Phylogenetic relationships and diversity of bat-associated *Leptospira* and the histopathological evaluation of these infections in bats from Grenada, West Indies

Amanda I. Bevans, Daniel M. Fitzpatrick, Diana M. Stone, Brian P. Butler, Maia P. Smith, Sonia Cheetham

1 Department of Pathobiology, School of Veterinary Medicine, St. George’s University, Grenada, West Indies, 2 Department of Public Health and Preventive Medicine, School of Medicine, St. George’s University, Grenada, West Indies

These authors contributed equally to this work.

*scheetha@sgu.edu*

**Abstract**

Bats can harbor zoonotic pathogens, but their status as reservoir hosts for *Leptospira* bacteria is unclear. During 2015–2017, kidneys from 47 of 173 bats captured in Grenada, West Indies, tested PCR-positive for *Leptospira* bacteria. Sequence analysis of the *Leptospira rpoB* gene from 31 of the positive samples showed 87–91% similarity to known *Leptospira* species. Pairwise and phylogenetic analysis of sequences indicate that bats from Grenada harbor as many as eight undescribed *Leptospira* genotypes that are most similar to known pathogenic *Leptospira*, including known zoonotic serovars. Warthin-Starry staining revealed leptospiral organisms colonizing the renal tubules in 70% of the PCR-positive bats examined. Mild inflammatory lesions in liver and kidney observed in some bats were not significantly correlated with renal *Leptospira* PCR-positivity. Our findings suggest that Grenada bats are asymptotically infected with novel and diverse *Leptospira* genotypes phylogenetically related to known pathogenic strains, supporting the hypothesis that bats may be reservoirs for zoonotic *Leptospira*.

**Author summary**

Leptospirosis is a worldwide disease of humans and animals caused by pathogenic strains of *Leptospira* spp. These bacteria are transmitted in urine from infected and usually asymptomatic reservoir hosts. *Leptospira* have been detected in several species of animals in Grenada, and human cases of leptospirosis are reported annually. However, little is known about the source of infection, and while rats are the most commonly recognized reservoir host of *Leptospira*, there is growing evidence that bats may also be asymptomatic carriers in the wild. To examine this, we captured different species of bats in Grenada, West Indies, from 2015 to 2017, tested kidney tissue for *Leptospira* spp. bacteria by PCR, and performed histological examination to see whether there is any relationship between...
Leptospira infection and associated lesions. Our results suggest that two species of bats tested—Artibeus spp. and Glossophaga longirostris—are asymptotically infected with several types of Leptospira bacteria that may be new to science and that are phylogenetically related to strains that are known to cause disease.

Introduction

Leptospirosis, caused by spirochete bacteria, is the most frequently reported zoonosis worldwide with an estimated 1.03 million cases each year. Leptospirosis is a leading cause of morbidity and mortality, with tropical areas accounting for the majority of all human cases and deaths [1]. There are at least 35 recognized species and 250 serovars of Leptospira, and depending on the convention used, these species have been clustered into either three [2,3] or four [4] major monophyletic groups that correspond with their pathogenicity and niche. To date, at least seventeen species have been described in Group I, previously known as the “pathogenic” group. Several of these species cause severe disease and/or death in some animals and humans, while chronically infected reservoir hosts can be asymptomatic or have few symptoms of infection [5]. Humans can also become infected with some of the Leptospira species in Group II, also known as the “intermediately pathogenic” group, though infection is typically subclinical to mild [6,7]. Non-pathogenic, saprophytic Leptospira comprise either one or two clades depending on the convention used [3,4].

Pathogenic Leptospira colonize the kidneys of the infected host and are excreted in the urine. Infections occur through direct contact with the urine of infected animals or when this urine contaminates the environment. The bacteria enter the body through cuts or abrasions on the skin, or through the mucous membranes of the mouth, nose, and eyes [reviewed in [8]].

Bats are reservoir hosts for several important zoonotic pathogens including viruses and bacteria [9,10]. Although serological evidence of bat exposure to Leptospira spp. has been reported from several parts of the world [11–13], and molecular detection of infection by PCR has also been documented [14–20], the role of bats in the epidemiology of zoonotic Leptospira is not well-understood. Leptospira infection has been detected in over 50 bat species belonging to eight of the nine investigated bat families, representing bats from many geographical regions, including both the tropics and subtropics [21]. Importantly, it has also been documented that bats can carry Leptospira in the renal tubules and shed the spirochetes in their urine for at least five months [22]. Taken together, the global abundance of bats, their spatial association with humans and both domestic and wild animals, and evidence that bats can shed Leptospira in their urine suggest that bats may be epidemiologically significant for Leptospira transmission.

Grenada is a tropical island nation in the southern part of the West Indies. Between 2008 and 2014, Grenada reported from two to 22 cases of human leptospirosis annually [23]. This likely reflects a small fraction of actual infections, as most cases of leptospirosis in humans, regardless of geographic area, are not confirmed or reported [24,25]. Humans and animals in Grenada have tested seropositive for at least 17 serotypes of Leptospira. Leptospira-seropositive animals in Grenada include bats, cattle, chickens, goats, mongooses, pigs, sheep and toads [11,26–28]. Research performed in the 1970s in Grenada found that 13/61 (21%) Anoura spp. bats and 4/52 (8%) of Glossophaga spp. bats were Leptospira-seropositive. Reacting serovars included L. borgpetersenii serovar Tarassovi; L. interrogans serovars Autumnalis, Canicola, Hebdomadis, and Icterohaemorrhagiae; L. noguchii serovars Bataviae and Panama; and L. santarosai serovar Shermani [11]. However, Leptospira could not be cultured from the extracted kidney tissue of any seropositive bats. Thus, in Grenada, there are no reports to date
documenting active infection in bats with any *Leptospira* species whether by PCR or microscopic observation.

