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Domestic dogs and cats as potential reservoirs of pathogenic Leptospira in American Samoa

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Abstract

Background

Leptospirosis is a globally distributed zoonotic disease that disproportionately affects resource-limited populations in tropical regions. Annually, it is estimated that there are about 1.03 million cases with nearly 60,000 deaths and 2.90 million DALYs. Although rodents are considered the primary reservoirs of pathogenic *Leptospira* spp., many mammalian species, including companion animals like cats and dogs, can harbor and shed the pathogen, contributing to environmental contamination and human spillover. In settings such as American Samoa, where domestic and stray cats and dogs live in close proximity to households, farmland, and water sources, their roles as potential contributors to environmental contamination and human leptospirosis cases are of significant concern. Previous sero-epidemiological studies have revealed high human exposure, yet specific animal reservoirs that are maintaining the local transmission remain poorly understood. To address this gap, the present study employed molecular detection and genetic characterization to generate foundational knowledge on the presence of *Leptospira* strains in domestic cats and dogs of American Samoa.

Results

A total of 114 urine samples (83 from dogs and 31 from cats) in American Samoa were screened for pathogenic *Leptospira* using *lipL32* qPCR. Overall, 111 (97.36%) of the animals tested positive, with 98.79% positivity in dogs and 93.55% positivity in cats, with no significant sex-associated differences. The *secY* gene was amplified from 72 of 111 *lipL32* qPCR-positive samples using PCR and sequenced. Allele typing revealed that three *secY* genotypes (1, 5, and 16), all clustering within the pathogenic P1 clade, primarily to *Leptospira interrogans*, are prevalent. Spatial variation in allele distribution was observed across the Tutuila Island, indicating multiple circulating genotypes among companion animals.

Conclusions

Dogs and cats in American Samoa are actively shedding pathogenic *Leptospira*, indicating they play a role in maintaining environmental contamination and local transmission. These findings underscore the need for an expansion of molecular-level surveillance for pathogenic *Leptospira* spp. in animal reservoirs that can contribute to human spillover. Due to the high rate of carriage identified in cats and dogs, this work also supports a targeted vaccination strategy as part of a comprehensive One Health approach to control leptospirosis in American Samoa.

Introduction

Leptospirosis is a widespread zoonotic infectious disease affecting both tropical and temperate regions worldwide (1, 2). Despite its global prevalence, the greatest burdens are disproportionately conveyed on

resource-limited populations, particularly those in tropical regions, accounting for approximately 73% of total cases. Each year, almost 1.03 million cases of leptospirosis are reported globally, resulting in nearly 58,900 deaths (3, 4). This contributes to an estimated 2.90 million Disability Adjusted Life Years (DALYs), placing leptospirosis among the highest-burden zoonoses worldwide (5). Clinical manifestations of the disease range from a mild, often subclinical illness characterized by non-specific flu-like symptoms that is observed in 90–95% of cases, to a severe, life-threatening form marked by jaundice, acute renal failure, hepatic damage, pulmonary distress and hemorrhage, also known as Weil's disease which occurs in about 5–10% of the cases (6–9). Mild subclinical cases often go undiagnosed or are misdiagnosed as they resemble other tropical illnesses such as dengue or malaria, leading to substantial underreporting of the true global impact of the disease (10–14).

Leptospirosis is caused by a pathogenic spirochete in the genus *Leptospira*. Leptospires are slender, tightly coiled spiral bacteria, typically 6–20 μm long with 0.1–0.15 μm diameter, equipped with a pair of periplasmic endoflagella that drive their rapid cork-screw motility (9). *Leptospira* species are broadly classified into two major clades: the pathogenic (P) clade and the saprophytic (S) clade based on their pathogenicity. The P clade is comprised of the highly pathogenic P1 subclade, consisting of 17 species, and the intermediate pathogenic P2 subclade, consisting of 21 species. Similarly, the S clade, comprising free-living non-pathogenic (saprophytic) species, is subdivided into the S1 subclade with 21 species and the S2 subclade with 5 species (15–17). Among the recognized pathogenic species, *Leptospira interrogans* and *Leptospira kirschneri* of the P1 subclade account for the majority of human infection (18). In clinical and epidemiological studies, pathogenic *Leptospira* species are classified into different serogroups using the Microscopic Agglutination Test (MAT). Thirty serogroups with more than 250 pathogenic serovars have been described thus far (19–21).

Rodents, particularly species within the *Rattus* genus, are considered to be the primary reservoir of pathogenic *Leptospira* (8, 22). Nevertheless, almost every mammalian species is susceptible to the infection and can act as reservoirs for the bacteria, persistently maintaining the bacteria within the proximal renal tubule of the kidneys and shedding the leptospires in urine (23–26). Humans are incidental hosts within the leptospiral transmission cycle (27), with infection mediated by a sophisticated interplay of ecological, environmental, occupational, and socio-cultural determinants (28, 29). Humans are infected when the bacteria enter through the mucous membrane or damaged skin. Although direct inoculation is possible via contact with urine or tissue of animal reservoirs, the majority of human cases are from an indirect mode of exposure. Such indirect transmission typically involves interaction with water bodies or moist soil that have been contaminated by the urine of infected animals (30–33).

