

Quantitative PCR-based detection of pathogenic *Leptospira* in Hawai'ian coastal streams

Emily J. Viau and Alexandria B. Boehm

ABSTRACT

Pathogenic *Leptospira*, the causative agents of leptospirosis, are mainly associated with tropical freshwaters, but little is known about their fate in the environment. This study analyzed the distribution of pathogenic *Leptospira* genomes in 22 tropical coastal streams using quantitative polymerase chain reaction (qPCR). Statistical analyses were conducted to understand pathogenic *Leptospira* associations with water quality parameters, land use, microbial source tracking (MST) markers, and fecal indicators. Results indicated that pathogenic *Leptospira* genomes were widespread in O'ahu coastal streams during the Hawai'ian rainy season, with slightly higher concentrations in December when compared with March. *Leptospira* showed a strong positive association to turbidity, a finding consistent with studies showing increased *Leptospira* survival when aggregated to particles. Positive correlations to salinity may also indicate survival of *Leptospira* in relatively saline stream waters. A positive association to the human *Bacteroidales* fecal marker, no correlation to pig or cow *Bacteroidales* markers, and a negative association to agricultural land coverage may suggest human or other non-agricultural animal sources of *Leptospira* (e.g., rats or dogs). Future studies of *Leptospira* in the Hawai'ian environment are recommended to investigate *Leptospira* survival in saline waters, to determine both primary and secondary Hawai'ian animal hosts of *Leptospira*, and to correlate environmental exposures with epidemiological studies of leptospirosis.

Key words | emerging pathogen, leptospirosis, recreational water, zoonosis

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INTRODUCTION

Leptospirosis is a re-emerging zoonotic disease of global importance. It is acquired by humans through direct or indirect contact with the urine of animals shedding pathogenic *Leptospira* species; humans are an accidental host (Levett 2001). Pathogenic *Leptospira* are largely transmitted through tropical soil and freshwater environments, where they survive for months under warm and humid conditions (Henry & Johnson 1978). Long-term survival is associated with *Leptospira* cell aggregation, biofilm formation, and attachment to particles (Trueba *et al.* 2004; Ristow *et al.* 2008) as well as slow or stagnant freshwaters (Monahan *et al.* 2009). Leptospirosis outbreaks in the tropics typically occur during the rainy season, especially after extreme flooding events. A link has also been documented between the disease and increased urbanization in tropical locations

(Vinetz *et al.* 2005; Monahan *et al.* 2009). In general, at-risk populations include traditional wetland farming communities, workers in animal husbandry industries, adventure tourists, and urban residents exposed to contaminated freshwaters (Katz *et al.* 1991; Vinetz *et al.* 2005).

Hawai'i provides an ideal location for studying environmental risk factors of leptospirosis because it has the highest incidence of leptospirosis in the USA (Katz *et al.* 1991). Overall Hawai'ian leptospirosis rates are 2.5 infections/100,000 general population/year, 100 times higher than continental US leptospirosis rates of 0.02 infections/100,000 general population/year (Katz *et al.* 1991). Reporting of leptospirosis is likely underestimated due to poor diagnosis tools in endemic areas and the fact that disease symptoms resemble the common flu, including shivering, fever, headache, myalgia,

rashes, and nausea/vomiting (Katz *et al.* 1991; Ko *et al.* 2009). A previously conducted epidemiology study of leptospirosis in rural Kaua'i and Big Island populations of Hawai'i found that 12% of persons with these symptoms had leptospirosis – it was endemic and extremely common affecting 128 in 100,000 person/years during an outbreak (Sasaki *et al.* 1993).

Currently, there is little information in Hawai'i regarding the fate and occurrence of *Leptospira* in fresh and brackish surface waters, although signs are permanently posted by many coastal streams warning of the risk of leptospirosis. Direct studies of *Leptospira* occurrence in surface waters have not been undertaken (Vinetz *et al.* 2005). This situation may be because culture-based *Leptospira* assays currently do not differentiate between pathogenic *Leptospira* (e.g., *L. interrogans*, *L. borgpetersenii*, *L. kirschneri*, *L. noguchii*, *L. weilii*) and harmless waterborne saprophytic *Leptospira* species that occur naturally in tropical freshwater (e.g., *L. biflexa*, *L. wolbachii*) (Henry & Johnson 1978). Quantitative polymerase chain reaction (qPCR) assays that selectively target the pathogenic *Leptospira* group without detecting saprophytes offer advantages over the non-pathogen specific culture-based assays. Such an assay has been developed (Smythe *et al.* 2002) and subsequently used to study leptospirosis risk factors in urban and rural slums in Peru (Ganoza *et al.* 2006).