PCR-based tools have become essential in studying *Leptospira* biology, phylogeny, and pathogenesis, and are key to diagnosing active infection. Several genes have been used singly or in tandem to type *Leptospira* (*flaB, gyrB, rpoB, 16S rRNA rrs, secY, lipL32*) [29]. In particular, the *rpoB* gene is an ideal target for phylogenetic analyses: it allows for discrimination among *Leptospira* species better than most other gene sequences (e.g., *rrs, lipL32*), and all recognized *Leptospira* species to date have partial or whole *Leptospira rpoB* sequence entries in GenBank due to its use in many studies [30,31]. Previous research using serology to identify and name infecting *Leptospira* organisms (serovars) had major limitations. Although serology can reflect the epidemiology of circulating serovars, it does not identify species conclusively [29]. Furthermore, there is poor correlation between *Leptospira* serological (i.e., serovar) and genomic classification (i.e., species and strain), which complicates comparing current *Leptospira* genomic data with past and present serological data [29]. For instance, strains identified as belonging to serovar Bataviae have vast genetic heterogeneity, belonging to *L. borgpetersenii*, and *L. kirschneri, L. interrogans, L. noguchii, or L. santarosai*, according to genetic analyses [8]. *Leptospira* serological tests also have a history of inadequate sensitivity and specificity across a range of hosts, including instances where seronegative carriers had infectious leptospires in their urine and/or kidneys [32].

Accordingly, the purposes of this study are to determine whether bats in Grenada are actively infected with potentially pathogenic and possibly zoonotic species of *Leptospira* by PCR, determine genetic diversity of the leptospiral strains carried by bats through gene sequence analysis, and evaluate whether infection is associated with any gross or microscopic pathology. These data will provide insight into the diversity of bat-associated zoonotic leptospires in a tropical setting and establish the basis for determining the role of bats in transmitting *Leptospira* to humans.

**Materials and methods**

**Ethics statement**

All protocols for trapping, handling and euthanizing bats were approved by the Institutional Animal Care and Use Committee (IACUC-14008-R) at St. George’s University, School of Veterinary Medicine and with consent from the Grenada Ministry of Agriculture, Forestry, Wildlife and Fisheries, Grenada, West Indies. Bats were trapped using mist nets and hand nets. Both of these methods were approved as humane by the Animal Care and Use Committee of the American Society of Mammalogists [33].

**Bat trapping**

During 2015–2017, 173 clinically healthy bats representing both sexes and three abundant bat genera—several species of *Artibeus, Glossophaga longirostris* (GL), and *Molossus molossus* (MM)—were identified by morphology [34]. Due to the changing taxonomic status of bats in the *Artibeus jamaicensis* complex of bats [35], all potential *Artibeus jamaicensis, Artibeus planirostris, and Artibeus schwartzi* bats in this study are collectively identified as *Artibeus* spp. and abbreviated as (AS) (for *Artibeus* spp.), while *Artibeus literatus* (AL) bats in this study are treated as a separate taxon from AS bats.

Several mist nets (avinet.com) were used per site to ensure adequate monitoring and prompt removal of the bats. Mist nets were not left unattended at any time. Additionally, mist nets were not used in areas of high winds, as wind may contribute to stress and entanglement.
of bats. Captured bats were removed from the nets immediately, and all mist nets were removed immediately after the trapping period had ended.

Processing of bats

All processing was conducted with appropriate personal protective equipment (latex gloves, surgical masks and eyewear). Rabies virus has not been detected in bats in Grenada using viral detection by RT-PCR or direct immunofluorescence, but neutralizing antibodies to rabies virus have been observed previously [36]. Thus, all personnel handling the bats had completed the rabies vaccination series and demonstrated protective titers. Live bats were transported to the necropsy laboratory at St. George’s University, School of Veterinary Medicine (SGU SVM), Grenada, West Indies, in individual opaque cloth bags to prevent post-capture cross-contamination. Bats were euthanized in the necropsy lab using isoflurane followed by thoracotomy and cardiac exsanguination while under anesthesia. Tissue samples were stored in RNAlater at -20˚C and formalin.

PCR and sequencing

In 2017, DNA was extracted from 30 mg of kidney after tissue disruption in a bead-beater (Mini Beadbeater Biospec Products, Bartlesville, OK, USA) using QIAamp DNA Mini Kit spin columns (QIAGEN, Hilden, Germany) according to the manufacturer’s directions. Generic zoonotic Leptospira-specific primers for a ~600 bp region of the rpoB gene (beta-subunit of RNA polymerase) were used [31]. After electrophoresis in a 2% agarose gel, bands of the expected size were extracted and sent for direct Sanger sequencing. Resulting sequences were analyzed and edited using Chromas 2.6.4 and compared to known rpoB gene sequences in NIH-NCBI GenBank using the Basic Local Alignment Search Tool (BLAST). In cases of overlapping sequence data, cloning and transformation were performed on amplicons from those samples. DNA was extracted from several colonies, and PCR was conducted using primers T7 (5’-TAATACGACTCACTAT AGG-3’) and SP6 (5’-GATTTAGGTGACACTATAG-3’) in the plasmid which flank the inserted amplicon. Briefly, PCR was performed using 40 cycles with 55˚C annealing and one-minute extension. Amplicons from this PCR were then electrophoresed, extracted from gels, sent to Sanger sequencing, edited, and compared to GenBank entries as described above. Bats were considered PCR-positive for Leptospira infection after sequencing confirmation.

Phylogenetic analysis

Phylogenetic trees were constructed with bat-derived Leptospira sequences and rpoB sequences from all Leptospira species retrieved from GenBank (S1 Table) using MEGA X. Specifically, maximum-likelihood phylogenetic analysis was conducted with the Kimura’s 2-parameter nucleotide substitution model, gamma-distributed substitution rates, and an allowance for invariant sites (K2+G+I) and with 1000 bootstrapped replicates. Nodes with bootstrap confidence below 70% were condensed in the phylogenetic tree presented. Pairwise sequence alignments were obtained using MEGA X software (S2 Table).

Histopathology

Bat liver and kidney tissues were fixed by immersion in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, stained with Hematoxylin & Eosin (HE) and Warthin-Starry (WS) silver stain (kidneys only) using standard histological techniques, and examined
by light microscopy by a board certified veterinary pathologist. Staining with WS is an established method for the detection of Leptospira spp. within tissue sections [37,38].