Wild and domestic mammals, including companion species, can be reservoirs of pathogenic *Leptospira* and may introduce the bacteria into the environment or transmit them directly to humans (34). Cats and dogs are the two most widely kept domestic companion animals, and they have coexisted with humans for millennia (35). Domestic dogs (*Canis lupus familiaris*) and domestic cats (*Felis silvestris catus*) are recognized reservoirs of pathogenic *Leptospira*, and their roles in human leptospirosis have been widely studied (18, 36–45). Up to 48% of cats and up to 75% of dogs have been reported to carry anti-*Leptospira*

antibodies, underscoring their potential roles as reservoirs and disseminators of pathogenic *Leptospira* (46, 47). In cats, *Leptospira* infection is thought to occur primarily through predation on infected rats, and their clinical signs are often subtle or absent, and when present, they typically involve renal abnormalities. In contrast, dogs are commonly exposed to the pathogen through activities such as drinking or swimming in contaminated water bodies or ingestion of contaminated foods (40). Dogs frequently develop an acute clinical syndrome accompanied by hepatic or renal injury, pulmonary hemorrhage, uveitis, polyuria and polydipsia. (36, 40, 47).

In the United States alone, an estimated 63 million households have at least one dog, and 42 million have at least one cat (48). In settings such as American Samoa, where domestic and stray cats and dogs live in close proximity to households and frequently interact with human environments, including farmland and water sources, their roles as potential contributors to environmental contamination and human exposure to pathogenic *Leptospira* are of significant concern. The combination of abundant companion and stray animals and frequent encounters between them, limited animal control infrastructures, and regular animal-human interactions creates conditions in which cats and dogs may play a meaningful role in sustaining *Leptospira* transmission cycles and facilitating spillover to people. Since the first laboratory-confirmed human leptospirosis case reported in 2003, subsequent sero-epidemiological investigations have demonstrated a substantial burden of infection in American Samoa. A 2004 survey by the U.S. Centers for Disease Control and Prevention (CDC) reported leptospiral antibodies in 17% of the studied human population, and a 2010 study by Lau et. al. estimated a seroprevalence of 15.5% in a broader American Samoan population (49, 50). American Samoa has a humid climate, tropical terrain, and frequent flooding supportive of environmental persistence of pathogenic *Leptospira* from peri-domestic or domestic cats and dogs, rodents, and piggeries. Human behavioral and socioeconomic factors such as contact with animals, bathing in streams, agricultural practices, and low income, have been identified as major risk factors for human leptospiral infection in American Samoa (51). Despite these findings, there are no additional published studies assessing the prevalence of pathogenic *Leptospira* in humans, animals, and environmental sources. The specific animal reservoirs contributing to local transmission and maintenance of the disease remain uncharacterized. In this context, the present study aimed to generate foundational knowledge on the presence of *Leptospira* strains in domestic cats and dogs of American Samoa using molecular detection and characterization techniques. Herein, we demonstrated that the majority of cats and dogs are infected with pathogenic *Leptospira*, suggesting that they might serve as important reservoirs sustaining transmission and contributing to human leptospirosis in American Samoa.

Results

Detection of pathogenic Leptospira in cats and dogs urine samples.

Infected mammals can release pathogenic *Leptospira* species via urine, thereby contributing to either direct or indirect transmission to humans. As companion animals that live in proximity to people, cats and dogs may serve as important sources of environmental contamination and potential human

exposure. To investigate the presence of pathogenic *Leptospira* in the canine and feline population, urine samples collected during routine veterinary visits were screened for the presence of the *lipL32* gene, a gene unique to pathogenic *Leptospira* species (52). A total of 114 urine samples were collected from cats (n = 31, 27.19%) and dogs (n = 83, 72.81%) across different villages of Tutuila Island, American Samoa (Table 1). One hundred and eleven (97.36%) urine samples were positive for *lipL32* across all samples tested (Table 1, Fig. 1A), with bacterial load (genomic equivalence of *lipL32* gene) per ml of urine sample between 9 and $3.2 \cdot 10^7$ (GE/ml) (Table S1).

Table 1
Sample and screening results summary

Demography	Dogs				Cats			
	***lipL32*+* / Total**	**% positive**	***secY* (n†)**	***secY* Allele ID (n)**	***lipL32*+ / Total**	**% positive**	***secY* (n)**	***secY* Allele ID (n)**
Total Animals (N = 114)	82 / 83	98.80%	53	—	19 / 31	61.30%	19	—
EASTERN DISTRICT								
Afono	0 / 0	—	0	—	1/1	100%	0	—
Alao	3 / 3	100%	3	5 (2), 1 (1)	0 / 0	—	0	—
Alega	0 / 0	—	0	—	1/1	100%	1	5 (1)
Aua	1 / 1	100%	0	—	0 / 0	—	0	—
Faga□lu	2/2	100%	2	5 (1), 1 (1)	2/2	100%	2	5 (2)
Fagasa	3/3	100%	2	5 (2)	0 / 0	—	0	—
Fagatogo	2/2	100%	1	5 (1)	0 / 0	—	0	—
Masefau	1/1	100%	1	1 (1)	0 / 0	—	0	—
Nu□uuli	7/7	100%	6	5 (6)	2/2	100%	1	5 (1)
Pago Pago	1/1	100%	1	5 (1)	3/3	100%	2	5 (1), 1 (1)
Tula	2/2	100%	2	1 (1), 16 (1)	0 / 0	—	0	—
WESTERN DISTRICT								
Faleniu	3/3	100%	2	5 (1), 1 (1)	2/2	100%	1	5 (1)
Futiga	2/2	100%	0	—	3/3	100%	3	5 (3)
□lli□ili	4/4	100%	3	5 (3)	1/1	100%	1	5 (1)
Leone	11/11	100%	6	5 (5), 1 (1)	2/2	100%	1	5 (1)
Malaeloa Aitulagi	1/1	100%	1	5 (1)	3/3	100%	2	5 (2)

