Distribution and Diversity of Pathogenic *Leptospira* Species in Peri-domestic Surface Waters from South Central Chile

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Abstract

Background

Leptospirosis is a neglected zoonosis affecting animals and humans caused by infection with *Leptospira*. The bacteria can survive outside of hosts for long periods of time in soil and water. While identification of *Leptospira* species from human cases and animal reservoirs are increasingly reported, little is known about the diversity of pathogenic *Leptospira* species in the environment and how surveillance of the environment might be used for monitoring and controlling disease.

Methods and Findings

Water samples (n = 104) were collected from the peri-domestic environment of 422 households from farms, rural villages, and urban slums participating in a broader study on the eco-epidemiology of leptospirosis in the Los Rios Region, Chile, between October 2010 and April 2012. The secY region of samples, previously detected as pathogenic *Leptospira* by PCR, was amplified and sequenced. Sequences were aligned using ClustalW in MEGA, and a minimum spanning tree was created in PHYLOViZ using the goeBURST algorithm to assess sequence similarity. Sequences from four clinical isolates, 17 rodents, and 20 reference strains were also included in the analysis. Overall, water samples contained *L. interrogans*, *L. kirschneri*, and *L. weilii*, with descending frequency. All species were found in each community type. The distribution of the species differed by the season in which the water samples were obtained. There was no evidence that community-level prevalence of *Leptospira* in dogs, rodents, or livestock influenced pathogen diversity in the water samples.

Conclusions

This study reports the presence of pathogenic *Leptospira* in the peri-domestic environment of households in three community types and the differences in *Leptospira* diversity at the community level. Systematic environmental surveillance of *Leptospira* can be used for...
Author Summary
Leptospirosis is a zoonotic disease that is caused by either direct contact with the urine of animals infected with pathogenic forms of *Leptospira*, or indirectly, through contact with contaminated water or soil. Because many people become infected through the environment, where the bacteria can live for many months if the conditions are suitable, we tested water samples from the peri-domestic area in twelve different communities from Los Ríos region, Chile, to examine whether the *Leptospira* species were different in urban, rural village, and farm areas. We found that all three community types had *L. interrogans*, *L. kirschneri*, and *L. weilii*. No evident associations were seen between animal infection and the frequency or diversity of *Leptospira*. The proposed surveillance method has potential for systematic monitoring of surface waters that will help us better understand the importance of animal, climate, and environmental factors in the variation of *Leptospira* species present in a community in order to predict infection risk and inform prevention programs.

Introduction
Each year, an estimated 1.03 million cases of human leptospirosis occur worldwide, resulting in 2.9 million disability adjusted life years lost [1,2]. Transmission of *Leptospira* to animal hosts and humans occurs either through direct contact with the urine of an infected animal, or indirectly, through contact between mucosae or open skin and contaminated soil or water [3,4]. The resulting clinical disease in humans can range from asymptomatic infection to flu-like symptoms, jaundice, and pulmonary hemorrhaging, with a case fatality of 10–50% [5]. Risk factors associated with human infection include specific occupational hazards and recreational activities [4,6,7], but also commonplace exposures such as living in urban areas [8], contact with livestock and companion animals in the peri-domestic environment [9], and walking barefoot [10]. Many of these factors are related to living in resource-poor settings, and indeed, the impact of leptospirosis on a community depends heavily on where it rests on the socio-economic gradient [11,12].

Many studies have documented the impact that rainfall and flooding have on the incidence of leptospirosis in humans and animals [13–17]. With lasting humidity and warm temperatures, *Leptospira* can persist in the environment for several months [3]. Studies have also previously examined the concentration and species of *Leptospira* present in surface waters in tropical areas where heavy flooding occurs seasonally. In urban sites in Malaysia, water samples collected from public spaces including lakes, swamps, and effluent drains, as well as soil samples near households, were examined for *Leptospira* using culture [18]. Approximately 23% of all samples tested positive, however, many of the species found were intermediate and saprophytic (*L. wolffii*, *L. biflexa*, and *L. meyeri*) [18]. In Peru, surface waters in both urban and rural areas were assessed for *Leptospira* using PCR methods, revealing increased species diversity in rural areas compared with urban centers. Urban samples were primarily identified as *L. interrogans* while rural area samples were primarily *L. santarosai* and *L. noguchii* [19]. Both studies recommended regular monitoring of the environment for identifying contaminated areas where humans may be at higher risk for infection.
Recent developments in PCR methods have made it possible to more efficiently determine the presence of pathogenic *Leptospira* from environmental samples [20,21]. Presently, public health responses to *Leptospira*-contaminated bodies of water are reactionary. There can be a delay of several days or weeks from the start of an outbreak to messages reaching the community advising them to avoid the contaminated sources, by which point human infection has already occurred [22]. In high risk areas, it would be beneficial to have systematic surveillance of *Leptospira* in the environment so that meaningful changes in the amount or the geographic distribution of the pathogen can be detected in advance of human cases. Additional improvements to molecular sequencing are also allowing for rapid genetic classification of *Leptospira* which can be useful for integration of animal and human surveillance [23]. Shifts in the relative abundance of particular species in these settings may be indicative of changes in the local ecology and heightened risk for human infection.

The objective of this study was to describe the presence and species diversity of *Leptospira* in surface water samples from the peri-domestic environment in a region with endemic levels of leptospirosis in humans and animals. Of particular interest was examining the differences in the molecular makeup of the pathogen across rural, small village, and urban community types.

