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Detection of *Leptospira* spp. in domestic dogs (*Canis lupus*) from the riverbank of Napo River.

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DEDICATORIA

A mi amada madre, por su motivación y cariño en todo el trayecto. A mi padre, que fue el motor fundamental para poder cumplir este sueño, siempre estarás en mi corazón, ¡Este logro es para ti! A mis hermanos, Andrés y Fernando, por empujarme a seguir adelante y por nunca dejarme rendir.

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RESUMEN

La leptospirosis es una enfermedad zoonótica de distribución mundial y es producida por la bacteria del género *Leptospira*. Casi todas las especies de mamíferos y marsupiales pueden ser portadores crónicos y excretores de especies de *Leptospira* patógena, siendo un riesgo para las infecciones en otros animales incluidos los humanos. Los perros cumplen un rol importante en la transmisión del patógeno a los humanos debido a su cercanía y por que están constantemente expuestos. Estos animales pueden adquirir la infección por el contacto con animales infectados o superficies ambientales contaminadas, específicamente los perros que viven en las zonas tropicales como la Amazonía podrían estar altamente expuestos. El conocimiento sobre la leptospirosis en la Amazonía es muy limitado, sin embargo, es un lugar idóneo para la circulación de *Leptospira* debido a las condiciones climáticas y la alta diversidad de fauna silvestres. Por tal motivo, el objetivo de este estudio fue investigar si los perros domésticos que viven en 5 comunidades Kichwa de la Amazonía ecuatoriana localizadas en la ribera del Río Napo estaban expuestos a *Leptospira* spp. y excretando el patógeno en su orina. Se analizaron 48/53 muestras de suero de perros mediante la técnica de microaglutinación (MAT) y se recolectaron muestras de orina de 19 perros para determinar si los animales estaban excretando la bacteria. Para detectar el ADN de leptospiras patógenas se realizó una qPCR usando los ensayos *Lipl32* y SNP 111 y a las muestras positivas se les corrió un PCR para amplificar los genes s*ecY* y *16S rDNA* de la bacteria. La identificación de especies fue realizada mediante el análisis de las secuencias de los dos últimos genes, el secuenciamiento se realizó usando la plataforma MinION (Oxford Nanopore). Se evidenció anticuerpos anti-leptospira en el 75% sueros analizados (títulos ≥ 100) y los serovares predominantes fueron Tarassovi, Pyrogenes y Australis. Adicionalmente, se logró detectar ADN del patógeno en el 94.74% muestras de orina de perros se identificaron 3 especies de *Leptospira* patógena: *L. santarosai* (7), *L. noguchii* (7) y *L. interrogans* (1). Nuestros resultados muestran que los perros analizados se encuentran altamente expuestos a *Leptospira* y que un alto porcentaje de estos animales excretan a este patógeno en su orina. Esto sugiere que los perros juegan un papel importante como transmisores y reservorios del patógeno en la Amazonía Ecuatoriana y la interacción de estos animales son los seres humanos podría ser un riesgo para la leptospirosis humana.

Palabras clave: leptospirosis, *Leptospira*, perros, Amazonia, zoonosis.

ABSTRACT

Leptospirosis is a zoonotic disease of worldwide distribution and is caused by bacteria of the genus Leptospira. Almost all mammalian and marsupial species can be chronic carriers and excretors of pathogenic Leptospira species, posing a risk for infections in other animals including humans. Dogs play an important role in the transmission of the pathogen to humans because of their close proximity and because they are constantly exposed. These animals can acquire the infection by contact with infected animals or contaminated environmental surfaces, specifically dogs living in tropical areas such as the Amazon Basin may be highly exposed. Knowledge about leptospirosis in the Amazon is very limited, however, it is an ideal place for the circulation of Leptospira due to the climatic conditions and the high diversity of wildlife. Therefore, the aim of this study was to investigate whether domestic dogs living in 5 Kichwa communities of the Ecuadorian Amazon located on the banks of the Napo River were exposed to *Leptospira* spp. and excreted the pathogen in their urine. A total of 48/53 dog serum samples were analyzed by the microagglutination technique (MAT) and urine samples were collected from 19 dogs to determine if the animals were excreting the bacterium. To detect pathogenic leptospira DNA, qPCR was performed using Lipl32 and SNP 111 assays and positive samples were PCR amplified for the bacterial *secY* and *16S rDNA* genes. Species identification was performed by sequence analysis of the latter genes, sequencing was performed using the MinION platform (Oxford Nanopore). Anti-leptospira antibodies were evidenced in 75% of the sera analyzed (titers ≥ 100) and the predominant serovars were Tarassovi, Pyrogenes and Australis. Additionally, DNA of the pathogen was detected in 94.74% of dog urine samples and 3 pathogenic *Leptospira* spp. were identified: *L. santarosai* (7), *L. noguchii* (7) and *L. interrogans* (1). Our results show that the dogs tested are highly exposed to pathogenic *Leptospira* spp. and that a high percentage of these animals excrete this pathogen in their urine. This suggests that dogs play an important role as transmitters and reservoirs of the pathogen in the Ecuadorian Amazon and the interaction of these animals with humans could be a risk for human leptospirosis.