*, *lipL32* + = qPCR positive for *lipL32* gene; †, n = number of positive samples

Demography	Dogs				Cats			
	***lipL32*+* / Total**	**% positive**	***secY* (n†)**	***secY* Allele ID (n)**	***lipL32*+ / Total**	**% positive**	***secY* (n)**	***secY* Allele ID (n)**
Mapusagafou	1/1	100%	1	5 (1)	0 / 0	—	0	—
Pava□ia□i	2/2	100%	1	5 (1)	0 / 0	—	0	—
Petesa	0 / 0		0	—	2/2	100%	1	5 (1)
Tafuna	15 / 16	93.80%	9	5 (8), 1 (1)	4 / 6	66.70%	3	5 (3)
Vaitogi	8/8	100%	5	5 (5)	0 / 0	—	0	—
Vailoatai	10/10	100%	5	5 (3), 1 (2)	2/2	100%	0	—
Amanave	1/1	100%	0	—	0 / 0	—	0	—
Aoloau	1/1	100%	1	5 (1)	0 / 0	—	0	—
Village Unreported	1/1	100%	1	5 (1)	1/1	100%	1	5 (1)
SEX								
Male	28 / 29	96.60%	19	5 (15), 1 (4)	11/11	100%	7	5 (7)
Female	49 / 49	100%	31	5 (26), 1 (5)	18 / 20	90.00%	12	5 (11), 1 (1)
Missing / Unreported	5/5	100%	3	5 (2), 16 (1)	0 / 0	—	0	—

*, *lipL32* + = qPCR positive for *lipL32* gene; †, n = number of positive samples

Table 1. **Summary of urine sample testing results from villages of Tutuila.** Distribution of *Leptospira lipL32* positive dogs and cats across villages in the Eastern and Western districts of Tutuila Island, American Samoa, including total animals tested, proportion of positive samples, number of *secY*-amplified samples, corresponding *secY* allele IDs with frequencies, and sex-stratified results summary.

A total of 82 of 83 (98.79%) canine urine samples were positive for *lipL32*. Among the positive samples, 28 of 82 (34.15%) were from male dogs, 49 of 82 (59.75%) from female dogs, and 5 (6.1%) from dogs with undetermined sex. Among the dogs with known gender, 28 of 29 male dogs were positive (96.55%; 95% CI: 83.0-99.4%) and all 49 female dogs were positive (100%; 95% CI: 93.0–100.0%) (Fig. 1A). Although the proportion of positive female samples were slightly higher than that of males, Fisher's exact test indicated no significant difference between male and female positivity rates (p = 0.3718).

Similarly, 29 of 31 (93.55%) feline urine samples were positive, comprising 18 of 20 females (90.0%; 95% CI: 69.0–97.2%) and 11 of 11 males (100%; 95% CI: 74.1–100.0%) (Fig. 1A). As with the canine samples, the positivity rates did not differ significantly between male and female cats (Fisher's exact test, p = 0.5269).

Phylogenetic analysis using secY gene sequence.

Seventy-two of 111 urine samples that tested positive for *Leptospira lipL32* by qPCR yielded ~ 550 bp *secY* amplicon amenable to sequencing and phylogenetic analysis (Table 1). Phylogenetic analysis from the 540 bp *secY* region revealed that all recovered sequences grouped within a lineage corresponding to *Leptospira interrogans* and *Leptospira weilli* in the P1 clade (Fig. 3A). A neighbor-joining phylogenetic tree was reconstructed from these 72 *secY* sequences generated in this study and 57 reference *secY* gene sequences representing diverse serogroups of different pathogenic *Leptospira* spp.

PubMLST allele typing using the secY_3 scheme (53) revealed three allele groups including allele 1 for ten sequences, allele 5 for sixty-one sequences, and closest to allele 16 for the remaining one sequence (Fig. 1B and 1C). Phylogenetic analysis was concordant with the allele-typing revealing that one allele-1 sequence clustered closely with *L. weilii* serovar Heyan, ten allele-5 sequences grouped with *L. interrogans* serovars Bratislava, Canicola, Copenhageni, Wolffi, and Pyrogenes, and the remaining 61 allele-4 sequences formed a well-supported cluster with *L. interrogans* serovars Bataviar, Bulgarica and Australis (Fig. 2A). Distribution of the *secY* allele types (genotype, GT) was not uniform across the Tutuila island. Instead, a clear pattern of spatial heterogeneity, with more genetic variation in Eastern districts, was observed (Fig. 2B).

Principal component analysis (PCA) of secY allele positive samples.