### Statistical analysis

Relative risk was calculated to critically examine the association between bats that tested positive for Leptospira and the presence of histopathological lesions in the kidney. Sensitivity and specificity values were calculated to analyze the utility of WS staining technique relative to PCR testing.

Prevalence of Leptospira was compared between the two positive bat taxa and across years. Comparisons between species were done using chi-squared tests, both year by year except where counts were less than five for a given year, and overall. Comparisons across years were done using Spearman’s rank correlation to establish increasing or decreasing trend.

### Results

#### Characteristics of Leptospira positive and negative bats

The 173 bats evaluated in this study represent three genera: 51 Glossophaga longirostris (GL), 35 Molossus molossus (MM), 2 Artibeus literatus (AL), and 85 non-Artibeus literatus species of Artibeus (AS) (Table 1). Bats from all six parishes of Grenada were among the Leptospira PCR-positive (Fig 1). All Leptospira PCR-positive bats were either AS or GL (Table 1). Prevalence of Leptospira varied significantly among bat species. No MM or AL tested positive. Positive AS and GL bats were trapped in all three years of trapping (Table 1). Across all three years, significantly more GL bats were Leptospira-positive (33/51; 65%) than were AS bats (14/85; 16%) (p<0.0001). Furthermore, Leptospira infection rates in GL were significantly higher than those of AS in 2015, the only year in which both species were captured in abundance (GL: 31/43 [72%]; AS: 7/52 [13%]; p<0.0001.) Prevalence of Leptospira in GL bats decreased significantly (p = 0.01) from 72% in 2015 to 25% in 2016 and 2017; however, only 8 GL bats were caught in 2016–2017. Bats collected in both the dry and rainy seasons were found to be positive, but the prevalence of diseases did not differ between seasons (S1 Fig). Five of the 173 bats were pregnant, and three of them were PCR-positive for Leptospira (2 AS and 1 GL).

#### Table 1. Species of bats testing PCR-positive for Leptospira by year trapped.

| Bat Species       | 2015 Positive/ Test (%) | 2016 Positive/ Test (%) | 2017 Positive/ Test (%) | Total Positive/ Test (%) | P for difference by year |
|-------------------|-------------------------|-------------------------|-------------------------|--------------------------|--------------------------|
| Artibeus literatus| 0/2 (0%)                | -                       | -                       | 0/2 (0%)                 | -                        |
| Artibeus spp.     | 7/52 (13%)              | 2/17 (12%)              | 5/16 (31%)              | 14/85 (16%)              | 0.20                     |
| Glossophaga longirostris | 31/43 (72%) | 1/4 (25%)               | 1/4 (25%)               | 33/51 (65%)              | 0.01                     |
| Molossus molossus | 0/26 (0%)               | -                       | 0/9 (0%)                | 0/35 (0%)                | -                        |
| Total             | 38/123 (31%)            | 3/21 (14%)              | 6/29 (21%)              | 47/173 (27%)             | 0.11                     |

P for difference by species, AS vs. GL

*Artibeus literatus* was not collected in 2016/2017.

Molossus molossus was not collected in 2016.

**P for difference in Leptospira infection rates for each species, and then for total overall years, from Spearman’s rank correlation (P for increasing or decreasing trend over time).

***P for difference in Leptospira infection rates by species for each year, and then for total over all species, from chi-squared test (P that at least one species differs from at least one other).

- Chi-squared test was not used for comparing Artibeus literatus or Molossus molossus differences by year because bats caught have expected values below 5.

https://doi.org/10.1371/journal.pntd.0007940.t001
PCR-based identification and phylogenetic analysis of *Leptospira* spp.

Consensus sequences of over 450 bp of the *rpoB* gene were obtained from forward and reverse reactions from 31 of 47 *Leptospira*-positive samples. These Grenada bat derived *Leptospira* sequences were 87–91% identical to known *Leptospira rpoB* gene sequences in GenBank (Table 2). Isolate GBL-AS-x6 matches best with *Leptospira* sp. strain ADMAS 2667, a pathogen isolated from an infected dog in India for which the serovar or species was not determined. The best matches for the other 30 isolates are known pathogenic *Leptospira* species, including species known to cause disease in humans (*L. noguchii*, *L. santarosai*) [5].

Pairwise comparisons of all the Grenada bat derived *Leptospira* sequences ranged from 79–100% (S2 Table). The bat-derived *Leptospira* identified herein likely comprise several *Leptospira* species; this is corroborated by comparing overlapping regions of the Grenada bat-derived *Leptospira rpoB* gene sequences with known *Leptospira* sequences in GenBank using MEGA X (Fig 2).

All Grenadian bat *Leptospira* isolates fall within the pathogenic branch and form eight discrete clusters that are distinct from all previously identified *Leptospira* serovars. We designated these clusters Clades A–H (Fig 2) based upon their similarity to each other: specifically, for...
Table 2. Best matches to bat derived *Leptospira* rpoB gene sequences found in GenBank.

