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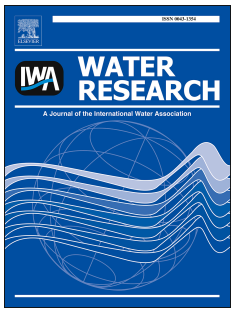

Spatial and temporal dynamics of pathogenic *Leptospira* in surface waters from the urban slum environment

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High risk urban slum

Surface water sampling

- ✓ Sewage and standing water
- ✓ Dry, intermediate and rainy seasons
- ✓ Morning and afternoon
- ✓ Different areas of the valley

Leptospira quantification

Sewage

Standing water

>33% positive

~150 GEq/mL

Spatiotemporal predictors

More risk in:

- Rainy season (sewage)
- Bottom of the valley

Association with leptospirosis outbreaks after rainfall

Spatial and Temporal Dynamics of Pathogenic *Leptospira* in Surface Waters from the Urban Slum Environment

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ABSTRACT

Leptospirosis has emerged as an important urban health problem as slum settlements have expanded worldwide. Yet the dynamics of the environmentally transmitted *Leptospira* pathogen has not been well characterized in these settings. We used a stratified dense sampling scheme to study the dynamics of *Leptospira* abundance in surface waters from a Brazilian urban slum community. We collected surface water samples during the dry, intermediate and rainy seasons within a seven-month period and quantified pathogenic *Leptospira* by quantitative PCR (qPCR). We used logistic and linear mixed models to identify factors that explained variation for the presence and concentration of *Leptospira* DNA. Among 335 sewage and 250 standing water samples, *Leptospira* DNA were detected in 36% and 34%, respectively. Among the 236 samples with positive results geometric mean *Leptospira* concentrations were 152 GEq/mL. The probability of finding *Leptospira* DNA was higher in sewage samples collected during the rainy season when increased leptospirosis incidence occurred, than during the dry season (47.2% vs 12.5%, respectively, p=0.0002). There was a marked spatial and temporal heterogeneity in *Leptospira* DNA distribution, for which type of water, elevation, and time of day that samples were collected, in addition to season, were significant predictors. Together, these findings indicate that *Leptospira* are ubiquitous in the slum environment and that the water-related risk to which inhabitants are exposed is low. Seasonal increases in *Leptospira* presence may explain the timing of leptospirosis outbreaks. Effective prevention will need to consider the spatial and temporal dynamics of pathogenic *Leptospira* in surface waters to reduce the burden of the disease.

Keywords: *Leptospira*, leptospirosis, surface water, public health, sewage, urban slum

1. INTRODUCTION

Leptospirosis is a widespread zoonotic disease that causes more than 1 million cases and 50,000 deaths each year (Costa et al., 2015; Torgerson et al., 2015). The disease ranges from mild flu-like symptoms to severe complications, such as Weil's disease and pulmonary hemorrhagic syndrome, for which case fatality is 5 to >40% (Haake and Levett, 2015; Ko et al., 2009). Pathogenic *Leptospira* colonize the kidneys of a broad range of mammalian species and are shed in the urine into the environment where they survive for periods that range from a few hours to several months depending on the species, serovar and the characteristics of the environmental matrix (Hellstrom and Marshall, 1978; Khairani-Bejo et al., 2004; Okazaki and Ringen, 1957; Thibeaux et al., 2017; Trueba et al., 2004). Leptospirosis is an environmentally-transmitted disease: human infection occurs primarily through contact of abraded skin or mucous membranes with contaminated environment, most notably water (Ko et al., 2009). However, there is a lack of knowledge regarding the abundance and distribution of pathogenic *Leptospira* in surface waters that serve as a transmission source in endemic areas. Moreover, the environmental factors that influence their abundance and distribution, and therefore the risk of infection, are poorly understood.

Leptospirosis has recently emerged as a major public health problem among impoverished urban settlements in tropical and subtropical developing countries (Karande et al., 2002; Ko et al., 1999; Kyobutungi et al., 2008; Riley et al., 2007). Inadequate sanitation in these settings, specifically precarious sewer systems and trash accumulation, promotes the thriving of rodents, which are major reservoirs of pathogenic *Leptospira* (Costa et al., 2014; Panti-May et al., 2016; Riley et al., 2007; Unger et al., 2016). 865 million people resided in urban slums in 2012 and this number is expected to double by

2025 (UN-HABITAT, 2013). Consequently, the burden of the disease will continue to increase in the coming years.

Exposure to contaminated water is a well-recognized risk factor for leptospirosis in urban slums. Climatic conditions leading to an increased human exposure to water appear to be important drivers for disease transmission. Leptospirosis outbreaks frequently occur during periods of seasonal rainfall and flooding in the urban slum setting (Ko et al., 1999; Tassinari et al., 2004), as well as in other epidemiological situations where transmission is endemic (Desvars et al., 2011; Ko et al., 2009; Lau et al., 2016; Smith et al., 2013; Tangkanakul et al., 2005; Weinberger et al., 2014), or following extreme weather events (Agampodi et al., 2014; Amilasan et al., 2012; Karande et al., 2002; Trevejo et al., 1998). In addition, the proximity of households to open drainage systems and direct contact with sewage, flooding water and runoff have been associated with increased risk of infection in prospective, cross-sectional and case control studies (Barcellos and Sabroza, 2001; Felzemburgh et al., 2014; Navegantes de Araújo et al., 2013; Oliveira et al., 2009; Reis et al., 2008; Sarkar et al., 2002). Furthermore, pathogenic *Leptospira* have been detected in sewers, streams and puddles from endemic areas (Ganoza et al., 2006; Kurilung et al., 2017; Muñoz-Zanzi et al., 2014; Saito et al., 2013; Sumanta et al., 2015). Altogether, this highlights the key role of surface waters in the transmission of leptospirosis in urban slums.