To understand the factors shaping the distribution of *secY* allele types, PCA was applied using host species, host demographics, spatial characteristics, and infection intensity (calculated genomic equivalence (GE) of *lipL32* per mL urine sample). The analysis explained 64.1% of the total variance across the first two axes, with PC1 and PC2 accounting for 36.3% and 27.8% of the variance, respectively (Fig. 3). Although there is significant overlap between 95% confidence ellipses for the distinct *secY* alleles, clear separation was seen by host species. Feline samples (triangles) clustered tightly in the upper-left quadrant, while canine samples (in circle) were distributed more broadly across the lower-right quadrant. Analysis of the variable loadings indicated that the host demographics and infection intensity were driving the variation along PC1. Host body weight, age, and sample collection month were positively correlated, and they collectively placed the canine samples towards the positive PC1 axis. In contrast, *lipL32* GE/mL showed a strong negative relationship with these demographic variables, pulling the samples toward the negative PC1 axis and coinciding with the tightly grouped feline samples. Furthermore, latitude and longitude were strongly correlated, and they contributed to variation along the positive PC2 axis, indicating that geographic position shaped the variation along this axis. The *secY* allele 5 distribution was seen even across the geographic gradient indicating that it was prevalent across

the whole island of Tutuila (Fig. 2B, Fig. 3). However, the *secY* allele 1 distribution seems to be heavily influenced by geographic location, with clustering in the eastern districts (Fig. 2B, Fig. 3).

Discussion

Leptospirosis has high prevalence across the Pacific Islands, including American Samoa, where the annual case rate has been estimated at 66.4 per 100,000 population (50). American Samoa provides an ideal ecological setting for the maintenance and circulation of pathogenic *Leptospira* species as its high rainfall, frequent flooding events, socio-economic conditions, and lifestyle and agriculture-related exposures collectively heighten the population's vulnerability to this disease. Despite the first investigation of human leptospirosis in American Samoa in 1948, only a few studies have been done in this region. Additionally, animal and environmental research have been extremely limited in this region. To our knowledge, only two small-scale animal studies that lacked molecular methods, culture-based confirmation, and phylogenetic resolution have been conducted (54). Even with these constraints, evidence of infection or exposure was noted in dogs, rats, pigs, and cattle, suggesting multiple potential reservoir hosts (54). This is the first study in American Samoa to employ molecular diagnostics to detect pathogenic *Leptospira* species in urine from companion animals. The high *lipL32* positivity rate indicates that most sampled pet animals were shedding the pathogen in their urine. These findings identify a plausible role for domestic cats and dogs as reservoirs capable of contaminating the environment and contributing to the spillover of leptospirosis to humans and other mammals.

Leptospirosis was first described in dogs in 1899, long before it was recognized in humans. Dogs are well-defined reservoir hosts for *Leptospira interrogans* serovar Canicola, although they can be infected with multiple serovars, most commonly Canicola, Icterohaemorrhagiae, Grippotyphosa, Bratislava, and Pomona (34, 40, 55). In this study, we found that 98.79% (n = 82) of dog urine samples tested positive for the *lipL32* gene using a qPCR assay. This molecular prevalence (98.79%) is markedly higher than the 8.7% to 12% national canine seropositivity reported by MAT surveys conducted in the continental US between 2002 and 2004 (43, 56), highlighting a substantially greater burden of leptospirosis in the canine population in American Samoa. The published MAT data is two decades old and contains the data from the US mainland provided by the Antech Diagnositc Veterinary Laboratory (56). Regions such as American Samoa, because of its environmental conditions, human-animal interaction, and social and ecological factors, may experience inherently higher endemic levels of infection.

A similar result was reported in domestic dogs from Kichwa communities living along the riverbanks of the Napo River in the Ecuadorian Amazon, where MAT detected 75% seropositivity, whereas molecular assays revealed 94.7% (18 of 19 animals) positivity in dog urine sampled (46). These findings arise from communities featuring tropical environments characterized by significant rainfall, persistent heat and humidity, and proximity to water bodies, reinforcing the fact that ecological pressures contribute to a high level of *Leptospira* exposure, maintenance in reservoir hosts, and shedding in the environment.

Feline infection with *Leptospira* has also been documented for nearly a century, beginning with the first isolation from a cat in Indonesia in 1938 (34). *Leptospira interrogans* serovars Icterohaemorrhagiae, Pomona, Bratislava, Autumnalis, and Canicola, *L. kirschneri* serovar Grippotyphosa, and *L. borgpetersenii* serovars Hardjo and Ballum serovars have been reported in cats (41). Among 31 cat urine samples tested, 93.55% (n = 29) were qPCR positive for the *lipL32* gene. This molecular prevalence is exceptionally high compared with global estimates of ~ 10% − 12% feline leptospirosis seropositivity (44, 57). In the USA, reported feline seroprevalence has been relatively low, including 4.8% (n = 3.63) in Massachusetts, 8.6% (n = 12/139) in Iowa, 17% (n = 9/52) in California, and 11% (n = 8/75) in Texas (58). The differences observed between the continental USA and American Samoa are twofold. First ecological, climatic, disease endemicity, and socio-economic factors such as contact with water bodies, abundant animal reservoirs, human-animal interactions, farming practices, education, social practices, and economic status may elevate the prevalence in American Samoa (28, 29, 59, 60). Secondly, the differences also reflect methodology divergence, where the past work in the continental USA relied on serological assays, and the present study focused on molecular targeting of the *lipL32* gene in urine to directly detect pathogenic *Leptospira* carriage.

Phylogenetic analysis of the *secY* gene sequences revealed that 61 samples clustered mostly closely with *L. interrogans* serovar Australis, 10 samples grouped most closely with *L. interrogans* serovars Wolffi, and only one sample was closely related to *L. weilii.* These results indicate that *L. interrogans* is the most abundant species circulating among the dogs and cats, with multiple serovars likely persisting within these communities. Notably, the presence of serovars Australis and Pyrogenes that were previously implicated in human leptospirosis (49) in this region suggests that the dogs and cats might be responsible for environmental contamination and transmission of the pathogenic *Leptospira* to humans.