| Bat Species           | Bat Leptospira ID number | Species and serovar of closest match in GenBank | Accession number of closest match | Percent identity | Accession number of *Leptospira* isolate |
|-----------------------|--------------------------|-------------------------------------------------|-----------------------------------|------------------|----------------------------------------|
| *Artibeus* spp.       | GBL-AS-x3                | *Leptospira kmetyi* strain LS 001/16             | CP033614.1                        | 497/548 (91%)    | MG981094                               |
| GBL-AS-x4             | GBL-AS-x9                |                                                 | 486/548 (89%)                     | MG981095         |
| GBL-AS-x30            | GBL-AS-x7                | *Leptospira mayottensis* strain MDI222          | CP030144.1                        | 485/548 (89%)    | MG981109                               |
| GBL-AS-x7             | GBL-AS-x8                |                                                 | 493/557 (89%)                     | MG981097         |
| GBL-AS-x2             | GBL-AS-x8                | *Leptospira noguchii* strain Cascata            | EU349502.1                        | 483/548 (88%)    | MG981093                               |
| GBL-AS-x29            | GBL-AS-x7                | *Leptospira santarosai* strain U160             | CP027843.1                        | 476/548 (87%)    | MG981108                               |
| GBL-AS-x6             |                          | *Leptospira sp.* ADMAS 2667                     | JN388649.1                        | 498/548 (91%)    | MG981096                               |
| *Glossophaga* longirostris | GBL-GL-x1'          | *Leptospira kmetyi* strain LS 001/16             | CP033614.1                        | 427/479 (89%)    | MG981092                               |
| GBL-GL-x15            | GBL-GL-x18               |                                                 | 492/548 (90%)                     | MG981101         |
| GBL-GL-x23            | GBL-GL-x24               |                                                 | 492/548 (90%)                     | MG981103         |
| GBL-GL-x23            | GBL-GL-x24               |                                                 | 492/548 (90%)                     | MG981106         |
| GBL-GL-x33            | GBL-GL-x36               |                                                 | 492/548 (90%)                     | MG981107         |
| GBL-GL-x34            | GBL-GL-x36               |                                                 | 497/548 (91%)                     | MG981111         |
| GBL-GL-x36            | GBL-GL-x37               |                                                 | 459/517 (89%)                     | MG981112         |
| GBL-GL-x37            | GBL-GL-x38               |                                                 | 484/548 (88%)                     | MG981113         |
| GBL-GL-x38            | GBL-GL-x47               |                                                 | 492/548 (90%)                     | MG981114         |
| GBL-GL-x47            | GBL-GL-x48               |                                                 | 460/517 (89%)                     | MG981115         |
| GBL-GL-x47            | GBL-GL-x48               |                                                 | 492/548 (90%)                     | MG981117         |
| GBL-GL-x48            | GBL-GL-x48               |                                                 | 492/548 (90%)                     | MG981118         |
| GBL-GL-x51            | GBL-GL-x51               |                                                 | 492/548 (90%)                     | MG981119         |
| GBL-GL-x73'           | GBL-GL-x75               |                                                 | 381/426 (89%)                     | MG981120         |
| GBL-GL-x76            | GBL-GL-x75               |                                                 | 485/548 (89%)                     | MG981121         |
| GBL-GL-x76            |                          |                                                 | 492/548 (90%)                     | MG981122         |

(Continued)
each bat-derived isolate, it is (A) 96% or more identical to all other isolates within its clade and (B) 92% or less identical to any other isolates outside of its clade used in the phylogenetic analysis.

Histopathological examination of bat tissue

In general, all of the examined bats appeared to be healthy based on postmortem examination, e.g., adequate body condition, mild to moderate parasite burdens, no lesions that suggest significant overt disease within the examined organ systems.

Liver and kidney sections from 124 bats in this study were examined for histopathological lesions that may be associated with leptospirosis. Mild or moderate chronic non-suppurative interstitial nephritis was observed in 29/124 (23%) of the examined bats. Inflammatory lesions accompanied by renal tubular degeneration and necrosis, indicative of mild tubulointerstitial nephritis, was observed in 6/124 (5%) of the examined bats. Relative risk (RR) was calculated to examine the association between PCR-positive bats and the presence of histopathological lesions in the kidney. The probability of a wild captured bat to have interstitial nephritis and be PCR-positive for *Leptospira* is 24% with a RR of 31%. Thus, PCR-positivity for *Leptospira* is not strongly associated with the presence of interstitial nephritis.

Warthin-Starry (WS) silver stain was applied to the kidney sections of 44 bats that tested PCR-positive for *Leptospira* with 31/44 (70%) testing WS-positive (Fig 3). Conversely, WS stain was applied to the kidneys of five bats that tested PCR-negative for *Leptospira* which resulted in 0/5 (0%) WS positives. Thus, the WS staining technique performed with 70% sensitivity and 100% specificity when compared with conventional PCR methods used in this study.

Discussion

The overall prevalence of *Leptospira* PCR-positive bats in this study (27%) was considerably higher than the 12% seroprevalence previously reported in Grenada bats. Although four taxa of bats were sampled in our study, only two were *Leptospira* PCR-positive: *Artibeus* spp. (AS) and *Glossophaga longirostris* (GL). Our findings in these two bat species are in agreement with

| Bat Species | Bat *Leptospira* ID number | Species and serovar of closest match in GenBank | Accession number of closest match | Percent identity | Accession number of *Leptospira* isolate |
|-------------|---------------------------|-----------------------------------------------|----------------------------------|----------------|-----------------------------------------|
| GBL-GL-x14  |  | *Leptospira kneyi* strain LS 001/16 *Leptospira mayottensis* strain MDI272 *Leptospira mayottensis* 200901116 | CP033614.1 CP030147.1 CP024871.1 | 482/548 (88%) | MG981100 |
| GBL-GL-x16  |  | *Leptospira mayottensis* strain MDI222 | CP030144.1 | 485/548 (89%) | MG981102 |
| GBL-GL-x20  |  |  | CP030149.1 | 485/548 (89%) | MG981105 |
| GBL-GL-x45  |  | *Leptospira noguchii* strain Cascata | EU349502.1 | 467/522 (89%) | MG981116 |
| GBL-GL-x19  |  |  | EU349519.1 | 491/548 (90%) | MG981104 |
| GBL-GL-x32  |  |  | CP030144.1 | 483/548 (88%) | MG981110 |

GenBank accession numbers and percent identity between the bat derived *Leptospira rpoB* gene sequence and its best match in GenBank, as determined by BLAST, are provided. For all sequences, the percent identity was calculated using over 99% of the Grenada bat *Leptospira*-derived sequences.