**Materials and Methods**

**Surface water sample collection**

The water samples collected for this study were obtained as part of a broader research project on the eco-epidemiology of leptospirosis in the Los Rios Region of South Central Chile between November 2010 and April 2012. Valdivia, the capital of the Los Rios Region (39° 48’ 50”S, 17° 14’ 45”W), receives an average of 2500 mm of rainfall annually, and experiences temperatures between 7.6° and 16.9°C throughout the year [24]. Water samples were collected from the peri-domestic environment of 422 households in twelve communities across three community types: marginalized or urban slum communities (U, n = 142 households), rural villages (C, n = 134 households) and rural farm areas (D, n = 146 households). Households were selected randomly from within each community as part of the larger research study and were enrolled based on their willingness to participate in a household questionnaire, collection of serum samples from pets, livestock and household members, rodent trapping efforts in and around the household, and water sample collection. A detailed description of the components of this cross-sectional study has been previously provided [25,26]. Reported results from these same communities document wide evidence of *Leptospira* exposure in animals and people. Overall seroprevalence in humans was 6%, ranging by community from 3% to 10% [27]. Additionally, 26%, 16%, and 37% of dogs, sheep, and cattle were seropositive, respectively, and 20% of trapped rodents were PCR positive [26–28] (S2 Fig).

Water samples were collected primarily from the peri-domestic environment, outside of the physical housing structure and within the household’s property limits. In farm areas and villages, this included the yard, and any livestock living spaces within approximately a 15-20m radius. In urban slums, because of the close proximity between houses, the sampling space was limited to small yards separating houses. Water sample sources included pails, buckets, large bins, animal drinking troughs, trash cans, small streams, ditches, puddles, and standing water. Based on additional usage information from study participants, these sources were further classified into five categories for analysis: flowing water sources, animal drinking troughs, puddles, containers, and household drinking water sources. Study staff collected water from as many of the water sources described above within a household’s peri-domestic area, up to a limit of 7 samples per household. At least 50mL of water, 1L when possible, was collected using sterile technique and poured into a labeled Whirlpak bag [29]. Samples were stored at 4°C for no more than 24 hours before processing through previously described methods [25].
Extraction and PCR detection of *Leptospira*

Extraction of whole DNA from water samples was conducted using a commercially available QIAamp DNA Mini Kit (Qiagen, Valencia, California, US), per the manufacturer’s instructions. DNA elution was performed with 200 μl of elution buffer. All water samples were tested using two PCR protocols, and all amplifications included a negative control, consisting of water, and a positive plasmidial control. The first PCR was a nested PCR protocol using the Lepat set of primers (Lepat1 and Lepat2) targeting the 16s rRNA gene found by Murgia et al. to identify pathogenic *Leptospira* [20]. A 510 base pair product was amplified in the first round using 16S13 (5'-CGG CGC GTC TTA AAC ATG-3') and 16S522 (5'-TCC GCC TAC TAC CCC TTT AC-3') primers. A second amplification round of a 330 bp product was obtained using Lepat1 (5'-GAGTCTGGGATAACTTT-3') and Lepat2 (5'-TCACATCGYTGCTTATT TT-3') primers. The second PCR protocol targeted a 242 base pair fragment of the lipL32 gene that also detects pathogenic *Leptospira*. This portion of the *Leptospira* genome was amplified with the LipL32-45F (5'-AAGCATTACCGCTTGTGGTG-3') and LipL32-286R (5'-GAACCTCCATTTCAGCGATT-3') primers [30,31]. GoTaq Flexi DNA polymerase was used in combination (5:1) with Pfu DNA Polymerase (Promega, Madison, Wisconsin, US) for all PCR protocols.

A sample was considered positive for pathogenic *Leptospira* if it tested positive through either PCR protocol. All of these positive samples as well as DNA from 20 reference *Leptospira* strains obtained from the Royal Tropical Institute, The Netherlands (S1 Table), four local clinical isolates (two humans, one horse, and one cow), and 17 rodents (7 *Rattus rattus*, 7 *Mus musculus* and 3 wild rodents) captured from the same 422 households [26] were then tested using a PCR that targets the secY gene. The secY primers used were SecYIVF (5'-GCGATTCAGTTT AATCCTGC -3') and SecYIV (5'-CTTAGATTTGAGCTCTAACTC -3'), with a target of 202 base pairs [32]. After amplification, the PCR products were purified and sequenced (Macrogen Inc., Seoul, Korea) and then used in a BLAST search of GenBank (National Center for Biotechnology Information, Bethesda, MD) to verify their similarity to other pathogenic *Leptospira* sequences. The chromatograms obtained from sequencing were cut at the start and end of the primers for the respective forward and reverse sequences, and examined visually for mismatches in the nucleotide bases using Sequencher 4.10 (Gene Codes Corporation, Ann Arbor, Michigan, USA). A sample was considered suitable for phylogenetic analysis if the forward and reverse sequences could be aligned, and any mismatches between base pairs could be resolved based on visual inspection of the chromatogram. Sequences are available in GenBank (Accession numbers: KX444622-KX444627, KX513514—KX513524, KX513410—KX513513).

Statistical and phylogenetic analyses

Sample positivity was tabulated and described by community type (rural farms, rural villages, or urban slum areas), year, and season of sampling (Spring: August-November, or Summer: December-April). A mixed-effects logistic regression model with random intercepts at the community level was used to examine associations between these variables and sample PCR-positivity. Correlations between the proportion of seropositive dogs, livestock, PCR-positive rodents, and the proportion of PCR-positive water samples within a community were also calculated. Data for the community-level seroprevalence of *Leptospira* in livestock and dogs, and the PCR data for *Leptospira* carriage in rodents were obtained from the data collected for the broader study in the region [25,28] (S2 Fig).

The secY sequences from the PCR-positive samples were compared to the sequences from the reference strains using the goeBURST algorithm in PHYLViZ at the single-, double- and triple-locus variant levels, corresponding to the number of base pair differences between
samples [33,34]. Each water, rodent, and clinical sample was classified as a particular *Leptospira* species based on the reference sequence to which it shared the most genetic similarity. The resulting minimum spanning tree was paired with data from the water sample collection sites to visually assess associations between household characteristics and the way in which samples clustered on the minimum spanning tree. Fisher’s exact tests were used to examine whether the distribution of *Leptospira* species differed across community types (rural farms, rural villages, or urban slum areas), year, and season of sampling (Spring: August-November, or Summer: December-April) [35]. Differences in the distribution of *Leptospira* species by sample type, average temperature of the preceding seven days (°C), rainfall in the preceding 30 days (mm), and household-level prevalence of animals and rodents with *Leptospira* infection were also assessed because of their relevance in prior analyses [25,26,28].

