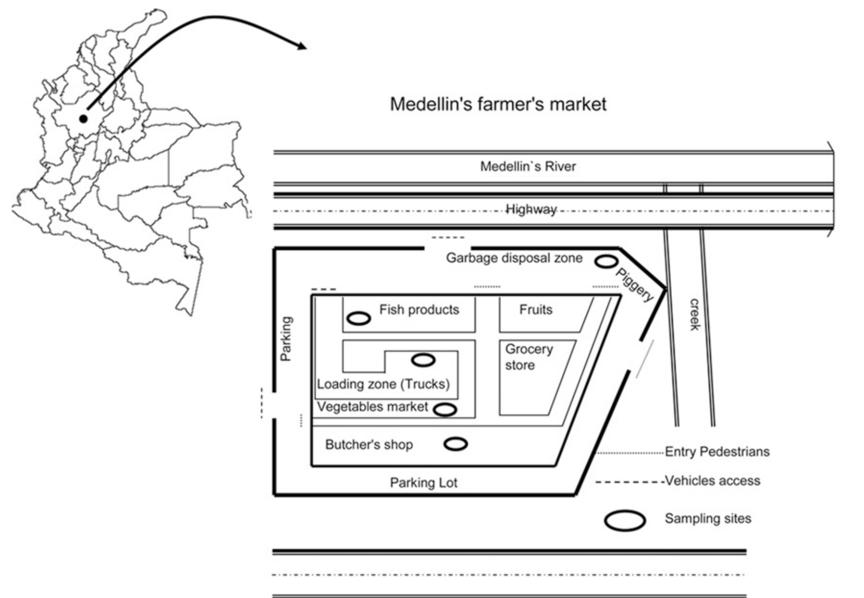

FIGURE 1. Study site diagram. The Medellin farmers market is shown in the center of the city, near the river and between popular roads and crowded sidewalks.

to the microbiology laboratory of the Veterinary School of the University of Antioquia for sample processing. In the laboratory, the dead animals were initially disinfected by immersion in an Iodined solution for 15 minutes and then passed into a Type 1 biosafety cabinet, where they were dissected to extract both kidneys and examine the internal organs for any signs of clinical illness. One of the kidneys was macerated in 3 mL of physiologic saline solution in a sealed plastic bag, and 50 µL of the supernatant for *Leptospira* culture was inoculated into a sterile tube containing semisolid Fletcher medium.

***Leptospira* culture.** Samples cultured in Fletcher medium were transported to the Instituto Colombiano de Medicina Tropical–CES University (ICMT-CES), where they were incubated at 28–30°C. The cultures were checked once a week, for up to 4 months, and weekly samples were taken for direct examination under a dark-field microscope.

Polymerase chain reaction detection from cultured samples. To determine the pathogenic status of *Leptospira* isolates, one sample of the positive culture was used for genomic DNA extraction using Qiamp DNA mini kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. This DNA was used in a polymerase chain reaction (PCR) reaction for pathogenic serovars described by Levett,[8] with primers LipL32/270F (CGCTGAAATGGGAGTTCGTATGATT) and LipL32/692R (CCAACAGATGCAACGAAAGATCCTTT). The primers were located between positions 270 and 692 of the *lipL32* gene, with an expected product size of 423 bp. The PCR reactions were done in a final volume of 25 µL containing: 2–5 µL (2–150 ng) of DNA, 0.25 µmol/L dNTPs, 1.0 IU of Taq DNA polymerase (Fermentas, Vilnius, Lithuania), 0.1 µmol/L of each primer, and 30 mmol/L $MgCl_2$. Negative and positive controls were used in each PCR reaction; for positive controls, we used DNA from reference pathogenic leptospires (Icterohaemorrhiae) in culture, and for negative controls, we used sterile water. The PCR program consisted of an initial cycle at 95°C for 5 minutes, followed by 35 cycles at 94°C for 1 minute and 55°C for 1 minute, and a final extension step at 72°C for 5 minutes.