Key words: leptospirosis, *Leptospira*, dogs, Amazon Basin, zoonosis.

CONTENT INDEX

TABLE INDEX

FIGURE INDEX

PART 1: GENERAL INTRODUCTION

General information about *Leptospira* spp.

Leptospira spp. is a gram-negative spirochete bacteria (Holt, 1978), that causes an emerging zoonotic disease known as "leptospirosis" (Levett, 2001). Leptospirosis has a worldwide distribution due to the wide variety of mammalian hosts that excrete leptospires through urine, and it is established as a global public health problem (Vijayachari et al., 2008). In addition, the mortality and morbidity caused by leptospirosis are common and underestimated because it is a neglected disease in the world. It causes about 1 million human cases each year (Costa et al., 2015).

Incidental or accidental infections in humans and animals are commonly found in countries and regions with tropical climates, which show an incidence of 73% in leptospirosis cases worldwide, poor sanitation, constant heavy rainfall, low rodent control, and poor-quality domestic animal production (Costa et al., 2015; Everard & Everard, 1993; Muñoz-Zanzi et al., 2014). All these conditions may favor the dispersion of the pathogen in the environment and contribute to its survival (Costa et al., 2015).

Leptospira spp. transmission cycle

Almost all species of mammals and marsupials can be carriers and shedders of pathogenic species in the genus *Leptospira*, which makes them a probable cause of infection for humans and animals (Ellis, 2015). Rodents are identified as the most important reservoir of leptospires; they are asymptomatic carriers and can excrete the bacteria in their urine for long periods (Barragan et al., 2017; Ellis, 2015; Levett, 2001). Leptospirosis occurs after exposure to contaminated environmental surfaces or direct contact with urine of infected carriers (Ganoza et al., 2006; Haake & Levett, 2015; Johnson et al., 2004). Its spirochetal shape favors

the entry of the pathogen to the body through lacerated skin or mucosal surfaces of the host, then leptospires are disseminated through the blood and persist there during the leptospiremic phase of the disease. After entering the blood stream, leptospires move to different target organs such as the lungs, liver, and they colonize the renal tubules and survive there for several months (Silverstein & Atlanta, 1953). Finally, they are excreted in urine for weeks or even months (Fig 1.) (Haake & Levett, 2015; Levett, 2001).

Human leptospirosis causes mild disease, which occurs in 90% of the cases and it can also cause severe disease in 5–10 % of the cases (Togal et al., 2010). Mild disease is characterized by febrile flu like symptoms, nausea, vomiting, diarrhea, malaise, and myalgia. Leptospirosis misdiagnosis is very common due to the similarity of its symptoms to a common flu, dengue, malaria, and other tropical diseases(Haake & Levett, 2015; Levett et al., 2000). Severe disease can produce liver and kidney damage, pulmonary hemorrhage, or even death (Haake & Levett, 2015; Smith & Hammond, 1952). Asymptomatic cases of human leptospirosis occur in 9-48% of the cases (Bovet et al., 1999), and there are reports showing that asymptomatic humans can excrete leptospires in their urine for long periods contributing to leptospirosis transmission (Ganoza et al., 2010).

