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Leptospira In The Environmental Reservoir: Quantification And Persistence Of The Pathogen In An Urban Slum Setting Of Endemic Transmission

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ABSTRACT

Introduction

The ecology of *Leptospira*, the spirochete agent for leptospirosis, in its environmental reservoir is poorly understood. We quantified the presence of pathogenic leptospires in soil and sewage from a high-risk slum community for leptospirosis, and characterized its persistence in this environment.

Methods

We collected soil and sewage samples from three sites within a slum community in the city of Salvador, Brazil, and measured the concentration of leptospires using real-time quantitative PCR targeting *lipL32*, a gene exclusively present in pathogenic *Leptospira*. Additionally, we investigated the persistence of *L. interrogans* serovar Copenhageni, the agent for urban leptospirosis, in soil and sewage by performing time-course experiments with *in situ* mesocosms spiked with live pathogen.

Results

Among 70 soil samples obtained from 41 sites, *lipL32* was detected in 22 (31%), with a mean concentration of 11.6 (95% CI: 4.6, 29.2) genome-equivalents (GEq) per g for PCR-positive samples. *LipL32* was also detected in seven of the eight sewage samples, with a mean of 0.55 (95% CI: 0.38, 0.81) GEq per mL among PCR-positive samples. Specific attributes of the surrounding environment were not found to be significantly associated with sample positivity. The concentration of *lipL32* GEq/g, however, was positively correlated with soil moisture (r^2 = 0.50) among PCR-positive samples. In experiments of spiked mesocosms, surface-soil

concentrations of *Leptospira* decayed rapidly over the course of six days, while bacteria situated 5-10cm below the surface persisted at 100-fold higher concentrations during the same time period.

Conclusions

Pathogenic *Leptospira* are widely distributed in the urban slum environment where endemic transmission of leptospirosis occurs. Specific features of the microenvironment, such as soil moisture, promote abundance and persistence. Yet the overall pathogen load in soil and sewage is low, suggesting that during environmental exposures the infecting inoculum dose may be small. Control measures should therefore emphasize reducing the frequency and duration of contact with contaminated environment among slum residents, in addition to decontaminating and removing transmission sources.

INTRODUCTION

Leptospirosis is a life-threatening zoonotic disease in humans caused by bacteria of the genus *Leptospira*, an aerobic spirochete that induces high-grade renal carriage in a wide range of animals. Humans are accidental hosts and often acquire the infection during environmental exposures, when the spirochete penetrates abraded tissue after contact with soil, mud, or water contaminated with the urine of animal reservoirs (Diesch et al., 1966; Athanazio et al., 2008; de Faria et al., 2008; Geoijenbier et al., 2013). More than 500,000 severe cases of leptospirosis and 50,000 deaths in humans occur annually; the majority in resource-poor countries situated in tropical settings (WHO, 1999; Pappas et al., 2008; Ko et al., 2009). Human infections range from asymptomatic or a mild influenza-like illness, to serious manifestations such as Weil's disease, characterized by jaundice, bleeding, and acute renal failure (McBride, et al., 2005; Ko, et al., 2009; Goeijenbier et al., 2013). Severe pulmonary hemorrhage syndrome (SPHS) can occur in the most serious cases, resulting acute respiratory distress syndrome with a case-fatality ratio greater than 50% (McBride, et al., 2005; Gouvela et al., 2008).

Despite the critical role that the environment plays in the maintenance of the bacterium among animal reservoir and transmission of the disease to humans, the distribution and dynamics of the pathogen in soil and water reservoirs are still largely unknown. In areas of endemic leptospirosis or reported leptospirosis outbreaks, the bacterium has been detected in puddles and streams (Diesch et al., 1966; Ganoza et al., 2006; Muñoz-Zanzi et al., 2014). *Leptospira spp.* has also been isolated from soil samples, especially those near bodies of water, and it has been proposed that they migrate between transient water bodies and the underlying soil during periods of drying and re-wetting (Smith & Self, 1955; Henry & Johnson, 1978; Saito et al., 2013). Previous studies

have reported that pathogenic *Leptospira spp.* persists in aquatic environments from a few hours to several weeks, depending on the characteristics of the water and the species and serovar evaluated (Chang et al., 1948; Smith and Turner, 1961; Khairani-Bejo et al., 2004; Saito et al., 2014). In soils the ranges are even wider: from few hours to 193 days, with increased soil moisture identified as a factor related to longer survival (D. J. W. Smith and Self, 1955; Kirschner and Maguire, 1957; Okazaki and Ringen, 1957; Hellstrom and Marshall, 1978; Saito et al., 2013). Most of these studies, however, consisted of determining only the presence or absence of the spirochete, and less is known about how environmental interactions affect the bacteria's concentration and persistence. In addition, they relied on culturing and microscopy in order to assess the spirochete's characteristics, and modern molecular genetic methods can expand our understanding of *Leptospira* in the environment.

Real-time quantitative PCR (qPCR) allows the enumeration of specific genetic sequences within a sample, which provides a method for measuring the quantity of leptospira in the environment. Ganoza et al. (2006) employed this technique to detect *Leptospira spp*. in environmental water samples from the city of Iquitos, Peru. Their data suggested that leptospira were widespread and abundant in the environment, although there is some question as to the specificity of the *16S* rRNA gene qPCR assay that they employed. To date, more than 20 species have been identified in the *Leptospira* genus, the majority of which are non-pathogenic or have an undetermined potential for pathogenicity (Musso & La Scola, 2013). The *16S* gene is similar for many of these species, and it is possible that the quantities that Ganoza et al. found included a large proportion of non-pathogenic spirochetes. By employing a more specific qPCR assay, such as for the

exclusively-pathogenic *lipL32* gene, we can measure the presence and persistence of pathogenic leptospira in the environments.