The PCR products were visualized by electrophoreses in 1.5% agarose gels stained with SYBR safe (Invitrogen, Carlsbad, CA) and visualized with Epichemi camera. Furthermore, some of the LipL32 PCR products were purified with a filter for single sample PCR clean up (Microcon Centrifugal Filter Devices; Millipore, Billerica, MA) and used as templates for cycle sequencing that were run on a 3130 DNA sequencer (Applied Biosystems, Foster City, CA) to confirm whether they really were *L. interrrogans* sequences.

Microagglutination test. Blood samples were centrifuged, and serum was obtained and stored at −20°C until the end of the study when all the samples were screened for antibodies against generic *Leptospira* antigen, using the strain Patoc 1. In general, a microagglutination test (MAT) was performed starting with a serum dilution of 1:12.5 up to 1:400. The highest dilution of serum showing 50% reduction in free-moving leptospires was considered the endpoint. A titter of 1:50 or more was considered positive.

The sera that tested positive were assayed against a panel of 11 different *Leptospira* pathogenic serovars (Grippotyphosa, Canicola, Pomona, Hardjo, Bratislava, Ballum, Icterohaemorrhagiae, Shermani, Tarassovi, Australis, and Pyrogenes). Five percent of seronegative sera were also tested for the same panel of 11 *Leptospira* pathogenic serovars to confirm the validity of the screening with the Patoc 1 strain.

Statistical analyses. Data and variables of captured animals and samples tested for *Leptospira* were analyzed through descriptive statistics. Because of the fact that all the variables studied were qualitative, we obtained from them mainly proportions and rates, and thus, we established the frequency of animals captured according to species, sex, age, and infectious status. Infection was determined first by bacterial isolation or direct observation (dark-field microscope detection) and then by antibody detection. Likewise, we determine the correlation between these two tests. Additionally, we did χ^2 tests to establish the association between each of the variables studied and the presence of antibodies against *Leptospira* in captured rodents. The data were analyzed using the statistical program EPI INFO 2000.

RESULTS

We collected 254 rodents, all of them *Rattus norvegicus,* averaging two captures per week for a total of 28 nights of work during 7 months, for a capture success rate of ~25%. Taking into account an infinite population of unknown size with 50% prevalence (actually unknown also), we calculated a sampling error of 8% with a confidence level of 95%. Of the captured animals, 156 (61%) were male, 179 (71%) were adults, and 234 (92%) were found apparently healthy when examined clinically under anesthesia. The remaining 20 rodents showed unspecific symptoms (bruises, scratches, listlessness). After being dissected, some rat livers (7/254, 3%) showed a variety of signs such as petechia, hemorrhage, edema, jaundice, cysts, enlargement, or atrophy (data not shown).

Sixty-three rats were positive with the genus-specific MAT, using a 1:50 dilution against *Leptospira biflexa* strain Patoc 1, and one of the seronegatives tested with the 11 serovars (13/254, 5%) was also positive for serovars Pyrogenes, Tarassovi, and Bratislava, for an estimated accumulated seroprevalence of 25% (64/254, 95% confidence interval [CI]: 19.5%; 30.1%). Fifty-two were also positive for culture, for a prevalence of infection of 21% (95% CI: 15.5%; 25.4%). All isolates showed typical morphology and characteristic motility of the genus *Leptospira* through direct examination under the dark-field microscope (data not shown). Unfortunately, because of the polluted source of the samples, none of the isolates survived long enough to thrive and be characterized; therefore, we do not know yet what serovars prevailed in the studied rats.

All the samples that were positive were also tested against a panel of 11 serovars, and 63 of 64 (1 of the samples tested in the first assay was insufficient for the second assay) reacted with one (22%), two (48%), or more serovars (30%). The most frequent co-agglutination for sera that reacted against two serovars was Icterohaemorrhagiae-Grippotyphosa (8/30), followed by Canicola-Grippotyphosa (6/30).

All the samples had titers of 1:50, except two that had titters of 1:100 for serovars Canicola and Grippotyphosa. The distribution of sera reactive for each serovar is shown in Figure 2. There were also samples from 12 rats (19%) that were positive only to other serovars not commonly found in rodents: Canicola, 7/63 (11%); Bratislava, 1/63 (1.6%); Hardjo, 2/63 (3.2%); Pomona, 2/63 (3.2%).