Yet the abundance and distribution of pathogenic *Leptospira* in the surface waters of endemic areas have not been well characterized. To date, only one study performed in Peru has succeeded in quantifying pathogenic *Leptospira* in the waters of an urban slum, reporting mean concentrations around 1,000 leptospires/ml (count range 2-1,286) (Ganoza et al., 2006). In this study, we aimed to provide high-resolution information on the presence and concentration of pathogenic *Leptospira* in an urban slum at high-risk for leptospirosis,

and to evaluate whether the spatiotemporal dynamics of the pathogen explained the variation in risk of infection. To this end, we performed a dense sampling of the surface waters from a Brazilian urban slum with high infection rates (37.8 per 1,000 individuals per year) (Hagan et al., 2015) where leptospirosis outbreaks occur each year in the rainy season (Ko et al., 1999; Sarkar et al., 2002). We collected 585 samples of sewage and standing water from different elevations within this urban slum across a seven-month period that spanned the dry, intermediate and rainy seasons. Presence/absence of pathogenic *Leptospira*, and concentrations in positive samples, were estimated by quantitative-PCR (qPCR) and subsequently modeled using logistic and linear mixed models, respectively, to identify the factors that explained their spatial and temporal variation.

2. METHODS

2.1 Study site

The study was conducted in Pau da Lima, an urban slum community located in the city of Salvador, Brazil (Fig. 1A). The study site has been previously described in detail (Reis et al., 2008; Unger et al., 2016). Briefly, the community consists of four valleys with an area of 0.46 km^2 (Fig. 1B) and has a population of 12,651 inhabitants (Felzemburgh et al., 2014). The slum has a precarious sanitary infrastructure with open sewers and rainwater drainage that overflow during heavy rainfall events, leading to frequent flooding in valley bottoms during the rainy season. Salvador has a typical tropical rainforest climate (Köppen classification: *Af*) with relatively stable temperatures throughout the year daily mean, and average high and low values; 25.3 °C, 28.2 °C, and 22.7 °C, and high relative humidity (average, 80.9%). The average annual precipitation is 2,144 mm, with a monthly average rainfall of over 60 mm, indicating that there is no authentic dry season. However, the period

from April to July has an average rainfall of over 200 mm/month (Brazilian National Institute of Meteorology, 2015) and it is locally considered as the rainy season.

2.2 Sampling design and collection

One of the valleys in the Pau da Lima community with similar environmental features and risk factors for leptospirosis than the other valleys (Felzemburgh et al., 2014; Hagan, 2016) was selected for the longitudinal survey of surface waters. The valley selected had a slightly smaller surface and a lower incidence of violence, which allowed for a denser sampling and facilitated the access to the sampling sites. The stratified sampling scheme was designed to collect 672 water samples from three strata of sampling sites based on elevation (valley top, middle and bottom) and three collection periods (rainy, intermediate and dry) during the seven-month period from July 2011 to January 2012 inclusive. The valley was divided into three sections of approximately 30,000 m^2, which corresponded to above 52 m (valley top), between 38 and 52 m (valley middle), and below 38 m (valley bottom), measured from the lowest point of the valley. We stratified sites according to elevation since previous studies found that leptospirosis infection risk was inversely associated with household elevation (Hagan et al., 2015). Fourteen paired sampling sites were selected along a continuous section of the major open sewer that flows from the top to the bottom of the valley. Among the 14 paired sampling sites, four, eight and sixteen sites were distributed at the valley top, middle and bottom sections, respectively. Within each valley section, paired sampling sites were approximately 30 m apart from each other. For each paired site, sampling was performed at two locations that were 5 m apart between sewer confluences (Fig. 1C). At each of the 28 sampling points, samples were collected from the open sewer and from standing water located in an area

142 contiguous to the sewer. Standing water was defined as any accumulation of water without
143 connection to a sewer or other water flow. If standing water was not available in the area
144 adjacent to the sewer, the sample was collected within a radius of 15 m from the established
145 site, or otherwise not collected.

146 Samples were collected during three sampling campaigns: July 2011, November
147 2011 and January 2012 (Fig. 2). The sampling months were selected based on the historical
148 average monthly rainfall (1996-2009): those months with an average precipitation higher
149 than 200 mm were considered as the rainy season, those with less than 100 mm were the
150 dry season, and those with a precipitation between 100 and 200 mm were the intermediate
151 season (Fig. S1). Measures of daily rainfall were obtained from a municipal weather station
152 located 0.9 Km away from the study site. Within each sampling period, samples were
153 collected at each of the 28 sampling points on three days each week, both in the morning
154 (from 8 am to 10 am) and in the afternoon (from 4 pm to 6 pm). Because of the correlation
155 between leptospirosis incidence and seasonal rainfall (Ko et al., 1999), samples were
156 collected for two consecutive weeks in July 2011, but only one week in November 2011
157 and January 2012. Sample collection points were georeferenced and entered in a
158 Geographic Information System (GIS) database (Reis et al., 2008) during the first sampling
159 campaign to facilitate the return to the same sites in the subsequent campaigns. Aliquots of
160 50 mL of sewage or standing water were collected in sterile polyethylene containers using
161 aseptic techniques at the selected sites and times, and refrigerated at 4 °C up to 18 h before
162 processing.
163

164 **2.3 Quantification of *Leptospira* DNA in surface water**

DNA was extracted following a procedure described previously (Riediger et al., 2016). Briefly, samples were homogenized by inversion and a 40-mL aliquot was centrifuged at 15,000 × *g* for 20 min at 4°C. The supernatant was discarded and the pellet was recovered and frozen at -80 °C. Pellets were then thawed in batches of 23 samples and DNA was extracted using the PowerSoil® DNA Isolation kit (MoBio) following the manufacturer's instructions. An extraction blank consisting of ultrapure water was added to each extraction batch to monitor for cross-contamination.