The purpose of the present study was to characterize *Leptospira* distributions in tropical streams near the point at which they discharge to the coastal ocean. Twenty-two O'ahu streams were tested for pathogenic *Leptospira* using a 16S rRNA qPCR assay (Smythe *et al.* 2002) during two sampling campaigns ($n = 4$ samples per stream). The study also investigated statistical associations between *Leptospira* and previously published data on water quality, land cover, microbial source tracking (MST) markers and fecal indicators.

METHODS

Sample collection

Water samples were collected at 22 O'ahu coastal streams, during two sampling campaigns in the Hawai'ian rainy season (Nov–Apr). Briefly, 20-L water samples were taken

twice (once before sunrise, and once after high noon) from each stream directly upstream of its ocean outfall in December (14–18 Dec 2009) and March (28 Mar–3 Apr 2010), as described previously (Viau *et al.* 2011a). Water was collected in DNA-/RNA-free containers, transported on ice, and processed within 6 hours of collection in accordance with standard procedures (AWWA 2005). Water samples were filtered through 0.45 µm-pore size nitrocellulose filters (HA type, Millipore, Billerica, MA, USA) and stabilized with RNALater (Ambion, Austin, TX, USA) for downstream molecular analyses (0.2–1 L per filter) as described previously (Viau *et al.* 2011b).

DNA extraction and *Leptospira* qPCR

Molecular methods were carried out in accordance with the Minimum Information for publication of Quantitative real-time PCR Experiment (MIQE) guidelines (Bustin *et al.* 2009). DNA was extracted from filters with the MoBio PowerWater[®] DNA kit (MoBio Laboratories, Carlsbad, CA, USA) with a modified protocol described previously (Viau *et al.* 2011b). A negative extraction blank was included each day that filters were extracted.

A previously validated 16S rRNA TaqMan[®] qPCR assay targeting pathogenic *Leptospira* was used to test each stream sample in triplicate (Smythe *et al.* 2002). All qPCR reactions consisted of 6 µl of extracted DNA with a 14 µl reaction buffer containing 1× FastStart universal probe master mix with ROX (Roche Diagnostics, Indianapolis, IN, USA), 0.6 mg/ml bovine serum albumin fraction V (BSA, Invitrogen), 1.5% polyvinylpyrrolidone 25,000 (PVP, Sigma-Aldrich, St. Louis, MO, USA), 0.2 µM of primers Lepto171F (5'-CCC GCGTCCGATTAG-3') and Lepto 258R (5'-TCCATTGTGGCCGR(A/G)ACAC-3'), and 0.5 µM Lepto 205 commercial hydrolysis probe (5'-FAM-CTCACCAAGGC GACGATCGGTAGC-BHQ1-3', Biosearch Technologies, Novato, CA, USA). Reactions were loaded into a 96-well microtiter plate in a StepOnePlus[™] Real-Time PCR system (Life Technologies, Carlsbad, CA, USA) and incubated at 95 °C for 10 min followed by 45 cycles of 15 s at 95 °C and 60 s at 60 °C.

To quantify unknown *Leptospira* DNA levels, a 'pooled' calibration curve was used to convert qPCR quantification cycle (C_q) to genome concentration/reaction (Sivaganesan

et al. 2010). *Leptospira* standards were created from purified *Leptospira interrogans* strain Fiocruz L1-130 genomic DNA (ATCC BAA-1198, Manassas, VA, USA). DNA concentrations were measured on a NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA concentrations of 10^0 – 10^6 genomes/reaction were made in five independent dilution series based on a *Leptospira interrogans* genome size of 4.691 Mbp and two copies of 16S rRNA gene/*Leptospira* genome. The 'pooled' calibration curve was developed by pooling the dilution series results from multiple instrument runs, which included the targeted unknown samples (Figure 1). A 'no template control' was also included in each plate run.

Leptospira genome concentrations were determined for each stream sample as follows. First, the number of genomes per reaction was determined using the pooled standard curve. This concentration was then converted to units of genomes per 100 mL water sample by taking into account the volume of template in the reaction, the total volume of DNA eluted during extraction, and the volume of water sample filtered onto the filter that was extracted. Then, the mean concentration of the triplicate reactions was calculated. Based on the pooled standard curve (Figure 1), the lowest possible quantity we could quantify per reaction was 0.5 genomes or one copy of the

16S rRNA gene. This quantity is designated as the lower limit of quantification (LOQ). If the C_q for a reaction was higher than the LOQ, it was considered positive but not within the range of quantification. For these cases, the replicate was assigned a copy number equal to the LOQ. If a sample replicate was negative (no amplification), the replicate was assigned a copy number equal to 0.5 LOQ for statistical analyses.