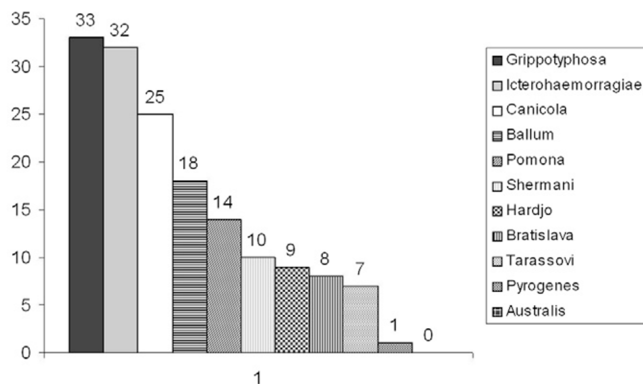

FIGURE 2. Frequency of infection of samples positive to each serovar tested. The number of samples positive to each serovar adds up to more than the actual total number of samples tested because many samples were positive to more than one serovar.

Samples from 52 rodents that were positive in cultures were tested by PCR for pathogenic serovars, and 12 of those were positive. A single PCR fragment of 423 bp was obtained in the positive control and in the DNA samples isolated from the cultures, confirming the pathogenic status for these isolates (Figure 3). Additionally, four of the LipL32 PCR fragments purified and sequenced showed specific products for *L. interrogans* (data not shown).

We did not find a statistically significant association between the independent variables sex, age, and health condition and the dependent variables culture and MAT ($P > 0.05$ by logistic regression).

DISCUSSION

This study showed for first time the role of rodents as reservoir hosts in the urban epidemiology of leptospirosis in Colombia. We showed that 23% (12/52) of the rats that were positive by culture (20%, 52/254) were shedding pathogenic *Leptospira* into the environment according to the PCR results, likely contaminating water and food with the consequent risk of producing human infection and disease. The high murine antibody prevalence obtained in this study for the serovars Grippotyphosa (13%, 33/254) and Icterohaemorrhagiae (12%, 31/254), commonly carried by rodents, coincides with a previous report of human leptospirosis prevalence for the same serovars in Medellin City (11%),[5] and Urabá area (12.5%)[7] and supports the role of domestic rats as an important reservoir for human pathogenic leptospiras. Despite that, we understand that case-control studies are needed to identify the real risk associated with the leptospirosis incidence in the human population from the study area.

Although we were unable to characterize our leptospiral isolates to serovar level, we established by PCR that several of this isolates were pathogenic and confirmed their identity as *L. interrogans* through sequencing. In contrast with our data, previous research performed on swine farms of the central rural area of the country (Quindio, Caldas y Risaralda, Colombia) showed 81% rodent samples positive by culture, but in this study, they used not only the kidney but also the lung and liver.[9] On the other hand, using kidney macerate from rodents captured in a western agricultural region (Valle del Cauca, Colombia), Morales and others[10] found 7% of samples positive by culture, which seems to be more in agreement with our results. Nevertheless, we consider that it is difficult to draw firm comparisons among the described Colombian reports because of the differences observed among them.

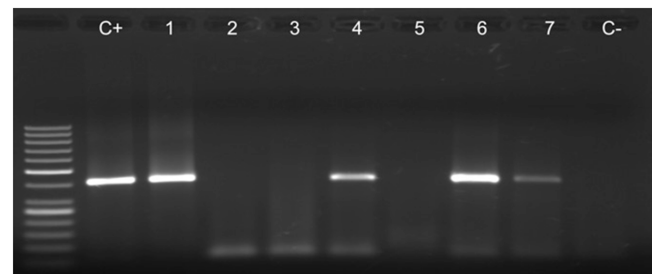

FIGURE 3. SYBR safe–stained 1.5% agarose gel showing the specific pathogenic Levett-primers product. Lane 1, molecular size marker (50 bp); Lane 2, C+, positive control of the PCR reaction; Lanes 1–7, isolates tested; Line C–, negative control.

Accordingly, in the first place, the two studies described above were performed in rural areas, whereas ours was carried out in an urban setting; second, they did not show the pathogenic nature of the *Leptospira* that they isolated. To the best of our knowledge, this is the first Colombian survey where the pathogenic nature from the *Leptospira* isolate is determined by PCR and sequencing.