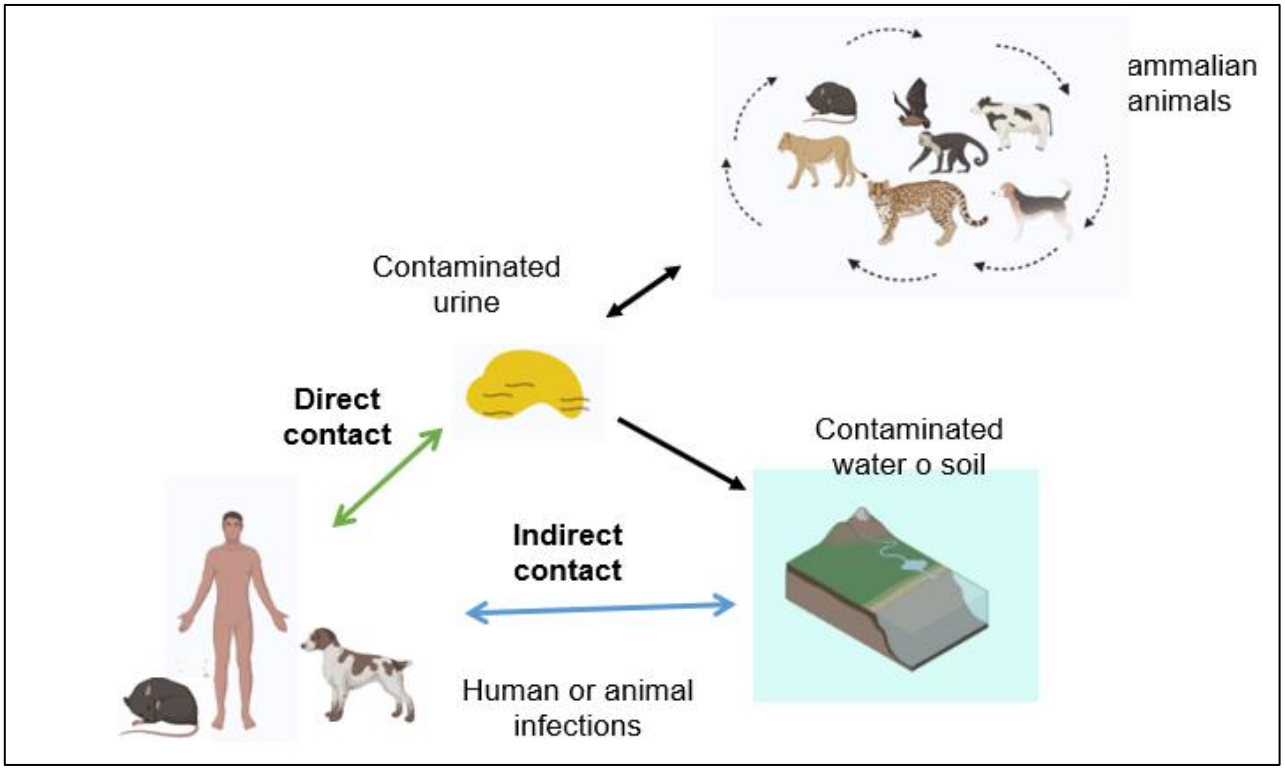

Fig 1. *Transmission cycle of Leptospirosis*

Animal leptospirosis signs depend on the infecting serovar and the animal species. Many serovars are generally linked with their animal reservoir, such as Canicola in dogs, Pomona in pigs and cows, Icterohaemorrhagiae in rats, Bratislava in horses, etc. Despite this association, animals can be incidental hosts of different species and serovars of *Leptospira*. (Bharti et al., 2003). The incubation period, before the development of clinical manifestations, has been shown to be about 7 days in experimental studies, but it may vary depending on host immunocompetence, animal species, infecting dose, and serovar (Greenlee et al., 2005b). Infections in cattle are mainly caused by Hardjo serogroup, while in pigs it is common to identify Pomona, Australis, and Tarassovi serogroups; in goats Australis, Grippotyphosa, Hebdomadis, Sejroe, and Pomona serogroups. These infections can cause abortions, stillbirths, and premature birth. Other less common symptoms, such as

fever, agalactia, vomiting, diarrhea, jaundice, decreased milk production, loss of appetite, meningitis, among others (Ellis, 2015). In dogs, the most common serovars are Canicola and Icterohaemorrhagiae serogroups; however, other serovars have been recently found to also cause disease (Ellis, 2015; Greenlee et al., 2005a). Leptospirosis in dogs is very similar to human leptospirosis. Mild disease can produce anorexia, vomiting, lethargy, abdominal pain, diarrhea, jaundice, fever, hypothermia and weakness while acute leptospirosis can produce acute renal failure, and liver failure (Schuller et al., 2015). Finally, in reptiles, e.g. snakes, the persistence of Pomona serogroup has been observed, without apparent signs of the disease (Ellis, 2015).