**Ethics statement**

The study protocol was approved by the University of Minnesota’s Institutional Review Board (No. 0903M62042), the Institutional Animal Care and Use Committee (No. 0904A63201), and Austral University’s Human and Animal Ethics Committee (No. 01/09). The Public Health Service Policy on Humane Care and Use of Laboratory Animals in testing, research, and training provides the core of the operational guidelines for the University of Minnesota Institutional Animal Care and Use Committee. Head of households, all adults, provided written informed consent prior to participation in this study.

**Results**

**Presence of pathogenic *Leptospira***

From the 422 households in the Los Rios Region, 359 (85.1%) had at least one environmental water sample collected. In those 359 households, 816 water samples were obtained, of which, 153 (18.8%) were PCR positive for pathogenic *Leptospira* by at least one of the two PCR protocols (Table 1). Farm areas had the most water samples collected (n = 359) and also the highest proportion of PCR-positive samples (20.6%). Of the different water sample types, puddles were most commonly contaminated with pathogenic *Leptospira* (27.3%). In the mixed model with the random intercept for community, community type was not associated with whether a water sample was PCR-positive (D vs C p-value = 0.94 and U vs C p-value = 0.71). This is likely due to the distinct differences in the proportion of positive water samples at the community level, with large differences across communities of the same type. Among the rural villages, between 10.5% and 37.5% of samples tested positive, 6.1% and 43.1% of samples tested positive in rural farm areas, and between 4.9% and 33.3% in urban slum communities. The mixed regression model also indicated an interaction between season and year of sampling. In the second year of the study, more samples collected in summer (December-April) tested positive (39.3%) compared with samples collected in spring (August-November) (20.3%, p-value = 0.04). This was not observed in the samples collected during the first year (p-value = 0.77). No trends were observed between the proportion of PCR-positive water samples and the proportions of livestock or dogs seropositive for *Leptospira* or the proportion of PCR-positive rodents (S2 Fig). Seropositivity ranged from 4.5%–22.6% for sheep, 10.7%–75.0% for cows, and 4.8%–68.0% for dogs. The proportion of PCR-positive rodents ranged from 0–41% at the community level.

**Leptospira species from water samples**

From the 153 PCR positive samples, 104 (12.7% of all water samples) had a sequence suitable for phylogenetic analysis using the *secY* gene (S1 Fig). In the goeBURST analysis of the
sequences from the secY gene, all sequences were not connected in the network until 18 variant loci were considered. Because the DNA from water samples were not obtained from isolates, some variation in the genetic makeup of the sequences relative to the reference sequences was expected, and therefore the triple-variant locus Minimum Spanning Tree (MST) was used for classification of samples. Fewer than three base pair differences from a reference sequence were considered to be indicative of the correspondent species. Based on this classification, the most common Leptospira species in water samples was *L. interrogans* (75.0%, n = 78), followed by *L. kirschneri* (15.4%, n = 16), and *L. weilii* (2.9%, n = 3) (Fig 1). Seven water samples (6.7%) had three or more base pair differences with any reference sequence and were not classified. Using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST), the unclassified were confirmed to be pathogenic Leptospira. The seven unclassified sequences matched with pathogenic Leptospira in the NCBI BLAST database at 97% or above as either *L. interrogans* (n = 6) or *L. borgpetersenii* or *L. kirschneri* (n = 1). The majority of rodent samples were most similar to *L. kirschneri* (70.6%, 12/17 rodents), followed by *L. borgpetersenii* (11.8%, 2/17 rodents), *L. interrogans* (11.8%, 2/17 rodents) and one sample was unclassified (5.9%, 1/17). The sequences from the cow, horse, and from one of the human cases were most similar to *L. interrogans*, while the sequence from the second human case aligned most closely with *L. kirschneri* (Fig 1, S1 Fig).

Community type was not associated with the presence of a particular *Leptospira* species (p-value = 0.27) (Table 2). A visual representation is seen in Fig 2 where the community types are equally distributed across the three known *Leptospira* species in the MST produced in PHYLOViZ. However, at the community level, there were notable differences in distribution of species.

### Table 1. *Leptospira* PCR results by community type and sample characteristics for water samples collected from households in the Los Rios Region, Chile (2010–2012).

| Sample Characteristic | Subtotals | Animal drinking trough | Puddle | Human drinking water | Flowing source | Container | Year of Sample | Sample Type | Presumptive Species |
|-----------------------|-----------|------------------------|--------|----------------------|---------------|-----------|----------------|-------------|---------------------|
|                       | PCR+ (%)  | 5/28 (17.9%)           | 18/84 (21.4%) | 0/10 (0%)            | 5/45 (11.1%)  | 11/50 (22.0%) | 2010–2011 (Year 1) | Rural Village (C) n = 217 | *L. interrogans* |
|                       |           | 9/55 (16.4%)           | 35/104 (33.7%)| 11/47 (23.4%)        | 6/62 (9.7%)   | 13/91 (14.3%) | 2011–2012 (Year 2) | Farm Area (D) n = 359 | *L. kirschneri* |
|                       |           | 0/2 (0%)               | 31/120 (25.8%)| 3/38 (7.9%)          | 2/27 (7.4%)   | 4/53 (7.5%)   | 2010–2011 (Year 1) | Urban Slum (U) n = 240 | *L. weilii* |
|                       |           | 14/85 (16.5%)          | 84/308 (27.3%)| 14/95 (14.7%)        | 13/134 (9.7%) | 28/194 (14.4%) | 2011–2012 (Year 2) | Total n = 816 | Unclassified Leptospira sp. |
|                       |           | 74/359 (20.6%)         | 312/1248 (25.2%)| 312/1248 (25.2%)   | 59/183 (32.2%)| 91/120 (7.5%) | 2010–2011 (Year 1) |                       | *L. interrogans* |
|                       |           | 40/240 (16.7%)         | 312/1248 (25.2%)| 312/1248 (25.2%)   | 31/120 (25.8%)| 4/53 (7.5%)   | 2011–2012 (Year 2) |                       | *L. kirschneri* |
|                       |           | 153/816 (18.8%)        | 312/1248 (25.2%)| 312/1248 (25.2%)   | 116/436 (26.6%)| 28/194 (14.4%) |                       |                       | *L. weilii* |