In the South American context, our results contrast with those observed in Brazil that showed 80.3% positive culture from *R. norvegicus*[11] and 45.8% in Argentina in the same species.[12] These differences could be explained by diversity in the epidemiology of the leptospira infection in different countries.

Regarding the positive cultures for leptospira that were negative by PCR (40/52, 77%), among the several factors that might help to explain these contradictory results are 1) the PCR that we used was based on a real-time technique that is known to be much more sensitive than the classic one we used, 2) there might be extraction protocols "per se" more sensitive to be used, 3) the presence of PCR inhibitors in the cultures that, as reminder, were most of them contaminated with bacteria and fungi, and 4) *Leptospira* is thought as a microorganism of fastidious growth, therefore variability is expected when bacterial culture is used as a source of DNA for the PCR.

There was no statistically significant association ($P > 0.05$) between the independent rodents variables (sex, age, and health condition) and their infectious status that would indicate a particular relationship of any one of this factors with transmissibility of *Leptospira* spp. However, rodents have been shown to be the main carriers of pathogenic leptospires, and the urban abundance of these reservoirs could represent a risk factor for the potential transmission of *Leptospira* for humans and other species. The finding of antibodies in rats for serovars not usually found in this species, such as Canicola, Bratislava, and Hardjio y Pomona, suggests that rodents might have been in close contact with other animal species such as canine, equine, and even bovine and porcine in this urban environment. Interestingly, during our study, we witnessed not only the presence of homeless dogs but also noted that pigs were being raised in the facilities of this urban market, which could represent potential risk factors for people working or visiting the surroundings of this area. We also noticed that some regular cleaning workers distributed poisoned baits for rats and collected the carcasses from the sewer pipes afterward, placing themselves at the highest risk of exposure to the contaminated water and fumes from rodent excreta. Tasks like these should be avoided, given the level of pathogenic bacteria that can be released from these sources.

The broad range of reactivity detected through MAT against different serovars in the captured rodents could also be explained for the *Leptospira* shed by other animals, which ends up in the sewer and is spread by rain and floods. This has become even more real during the last years, because we experienced phenomena such as the climate change associated with stronger rainy seasons that might predispose to the transmission of rodent-borne diseases. In the same sense, it is worthwhile to highlight the closeness of the main river of the city (Rio Medellín) to the market where this work was performed and the possible risk borne by its polluted water.

There is also an old controversy about the lack of clear association between the active infection that is detected by microbiologic methods and the level of serum antibodies.[11] In agreement with this, our study did not show correlation between antibody titers and a positive culture. The low antibody levels that we detected in rats show, according to what has been reported in previous studies, that rodents are relatively resistant to the infection, and this could be explained by the long mutual relationship between these two species.[12,13]

To the best of our knowledge, this is the first study of *Leptospira* in rodents from an urban market in Colombia and shows the need for additional work, in order to identify the importance of leptospirosis in the context of public health for this kind of settings. Classically, leptospirosis has been considered a sporadic and occupational rural disease associated with livestock, sylvan reservoirs, and cultivation practices. Rural to urban migration and urban population growth have been accelerating in Latin American cities, creating new environments for leptospirosis transmission. Individuals at highest risk for severe leptospirosis are the ones in the poorest living conditions in the growing cities, and these areas often lack basic sanitation.[11,14,15] It is important to increase awareness, especially among health care providers, about the need to consider leptospirosis in urban areas within their differential diagnosis of diseases that show signs compatible with infection caused by these organisms.

We hope that, in the near future, this and other similar projects will provide the basis of an epidemiologic surveillance program, adapted to the particular conditions of our cities, which will establish the basis for prevention and control of these kinds of emerging urban diseases.

Received April 16, 2009. Accepted for publication July 12, 2009.

Acknowledgments: The authors thank Dr. Paul Levett for the sequences of leptospiral isolates and Dr. Thomas Yuill for his important suggestions to this manuscript.

Financial Support: This work was supported by the CODI EO 1206 (Comité para el Desarrollo de la Investigación, Universidad de Antioquia) and the ICMT-CES (Instituto Colombiano de Medicina Tropical–CES).