Species of *Leptospira*

Multiple species have been identified and isolated in mammalian animals from all continents except in Antarctica (Ben Adler & De la Peña Moctezuma, 2010). Within the genus *Leptospira*, 64 species and more than 260 serovars have been identified to date, antigenically related serovars are clustered into serogroups. According to their pathogenicity, leptospires are classified in two clades: pathogenic (P1 and P2), which contains species responsible for infections in humans and animals, and non-pathogenic (S1 and S2), which includes species isolated in the natural environment that do not cause infections (Vincent et al., 2019). Additionally, there is evidence that some pathogenic species may have an affinity for some hosts. For instance, *L. interrogans,* which has been mainly isolated from humans, pigs, wild animals and rodents. *L. borgpetersenii* which is common in cattle, *L. kirschneri* and *L. mayottensis* have also been isolated from humans, *L. noguchii* from sheep, cats and dogs, *L. santarosai* from rats, humans and dogs, *L. alstonii* from amphibians, *L. fanei* in pigs, etc.

(Bourhy et al., 2014; Ellis, 2015; Grillová & Picardeau, 2020; Kallel et al., 2020; Medeiros et al., 2020; Miotto et al., 2016; Murillo et al., 2020; Philip et al., 2020; Silva et al., 2007).

Diagnosis of leptospirosis and identification of strains and serovars

Serological analysis.

Most leptospirosis cases are identified by serology by detecting specific antibodies against leptospira in blood serum. Antibodies anti leptospira can be detected up to the fifth or seventh day after the onset of symptoms. Among the commonly used tests to detect *Leptospira* spp. are the microscopic agglutination test (MAT) and Enzyme-Linked ImmunoSorbent Assay (ELISA).

MAT is considered the gold standard for *Leptospira* spp. diagnostics (World Health Organization, 2003) and it is performed by exposing different dilutions of the patient's serum to live strains of different serovars of the pathogen (Brandão et al., 1998). After incubation, the microagglutinations produced by the antibodies that bind to the antigen can be observed under a darkfield microscope, and finally the antibody titers are determined (Goris & Hartskeerl, 2014). An antibody titer ≥100 is considered positive for a previous infection. Serovars used in MAT should include those representatives of each serogroup and the most common serovars of the regions where the analysis is performed (Faine & World Health Organization, 1982). This technique has several limitations because of the need to use several live cultures of the different serovars as antigens. Also, bacterial culture of *Leptospira* is laborious and time consuming. Among other disadvantages, there is the presence of cross contamination of serovar cultures causing false results at the time of analysis; therefore, constant control of the serovars is required (Sykes et al., 2011; Turner, 1968). To confirm a

recent leptospirosis infection by MAT, it is necessary to obtain a paired sample, one or 2 weeks apart and this could improve the sensitivity in the interpretation of the results (Fraune et al., 2013; Schuller et al., 2015).

Additionally, the interpretation of MAT results has low sensitivity due to the occurrence of cross-reaction between serogroups, which is very common, especially in samples from patients in acute phase (Appassakij et al., 1995; Brandão et al., 1998; Levett, 2001). For example, samples from dogs with acute leptospirosis, the technique may not detect anti-Leptospira antibodies due to a delayed generation of serum antibodies. (Midence et al., 2012). ELISA assays are popular and there are commercial kits used for the detection of IgM and IgG antibodies against *Leptospira* spp. in samples. This assay is very sensitive and specific for the biological diagnosis of leptospirosis. However, it is important to consider that the detection of IgM antibodies could indicate recent and previous infection because these antibodies can be detected even after several years of infection. More importantly, ELISA does not identify serovars compared to MAT and the antigen used is general and it is found in both pathogenic and saprophytic leptospires (World Health Organization, 2003).

Detection of pathogenic Leptospira DNA

PCR assays allow direct detection of the pathogen and can be performed on blood, urine, and tissue samples**.** Several PCR tests have been developed for the recognition of pathogenic *Leptospira*; for example, protocols have been standardized to amplify fragments of the *Lipl32* (Stoddard et al., 2009), *hap1* (Sleight & Langham, 1962), and *23sRNA* genes (Harkin et al., 2003) in human and animal samples (Bolin, 2003).