Pathogenic *Leptospira* were quantified using a TaqMan® assay targeting a fragment of *lipL32* gene (Stoddard et al., 2009) with minor modifications on a 7500 Fast Real-Time PCR thermocycler (Applied Biosystems). Calibration curves based on genomic DNA from *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (Nascimento et al., 2004) were run on each plate and used to transform quantification cycles (Cq) to concentrations (genome equivalents (GEq)/reaction). Non-template controls were randomly included in all rows of each plate to discard the presence of contaminating DNA. Samples, controls and calibrators were run in duplicate. All negative controls (extraction blanks and non-template controls) were negative in all cases. qPCR inhibition was monitored using a specifically designed Internal Amplification Control (IAC) plasmid tested in singleplex reactions. See Supplementary Material for further details on the qPCR assay, calibrators, genome equivalent calculations, inhibition assay and estimation of the correction factor. DNA extractions and qPCR analyses were performed according to the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin et al., 2009).

To confirm the specificity of the qPCR in detecting pathogenic *Leptospira*, 15% of the samples with a positive result in each sampling season were randomly selected for DNA

189 sequencing. The qPCR products were loaded in a 2% agarose gel, submitted to
190 electrophoresis and then purified using the QIAquick Gel Extraction Kit (QIAgen)
191 following the manufacturer's instructions. Purified products were Sanger sequenced using
192 primer LipL32-45F, edited using BioEdit 7.2.5 (Ibis Biosciences) and compared to the
193 sequences available in GenBank using BLAST.
194
195 **2.4 Data treatment**
196 Samples were considered positive when both qPCR replicates showed amplification
197 before a Cq of 40. Samples with a single positive reaction were submitted to an additional
198 qPCR in duplicate. If in this second qPCR the sample amplified in either of the replicates, it
199 was considered positive. The GEq per reaction in all positive qPCR replicates were
200 averaged, normalized by the volume of water analyzed, and $\log_{10}$-transformed to obtain
201 concentrations in GEq/mL. To account for the DNA loss during sample processing and
202 DNA extraction, *Leptospira* GEq concentrations were corrected using a calibration curve
203 generated in sewage spiked with known concentrations of *L. interrogans* (Riediger et al.,
204 2016) (Supplementary Material).
205 Positive qPCR samples with concentrations below the 95% hit-rate lower limit of
206 detection of the qPCR (18 GEq/mL) (Riediger et al., 2016) were included in the positivity
207 analysis but were excluded in the concentration analysis. In addition, standing water
208 samples that could not be collected due to the absence of water were treated as negatives
209 for modeling purposes since this absence implied no risk for leptospirosis infection.
210
211 **2.5 Statistical analysis**

Logistic and linear mixed models were used to analyze the occurrence of a positive *Leptospira* sample and the $\log_{10}$ *Leptospira* concentration in positive samples, respectively. In both models, we accounted for the repeated-measure structure of the data by including random effect terms for the sampling location, week and day within week. Surface water type, season, period of the day and elevation were included as fixed effects. Elevation was treated as a three-level factor (top, middle and bottom). We first selected only variables that were statistically significant in their respective univariate random effect models (logistic or linear). After including these significant variables in a general model, all possible interactions were tested. As a last step of the modeling strategy, fixed and interactions terms that did not remain significant were eliminated. In all steps, likelihood ratio tests were used for the inclusion or elimination of variables ($p < 0.05$). Random terms were kept in the models even if their respective variances were relatively small given the intrinsic expected correlation in the space and time (location, week and day within the week). In the resulting logistic and linear mixed models, we calculated the predicted probability of finding a surface water sample with *Leptospira* DNA and the predicted *Leptospira* DNA concentration according to specific interactions by centering the remaining variables on their observed mean values (Fox, 2003). When a factor with more than two levels was included in the model according to the likelihood ratio criterion described above, we assessed the significance of differences between factor levels using post-hoc pairwise tests (Lenth, 2016). Analyses were conducted using the statistical software R v3.1 (R Core Team, 2013), with lme4 (Bates et al., 2015) , Lsmeans (Lenth, 2016), lmerTest (Kuznetsova et al., 2013) and Effects (Fox and Hong, 2009) packages. Cohen's kappa was used to estimate the strength of agreement between sewage and standing water samples

collected in the same site. Comparisons were made using Welch's t-test in GraphPad Prism v7.01.

2.6 Leptospirosis incidence

Severe leptospirosis cases in the metropolitan region of Salvador within the study period were identified from an active surveillance program at the state infectious diseases hospital (Couto Maia Hospital). The study team prospectively evaluated admissions to identify suspected cases who met the clinical definition for severe leptospirosis (Ko et al., 1999) and enrolled patients per written informed consent protocols approved by the ethics committees of the Oswaldo Cruz Foundation and Yale University.

3. RESULTS

3.1 Rainfall pattern and leptospirosis incidence

During the study period, the rainfall pattern differed from the historical pattern described for the city of Salvador, Brazil (Brazilian National Institute of Meteorology, 2015). We observed a higher mean cumulative monthly rainfall in November 2011 compared to July 2011 and January 2012 (329.1 mm vs 81.9 and 36.4 mm, respectively, Fig. S1). Therefore, the sampling period in July 2011 was defined as the intermediate season, November 2011 as the rainy season, and January 2012 as the dry season.

A total of 101 severe leptospirosis cases were reported citywide during the 7-months study period, with an incidence of 3.8 cases per 100,000 inhabitants. The number of cases peaked in the rainy season (November 2011) with 6 to 13 cases per week following intense rainfall events (Fig. 2). In the dry and intermediate seasons, 0 to 5 cases were reported each week.