Sequencing of *Leptospira* qPCR amplicons

Leptospira qPCR amplicons were sequenced after qPCR to determine which pathogen groups were amplified and to ensure that qPCR was not amplifying non-pathogenic saprophytes. qPCR reactions were purified with ExoSap-IT™ (USB Corp, Cleveland, OH, USA) and submitted for standard DNA sequencing at Eurofins MWG Operon (Huntsville, AL, USA). Sequences were edited and aligned with MEGA version 5 (Tamura *et al.* 2011). MEGA5 was also used to construct neighborhood-joining phylogenetic trees with Jukes–Cantor distance corrections and bootstrapping (10,000 replicates).

Additional stream water quality parameters

Additional stream measurements were taken at the time of each *Leptospira* stream sample and used here to evaluate statistical relationships to *Leptospira* concentrations; these data were published in two previous publications that characterized major gastrointestinal and skin bacterial pathogens (Viau *et al.* 2011a) and calculated gastrointestinal illness risks for each stream in this study (Viau *et al.* 2011b). Various water quality parameters were taken at the time of sampling (salinity, temperature, dissolved oxygen, log turbidity) and nutrients (log PO_4^{3-} , log NO_3^- , log NO_2^- , square-root NH_4^+) were measured and normalized as described previously (Viau *et al.* 2011a). For land cover analyses, the normalized percentage of agriculture, urban, and forested land cover in each stream watershed was determined with ArcGIS (ESRI, Redlands, CA) using the Hawai'i Land Cover 2001 data (NOAA 2001) as previously described (Viau *et al.* 2011a). Fecal source-tracking microorganisms human *Bacteroidales* and cow/pig *Bacteroidales* were detected with quantitative PCR and PCR, respectively (Viau *et al.* 2011b). Concentrations of enterococci

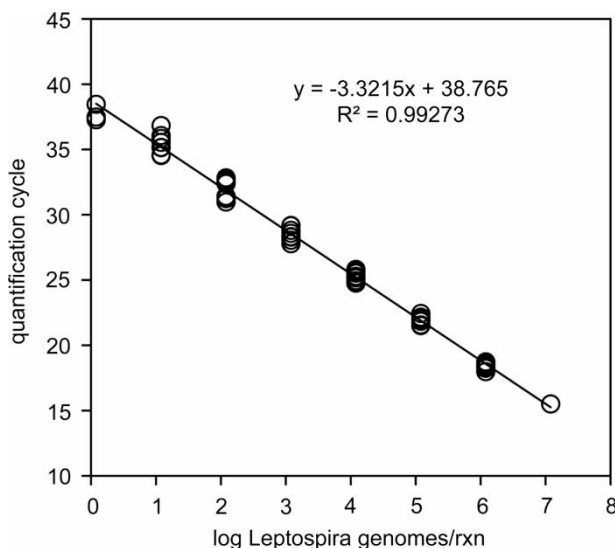


Figure 1 | *Leptospira* 16S qPCR calibration curve. Concentrations are in genomes/reaction and account for the two 16S rRNA genes in each *Leptospira* genome.

(colony forming units (CFU)/100 mL), *Escherichia coli* (CFU/100 mL), *Clostridium perfringens* (CFU/100 mL), and F+ coliphages (plaque forming units (PFU)/100 mL) were determined using membrane filtration of water samples and previously described cultivation methods (Viau *et al.* 2011a).

Statistical analyses

Statistics were calculated in Minitab version 15.0 or SPSS version 14.0. *Leptospira* concentrations were log-transformed prior to analysis and evaluated for normality with quantile-quantile plots. *Leptospira* quantities were then evaluated for differences between time of day and month using analysis of variance (ANOVA) followed by Tukey's post comparison tests. Associations between log *Leptospira* and water quality parameters, land cover, and log-transformed fecal indicator organisms were performed with binomial logistic regression for dichotomous dependent variables and linear regression models for continuous variables; these analyses utilized generalized estimating equations (GEE) to account for repeated measurements taken at each stream (Hardin & Hilbe 2003). Statistics were deemed significant if $P \leq 0.05$, although some marginally significant associations ($0.05 < P \leq 0.1$) were also discussed.