* Distribution of species among sequences obtained in each community type.
† Unclassified Leptospira sp. are sequences that were confirmed to be pathogenic Leptospira in a search of the NCBI database, but had more than a three base pair difference from reference strains used in the phylogenetic analysis.
(Fig 3). Community D-4, seemed to have *L. interrogans* only; 19 of 22 samples were classified as *L. interrogans*, and the other three, while unclassified, were also suggested to be *L. interrogans* based on results from the NCBI BLAST. Similarly, all sequences from community U-3 (n = 15) were classified as *L. interrogans*. In other communities, diversity was evident despite the small number of sequences. In community U-1, for example, among the three samples sequenced, all three *Leptospira* species were identified (L. interrogans, L. kirschneri and L. weilii).

In five communities (two rural villages and three farm areas) where *L. kirschneri* was found in rodents, it was also found in at least one water sample from the respective community. *L. kirschneri* was found in a rodent from an additional rural village but only one secY water sequence (classified as *L. interrogans*) was available for comparison. *L. interrogans* was found in two rodents (one from a rural community and one from a rural village) but it is also the
most commonly found species in water samples across all communities. Sequences classified as *L. borgpetersenii* were found in two rodents but in none of the water samples (Fig 1, S1 Fig). The diversity of *Leptospira* species in water samples in a community did not appear to be associated with the proportion of infected animals in the community (S2 Fig), and in general, *L. interrogans*, *L. kirschneri*, and *L. weilii* were found across the various communities (Fig 3).

### Sample characteristics associated with *Leptospira* diversity

Samples that were considered “not classified” in the MST procedure were excluded from this analysis. Sequences obtained in summer corresponded to a higher proportion of *L. kirschneri* (Table 2). The distribution of *Leptospira* species for each level of the variable investigated is shown in Table 2. The results correspond to sequences from households where both sequences and dogs, livestock, and rodents, respectively, were present.

Table 2. *Leptospira* species phylogenetic classification of secY (202 bp) sequences obtained from water samples collected from the peri-domestic environment and sample/household characteristics.

| Sample Type                        | L. interrogans | L. kirschneri | L. weilii | Total | p-value |
|------------------------------------|----------------|---------------|-----------|-------|---------|
| Animal drinking trough             | 8 (88.9%)*     | 1 (11.1%)     | 0         | 9     | 0.21    |
| Puddle                            | 42 (77.8%)     | 11 (20.4%)    | 1 (1.9%)  | 54    |         |
| Human drinking water               | 5 (55.5%)      | 2 (22.2%)     | 2 (22.2%) | 9     |         |
| Flowing source                     | 8 (100%)       | 0             | 0         | 8     |         |
| Container                          | 15 (88.2%)     | 2 (11.8%)     | 0         | 17    |         |
| Year of Sample                     |                |               |           |       | 0.07    |
| Year 1                             | 15 (65.2%)     | 7 (30.4%)     | 1 (4.4%)  | 23    |         |
| Year 2                             | 63 (85.1%)     | 9 (12.2%)     | 2 (2.7%)  | 74    |         |
| Season of Sample                   |                |               |           |       | 0.02    |
| Spring (August-November)           | 43 (86.0%)     | 4 (8.0%)      | 3 (6.0%)  | 50    |         |
| Summer (December-April)            | 35 (74.5%)     | 12 (25.5%)    | 0         | 47    |         |
| Community Type                     |                |               |           |       | 0.25    |
| Farms                              | 46 (86.8%)     | 5 (9.4%)      | 2 (3.8%)  | 53    |         |
| Rural villages                     | 10 (71.4%)     | 4 (28.6%)     | 0         | 14    |         |
| Urban slums                        | 22 (73.3%)     | 7 (23.3%)     | 1 (3.3%)  | 30    |         |
| Average Temperature Past 7 days    |                |               |           |       | 0.07    |
| <7°C                               | 17 (94.4%)     | 0             | 1 (5.6%)  | 18    |         |
| 7–14°C                             | 30 (76.9%)     | 7 (17.9%)     | 2 (5.1%)  | 39    |         |
| >14°C                              | 31 (77.5%)     | 9 (22.5%)     | 0         | 40    |         |
| Average Rainfall Past 30 days      |                |               |           |       | 0.12    |
| <50mm                              | 38 (76.0%)     | 11 (22.0%)    | 1 (2.0%)  | 50    |         |
| 50-100mm                           | 22 (78.6%)     | 5 (17.9%)     | 1 (3.6%)  | 28    |         |
| >100mm                             | 18 (94.7%)     | 0             | 1 (5.3%)  | 19    |         |
| Presence of Seropositive Dogs ‡    |                |               |           |       | 1.0     |
| No                                 | 11 (78.6%)     | 3 (21.4%)     | 0         | 14    |         |
| Yes                                | 27 (77.1%)     | 7 (20.0%)     | 1 (2.9%)  | 35    |         |
| Presence of Seropositive Livestock ‡|            |               |           |       | 0.04    |
| No                                 | 12 (66.7%)     | 5 (27.8%)     | 1 (5.6%)  | 18    |         |
| Yes                                | 21 (95.5%)     | 1 (4.5%)      | 0         | 22    |         |
| Presence of PCR Positive Rodents ‡ |                |               |           |       | 1.0     |
| No                                 | 8 (88.9%)      | 1 (11.1%)     | 0         | 9     |         |
| Yes                                | 70 (79.5%)     | 15 (17.0%)    | 3 (3.4%)  | 88    |         |

* Percentages correspond to the distribution of *Leptospira* species for each level of the variable investigated.