Samples with asterisks (bats GBL-GL-x1 and GBL-GL-x73) were not used in subsequent phylogenetic analyses because of degenerate nucleotides in the sequence data.

https://doi.org/10.1371/journal.pntd.0007940.t002
Detection of *Leptospira* in bats in Grenada

Reports detecting of *Leptospira* antigen or antibody reported in various countries including Brazil [39], Peru [15,18], and Grenada and Trinidad and Tobago [11]. We found 65% of GL bats were *Leptospira* PCR-positive, which is approximately eight-fold higher than the seroprevalence rate previously reported for this species of bats from Grenada (8%) [11]. In our study, only two *Artibeus littatus* (AL) bats were evaluated, both trapped in the same year and both *Leptospira* PCR-negative. While the negative findings may reflect the small number of AL bats tested, another study that sampled 22 bats of this species also failed to detect any *Leptospira* positive animals [15]. However, *Leptospira* PCR-positive AL bats have been reported in at least one study from Mexico (2/8 positive; 25%) [19]. The other bat species that tested *Leptospira* PCR-negative in our study was *Molossus molossus*. This species represented 26 of 173 bats trapped and included bats from two of the three trapping years. *Leptospira* PCR-positive *M. molossus* have previously been reported at low rates in Brazil (4/19 bats positive; 21%) and Trinidad (5/20; 25%) [11,39]. Accordingly, we may have missed finding *Leptospira*-positive MM bats simply due to the low number of captured MM and possibly low infection rates overall in this project.

Fig 2. Phylogenetic tree representing the relationships among *Leptospira* identified in this study (70% bootstrap cutoff). Maximum-likelihood phylogenetic trees were derived from partial rpoB gene sequences in GenBank for known species and by sequencing of partial rpoB gene from Grenadian bat-derived *Leptospira* spp. sequences. Bootstrap values are listed at each node. Nodes with bootstrap confidence values below 70% support are condensed. Clusters representing potentially novel *Leptospira* taxa are designated Clades A-H in the tree. Within each group, the range for the percent identities of each pairwise comparison is shown next to the group name in parentheses. Labels: GBL—Grenada Bat *Leptospira*; AS—*Artibeus* spp. complex bat; GL—*Glossophaga longirostris*; x##—specimen number. https://doi.org/10.1371/journal.pntd.0007940.g002
species. Also, of five pregnant bats collected, three tested *Leptospira* positive. Though this is a small sample size, this may reflect increased *Leptospira* exposure and/or susceptibility of pregnant bats, as has been reported elsewhere [40].

Importantly, *Leptospira* infection rates differed greatly between AS and GL bats (16% vs. 65%). Bat *Leptospira* infection rates based on molecular techniques or culture from other studies also show marked variation from 0% to over 80% depending on bat species and location [14–17,20,22,39–41]. Some speculate that the primary bat feeding habits (fruit, nectar, and insect) represented in most studies also explain some of the infection rate differences, but no statistically significant data have been published to confirm this. Grenada is an entirely semi-rural country, with no large densely populated urban centers. All the bats trapped in our study

**Table 3. Grenada bat *Leptospira* clade by year and bat species.**

| Grenada Bat Leptospira Clade | 2015 | 2016 | 2017 |
|-----------------------------|------|------|------|
| Clade A                     | 10 GL|      |      |
| Clade B                     | 1 GL | 1 GL |      |
| Clade C                     | 1 GL | 1 AS |      |
| Clade D                     | 1 AS, 3 GL |      | 3 AS |
| Clade E                     | 1 AS |      |      |
| Clade F                     |      | 1 AS |      |
| Clade G                     | 1 AS | 1 GL |      |
| Clade H                     | 1 AS, 4 GL | 1 AS | 1 AS |

AS–Artibeus spp. complex; GL–Glossophaga longirostris

https://doi.org/10.1371/journal.pntd.0007940.t003
lived in close proximity to human homes, roosting in covered porches of lived-in houses, abandoned structures, and in nearby orchards, forests, and caves. Thus, location, time of year and age of bat, together with Leptospira detection methods could have impacted observed infection rates in this study.

The closest match in GenBank for each of the Leptospira-positive samples belonged to Group I (i.e., pathogenic Leptospira). Based on recommendations by La Scola et al. (2006), Leptospira sequences with rpoB identity lower than 92% represent different species, and 97–100% identity between partial rpoB gene sequences suggests that isolates are conspecific [31]. All of the bat derived Leptospira partial rpoB sequences in this study were less than 92% identical to known Leptospira rpoB gene sequences in GenBank (Table 2) (range of best match identity: 87–91%). Thus, Leptospira genotypes described herein are presumably different species from all Leptospira with rpoB gene sequences catalogued in GenBank. Furthermore, our analysis suggests that the Grenada bats tested are infected with as many as eight separate undescribed Leptospira taxa, in which each genotype within a clade is 97–100% identical to all other members of its clade and <92% identical to other Leptospira in this study and in GenBank (Fig 2, S1 Table). Pairwise sequence comparison of overlapping regions, presented in S1 Table as percent identity, between each pair of Grenada bat derived Leptospira rpoB gene sequences demonstrated a 79–100% identity. Furthermore, for our phylogenetic analysis, we included at least one partial rpoB sequence from each of the pathogenic/Group I Leptospira species [4,42] and none are conspecific to the bat Leptospira isolates described herein by percent identity or phylogenetic analysis. Thus, based on the La Scola et al. (2006) recommendations, Grenadian bats harbor several distinct genotypes of Leptospira. Other studies have similarly demonstrated that even within a limited geographical range, bats are often infected with a diverse range of Leptospira [15,19,20] including potentially novel strains [16,43]. However, while the DNA sequences from PCR and phylogenetic analysis merely suggest that bats in Grenada have genetically diverse genotypes, of which several are possibly novel strains, DNA sequences alone are not considered sufficient to classify our unique bat-derived Leptospira genotypes reported herein as novel strains or species. DNA-DNA hybridization and more thorough genomic sequencing are necessary before we can deem our bat-derived Leptospira as novel taxa [4,29].

Clade H, the only genotype detected all three years of the study, was only detected in one bat in two of the three years surveyed (Table 3). Clade A, the predominant genotype in GL in 2015, was found in five of six parishes that year, but was not detected in 2016 or 2017. Annual changes in the dominant Leptospira serovars or strains have been observed in other studies of Leptospira-positive animals in the Caribbean islands [44] and elsewhere [45,46] but the prevalence changes observed over time in our study may also reflect the relatively small number of positive bats analyzed.