3.2 Specificity of *Leptospira* qPCR assay

To verify whether the qPCR reaction was specifically detecting pathogenic *Leptospira* in surface water samples, we partially sequenced the *lipL32* amplicon from 36 samples (15.3%) out of 236 qPCR-positive samples. These samples were randomly selected and came from all seasons, types of water, collection times and locations and comprised samples with all the range of estimated concentrations. All 36 sequenced samples showed their highest similarity to other *Leptospira lipL32* gene sequences deposited in GenBank (*Leptospira sp.* (24), *L. interrogans* (11) *and L. borgpetersenii* (1)), irrespective of the *Leptospira* DNA concentration of the sample (see Table S1 for sequence accession numbers and highest hits). This result confirmed that the *lipL32* qPCR method is highly specific for the detection of pathogenic *Leptospira* in complex environmental surface water matrices.

3.3 Distribution and quantification of *Leptospira* DNA in surface waters

A total of 585 samples (335 sewage and 250 standing water) were collected in Pau da Lima and tested by qPCR for the presence of pathogenic *Leptospira*. 86 standing water samples could not be collected because no accumulation of water was found in the designated sampling area and one sewage sample was lost during processing (Table 1)

Among 585 samples collected, 236 (40%) were positive for *Leptospira* DNA (36% of 335 sewage samples, and 46% of 250 standing water samples, respectively). Sewage showed the highest positive proportion in the rainy season, with up to 50% of 84 samples positive, and the lowest in the dry season with only 15% of 83 samples positive. In addition, more sewage samples were positive for pathogenic *Leptospira* DNA at the bottom

283 of the valley (41% of 191 samples) than in the middle and top areas of the valley. In
284 contrast, the proportion of positive samples in standing water was more stable across
285 seasons and elevations (Table 1). When accounting for non-collected samples the overall
286 positivity decreased to 34% for standing water. Standing water was less frequently found in
287 the middle of the valley and during the dry season with only 46% and 57% of samples
288 collected, respectively. As a result, the overall standing water positivity in the middle of the
289 valley and the dry season was particularly affected, with a reduction of approximately a
290 50% (Table 1). Furthermore, sewage and standing water samples collected in the morning
291 and afternoon had similar positivity ratios. Finally, the strength of agreement between the
292 results obtained for paired sewage and standing water samples collected in the same site
293 was only 'fair' (62% observed agreements; $\kappa = 0.21 \pm 0.06$) (Table S2).
294 Among 231 qPCR positive samples with concentrations above the lower limit of
295 detection, the geometric mean concentration and count range of *Leptospira* DNA was 152
296 [21-17,378] GEq/mL (143 [22 – 2,187] and 166 [20 – 17,378] GEq/mL in sewage and
297 standing water, respectively). Overall, mean geometric *Leptospira* DNA concentrations in
298 surface water from the urban slum surveys were generally low and did not vary
299 substantially with respect to type of water, season of collection, elevation in the valley and
300 period of collection (Fig. 3).
301

302 **3.4 Spatial and temporal predictors of *Leptospira* DNA presence and concentration**

303 The final logistic mixed model for the probability of finding a positive sample for
304 *Leptospira* DNA included fixed terms (elevation), fixed terms with interactions (surface
305 water type, season, and period of collection) and random effects (location, week, and day
306 within week) (Table 2). Elevation was included in the model as a fixed term indicating that

the localization of the sample in the valley modified their probability of being positive for *Leptospira* independently of the other variables. The modeled probability of finding a positive sample, with all variables other than elevation set at their observed mean values, was higher in the bottom of the valley (38%) than in the middle (22%) or the top (29%), although the differences with respect to the top section were not statistically significant ($p=0.0007$ and $p=0.269$, respectively) (Fig. 4A).

In addition, the model included two significant interaction terms: season and type of water, and season and period of collection. The analysis of the interaction between season and type of water showed that sewage samples in the rainy season and the intermediate season had higher probabilities to be positive (47% and 37%, respectively) than those in the dry season (13%; $p=0.0002$ and $p<0.0001$, respectively). In contrast, in standing water samples the probability of being positive did not vary significantly between seasons (Fig 4B). Furthermore, in the rainy season sewage samples showed significantly higher probabilities to be positive than standing water ones (47% and 27%, respectively; $p=0.0096$). On the contrary, in the dry season, standing water samples were more likely to show positive results, although the difference was not statistically significant (24% and 12%, respectively; $p=0.0553$) (Fig. 4B). However, when considering all seasons together no difference was found between the overall positivity of sewage and standing water (36% and 34%, respectively, $p=0.6028$). Regarding the interaction between the season and the period of collection, the model showed that in the rainy season samples had higher probabilities to be positive in the morning than in the afternoon (49% and 26%, respectively; ($p=0.0038$), whereas no differences were found between the intermediate and dry seasons (Fig. 4C). To sum up, the logistic mixed model revealed that elevation, season, type of water, and period

of collection were spatial and temporal predictors of the probabilities of finding *Leptospira* positive samples in the surface waters of the urban slum

The final linear mixed model for the concentration of *Leptospira* DNA in positive samples included only season and period of collection as fixed effects and random effects for location, week and day within week. The other variables (type of water and elevation) were not statistically significant in the final model (Table 2). In the rainy season, positive samples had significantly higher concentrations of *Leptospira* DNA when compared to the dry season (162 and 107 GEq/mL, respectively; $p=0.0429$). Moreover, samples collected in the morning showed higher concentrations than those collected in the afternoon (180 and 108 GEq/mL, respectively; $p<0.0001$). However, despite being statistically significant, these differences were small (less than 0.25 $\log_{10}$ units in all cases), which implied that the geometric means in positive samples were virtually the similar regardless of type of water, elevation, season or period of collection.