Leptospirosis affects slum communities around the globe (Himsworth et al., 2013). The disease exhibits an annual cyclicity within these communities, peaking during the rainy season. Poor sanitation is associated with leptospirosis, as sewage and uncollected refuse harbor both the pathogen and its primary rodent hosts in urban environments (Reis et al., 2008). In addition, specific infrastructure deficiencies can serve as transmission sources. Unpaved walkways expose people to contaminated dirt and mud, while open sewers and inadequate drainage systems allow contaminated water to pervade the surroundings. Proximity to open sewers and contact with mud are both identified risks for the disease (Sarkar et al., 2002; Costa et al., 2014). The environment affects the distribution of the pathogen and serves as the primary source for human infections, yet we know little of the bacteria's dynamics and specific interactions with its surroundings.

In the slum community of Pau da Lima in northeast Salvador, Brazil (Figure 1), the annual *Leptospira* infection rate among residents is 3% (Felzemburgh et al., 2014). The high burden of disease in Pau da Lima, coupled with the community's compact nature and relatively predictable pathogenic cycle, made this an excellent location for an environmental survey of the spirochete. We took advantage of this high-transmission setting to detect and quantify *Leptospira* in soil and sewage samples using *lipL32* qPCR, and performed time-course experiments in environmental mesocosms spiked with known quantities of cultured bacteria in order to assess the ecological controls on the pathogen. By identifying the distribution of the pathogen in the community as

well as likely pathways of transmission, the findings of this study can inform potential public health interventions.

METHODS

Field Site

The city of Salvador is a coastal city of 2.9 million inhabitants in Northeast Brazil (IBGE, 2014). It has a subtropical climate, with an average annual temperature of 25.3ºC and an average humidity of 81% (INMET, 2015). The community of Pau da Lima (12°55' S, 38°26' W) is on the periphery of the city, and encompasses four interconnected valleys within 0.46km^2 (Reis et al., 2008)(Figure 1). Residents receive piped non-potable municipal water, although there is no organized sewage system. Untreated wastewater drains to the bottom of the valley, where it combines with natural runoff to form open sewers. The soil around Salvador is generally a sandy lateritic ultisol, and large sections of the valley walls in Pau da Lima have been excavated to create stepped hillside spaces for new home construction as the community has expanded over the past four decades. Leptospirosis is endemic among the 50,000 residents of Pau da Lima, and cases tend to cluster in the low-lying parts of the community closest to the open sewers (Reis et al., 2008; Felzemburgh et al., 2014).

Environmental Survey

We selected three sites for environmental sampling (Figure 1). These locations encompassed the range of microenvironments present at the community, and varied with respect to key attributes of the slum environment such as proximity to open sewers, probability of rat, human, and domestic animal activity, vegetation coverage, and elevation. Site A (Figure 2A) was located along an open sewer at the valley bottom, and encompassed households, areas where domestic animals (pigs, chickens, goats) were raised, and thick vegetation. The steep, high banks of the sewer served as a barrier, separating households from the sewage and limiting flood potential.

Site B (Figure 2B) was situated at a higher elevation, and had closed sewage drains, paved stairs, and patios. It comprised households that were larger and better outfitted than at the other two sites. Site C (Figure 2C), like site A, was situated in a flood-risk region at a valley bottom and in proximity to an open sewer. This was a more densely-populated area than Site A, although thick vegetation and water-logged soil made much of the site inaccessible. At site C, the open sewer was shallow with low embankments, which, in contrast to site A, did not prevent frequent flooding of surrounding areas and was not a barrier for humans and animals. Sites A and B measured 30m x 30m, while Site C was reduced to 20m x 20m in order to accommodate the more enclosed space (Figure 2). At Sites A and C, the grids were partitioned into 5m x 5m squares. The squares in Site B were 10m x 10m because of the decreased number of sampling locations and the challenging terrain.

Soil and sewage samples were collected from Pau da Lima in July and August 2014. A plastic tracking board (approximately 30cm x 30cm) coated in ink was placed in an area of anticipated rat movement within each grid square. Tracking boards were evaluated daily over the course of three days for evidence of rat activity as ascertained by the identification of footprints, scrapes, and tail slides (Hacker et al., submitted). At the end of the three days, two soil samples of 100 to 200g were collected at a depth of 5 to 10cm from two non-adjacent spots within 30cm of the edge of each board. If a portion of the open sewer was included in the grid square and was accessible, two water samples of 50mL were collected for each sampling point. In addition to the samples, attributes related to the environment at each site, including the presence of vegetation, and proximity to open sewers, households and additional domestic structures, were recorded.

Processing of environmental samples. All samples were sealed in air-tight containers, labeled, and transported to the Gonçalo Moniz Research Center of the Oswaldo Cruz Foundation (Fiocruz) in Salvador. The soil moisture content and clay content of one sample from each grid square were measured. Briefly, 0.5 to 1.0g of soil were heated in an oven to 105°C for at least eight hours, and the difference in initial and final mass was attributed to water. For the clay content, approximately 10g of soil was vortexed in 40mL of distilled water for five minutes and allowed to settle for eight hours. The upper horizon was identified as clay particles, and its volume was measured relative to the total volume of the settled material

Detection of *Leptospira* DNA. Each soil sample (5g) was mixed with 40mL of sterile, double-distilled water and vortexed for two minutes. Samples were centrifuged at 100 x g for five minutes. The supernatant was recovered and centrifuged at 12,000 x g for 20 minutes at room temperature. The pellets were recovered, resuspended in 1.5mL of sterile, double-distilled water, and centrifuged at 12,000 x g for 20 minutes. Finally, the samples were decanted and the pellets were frozen at -80°C. The procedure for processing sewage samples was similar, with the exception that 45mL of the sewage was used, no sterile water was added, and the initial 100 x g centrifugation was not performed.