RESULTS

Leptospira qPCR

The *Leptospira* genome concentrations reported herein were obtained using a 16S rRNA qPCR assay with a 'pooled' calibration curve (Figure 1), where quantification cycle (C_q) = $-3.322x + 38.765$ ($R^2 = 99.3\%$) and $x = \log$ *Leptospira* genomes. The calibration curve had 100% efficiency based on five independent dilution series. For each environmental sample tested, a negative control was carried through from the: (1) field laboratory; (2) the nucleic acid extraction procedure; and the (3) qPCR plate. In all cases, negative controls were negative. No inhibition characterization studies were performed on the environmental samples and extraction efficiencies for the DNA extraction methods

used here are published elsewhere – these extraction efficiencies averaged $17 \pm 6\%$ for stream waters (Viau *et al.* 2011b).

Leptospira in O'ahu coastal streams

Table 1 shows *Leptospira* densities in 22 O'ahu coastal streams ($n = 4$ samples per stream). *Leptospira* was detected in 87 of 88 samples and ranged in concentrations from 0.7 to 4 log genomes/100 mL. While no differences were noted between AM and PM samples (ANOVA, $P > 0.05$), marginally significant decreases in *Leptospira* genomes were noted from December to March (ANOVA, $P = 0.053$). *Leptospira* concentrations averaged 2.0 ± 0.6 log genomes/100 mL in December samples while March samples averaged 1.8 ± 0.8 log genomes/100 mL. Overall, stream-to-stream *Leptospira* concentrations were similar, although ANOVA and post-hoc analyses did show that Punalu'u stream and the Kūhio storm drain had significantly lower values than Kapakahi stream, and that Kiikii stream had significantly lower values than Moanalua and Kalihi streams – these differences were approximately 1.5 log units in magnitude (ANOVA, $P = 0.000$).

To identify which *Leptospira* pathogen groups were amplified in O'ahu coastal streams, *post-hoc* sequencing analyses were performed on approximately one qPCR product from December and March for each stream ($n = 42$). The sequenced fragments were ~ 50 bp and included the region from the probe to the R-primer. Within this region, a single mismatch in the R-primer differentiated between the two pathogenic *Leptospira* groups while three mismatches differentiated between pathogenic *Leptospira* and saprophytic species. All of the sequenced amplicons ($n = 42$) were in the less-characterized *Leptospira* pathogen group (e.g. *L. wolffii*), not the *L. interrogans* pathogen group (Figure 1). It should be noted that four amplicons from three streams, including Punalu'u (two amplicons), Kahana (1 amplicon), and Paukauila (1 amplicon) showed amplification of both the *L. wolffi* and *L. interrogans* groups at the single mismatch site (data not shown), although the *L. wolffi* group amplification peak was greater and is represented on the phylogenetic tree (Figure 2). The last result should be interpreted with caution as it was

Table 1 | *Leptospira* genome concentrations measured in 22 O'ahu, Hawai'i coastal streams

Stream location ID	Stream name	Latitude, longitude of sample collection	<i>Leptospira</i> log genomes/100 mL				
			Associated beach	Dec AM	Dec PM	Mar AM	Mar PM
1	Ala Wai	21.288° N, 157.839° W	Ala Moana	2.29	2.87	1.73	1.16
2	Kūhiō Storm Drain	21.271° N, 157.824° W	Kūhiō	1.03	1.89	1.98	0.68
3	Kapakahi	21.270° N, 157.778° W	Wai'alaie	3.43	2.76	2.78	2.75
4	Wai'alaie Golf Course	21.273° N, 157.771° W	Kahala Hilton	2.44	2.71	2.14	2.19
5	Wailupe	21.278° N, 157.750° W	Wailupe	1.46	2.07	2.59	2.26
6	Moanalua	21.333° N, 157.894° W	Ke'ehi lagoon	1.57	2.47	3.16	3.33
7	Kalihi	21.332° N, 157.891° W	Ke'ehi lagoon	1.88	2.98	2.86	2.66
8	Mākua	21.530° N, 158.229° W	Mākua	2.15	1.93	1.98	2.76
9	Kaupuni	21.448° N, 158.193° W	Poka'i Bay	2.39	2.55	1.33	1.59
10	Mā'ili'iili	21.429° N, 158.180° W	Ma'ile	1.50	2.15	1.51	0.41
11	Mā'ile	21.409° N, 158.177° W	Ma'ile	2.31	2.53	2.14	1.02
12	Nānākuli	21.376° N, 158.140° W	Nānākuli	3.32	2.15	1.26	2.54
13	Mālaekahana	21.673° N, 157.936° W	Mālaekahana Bay	1.81	1.99	2.27	2.24
14	Waimea	21.641° N, 158.063° W	Waimea Bay	1.64	1.85	0.55	1.21
15	Anahulu	21.594° N, 158.103° W	Hale'iwa	1.17	1.51	1.08	2.09
16	Paukauila	21.580° N, 158.117° W	Kaiaka Bay	2.35	2.28	1.60	1.28
17	Kiikii	21.579° N, 158.120° W	Kaiaka Bay	1.31	1.32	1.19	1.15
18	Waimānalo	21.365° N, 157.709° W	Waimānalo	1.68	1.55	1.44	1.70
19	Ka'elepulu	21.398° N, 157.726° W	Kailua	1.80	2.69	2.39	1.02
20	Kawainui	21.426° N, 157.741° W	Kailua	1.74	1.08	0.97	1.30
21	Kahana	21.556° N, 157.869° W	Kahana Bay	2.02	2.10	2.25	BDL
22	Punalu'u	21.579° N, 157.885° W	Punalu'u	1.66	1.04	1.71	1.27