Notwithstanding the importance of this study, several limitations must be acknowledged. We assessed only the presence of the *lipL32* gene in urine samples and did not incorporate serological testing. We tried culturing the urine samples but due to remoteness and limited access to the samples, we were unable to follow up on the cultures. Phylogenetic inference relied solely on the *secY* gene, which, despite being widely used in phylogenetic analysis of *Leptospira* spp., provided limited resolution and restricted the classification to broader clusters rather than specific serovars. Additionally, sampling was limited to dogs and cats that were brought to the veterinary clinic, introducing potential sample selection bias and lack of generalizability to the broader companion animal population.

Despite these constraints, our study highlighted that domestic dogs and cats in American Samoa are susceptible to *Leptospira* spp., can be infectious, and may serve as important reservoirs contributing to the maintenance of the pathogen in local environments. The high molecular prevalence observed underscores their potential role in contaminating waterbodies and participating in transmission cycles that place both animals and humans at risk. The recent outbreak of leptospirosis in American Samoa which began in March 2026 was triggered following heavy rainfall; resulting in six human cases, including one fatality as of the writing of this manuscript (61–64). Collectively, these findings highlight

an urgent need for surveillance, targeted control measures, and implication of comprehensive public health strategies to limit the spread of leptospirosis among dogs, cats, humans, and the environment in American Samoa. The study also established the critical need for an effective and urgent vaccination program for dogs and cats and regular monitoring of their health in this region. In addition, there is a need for expanded serological and molecular studies in other reservoir populations like rodents, stray dogs and cats, and pigs, as well as monitoring of water bodies and human populations. A coordinated One Health approach integrating environmental monitoring, animal diagnostics, and human disease surveillance is imperative for mitigating the burden of leptospirosis in American Samoa.

Conclusion

In this study, we found that 97.36% of urine samples from dogs and cats were positive for pathogenic *Leptospira* using *lipL32* qPCR. The widespread detection of pathogenic *Leptospira* in cats and dogs in American Samoa indicates that companion animals may play a major role in maintaining *Leptospira* transmission and environmental contamination. There is an urgent need for consideration of vaccination programs for dogs, cats, and other potential reservoir species to effectively reduce leptospirosis transmission in American Samoa. Our findings emphasize the need for more in-depth studies, including pathogen isolation, comprehensive molecular characterization, and expansion of leptospiral surveillance in the environment and other potential reservoirs, including rodents, swine, and even local human populations.

Methods

Ethics statements

No animals were harmed during sample collection for this study. A urine sample obtained during routine veterinary diagnostic procedures at the American Samoa Department of Health Veterinarian Clinic was used for this study, thereby obviating the need for separate ethical committee approval. Verbal consent was obtained from the pet owner for samples collected from sick animals during this study. Additionally, no other clinical samples were taken for this study.

Study population and sample collection

Urine samples collected from cats and dogs attending the American Samoa Department of Health Veterinary Clinic from 2024 to 2025 for routine veterinary check-ups were used for this study. Approximately 400 µL of the urine sample was aliquoted into a sterile 2 mL microcentrifuge tube with 1200 µL of 1X DNA/RNA shield (Zymo Research, cat. no. R1200-125) in duplicate. For each animal, metadata recorded by the veterinarian, including date of collection, village of origin, age, sex, breed, health status (healthy or unhealthy), and urine color, were also obtained. The samples were stored at 4°C

and transported in batches of 20–30 to the University of Hawai□i at Mānoa for further molecular analysis.

DNA extraction and polymerase chain reaction

Each tube containing approximately 1600 µl of urine and DNA/RNA shield mix was centrifuged at 15,000 × *g* for 30 min at 4°C. 1400 µl was carefully discarded from the top to retain ~ 200 µl of the liquid with/without a small visible pellet. The remaining 200 µl was mixed well by gently vortexing, and DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's instructions. The DNA was eluted using 30 µl of sterile Milli-Q water. Extracted DNA was quantified using Qubit 1X dsDNA High Sensitivity (HS) assay kit (Thermo Scientific, Cat. No. Q33231) using the Qubit Flex fluorometer.

The extracted genomic material was analyzed using qPCR. The molecular detection was performed to verify the presence of pathogenic *Leptospira* in the extracted DNA by amplifying the *lipL32* gene, a genetic determinant characteristic of pathogenic strains (65, 66). The primers LipL32-45F (5'-AAG CAT TAC CGC TTG TGG TG-3') and LipL32-286R (5'-GAA CTC CCA TTT CAG CGA TT-3') were used to amplify a 242 base pair fragment of the *lipL32* gene. Detection of the amplicon was achieved using the hydrolysis probe LipL32-189P (FAM-5'-AA AGC CAG GAC AAG CGC CG-3'-BHQ1) (66). Amplification was performed in a total volume of 20 µl, including 10 µl of 2× PrimeTime™ Gene Expression Master Mix (Integrated DNA Technology, Cat. No. 105572), 0.8 µl of each of 10 µM forward and reverse primers (400 nM of each primer), 0.4 µl of 10 µM probe (200 nM of the probe), 1 µl of the template DNA, and sterile Milli-Q water to a final volume of 20 µl in BioRad CFX Connect Real-Time System. The condition for amplification consists of an initial denaturation step of 3 minutes at 95°C, followed by 45 cycles of 15 sec at 95°C, 30 sec at 60°C, and plate read.