In addition to bats, other animals in Grenada (cane toads, cattle, mongoose, and sheep) also have reacting antibodies to L. noguchii serovar Bataviae and L. borgpetersenii serovar Tarassovi [11,26,27], while Grenadian cane toads, cattle, chickens, mongooses, and pigs have reacting antibodies to L. santarosai serovar Shermani [11,26,27]. Several bats in this study produced Leptospira sequences that matched with L. noguchii and L. santarosai, albeit at low identities (below 90%). However, serology identifies serotypes and does not correlate with species in typing Leptospira [8,29], and hence we cannot compare our current genomic data with past serological data with any certainty. Thus, it is necessary to confirm Leptospira strains by PCR in these and other animals going forward in order to clarify Leptospira ecology and reservoir hosts in Grenada.

Interstitial nephritis and tubulointerstitial nephritis are renal lesions that are generally associated with leptospirosis. However, these findings, especially when mild, may also be nonspecific lesions that are commonly observed in many wildlife species and may have no direct correlation with leptospirosis. We conclude that bats are not likely to be adversely affected by
Leptospirosis infection, based on the lack of association between PCR-positivity for Leptospira and the presence of renal inflammatory lesions (RR = 31%). These results may also reflect that renal lesions of Leptospira-infected bats are transient and may not be detectable throughout the duration of chronic infection, similar to what has been described in rats, where subclinical chronic infection is marked by mild inflammatory renal lesions and no overt disease [47].

This is the first study to demonstrate the colonization of renal tubules by leptospires in wild captured bats using light microscopy with WS staining technique. Colonization of the renal tubules is a prerequisite for transmission of leptospires in the urine, and our findings provide further evidence that bats may indeed be an important reservoir host for zoonotic leptospirosis with potential for spread to humans via urine transmission. Future studies in experimental bat models are needed to determine the efficiency of urine shedding and transmission of Leptospira from bats to other individuals which will provide a better understanding of the zoonotic potential and public health risks posed by bats.

This study is the first report of molecular detection of Leptospira in bats in Grenada. Importantly, this is also the first report of phylogenetic analysis of Leptospira detected in any species of animal or humans from Grenada and a starting point for future comparative studies to improve our understanding of the epidemiology of Leptospira. Results show that Grenada bats are infected with novel and diverse Leptospira genotypes phylogenetically related to known pathogenic, including zoonotic, taxa. Further, our results suggest that infected bats are asymptomatic with concomitant renal Leptospira colonization that can be shed in urine. Together, these findings reinforce bats’ roles as potential reservoirs of Leptospira.

Supporting information
S1 Table. GenBank accession numbers for the rpoB gene sequences of Leptospira spp. and Turneriella spp. bacteria used in phylogenetic analysis of Grenada Bat Leptospira relatedness. (XLSX)

S2 Table. Pairwise alignments of Grenada bat Leptospira. Thirty-one Leptospira spp. isolates with at least 450 bp sequences were compared by pairwise sequence alignment using MEGA X. Values are presented as percent identity. (XLSX)

S3 Table. A Fisher’s exact test was performed to determine the significance of the number of Leptospira PCR positive bat tissues in the rainy season (July to December) compared to the dry season (January to June). There is not a significant difference between the number of positive Artibeus spp. and positive Glossophaga longirostris infected with Leptospira in the rainy season compared to the dry season (p = 0.1258). The analysis for Molossus molossus were excluded because of 0 values. Labels; AS–Artibeus spp. complex; GL–Glossophaga longirostris; MM–Molossus molossus. (XLSX)

S1 Fig. Number of Leptospira PCR positive bat tissues in the rainy season compared to the dry season. (TIFF)

Acknowledgments
Grenada Ministry of Agriculture, Fisheries and Wildlife for granting permission to trap bats; Dan Bradway from Washington State University for providing positive Leptospira controls;
Plant-Microbe Genomics Facility (The Ohio State University, Columbus, OH, USA) and the Molecular Cloning Laboratory (San Francisco, CA, USA) for their sequencing services; Vanessa Matthew-Belmar and Marcy Kanuka for their assistance in sample acquisition and processing.

Author Contributions
Conceptualization: Amanda I. Bevans, Daniel M. Fitzpatrick, Diana M. Stone, Sonia Cheetham.
Data curation: Daniel M. Fitzpatrick, Maia P. Smith, Sonia Cheetham.
Formal analysis: Maia P. Smith, Sonia Cheetham.
Funding acquisition: Sonia Cheetham.
Investigation: Amanda I. Bevans, Daniel M. Fitzpatrick, Brian P. Butler, Sonia Cheetham.
Methodology: Sonia Cheetham.
Supervision: Diana M. Stone, Sonia Cheetham.
Writing – original draft: Amanda I. Bevans, Daniel M. Fitzpatrick, Diana M. Stone, Brian P. Butler, Maia P. Smith, Sonia Cheetham.
Writing – review & editing: Daniel M. Fitzpatrick, Diana M. Stone, Sonia Cheetham.