Multilocus sequence typing (MLST).

MLST is an effective epidemiological tool, which allows identification of allelic variants between strains of a microorganism by sequencing housekeeping genes using SANGER technology, where the nucleotide substitutions that exist between isolated strains will be compared. Housekeeping genes are commonly sequenced from *Leptospira* isolates and they have been shown to have a low rate of genetic variability that allows us to elaborate an allelic profile that is able to identify strains of the same or different clone during an epidemic or outbreak in a population. (Maiden et al., 1998; Ranjbar et al., 2014). Two MLST schemes that use different sets of genes: scheme 1*: adk, icdA, secY, rrs2, Lipl41* and *Lipl3*2 (Ahmed et al., 2006), and scheme 2: *mreA, pfkB, pntA, sucA, tpiA, fadD,* and *glmU* (Romero et al., 2011); among others. By analyzing the nucleotide sequences from these schemes, it is possible to identify allelic differences between pathogenic *Leptospira* strains.

Next Generation Sequencing (NGS) for *Leptospira* species identification.

Next generation DNA sequencing technologies are highly cost-effective and fast compared to SANGER technology. Moreover, advantages of this technology are 1) to obtain sequence data from individual amplified DNA fragments, avoiding the need to clone DNA fragments; and 2) it allows the sequencing of many samples at the same time (Ansorge, 2009; Ansorge et al., 2017). Currently, NGS platforms have allowed us to perform Whole Genome Sequencing (WGS) in microorganisms, metagenomic analysis in different types of samples, sequencing of DNA or RNA fragments of interest, understanding epidemiology of bacteria including *Leptospira,* among others (Ansorge, 2009). In addition, NGS allows us to identify microorganisms present in a sample and one strategy is to sequence the gene of interest (Cox

et al., 2013), for example, for the identification of pathogenic *Leptospira* species, genes *Lipl32*, *secY*, *16s rDNA*, and *rpob* are used (Guernier et al., 2017; Scola et al., 2006).

In the present study, we used the Oxford Nanopore Technologies MinION sequencer. MinION identifies DNA bases and measures the electrical conductivity when DNA fragments pass through a biological pore. It is small and portable, allowing it to be easily transported and used in the field. Also, this technology is more economical compared to other existing sequencing technologies (Hayden, 2014). In *Leptospira*, this platform has been used previously to identify pathogenic species (Wilkinson et al., 2021), and for Whole Genome Sequencing of *Leptospira interrogans* Serovar Copenhageni (Llanes et al., 2020).

PART 2: SCIENTIFIC ARTICLE

Free-roaming domestic dogs (*Canis lupus*) are important reservoirs of pathogenic *Leptospira* spp. in Kichwa communities from the Ecuadorian Amazon Basin

Introduction

Leptospirosis is a zoonosis that continues to be an important neglected infectious disease affecting humans and animals around the world (Vijayachari et al., 2008). Humans contract leptospirosis when injured skin or mucous membranes are exposed to the bacteria through flesh meat, urine, or urine-contaminated environments (Ganoza et al., 2006; Haake & Levett, 2015; Johnson et al., 2004). Symptoms can vary from mild to severe and nonspecific clinical presentations of human leptospirosis makes misdiagnosis very common. Mild disease occurs in most of cases, and it is characterized by nonspecific febrile flu-like illness. On the other hand, severe leptospirosis can cause dysfunction of kidney, lungs, and liver, leading to the death of the patient in approximately 5% of cases (F. Costa et al., 2015; Togal et al., 2010). Unfortunately, the no specificity of symptoms and the co-circulation of other febrile diseases such as Dengue and Malaria, have meant that in many parts of the world the true prevalence of the disease is underestimated (Haake & Levett, 2015; Levett et al., 2000; Smith & Hammond, 1952)

Leptospirosis affects human populations living in conditions of poverty and poor sanitation (Bonner et al., 2007; Ko et al., 1999; Maciel et al., 2008; Muñoz-Zanzi et al., 2014). Despite the importance of leptospirosis worldwide, its eradication is not possible due to the great diversity of domestic and wild animals that can be reservoirs of the pathogen (Ellis, 2015;

Levett, 2001). Therefore, prevention may be the only way to reduce human infection. For leptospirosis prevention, it is important to understand the local epidemiology of the disease, including environmental particularities in which the pathogen circulates, and the diversity and density of animal reservoirs (Barragan et al., 2017). Defining the proximity and interaction of animal reservoirs with humans could also help to identify relevant risk factors for infection.