based on qualitative observation of the sequence data at the single mismatch site.

Leptospira associations with water quality parameters

Physical-chemical water quality

Relationships between stream physical-chemical parameters (Viau *et al.* 2011a) and *Leptospira* genomes were investigated by linear regression with GEE, which account for repeated observations at each sampling site. There were no significant associations between log *Leptospira* and temperature, dissolved oxygen (mg/L), or log nutrients (PO_4^{3-} , NO_2^- , NO_3^-). Log *Leptospira* showed a significant, positive association with log turbidity (GEE, $\beta = 0.861$, $P < 0.001$, Figure 3(a)). Salinity also showed a positive association to log *Leptospira* ($\beta = 0.015$, $P = 0.033$,

Figure 3(b)), as did square-rooted NH_4^+ ($\beta = 0.075$, $P = 0.010$, Figure 3(c)).

Land cover and microbial source tracking markers

To characterize potential sources of *Leptospira* in each stream, pairwise associations were tested with previously reported watershed percentages of urban, agricultural, and forested land cover (Viau *et al.* 2011a), as well as MST markers for human, pig, and ruminant *Bacteroidales* (Viau *et al.* 2011b). There was a negative correlation between log *Leptospira* and agricultural land cover (GEE, $\beta = -1.133$, $P = 0.005$, Figure 3(d)). No significant relationships emerged for urban or forested land cover percentages. For MST markers, positive associations were observed between log *Leptospira* and log human *Bacteroidales* concentrations (GEE, $\beta = 0.182$, $P = 0.004$,