References
1. Costa F, Hagan J, 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; 9:e0003898. https://doi.org/10.1371/journal.pntd.0003898. PMID: 26379143
2. Petrakovsky J, Bianchi A, Fisun H, Najera-Aguilar P, Pereira MM. Animal leptospirosis in Latin America and the Caribbean countries: Reported outbreaks and literature review (2002–2014). Int J Environ Res Public Health 2014; 11:10770–89. https://doi.org/10.3390/ijerph111010770. PMID: 25325360
3. Fouts DE, Matthias MA, Adhikarlal H, Adler B, Amorim-Santos L, Berg DE, et al. What makes a bacterial species pathogenic?: Comparative genomic analysis of the genus Leptospira. PLoS Negl Trop Dis 2016; 10:e0004403. https://doi.org/10.1371/journal.pntd.0004403. PMID: 26890609
4. Vincent AT, Schiettekatte O, Id CG, Neela VK, Bernet E, Thibeaux R, et al. Revisiting the taxonomy and evolution of pathogenicity of the genus Leptospira through the prism of genomics. PLoS Negl Trop Dis 2019; 13. https://doi.org/10.1371/journal.pntd.0007270.
5. Evangelista K V., Coburn J. Leptospira as an emerging pathogen: A review of its biology, pathogenesis and host immune responses. Future Microbiol 2010: 5. https://doi.org/10.2217/fmb.10.102.
6. Levett PN, Morey RE, Galloway RL, Steigerwalt AG. Leptospira broomii sp. nov., isolated from humans with leptospirosis. Int J Syst Evol Microbiol 2006; 56:671–3. https://doi.org/10.1099/ijs.0.63783-0. PMID: 16514048
7. Petersen AM, Boye K, Blom J, Schlichting P, Krogfelt KA. First isolation of Leptospira fainei serovar Hurstbridge from two human patients with Weil’s syndrome. J Med Microbiol 2001; 50:96–100. https://doi.org/10.1099/0090-3558-50-1-96. PMID: 11192512
8. Levett PN. Leptospirosis. Clin Microbiol Rev 2001; 14:296–326. https://doi.org/10.1128/CMR.14.2.296. PMID: 11292640
9. Moratelli R, Calisher CH. Bats and zoonotic viruses: Can we confidently link bats with emerging deadly viruses? Mem Inst Oswaldo Cruz 2015; 110:1–22. https://doi.org/10.1590/0074-02760150048.
10. Mühldorfer K. Bats and bacterial pathogens: a review. Zoonoses Public Health 2013; 60:93–103. https://doi.org/10.1111/j.1863-2378.2012.01536.x. PMID: 22862791
11. Everard COR, Fraser-Chanpong GM, Bhagwandin LJ, Race MW, James AC. Leptospirales in wildlife from Trinidad and Grenada. J Wildl Dis 1983; 19:192–9. https://doi.org/10.7589/0090-3558-19.3.192. PMID: 6644917
12. Smythe LD, Field HE, Barnett LJ, Smith CS, Dohnt MF, Symonds ML, et al. Leptospiral antibodies in flying foxes in Australia. J Wildl Dis 2002; 38:182–6. https://doi.org/10.7589/0090-3558-38.1.182. PMID: 11838213

13. Desvars A, Naze F, Vourc'h G, Cardina le E, Picardeau M, Michault A, et al. Similarities in Leptospira serogroup and species distribution in animals and humans in the Indian ocean island of Mayotte. Am J Trop Med Hyg 2012; 87:134–40. https://doi.org/10.4269/ajtmh.2012.12-0102. PMID: 22764304

14. Tulsiani SM, Graham GC, Dohnt MF, Burns M, Craig SB. Maximizing the chances of detecting pathogenic leptospires in mammals: the evaluation of field samples and a multi-sample-per-mammal, multi-test approach. Ann Trop Med Parasitol 2011; 105:145–62. https://doi.org/10.1179/136485911X12899838683205. PMID: 21396251

15. Matthias MA, Diaz MM, Campos KJ, Calderon M, Willig MR, Pacheco V, et al. Diversity of bat-associated Leptospira in the Peruvian Amazon inferred by Bayesian phylogenetic analysis of 16s ribosomal DNA sequences. Am J Trop Med Hyg 2005; 73:964–74. https://doi.org/10.4269/ajtmh.2005.73.964. PMID: 16282313

16. Ogawa H, Koizumi N, Ohnuma A, Mutemwa A, Hang’ombe BM, Mweene AS, et al. Molecular epidemiology of pathogenic Leptospira spp. in the straw-colored fruit bat (Eidolon helvum) migrating to Zambia from the Democratic Republic of Congo. Infect Genet Evol 2015; 32:143–7. https://doi.org/10.1016/j.meegid.2015.03.013. PMID: 25791930

17. Cox TE, Smythe LD, Leung LK-P. Flying foxes as carriers of pathogenic Leptospira species. J Wildl Dis 2005; 41:753–7. https://doi.org/10.7589/0090-3558-41.4.753. PMID: 16456164

18. Bunnell JE, Hice CL, Watts DM, Montruel V, Tesh RB, Vinetz JM. Detection of pathogenic Leptospira spp. Infections among mammals captured in the Peruvian Amazon basin region. Am J Trop Med Hyg 2000; 63:255–8. https://doi.org/10.4269/ajtmh.2000.63.255. PMID: 11421373

19. Ballados-Gonzalez GG, Sanchez-Montes S, Romero-Salas D, Colunga Salas P, Gutierrez-Molina R, Leon-Paniagua L, et al. Detection of pathogenic Leptospira species associated with phyllostomid bats (Mammalia: Chiroptera) from Veracruz, Mexico. Transbound Emerg Dis 2018; 65:773–81. https://doi.org/10.1111/tbed.12802. PMID: 29318786

20. Gomez Y, Dietrich M, Wieseke N, Ramaisindrazana B, Lagadec E, Goodman SM, et al. Malagasy bats shelter a considerable genetic diversity of pathogenic Leptospira suggesting notable host-specificity patterns. FEMS Microbiol Ecol 2016; 92:fiw037. https://doi.org/10.1093/femsec/fiw037.

21. Dietrich M, Muhldorfer K, Tortosa P, Markotter W. Leptospira and bats: Story of an emerging friendship. PLoS Pathog 2015; 11. https://doi.org/10.1371/journal.ppat.1005176. PMID: 4564183

22. Fennestad KL, Borg-Petersen C. Leptospirosis in Danish wild mammals. J Wildl Dis 1972; 8:343–51. https://doi.org/10.7589/0090-3558-8.4.343. PMID: 4564183

23. Grenada. Health Statistics for Communicable Diseases: Leptospirosis. http://health.gov.gd/images/PDF2/Communicable-Diseases/Leptospirosis%20cases%202008-2014. Minisit Heal n.d.

24. Peters A, Vokaty A, Portch R, Gebre Y. Leptospirosis in the Caribbean: a literature review. Rev Panam Salud Publica 2017; 41:e166. https://doi.org/10.26633/RPSP.2017.166. PMID: 31384278

25. Abela-Ridder B, Sikkema R, Hartskeerl RA. Estimating the burden of human leptospirosis. Int J Antimicrob Agents 2010; 36:S5–7. https://doi.org/10.1016/j.ijantimicag.2010.06.012. PMID: 20688484

26. Everard COR, Fraser-Chanpong GM, James AC, Butcher LV. Serological studies on leptospirosis in livestock and chickens from Grenada and Trinidad. Trans R Soc Trop Med Hyg 1985; 79:859–64. https://doi.org/10.1016/0033-9203(85)90138-5. PMID: 3832496

27. Leveit PN, Walton D, Waterman LD, Whittington CU, Mathison GE, Edwards COR. Surveillance of Leptospirosis carriage by feral rats in Barbados. West Indian Med J 1998; 47:15–7.