‡ Results correspond to sequences from households where both sequences and dogs, livestock, and rodents, respectively, were present.

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(25.5%, 12/47) compared to sequences obtained in the spring (8.0%, 4/50), and all three \textit{L. weilii} samples were obtained in the spring (Table 2, p-value = 0.02). In the subset of households with livestock, the water sequences from households with seropositive livestock contained a higher proportion of \textit{L. interrogans} (95.4%, 21/22) than water samples from households with seronegative livestock (66.7%, 12/18 of \textit{L. interrogans}) (p-value = 0.04).

The type of water sample did not appear to be associated with the presence of a particular \textit{Leptospira} species (p-value = 0.24), but all the sequences from flowing water sources (n = 8) were most closely related to \textit{L. interrogans} (Table 2). The temperature in the preceding week, and rainfall in the preceding month of sample collection were also not statistically significantly associated with the distribution of presumptive \textit{Leptospira} species (p-value = 0.07 and p-value = 0.12, respectively). Nonetheless, 94.4% (17) of samples were identified as \textit{L. interrogans} when the average temperature was below 7°C, while 76.9% (30) and 77.5% (31) of samples were identified as \textit{L. interrogans} at temperatures between 7°C and 14°C or above 14°C, respectively (Table 2). \textit{L. interrogans} was also more common at higher levels of precipitation with 94.7% (18) of samples identified when the rainfall in the preceding month was above 100 mm, compared to 78.6% (22 samples) when rainfall was between 50 and 100mm, and 76.0% (38 samples) when rainfall was less than 50 mm in the preceding month (Table 2).
Discussion

This study reports the presence of pathogenic *Leptospira* in the peri-domestic environment of households in several community types, and the substantial differences in *Leptospira* diversity at the community level. The development of PCR methods for detecting pathogenic *Leptospira* over the past decade has greatly improved the efficiency with which a specific site can be tested for *Leptospira* presence. Several studies have noted the success of these methods in establishing the presence of the bacteria in bodies of water such as rivers, canals and streams [21,36,37]. Fewer studies have examined the presence of pathogenic *Leptospira* in the daily human environment where regular contact with contaminated water sources may occur. In Peru, researchers examined surface water samples from puddles and gutters in an urban location, and wells, fish farms and streams in rural areas. *Leptospira* was detected in both urban and rural areas, but with higher concentration and more frequent contamination in the urban compared to the...
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rural sites [19]. More frequent contamination with pathogenic Leptospira was also observed in urban sites compared to rural areas in India [38]. In our study, greater differences in the proportion of water samples testing positive were observed at the community level than across community types (urban slums, rural villages or farms). This is indicative of the importance of small scale transmission dynamics in the ecology of leptospirosis.

This study also describes the difference in pathogenic Leptospira species obtained in environmental water samples in different communities, and across community types. Results in Peru suggested that non-L. interrogans species were more likely in samples from rural areas than from urban sites due to the presence of more animal species [19]. It is reasonable to expect a higher presence of non-L. interrogans species in locations where there is a higher diversity and density of animal species overlapping in the same environment; however, this was not generally reflected in our findings where the majority of sequences in rural areas were classified as L. interrogans (86.8%), which was higher than in urban sites (73.3%) (Table 2). This was primarily driven by the large number of samples with L. interrogans in farm communities D-3 (84.6%) and D-4 (86.4%). Furthermore, in some urban sites, U-1 for example, all three Leptospira species were present (L. interrogans, L. kirschneri, and L. weilii). A possible explanation of these findings is that the urban slum communities in the study were in close proximity to rural farms, and wild mammals may transmit these more diverse Leptospira species between the rural and urban areas. Nevertheless, the variation in diversity across all twelve communities highlights the importance of better understanding local transmission dynamics for assessing Leptospira risk in the peri-domestic environment. The seasonality of leptospirosis has been well-described; human and animal incidence increases in periods following warm temperatures and heavy rainfall [13,39]. Temporal patterns in species diversity was reported in a study in New Caledonia which found that black rats normally carried L. interrogans, but during the hot season, they were found to carry L. borgpetersenii [40]. In this study, most of the sequences corresponded to L. interrogans but, among water samples collected in spring, there was increase in the proportion that were classified as L. kirschneri compared to sequences obtained in the summer (Table 2). As a cross-sectional study with sampling that took place over two years, we cannot separate the effect of community differences from a potential true season effect. Future prospective studies can examine the joint influence of rainfall, temperature, and host population dynamics on the Leptospira diversity and determine seasonality patterns.