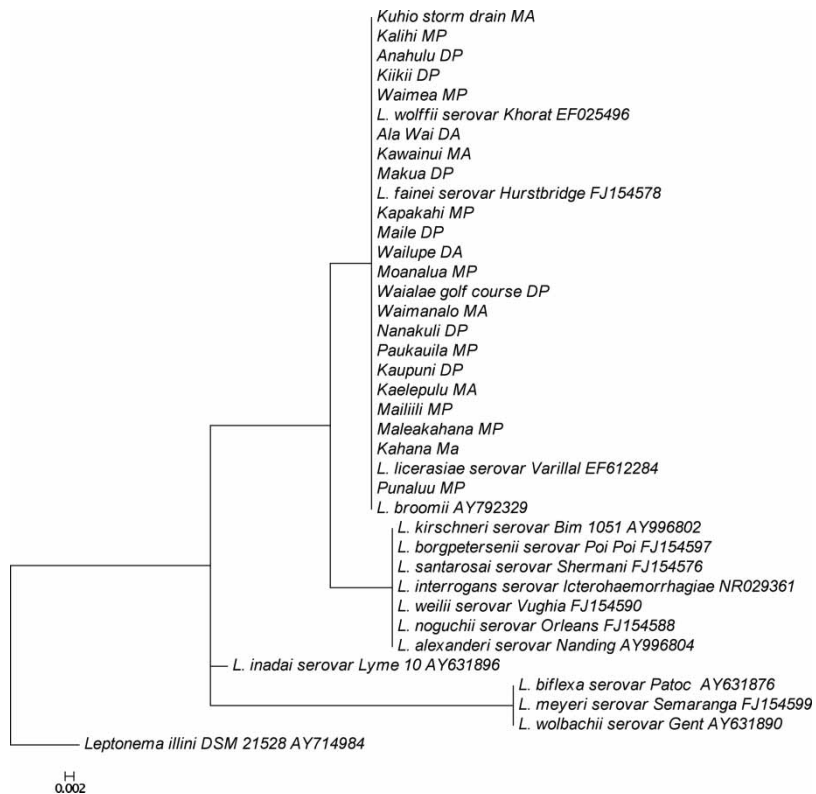


Figure 2 | Phylogenetic tree of 16S rRNA *Leptospira* amplicons from O'ahu Hawai'i coastal streams – stream IDs have stream name followed by D or M for December/March and A or P for AM/PM sample identification. Only one of two amplicons for each stream is shown in the tree for brevity. The tree is constructed using the neighbor-joining method with Jukes-Cantor distance matrix corrections rooted, with *Leptonema illini* as the out-group.

Figure 3(e)), but no associations were noted with log *Leptospira* and presence/absence data for pig and ruminant *Bacteroidales* (ANOVA, $P > 0.05$).

Fecal indicators

The ability of fecal indicator organisms to predict *Leptospira* in O'ahu streams was examined with pairwise associations to previously reported log concentrations of culturable enterococci, *E. coli*, *C. perfringens*, F+ coliphage (Viau *et al.* 2011a). Positive associations were found between log *Leptospira* and log *C. perfringens* (GEE, $\beta = 0.353$, $P < 0.001$, Figure 3(f)).

DISCUSSION

Researchers point out that climate change will have unknown consequences for transmission of leptospirosis;

the disease has high fatality rates, symptoms mimicking other bioterrorism infections, and potential for epidemics (Vinetz *et al.* 2005). They further suggest environmental measurements and quantitative analyses to better understand factors that relate environmental transmission with illness in places where leptospirosis is endemic. The present study is the first to report pathogenic *Leptospira* densities in coastal streams across the island of O'ahu, Hawai'i. Through evaluations of how this organism relates to water quality, land coverage, fecal sources, and fecal indicator parameters, the study then attempts to better understand environmental reservoirs of leptospirosis in tropical waters.

Leptospira are widespread in O'ahu coastal streams

Leptospira concentrations in streams ranged from 0.7–4 log genomes/100 mL (Table 1). These values were lower than *Leptospira* concentrations noted in Peruvian rural and

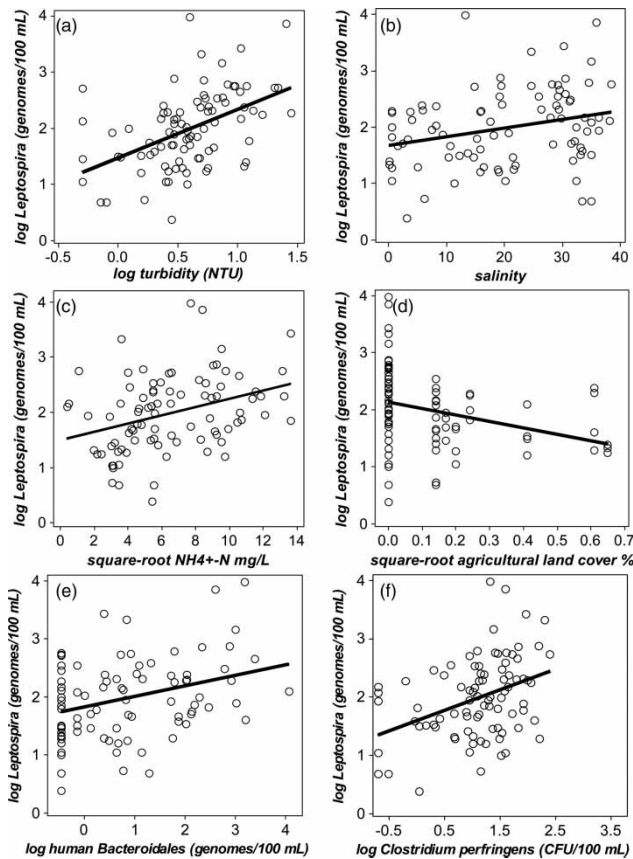


Figure 3 | Scatter plots of significant relationships ($P \leq 0.05$) between log *Leptospira* densities (genomes/100 mL) and (a) \log_{10} turbidity (nephelometric turbidity units (NTU)), (b) salinity, (c) square-root $\text{NH}_4^+\text{-N}$ mg/L, (d) square-root agricultural land cover %, (e) log human *Bacteroidales* microbial source-tracking marker (genomes/100 mL), and (f) log *Clostridium perfringens* fecal indicator (colony forming units (CFU)/100 mL).