Humans interact with multiple animal species of domestic animals, but one that is very close to humans is the dog, which is like a member of the family that sometimes sleeps and even eats from the same dish as its owners. The close interaction that dogs have with humans has suggested that these animals are important transmitters of human leptospirosis (Altheimer et al., 2020; Benitez et al., 2010; Brod et al., 2005; Gay et al., 2014; Rojas et al., 2010). Indeed, a recent study in Southern Brazil, showed that human leptospirosis was closely associated with dog seropositivity (Benitez et al., 2021). How exposed dogs are to pathogenic leptospira may depend on multiple factors, such as interaction or nearness to other animal species (rats, cows, wildlife, etc.), particularities in the environment surrounding the animals such as overcrowding in shelters and poor sanitary conditions, and local weather conditions such as rainy and tropical weather, among others (Kurilung et al., 2017; Lelu et al., 2015; Major et al., 2014; Miotto et al., 2018). Like humans, dogs can show mild and severe disease, being anorexia, lethargy, diarrhea, jaundice, fever, and weakness the most common signs. Severe leptospirosis in dogs can progress to renal failure, liver failure, shock and often death (Schuller et al., 2015). Despite this, they can also continuously excrete the bacteria in the urine without showing signs (Aslantaş et al., 2005; Mackintosh et al., 2011; Weekes et al., 1997; Zaidi et al., 2018). While the actual role of dogs in leptospirosis transmission remains

poorly documented, their close proximity to humans and urinary shedding in asymptomatic dogs suggest that these animals may have an important role in the epidemiology of the disease (Bal et al., 1994; Greenlee et al., 2005b).

As in other parts of the world, people from the Kichwa communities of the Ecuadorian Amazon Basin have domestic dogs. But, unlike dogs in the big cities, these animals are not confined indoors, they drink water from the river, roam freely in the forest and some of them hunt wild animals (Martins et al., 2012) (Manock et al., 2009). This behavior, together with the climatic conditions of the Amazon Basin that favor the occurrence of leptospirosis, increases the likelihood of these animals being exposed to the pathogenic leptospira species that circulate in wildlife. Thus, the close proximity that dogs have with people, especially children, may be an important risk factor for leptospirosis in this indigenous communities(Manock et al., 2009).

The aim of this study was to investigate whether domestic dogs from five Kichwa communities living on the riverbanks of the Napo River in the Ecuadorian Amazon were exposed to leptospirosis. In addition, we aimed to determine if these dogs were shedding leptospira in their urine and thus exposing their owners to leptospirosis. The results of this work suggest that domestic dogs from Kichwa communities could play an important role in the transmission of leptospirosis to their owner.

Materials and Methods

Study Site.

This study was carried out from during August 2019, in five rural communities inhabited by people of the Kichwa ethnic group living on the riverside of Napo River in the Ecuadorian

Amazon Basin (Fig 2). Urine and blood samples of dogs were collected from domestic dogs from the communities as follows.