L. interrogans was more common in households with seropositive livestock (sheep or cattle) than in households without seropositive livestock (Table 2), which is consistent with reports of sheep and cattle carrying L. interrogans in Chile [41] and in other animal species and rural water sources in other geographic locations [42–44]. Considering the large number of sequences classified as L. interrogans, it also possible that sequencing of the secY target may not provide enough discrimination to capture associations between other examined factors and molecular diversity. Alternatively, it is possible that water samples were contaminated with more than one Leptospira species, and that L. interrogans was more commonly amplified, since PCR is a competitive process that amplifies the most abundant species in the sample. This would result in less diversity than there really was mainly because the relative concentration of non-L. interrogans species is low. Furthermore, presence of multiples species in a sample could be a factor leading to the many sequences that were not able to be classified. The impact of contamination by multiple species on PCR detection and subsequent genotyping needs further investigation. Several methods have been developed for genetic classification of Leptospira isolates [45–48]; however, being able to use culture-free methods is essential for large-scale and systematic monitoring of environmental contamination. Recently, a culture-free high resolution melting method was proposed for identification of Leptospira strains in blood samples at the species and subspecies levels [47]. Similar methods need to be developed and optimized for environmental testing.
A limitation of culture-free methods for identification of *Leptospira* in the environment is the inability to differentiate between live and dead bacteria [49]. While the detection of DNA in the environment may not directly represent infection risk, the benefit of being able to test a large number of samples and obtain fast results may outweigh this limitation for the purpose of obtaining an indicator of contamination level and of the genetic makeup of the circulating strains. With the development of recent methods that target RNA and DNA in environmental samples, it may be possible to discern not only presence of *Leptospira* but also the relative abundance of viable *Leptospira* [45,46,50]. We expect that both detection limit, a likely problem when testing environment sample, and testing of mixed DNA contributed to our inability to sequence all the PCR positive samples or classify some of the secY sequences. Upon further review, seven of the eight unclassified samples (seven water samples, and one rodent sample) had some ambiguous base pairs in their chromatograms and the eighth sample had gaps at the end of the sequence where the final bases of the primer should have been. All eight sequences matched with *L. interrogans* in the NCBI BLAST database with at least 97% similarity. There

![Diagram](image)
were an additional 49 samples that were not included in this analysis because of the poor quality of the chromatograms. It is important to note that all these samples were detected as pathogenic *Leptospira* by at least one of the two detection protocols.

The environment is recognized as an important source of infection and development of proper methods for surveillance of *Leptospira* in soil and water can provide useful information for research and public health programs. An ideal environmental surveillance program may include detection and quantification of pathogenic *Leptospira* in environmental samples in a geographical area, followed by molecular classification and comparison with local strains from human and animal cases (Fig 4). Quantification of *Leptospira* contamination using qPCR methods, was beyond the original scope of this study, but would allow more accurate monitoring of high risk areas and environmental risk assessment [19]. This ideal environmental surveillance program could integrate human and animal genotyping data with the results of environmental sampling to allow for tracking the source of infections. Additionally, changes in the diversity of *Leptospira* detected may indicate a shift in the local epidemiology in the various animal hosts or lapses in control programs [44]. Monitoring changes in the level of contamination at appropriate scales, along with environmental features such as rainfall, temperature, pH, soil type, and geographic features [38,47] could potentially aid in outbreak prediction. This concept of environmental surveillance has been used to assess the safety of recreational waters for swimming in the United States. The primary pathogen observed is *E. coli*, where a protocol was developed to predict dangerously high concentrations in the environment. The model includes measurements pertaining to environmental conditions (wave height and log<sub>10</sub>turbidity), weather conditions (past 48 hour rainfall and wind direction), and animal factors (the presence of birds). After four years’ worth of *E. coli* measurements taken from the water source at least four consecutive days per week, the model was able to predict unsafe swimming days with 90.2–97.5% specificity, and 36.4–59.1% sensitivity [51]. Further development of laboratory methods is still needed to allow for a similar systematic, large-scale, and cost-effective identification of *Leptospira* in the environment.

**Conclusion**

This study demonstrated that PCR methods can be used to assess the presence and species diversity of pathogenic *Leptospira* in surface waters in several community settings. Presence and diversity of *Leptospira* species varied substantially at the community level, more than by community type, suggesting that targeted prospective monitoring may be appropriate to identify local mechanisms responsible for enhanced transmission, including critical periods of high risk, and key animal hosts.

**Supporting Information**

**S1 Fig.** Neighbor-joining tree of 104 water samples, 17 rodent samples, 20 reference strains and four clinical samples, from urban, rural village, and farm communities in the Los Rios Region, Chile, 2010–2012 using the secY gene. Samples labeled with a *Leptospira* species name are reference strains. Samples are coded to represent their community type of origin (C: rural village, D: farms, U: urban slum) and whether it is a water (B) or rodent (R) sequence. The clinical samples are listed by their types: Clinical-Human (blood sample), Human-Urine, Horse-Urine, and Cow-Blood.

(PDF)

**S2 Fig.** Community-level prevalence of *Leptospira* in dogs, livestock and rodents and distribution of *Leptospira* species obtained from water samples in the same communities. Dogs
and livestock show estimates of seroprevalence by MAT and rodents show the PCR positive proportion. Community types are represented by C: rural village, D: farms, and U: urban slum.

S1 Table. List of reference Leptospira species used in the phylogenetic analysis.

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Author Contributions

Conceived and designed the experiments: CMZ.

Performed the experiments: CMZ CE.

Analyzed the data: CMZ MRM SS.

Contributed reagents/materials/analysis tools: CMZ CE SS.

Wrote the paper: MRM CMZ SS CE.

References

1. Costa F, Hagan JE, Calcagno J, Kane M, Torgerson P, Martinez-Silveira MS, et al. Global morbidity and mortality of leptospirosis: a systematic review. PLoS Negl Trop Dis. 2015; 1–19. doi: 10.1371/journal.pntd.0003898

2. Torgerson PR, Hagan JE, Costa F, Calcagno J, Kane M, Martinez-Silveira MS, et al. Global burden of leptospirosis: estimated in terms of disability adjusted life years. PLoS Negl Trop Dis. 2015; 9: e0004122. doi: 10.1371/journal.pntd.0004122 PMID: 26431366

3. Levett PN. Leptospirosis. Clin Microbiol Rev. 2001; 14: 296–326. doi: 10.1128/CMR.14.2.296 PMID: 11292640

4. Farr RW. Leptospirosis. Clin Infect Dis. 1995; 21: 1–6. PMID: 7578715

5. McBride AJ, Athanazio DA, Reis MG, Ko AI. Leptospirosis. Curr Opin Infect Dis. 2005; 18: 376–386. doi: 10.1097/01.qco.0000178824.05715.2c PMID: 16148523