DNA was extracted from pellets using PowerSoil DNA Isolation Kits (Mo Bio Laboratories, Inc., Carlsbad, CA). Pathogenic *Leptospira spp.* was quantified using a TaqMan® assay targeting the *lipL32* gene, as described elsewhere (Stoddard et al., 2009) with minor modifications. Reaction mixtures (25µL) contained 12.5µL Platinum® qPCR SuperMix (Life Technologies, Grand Island, NY), 500nM of each primer, 100nM of TaqMan® probe, 0.2µg/µL

of bovine serum albumin (Ambion), and 5µL of DNA extract. The thermal-cycler conditions were as follows: an initial setup of two minutes at 50°C, followed by two minutes at 95°C, and 40 cycles of amplification (15s at 95°C and one minute at 60°C).

A second qPCR was conducted on DNA extracts of environmental samples using a TaqMan® assay, according to the procedure of Smythe et al. (2002). Instead of the *lipL32* gene, this quantification targeted the *16S* rRNA gene in accordance with Ganoza et al. (2006). Reaction mixtures (25µL) contained 12.5µL Platinum® qPCR SuperMix, 300nM of each primer, 200nM of TaqMan® probe, 0.2µg/µL of bovine serum albumin (Ambion), and 5µL of DNA extract. The thermal-cycler conditions were the same as for the *lipL32* assay.

Genomic DNA obtained from *L. interrogans* serovars Fiocruz L1-130 was used to construct standard curves with concentrations ranging from 10^7 to 10^0 genomic equivalents (GEq) (Nascimento et al., 2004). Samples and standards were run in duplicate, and non-template controls were included in each row of the plates to detect the presence of contaminating DNA. In the event of discordant results, samples were run again. All amplifications were performed in a 7500 Fast Real-Time PCR System (Life Technologies).

To confirm the specificity of *lipL32* and *16S* qPCR reactions, 25% of the samples showing a positive result for both reactions were randomly selected and Sanger sequenced. In addition, 25% of samples showing only a positve result for *16S* qPCR reaction were also Sanger sequenced. The products from the qPCRs reactions were loaded in a 2% agarose gel and purified after the electrophoresis using the QIAquick Gel Extraction Kit (QIAgen) following the

manufacturer's instructions. The products were sequenced using primers LipL32-45F for *lipL32* confirmation or Lepto-F for *16S* rRNA, corrected using BioEdit 7.2.5 (Ibis Biosciences) and compared using BLAST to the sequences available in the NCBI.

A fragment of the *secY* gene was amplified from a selection of 14 *lipL32* qPCR-positive samples using a nested PCR with primers SecY II (5′-GAATTTCTCTTTTGATCTTCG-3′) and SecY IV (5′-GAATTTCTCTTTTGATCTTCG-3′) in a first step, and internal primers G1 (5′-CTGAATCGCTGTATAAAAGT-3′) and G2 (5′-GGAAAACAAATGGTCGGAAG-3′) in a second step, modifying a protocol described previously (Victoria et al., 2013). The PCR reactions contained 10X PCR Buffer, 200 µM of each dNTP, 400 µM of the first or second pair of primers, 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. Both amplifications were conducted in a MyCycler™ Thermal Cycler (BioRad) using the following program: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 60 s; and a final step at 72°C for 7 min. The amplification products were subjected to gel electrophoresis in a 0.8% agarose gel, followed by ethidium bromide staining. The bands corresponding to the expected size of 285 bp were purified from the gel with the QIAQuick Gel Extraction Kit (Qiagen) and Sanger sequenced using primers G1 and G2. The sequences were assembled and corrected using BioEdit 7.2.5 (Ibis Biosciences) compared using BLAST to the sequences available in the NCBI.

Mesocosm Studies on* Leptospira *Persistence

The persistence of *L. interrogans* in the environment was measured with soil and sewage from Pau da Lima. For each of three independent experiments, approximately 20L of moist soil was collected from the floodplain at the bottom of the valley. Similarly, 20L of water was taken from the open sewer running through the valley. The same soil and sewage were used for Experiment #2 and #3, although they differed from those used for Experiment #1.

Cultures of *L. interrogans* serovar Copenhageni Fiocruz L1-130 were gown to mid-log phase (four to five days) at 30°C in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium. The cultures were centrifuged at 12,000 x g for 20 minutes and washed with Hanks' Balanced Salt Solution (HBSS). A Petroff-Hausser Chamber (Hausser Scientific Co., Horsham, PA) was used to determine the bacterial concentrations. Suspensions were adjusted with HBSS to achieve a final concentration of 10^7 bacteria/mL. The motility of leptospires was greater than 90% in all cases.

Mesocosms were constructed with 12-14kDa molecular-weight cutoff dialysis tubes (Fisher Scientific, Pittsburgh, PA), desalted following the manufacturer's instructions, and tied off at one end. For each soil sample, 1g of soil was placed into a dialysis tube. Similarly, 9mL of sewage were placed into a dialysis tube. For each soil sample, 100µL of the spiking solution was added to the dialysis tube in order to achieve a starting concentration of approximately 10^6 bacteria/g. Similarly, 1mL of the spiking solution was mixed into each tube containing the sewage in order to produce a concentration of 10^6 bacteria/mL. The open end of the dialysis tubes were then tied off, and each was inspected for leaks or tears.

The remaining unused soil was placed in an open-top, 20L plastic container, perforated to allow for drainage. Likewise, the sewage was poured into a similar, unperforated plastic container. Soil and sewage mesocosms were placed outdoors in a location that was uncovered and exposed to sunlight and precipitation. The dialysis tubes containing the sewage were weighted down and placed in the container filled with sewage, at least 10cm below the surface. Dialysis tubes containing soil were buried at least 5cm below the surface in the container of soil. For Experiment #1, samples were also placed on top of the soil surface in order to expose them directly to the sunlight. (See Figure 4A for a graphical depiction of the experimental design.) Soil and sewage samples were collected in triplicate on Days 0, 1, 2, 4, and 6 from each experiment. Upon collection, the entire sample was removed from the dialysis tube and processed as described above for the environmental samples.