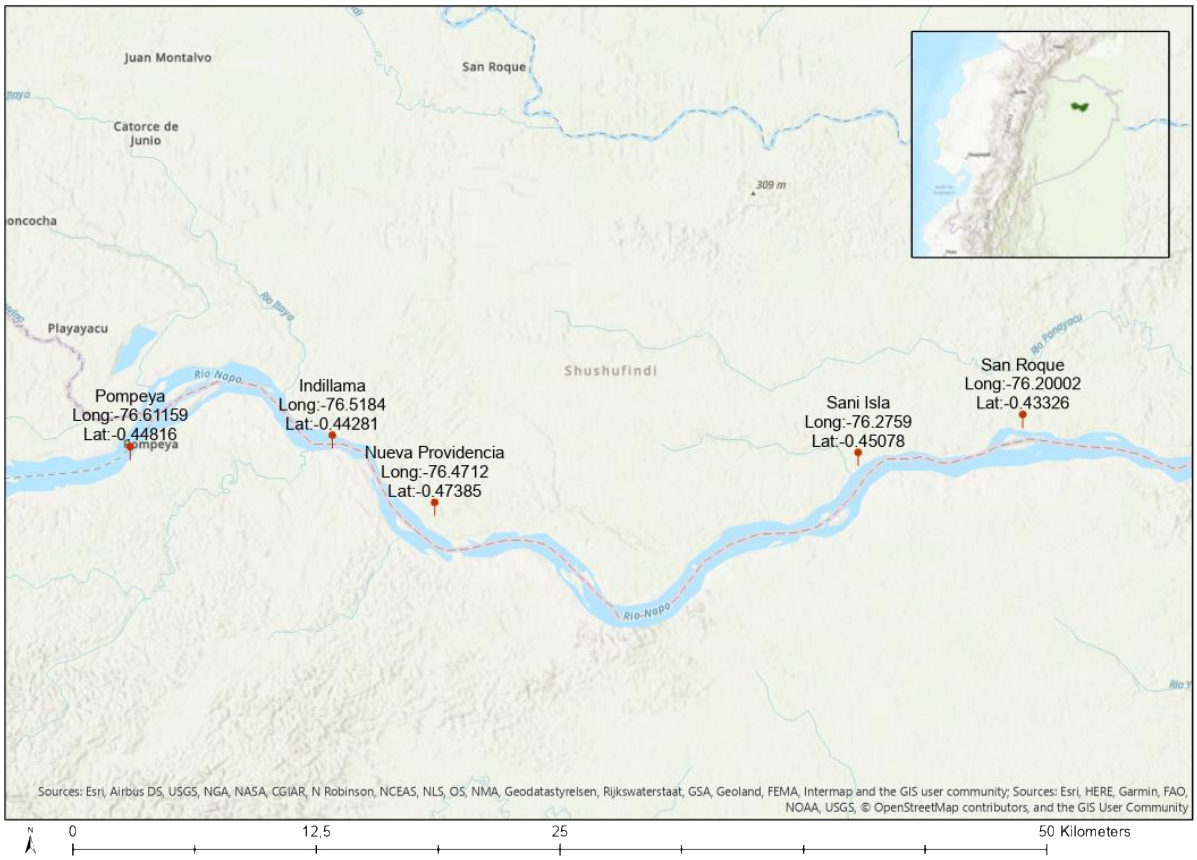

Fig 2. *Geographical location of sampled communities.*

Blood samples were collected from the cephalic vein of 53 domestic dogs from the communities of Pompeya (n=12), Sani Isla (n=12), San Roque (n=12), Indillama (n=5), and Nueva Providencia (n=12), samples were centrifuged to separate the blood serum and stored at -20°C. Urine samples obtained from 19 of the 53 dogs were collected by transurethral catheterization. To prevent DNA degradation, 4 ml of urine was mixed with 4 ml of 2X DNA/RNA Shield® (Zymo, USA). Serum and urine samples were kept and transported on ice to the Microbiology Institute at Universidad San Francisco and maintained at -20°C until

DNA extraction. Ethical approval of the sample collection procedures was issued by CEIDA-USFQ.

Serology and molecular detection of *Leptospira*.

Serum samples were analyzed by the Animal Leptospirosis Reference Laboratory (AGROCALIDAD) using MAT (Microscopic Agglutination Test) performed with a panel of 22 different available serovars (Bratislava, Autumnalis, Icterohaemorrhagiae, Canicola, Hardjo, Grippotyphosa, Wolffi, Saxkoebing, Shermani, Celledonis, Javanica, Tarassovi, Pyrogenes, Australis, Bataviae, Andamana, Castellonis, Sejroe, Copenhageni, Pomona, Hebdomadis, Djasiman). Forty-eight out of 53 serum samples were analyzed. Reactive samples with titers ≥1:100 were considered to have been exposed to leptospirosis in the past. The serovar with the highest titer was recorded in samples that reacted with multiple serovars. If a sample reacted with more than one serovar with the same titers and without showing a unique highest titer, samples were labeled as "cross reaction".

Molecular detection of pathogenic *Leptospira* sp. was performed on the 19 collected urine samples. Briefly, samples were thawed on ice and centrifuged at 4500 × g for 20 min at 4°C, then supernatant was discarded, and 200 μL of the pellet material was used for DNA extraction using DNeasy Blood and Tissue kit (Qiagen, CA, USA). DNA was stored at –20°C. Two previously described Taqman assays were used for molecular detection of pathogenic *Leptospira*: one assay that detects a fragment of *Lipl32* gen (Stoddard et al., 2009) and SNP 111 assay that detects the *16S rDNA* gene of pathogenic *Leptospira* (Barragan et al., 2016). A sample was considered positive when at least one of the assays (*Lipl32* or SNP 111) detected leptospiral DNA.