6. Watkins SA. Leptospirosis as an occupational disease. Br J Ind Med. 1986; 43: 721–725. PMID: 3790454

7. Jansen A, Schöneberg J, Frank C, Alpers K, Schneider T, Stark K. Leptospirosis in Germany, 1962–2003. Emerg Infect Dis. 2005; 11: 1048–54. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3371786&tool=pmcentrez&rendertype=abstract PMID: 16227797

8. Vinetz JM, Glass GE, Flexner CE, Mueller P, Kaslow DC. Sporadic urban leptospirosis. Ann Intern Med. 1996; 125: 794–8. Available: http://www.ncbi.nlm.nih.gov/pubmed/922895 PMID: 922895

9. Leal-Castellanos CB, García-Suárez R, González-Figueroa E, Fuentes-Alén JL, Escobedo-de la Peñal J. Risk factors and the prevalence of leptospirosis infection in a rural community of Chiapas, Mexico. Epidemiol Infect. 2003; 131: 1149–1156. doi: 10.1017/S0950268803001201 PMID: 14959783

10. Bovet P, Yersin C, Merien F, Davis CE, Perolat P. Factors associated with clinical leptospirosis: a population-based case-control study in the Seychelles (Indian Ocean). Int J Epidemiol. 1999; 28: 583–590. PMID: 10405868

11. Barcellos C, Sabroza PC. Socio-environmental determinants of the leptospirosis outbreak of 1996 in western Rio de Janeiro: a geographical approach. Int J Environ Health Res. 2000; 10: 301–13. doi: 10.1080/0960312002001500 PMID: 11260779
12. Barcellos C, Sabroza PC. The place behind the case: leptospirosis risks and associated environmental conditions in a flood-related outbreak in Rio de Janeiro. Cad Saude Publica. 2001; 17: 59–67. PMID: 11426266

13. Ward MP. Seasonality of canine leptospirosis in the United States and Canada and its association with rainfall. Prev Vet Med. 2002; 56: 203–13. Available: http://www.ncbi.nlm.nih.gov/pubmed/12441236 PMID: 12441236

14. Miller D, Wilson M, Beran G. Relationships between prevalence of *Leptospira interrogans* in cattle, and regional, climatic, and seasonal factors. Am J Vet Res. 1991; 52: 1766–68. PMID: 1785720

15. Carroll AG, Campbell RSF. Reproductive and leptospiral studies on beef cattle in central Queensland. Aust Vet J. 1987; 64: 1–5. PMID: 3593131

16. Barwick R, Mohammed H, McDonough P, White M. Risk factors associated with the likelihood of leptospiral seropositivity in horses in the state of New York. Am J Vet Res. 1997; 58: 1097–103. PMID: 9328661

17. Romero EC, Bernardo CCDM, Yasuda PH. Human leptospirosis: a twenty-nine-year serological study in São Paulo, Brazil. Rev Inst Med Trop Sao Paulo. 2003; 45: 245–8. Available: http://www.ncbi.nlm.nih.gov/pubmed/14743663 PMID: 14743663

18. Benacer D, Woh PY, Mohd Zain SN, Amran F, Thong KL. Pathogenic and saprophytic *Leptospira* species in water and soils from selected urban sites in Peninsular Malaysia. Microbes Environ. 2013; 28: 135–40. doi: 10.1264/jsme2.ME12154 PMID: 23363618

19. Ganoza CA, Matthias MA, Collins-Richards D, Brouwer KC, Cunningham CB, Segura ER, et al. Determining risk for severe leptospirosis by molecular analysis of environmental surface waters for pathogenic *Leptospira*. PLoS Med. 2006; 3: e308. doi: 10.1371/journal.pmed.0030308 PMID: 16933963

20. Murgia R, Riquelme N, Baranton G, Cinco M. Oligonucleotides specific for pathogenic and saprophytic *Leptospira* occurring in water. FEMS Microbiology Lett. 1997; 148: 27–34.

21. Tansuphasiri U, Thipsuk C, Phulsuksombati D. Duplex PCR-hybridization based detection of pathogenic *Leptospira* in environmental water samples obtained from endemic areas in northeast region of Thailand. Southeast Asian J Trop Med Public Health. 2006; 37: 729–741. PMID: 17121299

22. Jena AB, Mohanty KC, Devadasan N. An outbreak of leptospirosis in Orissa, India: the importance of surveillance. Trop Med Int Health. 2004; 9: 1016–21. doi: 10.1111/j.1365-3156.2004.01293.x PMID: 15361116

23. Perez J, Goarant C. Rapid *Leptospira* identification by direct sequencing of the diagnostic PCR products in New Caledonia. BMC Microbiol. BioMed Central Ltd; 2010; 10: 325. doi: 10.1186/1471-2180-10-325 PMID: 21176235

24. Balocchi OA, López IF. Herbage production, nutritive value and grazing preference of diploid and tetraploid perennial ryegrass cultivars (Lolium perenne L.). Chil J Agric Res. 2009; 69: 331

25. Muñoz-Zanzi C, Mason M, Encina C, Astroza A, Romero A. *Leptospira* contamination in household and environmental water in rural communities in Southern Chile. Int J Environ Res Public Health. 2014; 11: 6666–80. doi: 10.3390/ijerph110706666 PMID: 24972030

26. Muñoz-Zanzi C, Mason M, Encina C, Gonzalez M, Berg S. Household characteristics associated with rodent presence and *Leptospira* infection in rural and urban communities from Southern Chile. Am J Trop Med Hyg. 2014; 90: 497–506. doi: 10.4269/ajtmh.13-0334 PMID: 24445209

27. Munoz-Zanzi C, Mason M, Galloway R, Encina C, Salgado M, Berg S. Epidemiology of leptospirosis in distinct community settings. Proceedings of the 9th International Leptospirosis Society Meeting. Semarang, Indonesia; 2015.