4. DISCUSSION

In this study, we aimed to determine the abundance of pathogenic *Leptospira* in the surface waters of an urban slum with high risk for leptospirosis infection, and to evaluate how their presence and concentration varied across space and time. We found that pathogenic *Leptospira* are ubiquitous in sewage and standing water (>33% positivity) albeit in concentrations that are generally low (around 150 GEq/mL). Our results indicate that pathogenic *Leptospira* have a heterogeneous spatial and seasonal distribution in our study site, being more prevalent towards the lower areas of the valley and in the rainiest months. Nevertheless, despite the spatial and seasonal variation, there is a widespread and persistent but low environmental burden across the study site.

354 The probability of finding positive *Leptospira* samples in the sewage of the urban
355 slum presented a seasonal pattern. The number of positive samples increased during the
356 rainy season, reaching its minimum during the dry season (Table 1 and Fig. 4). This
357 increased positivity may be due to a combination of factors such as a mobilization of
358 pathogenic *Leptospira* from soil reservoirs because of rainfall, a dissolution of
359 environmental biofilms (Barragan et al., 2011), or an enhanced survival due to higher levels
360 of oxygen or the dilution of sewage toxic compounds (Chang et al., 1948), among others.
361 The specific dynamics of mobilization, dispersion and survival of pathogenic *Leptospira* in
362 water and soil deserve further studies. The seasonal pattern observed in our study site is
363 consistent with the increased number of severe leptospirosis cases reported in the
364 metropolitan area of Salvador, Brazil 1–4 weeks after intense rainfall events (Fig. 2). This
365 seasonal distribution has been reported in other settings around the world where large
366 epidemics occur in the rainy season preceded by episodes of heavy rainfall such as tropical
367 storms, typhoons or monsoons (Amilasan et al., 2012; Karande et al., 2002; Tangkanakul et
368 al., 2005). The increased contact with potentially contaminated water and soil due to
369 flooding and runoff has been hypothesized as the main driver of leptospirosis outbreaks
370 (Amilasan et al., 2012; Bourhy et al., 2012; Hagan et al., 2015; Karande et al., 2005, 2002).
371 Together with this exposure factor, our results provide the first empirical data showing that
372 in the rainy season surface waters, and sewage in particular, are more likely to contain
373 pathogenic *Leptospira* and thus, there is a higher environmental risk circulating in the urban
374 slum.

375 Both sewage and standing water samples were potential reservoirs of pathogenic
376 *Leptospira* in the environment. Up to 50% of sewage samples were positive in the rainy
377 season, which suggests that in the rainy periods, sewers and its overflow are drivers of

infection. In contrast, in the dry season standing water samples showed substantially - although not significantly- higher positivity ratios than sewage and, in general, they presented a diminished temporal variability (Table 1 and Fig. 4B). The differences between sewage and standing water were further accentuated by the weak positivity concordance observed in paired samples (Table S2). Taken together, these results lead us to hypothesize that sewage and standing water are two distinct ecological reservoirs of the pathogen. Consequently, the mechanisms that influence the presence of pathogenic *Leptospira* in sewage and standing water (input from the animal reservoir, effect of rainfall and run-off, survival kinetics, etc.) may have different spatiotemporal dynamics in each reservoir. Other studies in the Peruvian Amazon, Southern Chile, and Indonesia have also reported high positivity ratios in puddles (Ganoza et al., 2006; Muñoz-Zanzi et al., 2014; Sumanta et al., 2015). Puddles are abundant and ubiquitous in our study site, being found in areas such as the middle of the informal net of unpaved paths that connect the urban slum, and in the yards of houses. These areas are heavily used by community dwellers and may be a more accessible source of pathogenic *Leptospira* than the open sewers that, although precarious, have some degree of canalization. Since leptospirosis is endemic in the study site with cases occurring year-round (Fig. 2), we believe that standing water may play a role in leptospirosis transmission, particularly in between rainfall events when the accidental contact with sewage and runoff is diminished. Therefore, public health authorities need to consider standing water as a source of pathogenic *Leptospira* along with sewage when designing interventions aimed at reducing the transmission of the disease.

We identified a spatial distribution of positive samples with a higher environmental risk in the bottom of the valley, despite the small dimensions of our study site. Previous studies in this urban slum showed that lower household elevation was a risk factor for

leptospirosis infection presumably because lower elevations are a proxy for higher flooding risk during rainfall events (Hagan et al., 2015) and contact with mud after flooding is associated with higher risks of infection (Felzemburgh et al., 2014; Reis et al., 2008). Since open sewers, rainwater drainages, and non-canalized runoff converge towards the bottom of the valley, surface water in these areas and particularly sewage, may be receiving the influence from all the water basin increasing the probability of finding *Leptospira* positive samples. Overall, this spatial heterogeneity highlights that small-scale changes in the environmental features may substantially contribute to differences in the risk of infection.

The concentration of pathogenic *Leptospira* in positive surface water samples was predominantly low. The clear majority of samples had concentrations ranging from 20 to 1,000 GEq/mL, with an average around 150 GEq/mL. To date, there is only one other study that has succeeded in quantifying pathogenic *Leptospira* in surface waters of urban areas, where they found mean concentrations around 1,000 cells/mL (Ganoza et al., 2006). This discrepancy may be explained by the fact that the 16S rRNA gene-based qPCR used in that study (Smythe et al., 2002) was not completely specific for pathogenic *Leptospira* (Viau and Boehm, 2011), which resulted in the detection of *Leptospira* of unknown pathogenicity (Ganoza et al., 2006). On the contrary, the *lipL32* qPCR used in our study was highly specific for pathogenic *Leptospira* (Stoddard et al., 2009), which validates our results.