Statistical Analyses

Samples were considered qPCR-positive if both duplicates returned a quantifiable result. The mean CT value for a sample was interpolated against the standard curve calculated for each qPCR plate in order to estimate the concentration. These concentrations were then scaled based on the mass or volume of the original environmental sample. Unless otherwise noted, the geometric means of concentrations were used. The χ^2 test and unpaired *t*-test using Welch's correction were used to compare proportions of PCR positivity and log *Leptospira* DNA concentrations, respectively, between sites. Fisher's exact tests was used to assess the significance of associations between dichotomized environmental characteristics and the proportion of qPCR-positive samples. Additional *t*-tests using Welch's correction were used to

compare soil moisture content and clay component values between qPCR-positive and -negative samples. Cohen's kappa was used to analyze the degree of agreement between *lipL32* and *16S* PCR detection methods among environmental samples for which both were performed. A multivariate model was fit using the GENMOD procedure with a GEE model in SAS v9.3 (SAS Institute Inc., Cary, NC) to evaluate the relationship between the measured environmental characteristics and sample positivity.

RESULTS

Environmental Survey of Leptospira *DNA in the Urban Slum Field Site*

A total of 70 soil and eight sewage samples were collected and analyzed from the three sites within the Pau da Lima community (Table 1). These samples included 34 soil samples from 23 grids in Site A, 14 soil samples from 7 grids in Site B, and 22 soil and 8 sewage samples from 11 grids in Site C. Of the 70 soil samples, 22 (31%) had detectable pathogenic *Leptospira* DNA as measured by the *lipL32* qPCR assay (Figure 3A). There was no significant differences in the proportions of *lipL32* qPCR-positive samples from the three sites (range, 21-45%). Among the 22 qPCR-positive soil samples, the mean log concentration of *Leptospira* DNA was 1.07 GEq/g (95% CI: 0.66, 1.47). There was a significant difference between the mean *Leptospira* DNA concentrations in the qPCR-positive soil samples from Sites A and C (0.55 and 1.49 log GEq/g, respectively, p = 0.01). Of the eight sewage samples from Site C, the *lipL32* assay detected *Leptospira* DNA in seven (88%). The mean log concentration of *Leptospira* DNA among positive sewage samples was -0.26 GEq/mL (95% CI: -0.43, -0.09).

Although differences were observed between concentrations across sampling sites, *Leptospira* DNA was detected in soil samples from a widespread set of sampling grids within each of the sites (Figure 2). Table 1 shows the environmental attributes of the sample sites, separated by sample positivity. No significant associations between qPCR positivity and environmental characteristics in bivariate or multivariate analyses adjusted to account for the sampling design effect were identified. There was, however, a significant positive correlation between soil moisture and the log concentration of *Leptospira* DNA (r^2 = 0.50, p = 0.02) among qPCR positive samples (Figure S1).

Comparison of* lipL32 *and* 16S *RNA PCR methods to detect pathogenic Leptospira DNA

We found that, among soil and sewage samples, the *16S* rRNA qPCR detection method detected a higher proportion of positive samples and a higher concentration of estimated *Leptospira* DNA than the *lipl32* qPCR detection method. A total of 60 (86%) out of the 70 soil samples were positive when analyzed by qPCR using the *16S* assay (Figure 3B). The *16S* rRNA detection method found that Site C had significantly ($p < 0.01$) higher proportions of PCR positive samples (100%) than Sites A and C (88 and 57%). Among *16S* rRNA qPCR-positive soil samples, the estimated mean log concentration of leptospires was 2.47 log-GEq/g (95% CI: 2.25, 2.68), and was significantly higher ($p < 0.01$) than that estimated by the *lipL32* detection method. All eight sewage samples from Site C were positive when the *16S* assay was used, with a mean estimated log concentration of 2.28 GEq/g (95% CI: 2.13, 2.42).

Soil and sewage samples that were positive with the *lipL32* assay were also positive with the *16S* assay, yet there was poor overall concordance between results obtained by the two methods (Cohen's kappa = 0.16, 95% CI: 0.06, 0.26). We sequenced a 24% (7 of 29) of *lipL32* qPCR-positive samples. In all cases, the sequences strongly identified (>89%) with *lipL32* gene sequences deposited in the database. Since the *lipL32* gene is exclusive to pathogenic *Leptospira spp*., this result confirmed that the *lipL32* qPCR was highly specific for the detection of pathogenic *Leptospira spp*. In addition, we sequenced 15 *16S* rRNA qPCR-positive samples, of which 7 were also positive for *lipL32*. Although the sequences were found to be strongly (>95%) identified with *Leptospira 16S* rRNA sequences, we could not determine whether the sequences belonged to pathogenic species because the length of the amplicon (89 bp) did not allow for

sufficient discrimination. Interestingly, position 26 in the sequence of samples that were only positive by the *16S* qPCR presented double nucleotide peaks, and the selection of one nucleotide or the other changed the identity from pathogenic to intermediate species. These results suggest that *16S* qPCR may be detecting not only pathogenic *Leptospira spp*. but also intermediate species.

To confirm these findings, we amplified and sequenced a fragment of the *secY* gene from 14 samples. All amplified fragments from *lipL32* qPCR-positive and *16S* rRNA qPCR-positive soil samples (9 samples) were strongly identified (>90%) with *secY* genes from pathogenic species in the database. The samples with a positive result for the *16S* rRNA qPCR only, however, did not yield clean sequences, and the species could not be identified. More samples are being sequenced to confirm these results, although the information available to date suggests that *lipL32* qPCR is a specific method for pathogenic *Leptospira spp*., whereas *16S* rRNA qPCR detects both pathogenic and non-pathogenic species.