28. Keenan J, Sharma R, Dicker R, Rayner J, Stone D. Seroprevalence of Leptospirosis in Rattus Norvegicus in Grenada, West Indies. West Indian Med J 2009; 58:114–7. PMID: 21866595

29. Guernier V, Allan KJ, Goarant C. Advances and challenges in barcoding pathogenic and environmental Leptospira. Parasitology 2018; 145:595–607. https://doi.org/10.1017/S0031182017001147. PMID: 28716157

30. Cerqueira GM, McBride AJA, Hartskeerl RA, Ahmed N, Dellagiostin OA, Eslabão MR, et al. Bioinformatics describes novel loci for high resolution discrimination of Leptospira isolates. PLoS One 2010; 15: e15335. https://doi.org/10.1371/journal.pone.0015335.

31. La Scola B, Bui LTM, Baranton G, Khamas A, Raoult D. Partial rpoB gene sequencing for identification of Leptospira species. FEMS Microbiol Lett 2006; 263:142–7. https://doi.org/10.1111/j.1574-6968.2006.00377.x. PMID: 16978348

32. Fang F, Collins-Emerson JM, Cullum A, Heuer C, Wilson PR, Benschop J. Shedding and seroprevalence of pathogenic leptospira spp. in sheep and cattle at a New Zealand abattoir. Zoonoses Public Health 2015; 62:258–68. https://doi.org/10.1111/zph.12146. PMID: 25043226
33. Sikes R, Gannon W, Mammalogists and the animal care and use committee of the AS of. Guideline of the American Society of Mammalogists for the use of wild mammals in research. J Mammal 2011; 92:235–53. https://doi.org/10.1644/10-MAMM-F-355.1.

34. Gomes GA, Reid F, Tuttle MD. Bats of Trinidad and Tobago: A field guide and natural history. 2015.

35. Larsen PA, Bozeman MC, Pedersen SC. Phylogenetics and Phyleogeography of the Artibeus jamacensis Complex Based on Cytochrome - b DNA Sequences. J Mammal 2007; 88:71.2–7.

36. Ziegler U, Cheetham S, Santana SE, Leiser-miller L, Matthew-belmar V. Natural exposure of bats in Grenada to rabies virus. Infect Ecol Epidemiol 2017; 7. https://doi.org/10.1080/20008686.2017.1329393.

37. Fornazari F, Costa da Silva R, Richini-Pereira VB, Beserra HEO, Luvizotto MCR, Langoni H. Comparison of conventional PCR, quantitative PCR, bacteriological culture and the Warthin Starry technique to detect Leptospira spp. in kidney and liver samples from naturally infected sheep from Brazil. J Microbiol Methods 2012; 90:321–6. https://doi.org/10.1016/j.mimet.2012.06.005. PMID: 22713608

38. Agudelo-Flórez P, Murillo VE, Londoño A, Rodas JD. Histopathological kidney alterations in rats naturally infected with Leptospira. Biomédica 2013; 33:82–8. https://doi.org/10.7705/biomedica.v33i0.686. PMID: 24652252

39. Mayer FQ, Dos Reis EM, Bezerra AVA, Cerva C, Cibulski SP, et al. Pathogenic Leptospira spp. in bats: Molecular investigation in Southern Brazil. Comp Immunol Microbiol Infect Dis 2017; 52:14–8. https://doi.org/10.1016/j.cimid.2017.05.003. PMID: 28673456

40. Dietrich M, Wilkinson DA, Benlali A, Lagadec E, Ramasindrazana B, Dellagi K, et al. Leptospira and paramyxovirus infection dynamics in a bat maternity enlightens pathogen maintenance in wildlife. Environ Microbiol 2015; 17:4280–9. https://doi.org/10.1111/1462-2920.12766. PMID: 25580582

41. Mateus J, Gómez N, Herrera-Sepúlveda MT, Hidalgo M, Pérez J, Cuervo C. Original Article Bats are a potential reservoir of pathogenic Leptospira species in Colombia. J Infect Dev Ctries 2019; 13:278–83. https://doi.org/10.3855/jidc.10642.

42. Thibeaux R, Girault D, Bierque E, Soupe-Gilbert ME, Rettinger A, Douyère A, et al. Biodiversity of environmental Leptospira: Improving identification and revisiting the diagnosis. Front Microbiol 2018; 9. https://doi.org/10.3389/fmicb.2018.00816.

43. Han HJ, Wen HL, Liu JW, Qin XR, Zhao M, Wang LJ, et al. Pathogenic leptospirosis species in insectivorous bats, China, 2015. Emerg Infect Dis 2018; 24:1123–6. https://doi.org/10.3201/eid2406.171585. PMID: 2974833

44. Pratt N, Rajeev S. Leptospira seroprevalence in animals in the Caribbean region: A systematic review. Acta Trop 2018; 182:34–42. https://doi.org/10.1016/j.actatropica.2018.02.011. PMID: 29457993

45. Vasylyeva N, Andreychyn M, Kravchuk Y, Chervinska O, Isyk I. Changes in leptospirosis etiology in animals and humans. Ann Agric Environ Med 2017; 24:671–5. https://doi.org/10.26444/aaem/78031. PMID: 29284246

46. Jori F, Galvez H, Mendoza P, Cespedes M, Mayor P. Monitoring of leptospirosis seroprevalence in a colony of captive collared peccaries (Tayassu tajacu) from the Peruvian Amazon. Res Vet Sci 2009; 86:383–7. https://doi.org/10.1016/j.rvsc.2008.09.009. PMID: 1900627

47. Tucunduva de Faria M, Athanazio DA, Gonçalves Ramos EA, Silva EF, Reis MG, Ko AI. Morphological alterations in the kidney of rats with natural and experimental Leptospira infection. J Comp Pathol 2007; 137:231–8. https://doi.org/10.1016/j.jcpa.2007.08.001. PMID: 17996544