However, the low surface water loads detected in our study contrasted with the high infection rates reported in the community (35.4 to 37.8 per 1,000 individuals per year) (Felzemburgh et al., 2014; Hagan et al., 2015). The inoculum doses required for human infection are still unknown, but our findings indicate that the concentration circulating in the water is rarely higher than 1,000 GEq/mL. This concentration is several orders of magnitude lower than the doses required to cause infection through natural routes in animal

models of infection. The conjunctival route shows LD_{50} values of 2×10^5 in Guinea Pigs (Lourdault et al., 2009) and doses as high as 10^8 leptospires to cause 100% death in Golden Syrian hamsters (Wunder et al., 2016a, 2016b). Notably, cuts and abrasions in the skin are an effective route of infection in grivet monkeys and Guinea Pigs (Palmer et al., 1987; Zhang et al., 2012) and have been associated with increased risks for human infection in multiple epidemiological studies (Chusri et al., 2012; Hochedez et al., 2011; Leal-Castellanos et al., 2003). While we cannot rule out the presence of additional infection sources with higher concentrations, previous epidemiological studies performed in this site have consistently pointed out to open sewers as main drivers of infection (Felzemburgh et al., 2014; Hagan et al., 2015; Reis et al., 2008). Thus, we speculate that a mechanism by which the infectious dose substantially decreases, possibly the disruption of skin barriers, enables the transmission of *Leptospira* in waters with low concentrations. Further epidemiological and experimental studies are required to confirm this hypothesis and to determine whether this route of transmission is the main source of the disease in the study site.

As a limitation of our study, the *lipL32* qPCR assay used in our experiments had a detection limit of 18 cells/mL (Riediger et al., 2016). Based on our results, it is possible that concentrations under this limit may be occurring in the surface waters of our study site. If that is the case, the positive proportions reported here might be underestimated. Nevertheless, qPCR does not provide information regarding the viability of bacteria because DNA from metabolically inactive or dead cells can persist for a variable time in the environment (Nocker and Camper, 2009). Since only viable cells have the potential to cause infection, quantitative qPCR-based results may be overestimating the environmental risks. Furthermore, although we identified a higher prevalence of *Leptospira* positive

samples in the rainy season, this study was not designed to explore the specific effect of rainfall events in the dynamics of the pathogen. Thus, we only captured big seasonal differences and not the short-term variability in positivity and concentration that is likely occurring due to mobilization and runoff after rainfall. Such study is needed to understand the immediate impact of rain intensity and frequency in the environmental load and the risks of infection. Finally, this study focused on the surface water reservoirs. Soil and mud are other environmental reservoirs of pathogenic *Leptospira* that have received little attention in the literature and, may be essential to understanding the global dynamics of pathogenic *Leptospira* in the environment.

5. CONCLUSIONS

- The presence of pathogenic *Leptospira* exhibited a clear seasonal pattern in the surface waters of the urban slum, particularly in sewage, an epidemiologically proven source of infection. This is the first empirical evidence that the water-related risk to which inhabitants of an endemic area are exposed increases in the rainy season. Thus, the seasonal peaks of severe leptospirosis may be not only due to an increased exposure to contaminated sources, but also to a higher environmental risk, which modifies the current view on leptospirosis transmission after rainfall events.
- The water-related risk for leptospirosis was spatially heterogeneous, being more prevalent in sewage samples towards the bottom of the valley. This finding is remarkable when considered the small size of the study site. Furthermore, it indicates that preventive measures need to account for the spatial variation for the risk of the disease.

- In addition to sewage, standing water is a reservoir of pathogenic *Leptospira* in the urban slum environment. Their relatively stable positivity across seasons and elevations, suggests that standing water may be relevant for the transmission of the disease, especially in between rainfall events. Consequently, the closing of open sewers alone, a common public health measure, may not be sufficient to eliminate the water-related transmission of the disease.
- The concentration of pathogenic *Leptospira* in surface waters was generally low (mean concentration 152 GEq/mL) which contrasts with previous environmental studies and the high infection rates reported in this urban slum. Further epidemiological and experimental research is necessary to understand the natural history of leptospirosis infection and its correlation with low infectious doses.
- Pau da Lima, our study site in Salvador, Brazil, has similar characteristics to other marginalized communities around the world. Hence, our results may help to understand the drivers of the temporal and spatial variability in urban leptospirosis epidemics. This knowledge is essential to implement timely and efficient measures to reduce the burden of leptospirosis worldwide.

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FIGURE CAPTIONS

Figure 1. Pau da Lima community in the city of Salvador, Brazil. (A) Location of Salvador in South America. **(B)** Location of the study site (red) within the city. **(C)** Sampling sites along the open sewer in the studied valley. In orange, yellow and red, areas of the valley above 52 m (valley top), between 52 and 38 m (valley middle), and below 38 m (valley bottom), respectively, as measured from the lowest point of the valley. **(D)** Photograph of a representative section of the open sewer at the bottom of the valley.

Figure 2. Weekly severe leptospirosis cases identified at the state infectious diseases hospital (orange) and precipitation (blue) during the study period. The shaded areas denote the three sample collection campaigns during the intermediate, rainy and dry seasons. The vertical dashed lines indicate the collection days in each sampling campaign.

Figure 3. Concentration of pathogenic *Leptospira spp.* in sewage and standing water samples from Pau da Lima stratified by season, elevation and time of collection. The geometric mean and standard deviation are shown for each group of samples.

Figure 4. Predicted probability of finding a *Leptospira* DNA positive sample in the final logistic mixed model according to specific interactions. **(A)** Elevation **(B)** Interaction of season and type of water **(C)** Interaction of season and period. Probabilities were calculated by centering the remaining variables on their observed mean values and are expressed as decimals with 95% confidence intervals. (**) $p \leq 0.01$; (***) $p \leq 0.001$.