Persistence of* Leptospira *DNA in Mesocosms

The concentrations of the *L. interrogans* in each dialysis tube at subsequent time points as measured using the *lipL32* PCR detection method were scaled against the initial concentrations measured on Day 0. Data from three independent experiments were combined to produce a summary plot of the changes in *Leptospira* DNA concentrations over time (Figure 4B). A one-phase decay curve was found to best fit the data from each sample type. The decay rate constant (K) of *Leptospira* DNA in buried soil was 6.2×10^{-2} ($r^2 = 0.96$) and was significantly lower than

the decay rate constant for soil exposed on the surface ($7.5x10^{-1}$, $r^2 = 0.99$). There was greater variability in the sewage data, where the rate constant was $5.4x10^{-1}$ ($r^2 = 0.81$).

DISCUSSION

Our findings indicate that pathogenic *Leptospira* were widely distributed in the environment of an urban slum community where high endemic transmission of leptospirosis occurs. Among soil collected from diverse microenvironments within this specific slum setting, pathogenic *Leptospira* DNA was detected in 31% of the samples with the *lipL32* qPCR detection assay. Log concentrations of *Leptospira* DNA among positive samples varied over three orders of magnitude and had a mean of 1.07 GEq/g (Figure 3A). This finding was more pronounced in the sewage samples, where 88% were positive and had an average log concentration of -0.26 GE/mL, albeit the small number of samples collected limits the accuracy of these estimates. The positivity rates and leptospiral concentrations as measured with the *16S* rRNA detection assay were significantly greater than with the *lipL32* assay. Yet DNA sequencing revealed that the *16S* rRNA detection assay lacked specificity with respect to detecting pathogenic *Leptospira* and overestimated the true leptospiral load in environmental samples. Furthermore, in both unexposed soil and sewage, the *Leptospira* concentrations in the mesocosms lost around 2.5 logs over the course of six days (Figure 4B). The concentrations of the bacteria in soil that was exposed on the surface decreased rapidly, losing about 4 logs (and approaching the limit of detection) over the same time period.

Although these samples were collected from areas with high risk of leptospirosis, there was significant variation in *Leptospira* concentrations at the fine spatial scale and within the slum microenvironment. This observation, along with the low mean concentration of *lipL32* detected in the positive samples, indicates that the infectious inoculum dose associated with environmental exposures is low. Furthermore, the widespread distribution of pathogenic

leptospires in the slum environment suggests that interventions based on elimination or reduction of access to recognized transmission sources, such as open sewers, may not alone be effective. Promotion of barrier interventions, such as the use of protective clothing and footwear, and prevention of exposure of wounds and abrasions (which facilitate penetration of leptospires and subsequent infection) may be required to reduce the risk of spill-over transmission events among slum dwellers.

The mesocosm investigations found that the leptospiral pathogen persists in the environment for a few days. These findings suggest that the distribution and movement of the rat host is therefore a major factor in influencing the pathogen's presence and dispersal in the slum setting. Rats are the principal reservoir for urban leptospirosis, support high grade carriage in their kidneys, and shed leptospires into the environment in a spatial distribution dependent on their density and behavioral patterns. Once shed into the environment, our findings indicate that environmental factors such as sterilizing UV radiation play a major role in the persistence and survival of the bacteria. *Leptospira* do not survive well under dry conditions, and are more likely to be found in wetter microenvironments as demonstrated by the strong correlation ($r^2 = 0.50$) between soil moisture and detected *lipL32* concentration. Other researchers have identified similar relationships between soil moisture and *Leptospira* (Saito et al., 2013).

Together these findings suggest an additional mechanism to explain the increased risk of leptospirosis observed in urban settings and other settings of high endemic transmission in wet seasons. We found that survival of leptospires in the surface of soil mesocosms is markedly reduced, presumably due to UV radiation and soil moisture, compared to survival in buried soil.

Mobilization of leptospires from sub-surface soil, either by heavy rainfall, flooding or excavation, may therefore be required to yield environmental exposures with a sufficient inoculum dose to produce an infection in humans.

While this study did not identify any of the measured environmental characteristics as having a significant effect on detectable *Leptospira* DNA in samples, the association between leptospires and these factors may be more nuanced. Rats, for example, are a primary reservoir host for the pathogen, though we did not find a strong relationship between rat activity and detectable *Leptospira* in the soil. A potential explanation is that the tracking board method was not a sufficient proxy for assessing rat activity. It is also possible that a larger or more diverse sample size is necessary in order to detect the relationship in the complex microenvironments at the field site. A more detailed study of *Leptospira* concentrations along transects of open sewer lines, coupled with measures of rat abundance (e.g. trapping of rats or use of tracking plates to document rat activity) may provide the additional data that could clarify the importance of these study variables.

The use of qPCR for environmental samples present important challenges. In particular, the diversity of the autochthonous microbiota in soil and water matrices, as well as the potential presence of intermediate and saprophytic *Leptospira spp*., raises questions regarding the specificity of these qPCRs. In this study, we observed a large discrepancy between the results of the *lipL32* and *16S* rRNA qPCR assays (Figure 3). The reason for this difference might be the lack of specificity of *16S* rRNA qPCR, as suggested by the sequencing results. Studies are still

underway to sequence the gene for the SecY transmembrane protein in select positive samples in order to allow for a definitive comparison of the *lipL32* and *16S* assay's specificity.

Inherent limitations of real-time PCR detection of *Leptospira* DNA in environmental samples may influence the accuracy of our estimates and ability to evaluate risk associations. Although we optimized sample processing and DNA extraction and detection methodologies to reduce loss and PCR inhibition, the limit of detection for our *lipL32* qPCR detection assay is approximately 100 GEq/g. In addition, our experiments with mesocosms spiked with leptospires found that our procedure yields an estimated loss in *Leptospira* DNA log concentration of 0.08 ± 0.05 GEq/g in soil samples and 0.60 ± 0.50 GEq/mL in sewage samples. Our estimates may therefore be minimum values of leptospire concentration.