urban surface waters (from 4–5 log/100 mL) using the same qPCR assay (Ganoza *et al.* 2006). Sequencing of qPCR amplicons indicated that the majority of *Leptospira* measured using the assay were from the less-well documented *Leptospira* pathogen group (Figure 2), which includes *L. fainei*, *L. wolffii*, *L. licerasiae*, and *L. broomii*. Species from this group are only recently being identified in human infections around the world (Chappel *et al.* 1998, Matthias *et al.* 2008, Zakeri *et al.* 2010), but these species have not yet been associated with human leptospirosis cases in Hawai'i. This situation may be because these species are not included in common serological tests – Matthias *et al.* (2008) found that when *L. licerasiae* was included in serology testing for patients with acute febrile illness that leptospirosis incidence was far higher than previously

expected in Peru. Future studies are recommended that perform more intense DNA sequence typing efforts to determine the species and serovars of *Leptospira* present in streams and how that relates to human leptospirosis incidence in Hawai'i. This information will be important for future quantitative microbial risk assessments (QMRA), as recently developed dose–response curves for different *Leptospira interrogans* serovars show that infectious dose varies significantly even amongst a single species (Watanabe & Yamamoto 2010).

Positive *Leptospira* associations emerged with O'ahu stream parameters

Leptospira was strongly associated to turbidity (Figure 3(a)). This finding is congruent with the *Leptospira* ability to persist in the environment and its hosts by aggregating with other *Leptospira* and particles (Trueba *et al.* 2004) as well as forming biofilms (Ristow *et al.* 2008). Leptospirosis outbreaks worldwide are attributed to flooding in urban areas when large flushes of turbidity occur (Levett 2001; Monahan *et al.* 2009), including a 2006 outbreak on O'ahu associated with a stream flooding the University of Hawai'i (Gaynor *et al.* 2007). Given the association found here with turbidity and previous leptospirosis outbreaks during flooding, further studies are recommended that focus on *Leptospira* associations to turbidity in Hawai'iian streams throughout the rainy season. These studies would be especially useful during extreme storm events when Brown Water Advisories are issued indicating that turbid stormwaters will discharge from streams to coastal areas (HDOH 2009).

In this study, we noted a positive association between *Leptospira* genomes and salinity (Figure 3(b)). This finding is interesting because only a few studies have attempted to understand *Leptospira* survival in natural surface waters with varying salinities. In a 1934 study, *Leptospira* were found to survive less than 24 hours in marine waters (~16,000 mg/L salts) and less than a week in river and lake waters varying from 70–6,530 mg/L salts (Schuffner 1934). Another study showed that pathogenic *Leptospira* survived 18–20 hours in marine waters spiked with 2,000 mg/L salts (Chang *et al.* 1948). Faine (1959) showed increased growth of *Leptospira* in 900 mg/L NaCl when incubated at 30 °C, temperatures comparable with those encountered in

Hawai'ian surface waters (Viau *et al.* 2011a). Incubation at higher temperatures of 37 °C was shown to be inhibitory. In the most recent *Leptospira* salt survival study, the authors reported that *Leptospira* incubated in NaCl solutions of 850 mg/L survived for 2 weeks while *Leptospira* in distilled water survived up to 110 days at 20 °C (Trueba *et al.* 2004). To the authors' knowledge, there are no human leptospirosis outbreaks associated with marine water exposures. The evidence here and in previous studies suggests that survival in salinities characteristic of brackish and marine waters is possible, even if for short time periods. Recent leptospirosis outbreaks have occurred in marine mammals, but researchers point out that does not necessarily mean that *Leptospira* are found in marine waters (Monahan *et al.* 2009). Instead, it is suggested that leptospirosis outbreaks in California sea lions are from exposures to other animals on the beach and contaminated feces (e.g., dogs) (Norman *et al.* 2008). Future field and microcosm studies are warranted to understand *Leptospira* survival in surface waters of various salinity ranges to shed light on these uncertainties and to quantify persistence.