A 549-bp region of the *secY* gene was amplified from all *lipL32*-positive samples using the primer set containing secY_FOR (5'-ATG CCG ATC ATT TTT GCT TC-3') and secY_REV (5'-CCG TCC CTT AAT TTT AGA CTT CTT C-3') (53, 67). PCR was carried out in triplicate in 25 µl reaction volumes containing 5 µl of 5× Q5 PCR master mix, 0.5 µl of each of 10 µM forward and reverse primers, 0.1 µl of dNTPs, 0.1 µl of Q5 polymerase, 3 µl of the template DNA, and sterile Milli-Q water to a final volume of 25 µl. The condition for amplification was: initial denaturation step at 95°C for 5 minutes, followed by 39 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 20 sec, and a final extension step of 72°C for 10 min.

Following amplification, the amplified PCR product was visualized on 1.5% (w/v) agarose gel. The ~ 550 bp band was excised and the amplicon was purified from the gel using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Cat. No. 740609.50) following the manufacturer's instructions. The purified product was sent for Sanger Sequencing using both forward and reverse primers to the Advanced Studies in Genomics, Proteomics, and Bioinformatics (ASGPB) University of Hawai□i at Mānoa. Raw forward and reverse reads for each *secY* amplicon were aligned using SnapGene software and the same software was used to generate a single consensus DNA sequence. The assembled consensus sequence

was queried against the *Leptospira* PubMLST database (68) for allele assignment, using the single-locus sequence typing scheme3 corresponding to the *secY*_3 reference set (53). The assembled *secY* gene sequences were deposited to GenBank (the accession numbers of each sample are compiled in supplementary Table S1) The phylogenetic relationship with other *Leptospira spp*. was examined using the Molecular Evolutionary Genetics Analysis (MEGA) software version 12.0.8 by aligning the *secY* sequences of the samples with the *secY* sequences of reference strains retrieved from the NCBI database (the accession number and strain names are compiled in supplementary Table S1).

Statistical analysis and data visualization

Data were organized in Microsoft Excel, and all statistical analyses were performed using GraphPad Prism version 11.0.0 (GraphPad Software, Boston, Massachusetts, USA, www.graphpad.com). Positivity rates for pathogenic *Leptospira* spp. were summarized as the proportion of urine samples testing positive for the *lipL32* gene. An exact binomial 95% confidence interval (CI) was calculated for each proportion. Because of the small sample size and unbalanced group distribution, Fisher's exact test was conducted to compare positivity rate between male and female canine and feline samples. A p-value less than 0.05 was considered statistically significant.

Principal component analysis (PCA) was conducted based on *secY* allele types identified in host species with available metadata, including geographic location, age, sex, weight, collection month, and calculated genomic equivalence of *lipL32* in the urine sample. R software for statistical computing was used for the PCA utilizing the built-in R function prcomp(), and the results were visualized with the ggbiplot() function in ggplot2 as previously described (69).

Abbreviations

ASGPB
Advanced Studies in Genomics
Proteomics
and Bioinformatics

Declarations

Ethics approval and consent to participate

UH IACUC exemptions were granted for use of overflow urine samples from visits to the AS DOH Veterinary Clinic.

Consent for publication

Not applicable.

Availability of data and materials

The gene sequence datasets generated and/or analyzed during the current study are available in GenBank repository and the accession number of each sample are compiled in supplementary Table S1.

Competing interests

There are no competing interests in this publication.

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Authors' contributions

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Thi Hai Au La – Methodology, investigation, data analysis, writing - review

Ian McMillan – Data analysis, writing - review, and editing

Liam F. Fressie - Methodology, investigation

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Saiata Fila - Sample collection, conceptualization