Our study was also limited by the sampling scheme which may not have completely captured the heterogeneity in the slum environment. Although we evaluated 3 study sites that were representative of the high-risk microenvironments within the slum site, there may have been additional heterogeneity with respect to soil type, climactic conditions, and land use which was not accounted for. The mesocosm model used to evaluate leptospiral persistence did not replicate all of the dynamic environmental processes that occur in the natural setting. For example, there may be significant differences in O_2 tension, pH, and microbiota between flowing open sewers and static sewage mesocosm, which may have influenced leptospiral survival and persistence. Nevertheless, the mesocosm model that we employed represents an improved model for studying leptospiral persistence over controlled microcosm models in the laboratory setting,

Leptospirosis is a disease of poverty, and we still have only a limited knowledge of the ecology of the pathogen. As urban population densities grow and force people into increasingly-crowded slum communities, the risk for disease transmission multiplies. Prevention of this disease relies on our ability to limit human exposure to the bacteria in the environment. This study has demonstrated that *Leptospira* are widespread in Pau da Lima, though at relatively low concentrations, and do not persist for long. Infections, therefore, occur in situations of close contact with rats and exposure of abraded tissue to the environment. Consequently, efforts need to be directed towards reducing direct, prolonged exposure of the residents to the contaminated environments. This may be accomplished through the construction of impervious barriers for the open sewer, covering the sewer, or perhaps best, constructing buried sewer lines to isolate it from the environment. These barriers may help prevent flooding and the mobilization of the pathogen in mud. Paved pedestrian paths or elevated walkways can also serve to reduce direct contact with the contaminated soil. Rat control programs, organized refuse disposal, and the reduction of rat habitats will limit the introduction of *Leptospira* into the environment through rat urine. Combined, these interventions have the potential to decrease the risk of leptospirosis in the community by reducing both the number of *Leptospira* that enter into the environment, and limiting the residents' contact with contaminated soil and sewage.

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TABLES

Table 1. qPCR detection of *Leptospira lipL32* in soil samples according to environmental characteristics of the sampling sites within the urban slum community.

Characteristic[a]		Soil Samples (n = 70) No. (%) or Mean (SD)	
		***lipL32* Positive**	***lipL32* Negative**
Site	**All**	22 (31%)	48
	A	9 (26%)	25
	B	3 (21%)	11
	C	10 (45%)	12
Adjacent Vegetation (No.)		14 (64%)	27 (56%)
Tracking Board Evidence of Rat Activity (No.)		16 (73%)	28 (58%)
Distance to Open Sewer[b] (m)		3.3 (4.6)	5.3 (5.6)
Distance to House (m)		6.8 (4.5)	5.9 (5.7)
Soil Moisture Content[c] (%)		27.5 (12.9)	27.6 (15.3)
Soil Clay Content[d] (%)		10.7 (4.2)	10.8 (5.5)

None of the measured variables were significant at the $\alpha = 0.05$ level in either a bivariate or multivariate analysis.

[a] Percent for the site variables are row percent, all of others are column percent.

[b] n = 56 at sites A and C. Site B did not have an open sewer.

[c] n = 35

[d] n = 56

FIGURES

Figure 1. The urban slum community site in Salvador, Brazil. (A) Aerial photograph of the Pau da Lima community site (red border) which comprises three valleys and is an area of high endemic transmission of leptospirosis (Riley et al., 2007). The locations of the three sites where environmental sampling were performed are indicated by white rectangles. (B) Topographic map that demonstrates differences in elevation at the site. Open sewage draining systems, identified by survey, are depicted in dark red. (C) Photograph of the community site which depicts a representative valley and squatter households.

Figure 2. Environmental sampling and qPCR detection of *Leptospira* DNA at the slum community site. Sampling of soil and sewage (circles) was performed at three sites (A, B, and C) within the Pau da Lima field site. Site A was located in a valley bottom that had an open sewer, thick vegetation, and areas containing households and space used to raise domestic animals. The region had limited flood risk due to the sharp embankments encompassing the open sewer. Site B was situated in a more densely populated area of higher elevation and improved land quality with enclosed sewage and rainwater drainage systems. Site C was located in a flood-prone region at the valley bottom that had a shallow open sewer. Sites where qPCR positive and negative samples were obtained are shown as red and open circles, respectively.

Figures 3. qPCR measurement of *Leptospira* DNA concentrations in environmental samples. Soil units are on the left axes, sewage on the right. (A) The concentrations measured using the *lipL32* assay. (B) The *16S* assay identified a significantly greater proportion of positive soil samples. N.D. = PCR negative.

Figure 4. *Leptospira interrogans* persistence in mesocosms. Figure 4A shows the experimental design for which soil and sewage mesocosms were spiked with live *L. interrogans* serovar Copenhageni to achieve a concentration of 10^6 bacteria/g or bacteria/mL and then sampled to measure the *L. interrogans* DNA during a six-day time course. Figure B shows the concentration of recovered *L. interrogans* DNA during the time course. Units are normalized to the initial measurement of bacteria from Day 0. Mean values and standard deviations are shown for data that was obtained from three independent time course experiments, except for surface soil which was evaluated in one experiment.

Figure 1.