Authors' addresses: Piedad Agudelo-Flórez and Natalí Moreno, Instituto Colombiano de Medicina Tropical–CES, Cra 43A No 52 Sur-99, Sabaneta, Colombia, Tel: 574-305-3500, Fax: 574-301-4258, E-mails: pagudelo@ces.edu.co and natalimoreno@hotmail.com. Andrés F. Londoño, Víctor H. Quiroz, Juan C. Ángel, Erica T. Loaiza, Luis F. Muñoz, and Juan D. Rodas, Sede de Investigación Universitaria (SIU) T2–233, Universidad de Antioquia, Calle 62 52-59, Medellín, Colombia, Tel: 574-219-6593, Fax: 574-219-9104, E-mails: pipelb@gmail.com, pionopsitta1@yahoo.com.mx, cangelca13@hotmail.com, etloaiza@gmail.com, luisfemu@gmail.com, and juandavid.rodas@gmail.com.

REFERENCES

1. Levett PN, 2007. Sequence-based typing of Leptospira: epidemiology in the genomic era. *PLoS Negl Trop Dis 1:* e120.
2. Ko AI, Galvão Reis M, Ribeiro Dourado CM, Johnson WD Jr, Riley LW, 1999. Urban epidemic of severe leptospirosis in Brazil. Salvador Leptospirosis Study Group. *Lancet 354:* 820–825.
3. Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, Levett PN, Gilman RH, Willig MR, Gotuzzo E, Vinetz JM; Peru-United States Leptospirosis Consortium, 2003. Leptospirosis: a zoonotic disease of global importance. *Lancet Infect Dis 3:* 757–771.
4. Epsein PR, Calix O, Blanco J, 1995. Climate and disease in Colombia. *Lancet 346:* 1243–1244.
5. Sebek Z, Sixl W, Valova M, Marth E, Dock M, Reinthaler FF, 1989. Serological investigations for leptospirosis in humans in Columbia. *Geogr Med Suppl 3:* 51–60.

6. Ferro BE, Rodriguez AL, Perez M, Travi BL, 2006. Seroprevalence of Leptospira infection in habitants of peripheral neighborhoods in Cali, Colombia. *Biomedica (Bogota) 26:* 250–257.
7. Agudelo-Flórez P, Restrepo BN, Arboleda M, 2007. Situación de la leptospirosis en el Urabá antioqueño colombiano: estudio seroepidemiológico y factores de riesgo en población general urbana. *Cad Saude Publica 23:* 2094–2102.
8. Levett PN, Morey RE, Galloway RL, Turner DE, Steigerwalt AG, Mayer LW, 2005. Detection of pathogenic leptospires by real-time quantitative PCR. *J Med Microbiol 54:* 45–49.
9. Giraldo de León G, Orrego A, Betancurth M, 2002. Los roedores como reservorios de leptospiras en planteles porcinos de la zona central cafetera de Colombia. *Arch Med Vet 34:* 69–78.
10. Morales GA, Guzmán VH, Beltrán LE, 1978. Leptospirosis in Colombia: isolation of *Leptospira* spp. from the kidneys of brown rats (*Rattus norvegicus*) trapped on infected piggeries. *Trop Anim Health Prod 10:* 121–123.
11. Faria MT, Calderwood MS, Athanazio DA, McBride AJA, Hartskeerl RA, Pereira MM, Ko AI, Reis MG, 2008. Carriage of *Leptospira interrogans* among domestic rats from an urban setting highly endemic for leptospirosis in Brazil. *Acta Trop 108:* 1–5.
12. Arango J, Cittadino E, Agostini A, Dorta de Mazzonelli G, Alvarez C, Colusi M, Koval A, Cabrera A, Kravetz F, 2001. Prevalencia de leptospiras en *Rattus rattus* y *Rattus norvegicus* en el Gran Buenos Aires, Argentina. *Ecologia Austral 11:* 25–30.
13. Natarajaseenivasan K, Ratnam S, 1997. Experimental leptospirosis in laboratory mice and rats. *J Commun Dis 29:* 291–293.
14. Riley LW, Ko AI, Unger A, Reis MG, 2007. Slum health: diseases of neglected populations. *BMC Int Health Hum Rights 7:* 2.
15. Reis RB, Ribeiro GS, Felzemburgh RD, Santana FS, Mohr S, Melendez AX, 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 23:* e228.