Shawntiarre Faumui - Data entry, conceptualization

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Figures

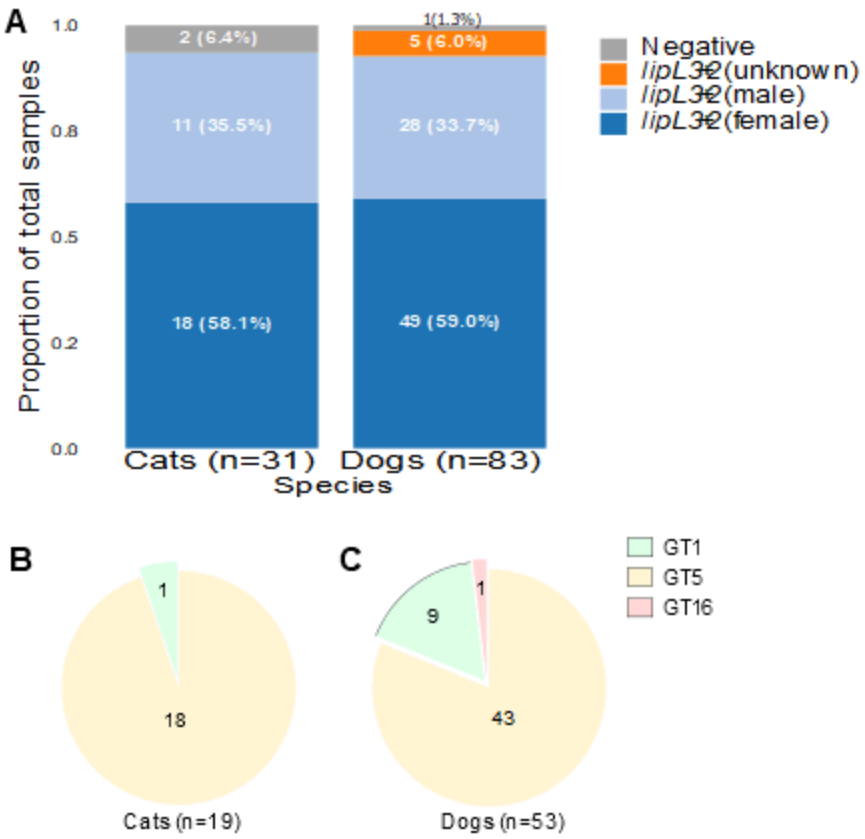


Figure 1

***lipL32* positivity in urine samples of dogs and cats from American Samoa and corresponding *secY* allele diversity. A)** Bar graph showing proportion of cats and dogs urine samples stratified by sex and *lipL32*positivity. Canine urine samples (n = 83), of which 82 (98.79%) were positive. Among positive samples, 28 of 29 males were positive (96.55%; 95% CI: 83.0-99.4%), 49 of 49 females were positive (100%; 95% CI: 93.0 – 100%), and 5 samples were from dogs of undetermined sex. No significant difference in positivity was observed between male and female dogs (Fisher's exact test, p = 0.3718). Feline urine samples (n = 31), of which 29 (93.55%) were positive. Among positive samples, 18 of 20 females were positive (90.0%; 95% CI: 69.0 – 97.2%), and 11 of 11 males were positive (100%; 95% CI: 74.1 – 100.0%). Positivity rates did not differ significantly between male and female cats (Fisher's exact test, p = 0.5269). **B and C)** Pie-chart showing distribution of three different allele types of *secY* sequenced from the urine samples of cats and dogs respectively, from American Samoa. The colors within the pie chart correspond to the *secY* genotype identified at that region, where green, yellow, and red represent GT1, GT5, and GT16, respectively.