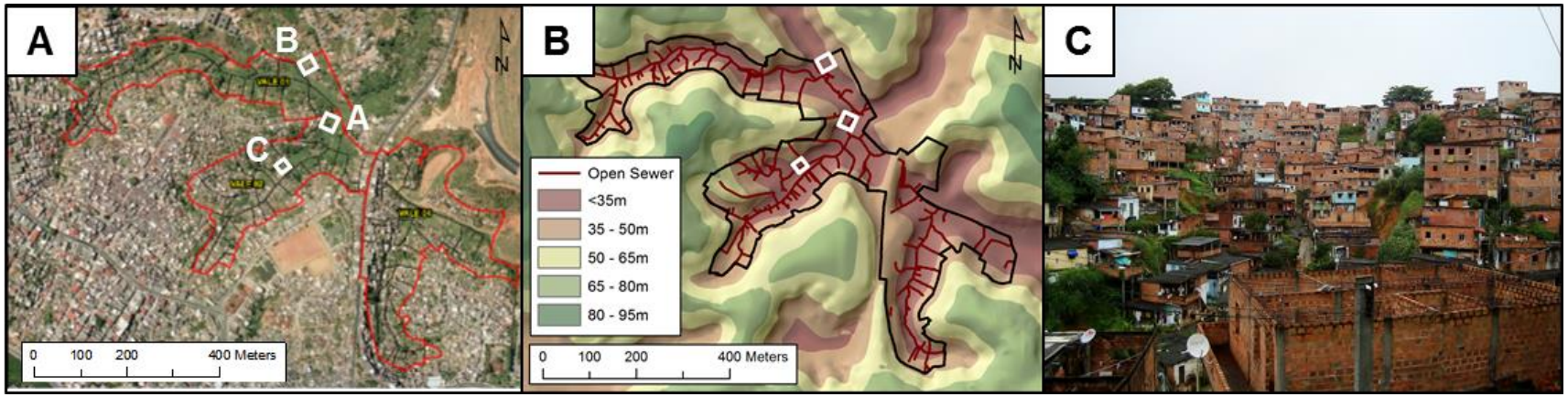

A
B
A
C
N
0 100 200 400 Meters
B
Open Sewer
<35m
35 - 50m
50 - 65m
65 - 80m
80 - 95m
N
0 100 200 400 Meters
C

Figure 2.

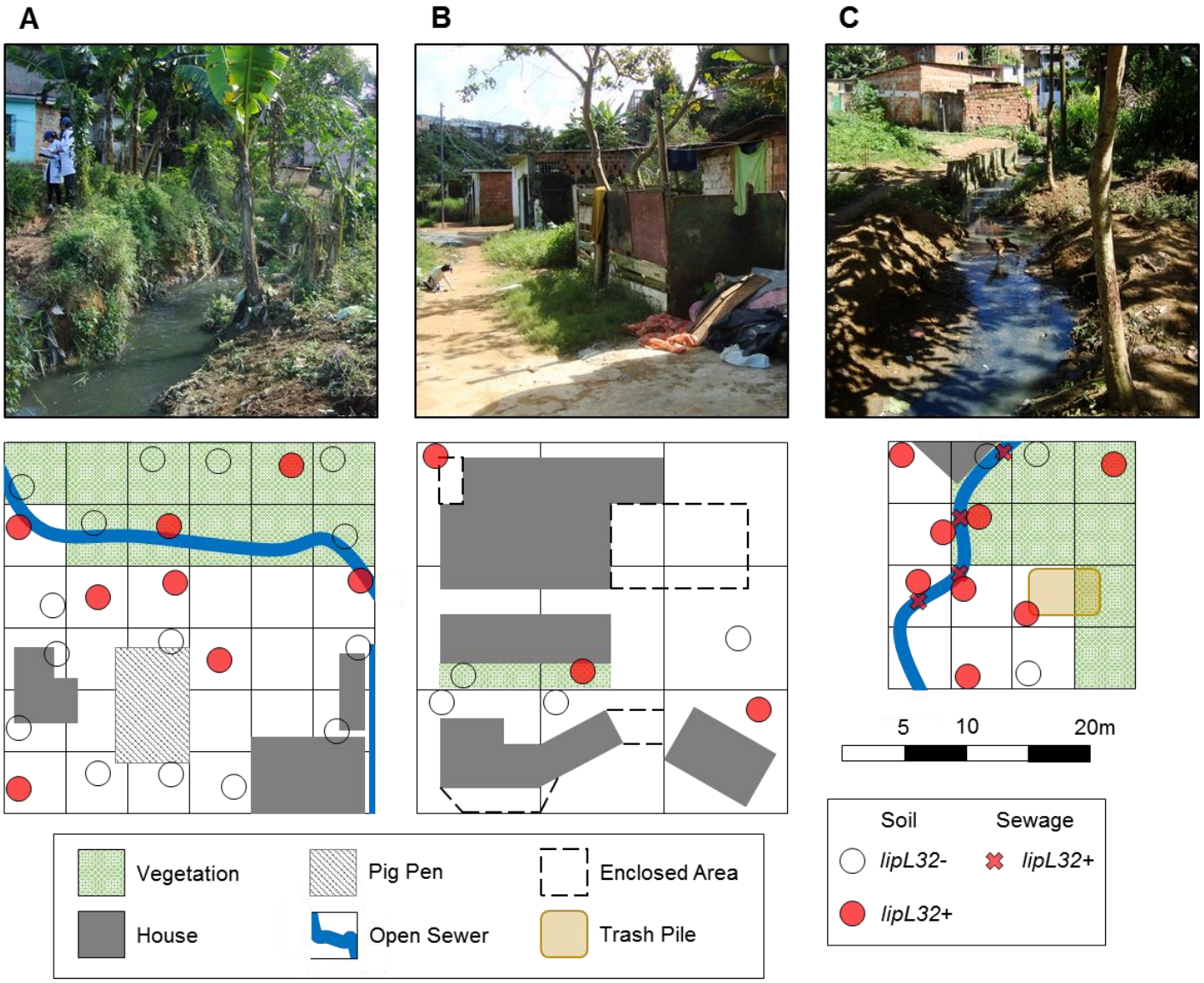

A
B
C
5
10
20m
Soil
Sewage
lipL32-
lipL32+
lipL32+
Vegetation
Pig Pen
Enclosed Area
House
Open Sewer
Trash Pile

Figure 3.