28. Lelu M, Muñoz-Zanzi C, Higgins B, Galloway R. Seroepidemiology of leptospirosis in dogs from rural and slum communities of Los Rios Region, Chile. BMC Vet Res. 2015; 11: doi: 10.1186/s12917-015-0341-9

29. Nasco. Instruction Sheet for Whirl-Pak Sample Bags. Fort Atkinson, Wisconsin; 2006.

30. Stoddard RA, Gee JE, Wilkins PP, McCausland K, Hoffmaster AR. Detection of pathogenic *Leptospira* spp. through TaqMan polymerase chain reaction targeting the Lpl32 gene. Diagn Microbiol Infect Dis. Elsevier B.V.; 2009; 64: 247–55. doi: 10.1016/j.diagmicrobio.2009.03.014 PMID: 19395218

31. Levett PN, Morey RE, Galloway RL, Turner DE, Steigerwalt AG, Mayer LW. Detection of pathogenic leptospires by real-time quantitative PCR. J Med Microbiol. 2005; 54: 45–49. doi: 10.1099/jmm.0.45860–0 PMID: 15591124

32. Ahmed A, Engelberts MFM, Boer KR, Ahmed N, Hartskeerl RA. Development and validation of a real-time PCR for detection of pathogenic *Leptospira* species in clinical materials. PLoS One. 2009; 4: e7093. doi: 10.1371/journal.pone.0007093 PMID: 19763264
33. Francisco AP, Bugalho M, Ramírez M, Carriço JA. Global optimal eBURST analysis of multilocus typing data using a graphic matroid approach. BMC Bioinformatics. 2009; 10: 152. doi: 10.1186/1471-2105-10-152 PMID: 19450271

34. Dehghan P, Bui T, Campbell LT, Lai Y-W, Tran-Dinh N, Zaini F, et al. Multilocus variable-number tandem-repeat analysis of clinical isolates of Aspergillus flavus from Iran reveals the first cases of Aspergillus miscelerotigenes associated with human infection. BMC Infect Dis. 2014; 14: 358. doi: 10.1186/1471-2334-14-358 PMID: 24986045

35. Blair RC, Taylor R. Biostatistics for the Health Sciences. Pearson; 2007.

36. Ridzlan FR, Bahaman AR, Khairani-Bejo S, Mutalib AR. Detection of pathogenic Leptospira from selected environment in Kelantan and Terengganu, Malaysia. Trop Biomed. 2010; 27: 632–638. PMID: 21399605

37. Viau EJ, Boehm AB. Quantitative PCR-based detection of pathogenic Leptospira in Hawaiian coastal streams. J Water Health. 2011; 9: 637–46. doi: 10.2166/wh.2011.064 PMID: 22048423

38. Desvars A, Jégo S, Chiroleu F, Bourhy P, Cardinale E, Michault A. Seasonality of human leptospirosis in Reunion Island (Indian Ocean) and its association with meteorological data. PLoS One. 2011; 6: e20377. doi: 10.1371/journal.pone.0020377 PMID: 21655257

39. Perez J, Brescia F, Becam J, Mauron C, Goarant C. Rodent abundance dynamics and leptospirosis carriage in an area of hyper-endemicity in New Caledonia. PLoS Negl Trop Dis. 2011; 5. doi:10.1371/journal.pntd.0001361

40. Zamora J, Riedemann S, Tedich N. A Serological Survey of Leptospirosis in Sheep in Chile. Rev Latinoam Microbiol. 1999; 41: 73–76. PMID: 10932754

41. Calderon A, Rodriguez V, Mattar S, Arrieta G. Leptospirosis in pigs, dogs, rodents, humans, and water in an area of the Colombian tropics. Trop Anim Health Prod. 2014; 46: 427–432. doi: 10.1007/s11250-013-0508-y PMID: 24254419

42. Sójima T, Iida K, Qin T, Tanai H, Seki M, Yoshida SI. Method to detect only live bacteria during PCR amplification. J Clin Microbiol. 2008; 46: 2305–2313. doi: 10.1128/JCM.02171-07 PMID: 18448692

43. Bailly J, Fraissinet-Tachet L, Verner M-C, Debaud J-C, Lemaire M, Wéoslovski-Louvel M, et al. Soil eukaryotic functional diversity, a metatranscriptomic approach. ISME J. 2007; 1: 632–642. doi: 10.1038/ismej.2007.68 PMID: 18043670

44. Naze F, Desvars A, Picardeau M, Bourhy P, Michault A. Use of a new high resolution melting method for genotyping pathogenic Leptospira spp. PLoS One. 2015; 10: 1–15. doi: 10.1371/journal.pone.0127430

45. Hamond C, Pinna M, Medeiros MA, Bourhy P, Lilenbaum W PM. A multilocus variable number tandem repeat analysis assay provides high discrimination for genotyping Leptospira santarosai strains. J Med Microbiol. 2015; 64: 507–12. doi: 10.1099/jmm.0.000045 PMID: 25721051

46. Henry RA, Johnson RC. Distribution of the genus Leptospira in soil and water. Appl Environ Microbiol. 1978; 35: 492–499. PMID: 637546

47. Tourier E, Amenc L, Pablo AL, Legname E, Blanchart E, Plussard C, et al. Modification of a commercial DNA extraction kit for safe and rapid recovery of DNA and RNA simultaneously from soil, without the use of harmful solvents. MethodsX. Elsevier B.V.; 2015; 2: 182–191. doi: 10.1016/j.mex.2015.03.007 PMID: 26150987

48. Francy DS, Damer RA. Procedures for developing models to predict exceedances of recreational water-quality standards at coastal beaches. Reston, VA; 2006. Available: https://internal.mdc.usgs.gov/glirwq/RefLinks/francy_2006_procedures_models_predict_exceedences_QW_coastal_beaches.pdf