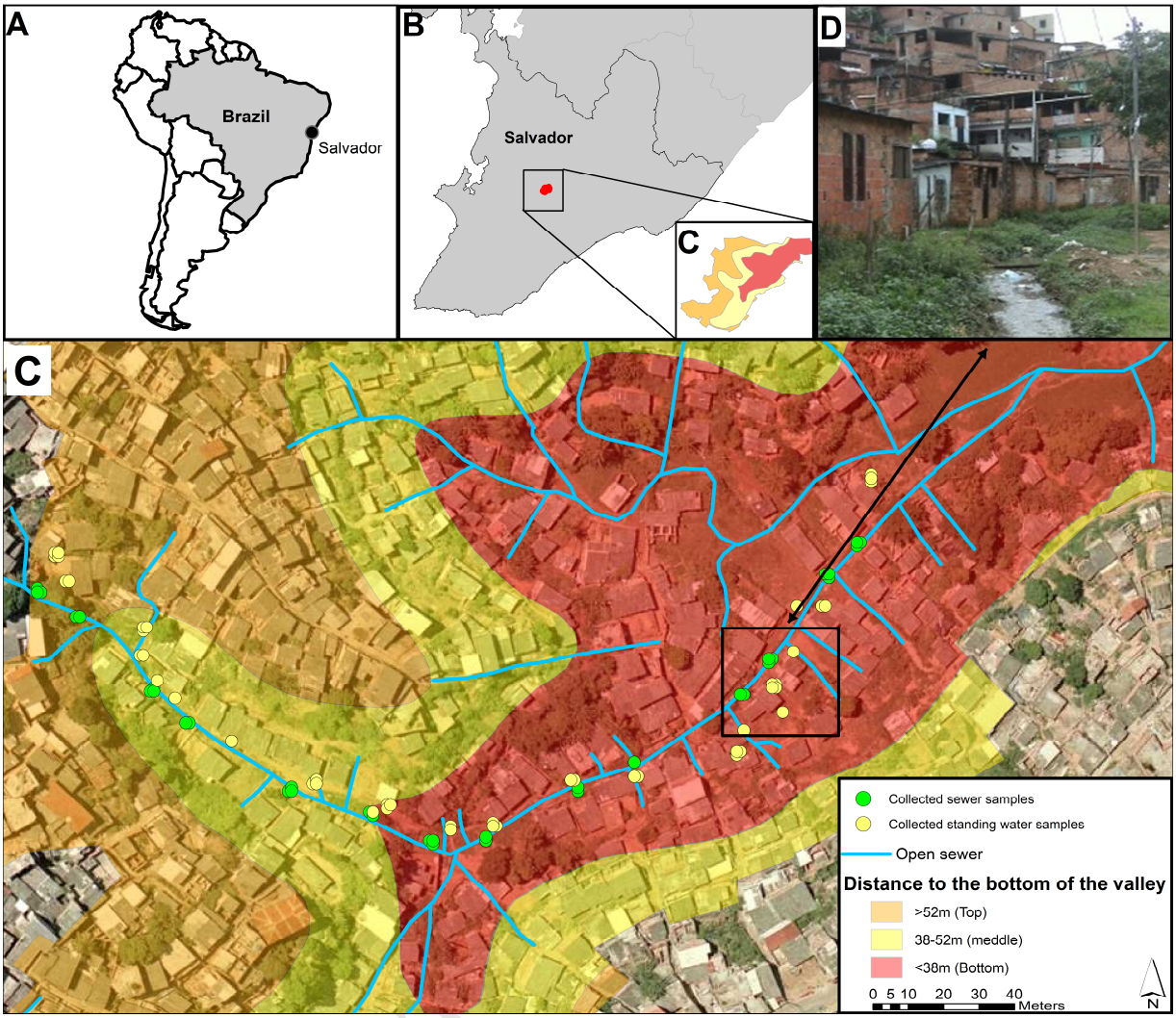
Table 1. Collection success and occurrence of pathogenic *Leptospira* in sewage and standing water samples from the urban slum community. The samples are stratified by season, elevation, and period of collection. The overall positivity used for modeling purposes was calculated by considering non-collected standing water samples as negative.

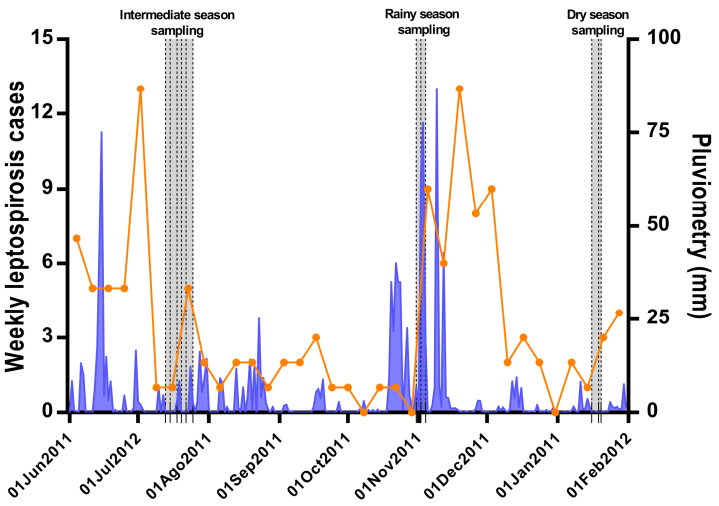
	Sewage samples				**Standing water samples**			
	Targeted	**Collected***	**Positive**	**Overall positivity**	**Targeted**	**Collected**	**Positive**	**Overall positivity**
TOTAL	336	335	121 (36%)	**36%**	336	250 (74%)	115 (46%)	**34%**
Seasons								
Intermediate	168	168	67 (40%)	**40%**	168	141 (84%)	67 (48%)	**40%**
Rainy	84	84	42 (50%)	**50%**	84	61 (73%)	26 (42%)	**31%**
Dry	84	83	12 (15%)	**15%**	84	48 (57%)	22 (46%)	**26%**
Elevation								
Top	48	48	14 (29%)	**29%**	48	34 (71%)	17 (50%)	**35%**
Middle	96	96	28 (29%)	**29%**	96	44 (46%)	21 (48%)	**22%**
Bottom	192	191	79 (41%)	**41%**	192	172 (90%)	77 (45%)	**40%**
Period								
Morning	168	168	65 (39%)	**39%**	168	132 (79%)	64 (49%)	**38%**
Afternoon	168	167	56 (34%)	**34%**	168	118 (70%)	51 (43%)	**30%**