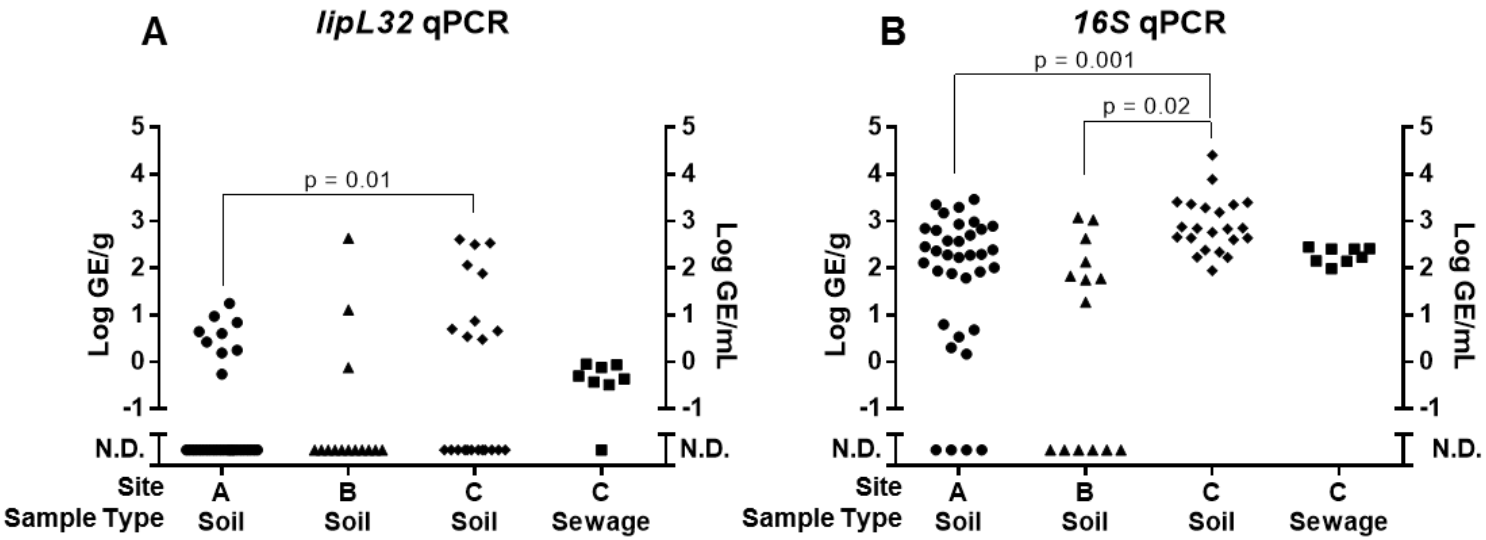
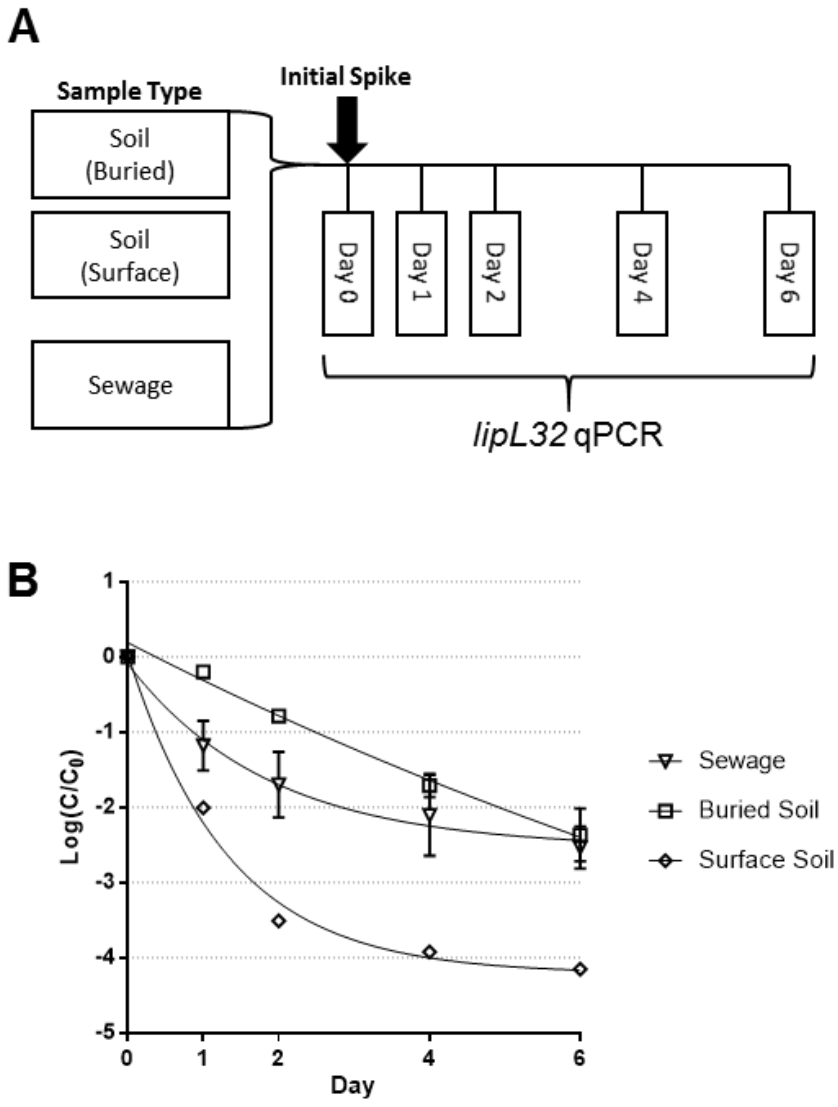

A
lipL32 qPCR
p = 0.01
Log GE/g
Log GE/mL
5
4
3
2
1
0
-1
N.D.
Site
Sample Type
A
Soil
B
Soil
C
Soil
C
Sewage
B
16S qPCR
p = 0.001
p = 0.02

Figure 4.


A
Sample Type
Initial Spike
Soil (Buried)
Soil (Surface)
Sewage
Day 0
Day 1
Day 2
Day 4
Day 6
lipL32 qPCR
B
Log(C/C0)
1
0
-1
-2
-3
-4
-5
0
1
2
4
6
Day
Sewage
Buried Soil
Surface Soil

SUPPLEMENTAL DATA

S1. Soil moisture content and *lipL32* concentration in positive environmental samples.