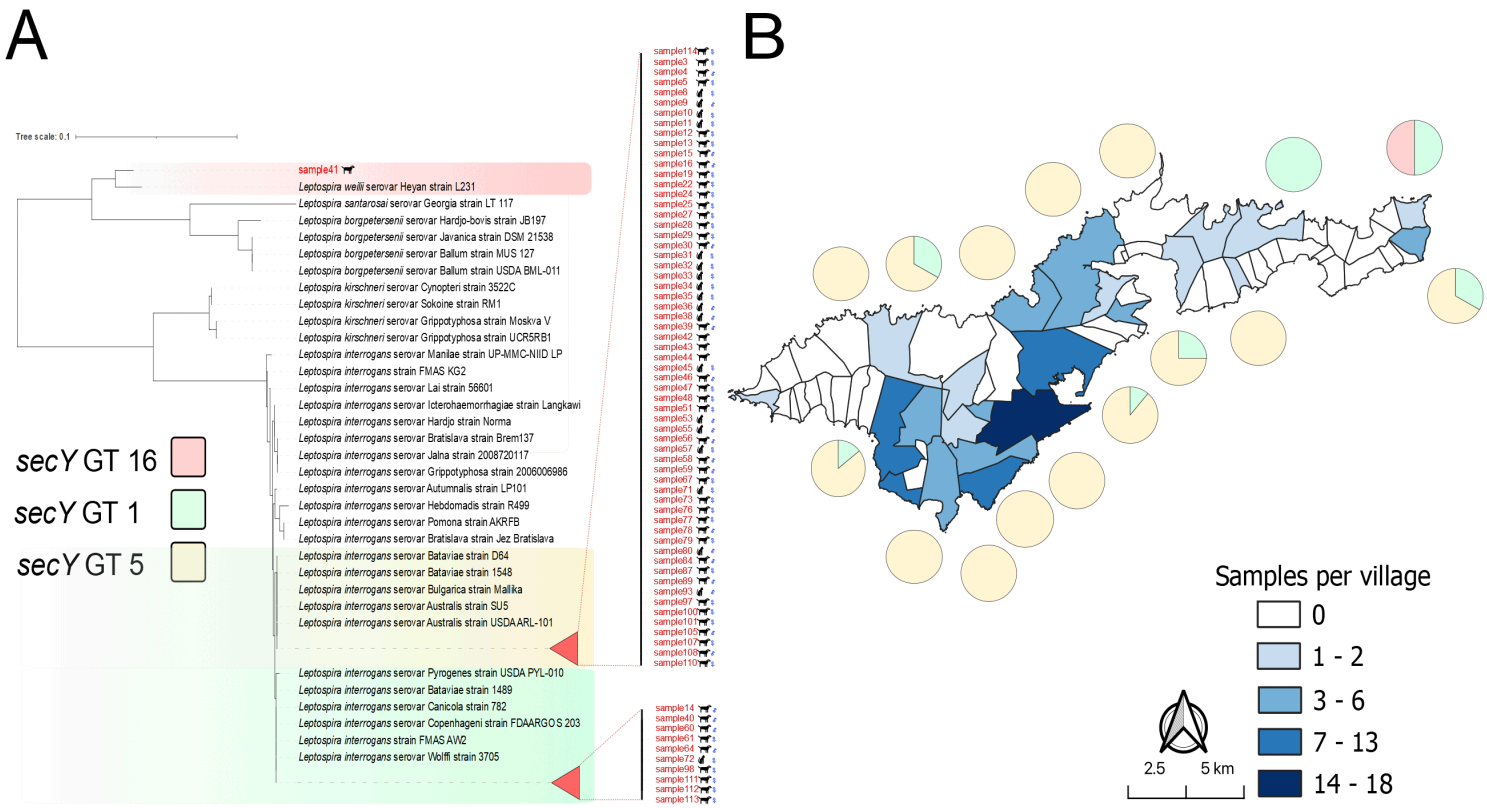


Figure 2

Phylogenetic relationship and spatial distribution of *Leptospira secY* genotypes detected in American Samoa. A) Neighbor-joining phylogenetic tree constructed from *secY* gene sequences from canine and feline urine samples. Sequences generated in this study are shown in red, while reference strains are labelled in black. Symbols at the end of each sample ID indicate the host species (cats or dogs), and blue male or female symbols denote the sex of the host species. Samples without a sex symbol represent individuals for whom sex could not be determined. Clusters corresponding to *secY*genotype 1, genotype 5, and genotype 16 are highlighted in green, yellow, and red, respectively. **B)** Map of Tutuila Island, American Samoa, with villages showing the spatial distribution of *secY* allele types (genotype, GT) detected in canine and feline urine samples. Villages are color-coded in shades of blue, where each shade represents the number of samples collected from each village, ranging from white (zero samples) to dark blue (14-18 samples). Each pie chart around the map of Tutuila represents the general geographic region (directional rather than exact location) of sample collection, and the colors within each pie chart correspond to the *secY*genotype identified at that region, where green, yellow, and red represent GT1, GT5, and GT16, respectively.

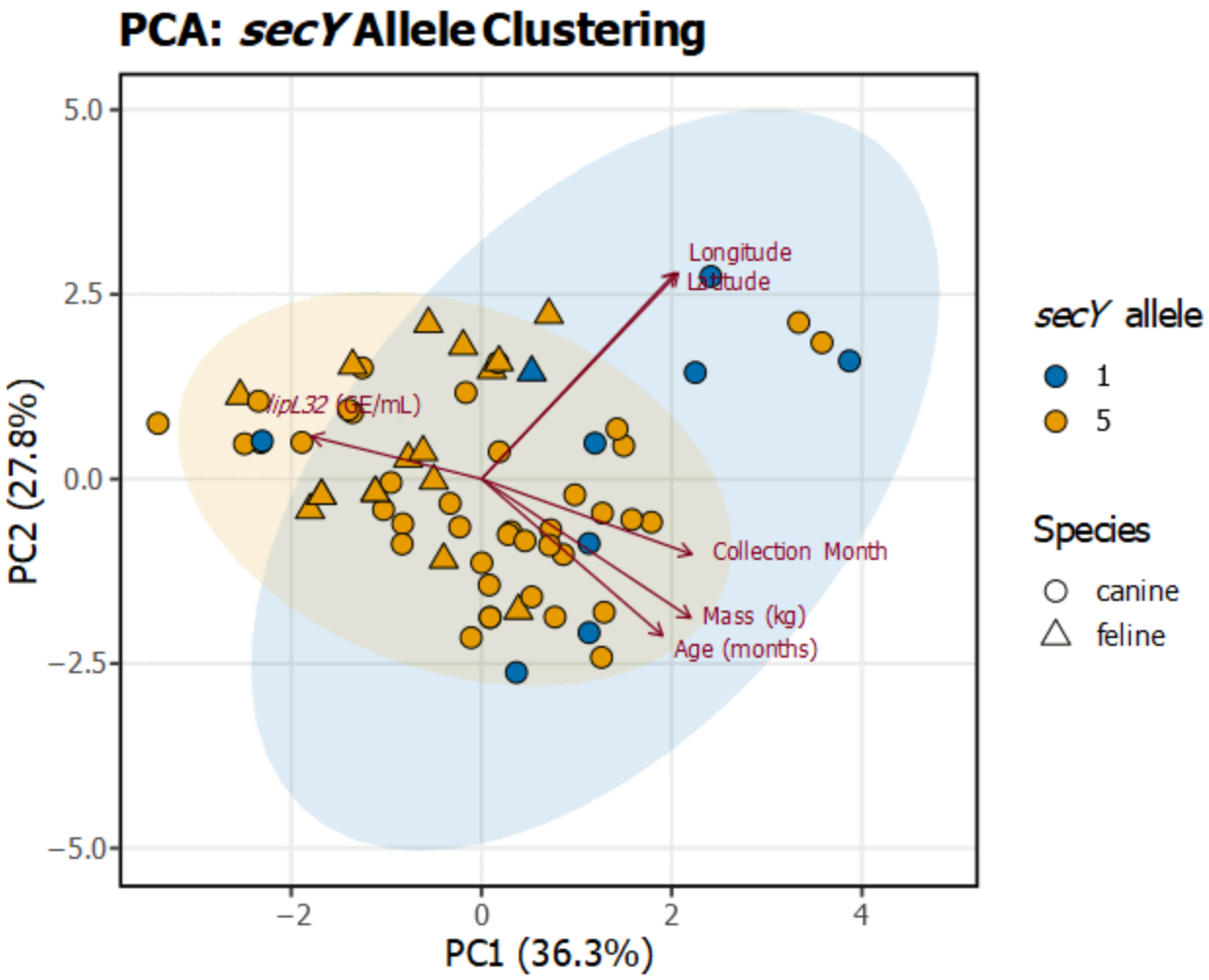


Figure 3

Principal component analysis (PCA) biplot representing the distribution pattern of the *secY* allele. Ellipses represent the 95% confidence intervals of the *secY* alleles. Canine samples are represented in circles and feline samples are represented in triangles, while blue represents *secY* allele type 1 and yellow represents *secY* allele type 5.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTableS1.xlsx