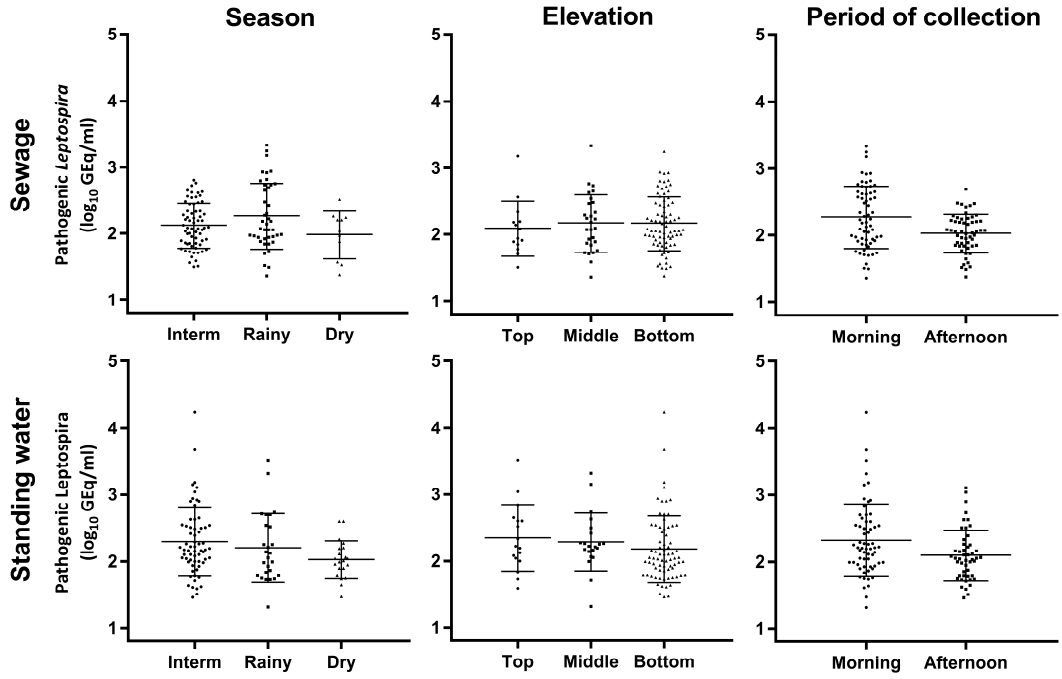
*The percentages of collected sewage samples are omitted because only one sample could not be collected and tested for *Leptospira* presence.

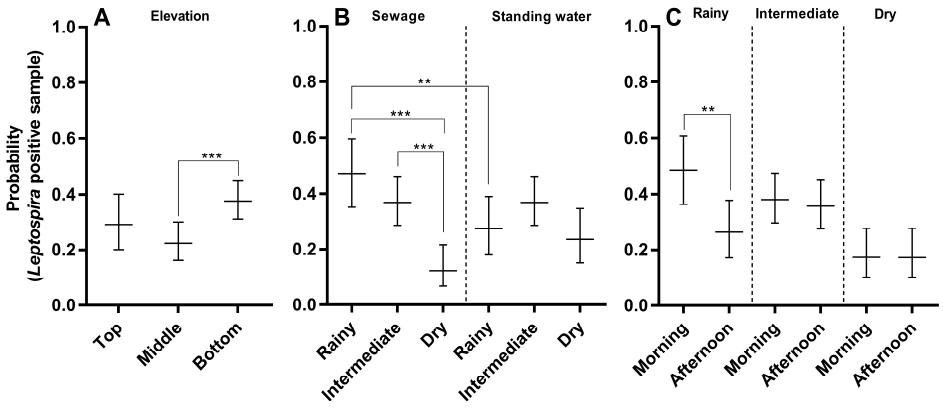
Table 2. Estimated regression parameters and standard errors in the final logistic and linear mixed models on the probability of finding a positive sample and $\log_{10}$ concentration for *Leptospira* DNA, respectively. (*) p≤0.05 (**) p≤0.01; (***) p≤0.001

	Coefficient estimate (SE)	
	Logistic model for probability	**Linear model for concentration**
Intercept	-1.57 (0.39) ***	1.92 (0.09) ***
Intermediate season	1.36 (0.42) **	0.16 (0.08)
Rainy season	1.35 (0.47) **	0.18 (0.09) *
Standing water	0.77 (0.40)	-
Morning period	0.00 (0.39)	0.09 (0.15) ***
Top elevation	-0.39 (0.20)	-
Middle elevation	-0.74 (0.25) ***	-
Interaction terms		
Intermediate season X Standing water	-0.77 (0.46)	-
Rainy season X Standing water	-1.64 (0.52) **	-
Intermediate season X Morning period	0.10 (0.45)	-
Rainy season X Morning period	0.97 (0.52)	-









- Sewage and standing water are a source of pathogenic *Leptospira* in urban slums
- *Leptospira* were ubiquitous in this setting, detected in 33% of sampled surface water
- Pathogen concentrations were low (~150 GEq/mL) in positive surface water samples
- Seasonal leptospirosis risk is associated with increased pathogen detection in water
- Prevention needs to account for the spatiotemporal dynamics of pathogenic *Leptospira*