While *Leptospira* were not associated with urban land cover, we did observe a positive correlation to human *Bacteroidales*, a marker of human fecal contamination (Figure 3(e)). There were no *Leptospira* associations to pig or cow MST marker or forested land, and a negative correlation was noted for agricultural land cover (Figure 3(d)). These findings may suggest that humans or other non-agricultural animals (e.g., dogs, rats) (Brown & Prescott 2008) could contribute *Leptospira* to the streams. The *Leptospira* species detected here have been identified in human infections associated with urban animal carriers, including dogs that had *L. wolffii* (Zakeri *et al.* 2010) and rats that had *L. licersiae* (Matthias *et al.* 2008). In a 1946 study of leptospirosis in urban areas of Honolulu, O'ahu, seropositivity was 3.78% in humans, 8.2% in rodents, and 25% in mongooses (Alicata & Breaks 1943). This finding matched studies of leptospirosis carriers at O'ahu streams – Higa & Fujinaka (1976) found that 24 and 23% of rodents and mongooses tested, respectively, were positive for *Leptospira* in urban areas and streams of O'ahu (Higa & Fujinaka 1976). Interestingly, streams tested here were also included in that study. The researchers found that 16% of rats at Kalihi stream were infected with leptospirosis and that infected

mongooses were most frequently captured at Kailua and Waimānalo streams. Both human and animal sources of *Leptospira* in the streams tested here should be further investigated for leptospirosis incidence.

Research vistas for *Leptospira* monitoring in the tropics

In 1995, the USA relaxed a 20-year-old leptospirosis reporting requirement due to a lack of zoonotic disease control systems and low incidence rates (Vinetz *et al.* 2005). To date, leptospirosis incidence remains 100 times higher in Hawai'i relative to the rest of the USA (Katz *et al.* 2002). Based on the study results here, leptospirosis monitoring in Hawai'i and other tropical locations of the USA would be beneficial for better understanding leptospirosis occurrence and associated environmental factors that could be controlled. Monitoring could be performed either through epidemiological studies and/or QMRA of *Leptospira* in the environment.

The study results here suggest that stream waters could be an important exposure pathway to investigate in future QMRA and epidemiological studies of leptospirosis transmission. It should be noted that Hawai'ian leptospirosis cases from 1978–1998 were predominantly male and interestingly, occupational disease decreased over time while recreational water-based exposures increased (Katz *et al.* 2002). Human exposure groups to investigate by QMRA could include recreational users entering streams as well as high risk groups such as taro farm workers, adventure tourists (Katz *et al.* 2002; Vinetz *et al.* 2005; Monahan *et al.* 2009), and low socio-economic urbanites living in poor sanitary conditions (e.g. homeless populations) (Reis *et al.* 2008). QMRA risks could be estimated by linking stream concentrations and exposure pathways with dose–response data for the primary infection route of *Leptospira*, the subcutaneous skin wound infection, using recently developed animal models (Watanabe & Yamamoto 2010). These risks could be compared to leptospirosis epidemiology studies that pursue an understanding of seropositivity, including newly identified *Leptospira* pathogens, among various Hawai'ian human and animal populations. Studies could then better understand where control systems may be different in urban and rural tropical conditions. A Hawai'ian epidemiology study of leptospirosis in rural

settings showed that environmental factors contributing to the disease included the use of rainwater systems, presence of wounds, and contact with animal tissues and urine, but did not include swimming in streams or having pets (Sasaki *et al.* 1993). Unfortunately, this study did not link epidemiological results to environmental measurements. Furthermore, it is unknown whether the environmental factors important to a study in a rural Hawai'ian setting would be the same as those on O'ahu, the most urbanized Hawai'ian Island.

CONCLUSIONS

This study showed that *Leptospira* genomes were widespread in O'ahu coastal streams, with higher concentrations in December when compared to March. Molecular-based typing of *Leptospira* in streams is recommended to understand species prevalence and further temporal studies are recommended to understand annual variations. The positive association found here with *Leptospira* and turbidity is congruent with other studies reporting increased *Leptospira* survival with particle aggregation. Positive *Leptospira* relationships with salinity suggest persistence of the organism in brackish to saline waters. Further work should investigate die-off rates as a function of salinity. Finally, the positive association to human *Bacteroidales* fecal markers and negative association with agricultural land cover may indicate that human or non-agricultural animal sources contribute to *Leptospira* contaminated runoff to the streams. Further MST using, for example, dog-, mongoose- or rat-specific markers could pinpoint specific Hawai'ian animal hosts of *Leptospira*. Future studies of *Leptospira* in the Hawai'ian environment are recommended to investigate *Leptospira* survival in saline waters, to understand both primary and secondary Hawai'ian animal hosts of *Leptospira*, and to correlate environmental exposures with human epidemiological studies of leptospirosis.

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