Species identification of pathogenic *Leptospira* by using Oxford Nanopore MinION.

Primers rrs2 and SecYIV described by Ahmed et al. (Ahmed et al., 2009; Ahmed et al., 2006), were used to amplify a fragment of the *16s rDNA* and *SecY* genes. Amplicons were sequenced using Oxford Nanopore Technologies. Briefly, amplicons were obtained by using the Q5 High-Fidelity Master Mix (New England, BioLabs), 0.4 μM each primer, and 2.5 μL of DNA template in a final reaction volume of 25 μL. PCR protocol consisted in an initial step at 98°C for 30 seconds, followed by 30 cycles of 10 s at 98°C, 30 s at 58°C or 54°C for rrs2 and SecYIV respectively, and 30 s at 72°C, followed with a final extension 2 minutes step at 72°C. Amplicons were purified using AMPure XP magnetic beads (Beckman Coulter, USA) following manufacturer instructions, and then quantified in a Qubit 3.0 equipment (Thermo Fisher Scientific) using the Qubit™ 1X dsDNA, high sensitivity kit (Thermo Scientific, Invitrogen, USA). The quantified samples were normalized to a concentration of 3,0 ng/uL. The normalized amplicons were sequenced following the Oxford Nanopore Library preparation protocol of the Ligation sequencing kit (SQK-LSK109) (Oxford Nanopore Technologies, UK). Finally, total of 5,72 ng of the library was loaded into a MinION flow cell (FLO-MIN 106). Most of the reads were obtained during the first 12 hours of the run. Reads were basecalled and demultiplexed using the Guppy software (version 3.4.5) (Oxford Nanopore Technologies, UK) (De Coster et al., 2018) and Porechop (version 0.2.4) (https://github.com/rrwick/Porechop) respectively.

Sequences analysis and *Leptospira* spp. identification.

Leptospira sequences were initially screened using BLASTn command line software (version 2.9.0-2), this step was implemented to filter only leptospira sequences due to the high amount

of non-leptospira reads obtained during sequencing (Bethesda (MD): National Center for Biotechnology Information (US), 2008). Then, sequences were aligned with minimap2 software (version 2.22) (Li, 2018), and visualized in Tablet software (version 1.21.02.08) (Milne et al., 2016). *L. interrogans* serovar Copenhageni FioCruz L1-130 chromosome 1 (NC_005823.1) was used as reference genome for the alignment. All the reads that were properly mapped with the corresponding genes (*16s rDNA* and *SecY*) were filtered to a new file, and aligned in MEGA-X software (version 10.1.8) (Kumar et al., 2018). The consensus sequences was obtained by using the EMBOSS cons online tool https://www.ebi.ac.uk/Tools/msa/emboss_cons/ (Madeira et al., 2019). Sequences from both genes were concatenated and compared with a representative sequence of each species of *Leptospira* obtained from GeneBank. A phylogenetic tree was built in MEGA-X software by using the Neighbor-Joining Tree (NJT) method (Saitou & Nei, 1987), with the Maximum Composite Likelihood model (Tamura et al., 2004), and 500 bootstraps. Finally, the tree was visualized using iTOL (Letunic & Bork, 2021).

All raw sequence reads were deposited in the Sequence Read Archive (SRA) of the NCBI under Bioproject Number PRJNA758395 and SRA accession numbers SRX12007895 - SRX12007909.

Results

Domestic dogs from Kichwa communities are highly exposed to *Leptospira* serovars.

Anti-leptospira antibodies were registered in 36 dogs (75%, n=48) with titers of 1:100 or greater. Tarassovi was the predominant serovar (n=5) followed by Australis (n=3), Pyrogenes (n=3), Saxkoebing (n=2), Wolffi (n=2), Canicola (n=2), Hardjo (n=1), Grippotyphosa (n=1),

Sejroe (n=1) and Shermani (n=1), and fifteen samples cross-reacted with more than one serovar. MAT titers for each sample are detailed in Table S1. Indillama community showed the highest positivity (100%, n=5), followed by San Roque (92%, n=12), Nueva Providencia (83%, n=12), Sani Isla (58%, n=12) and Pompeya (57%, n=7) communities (Fig. 3).

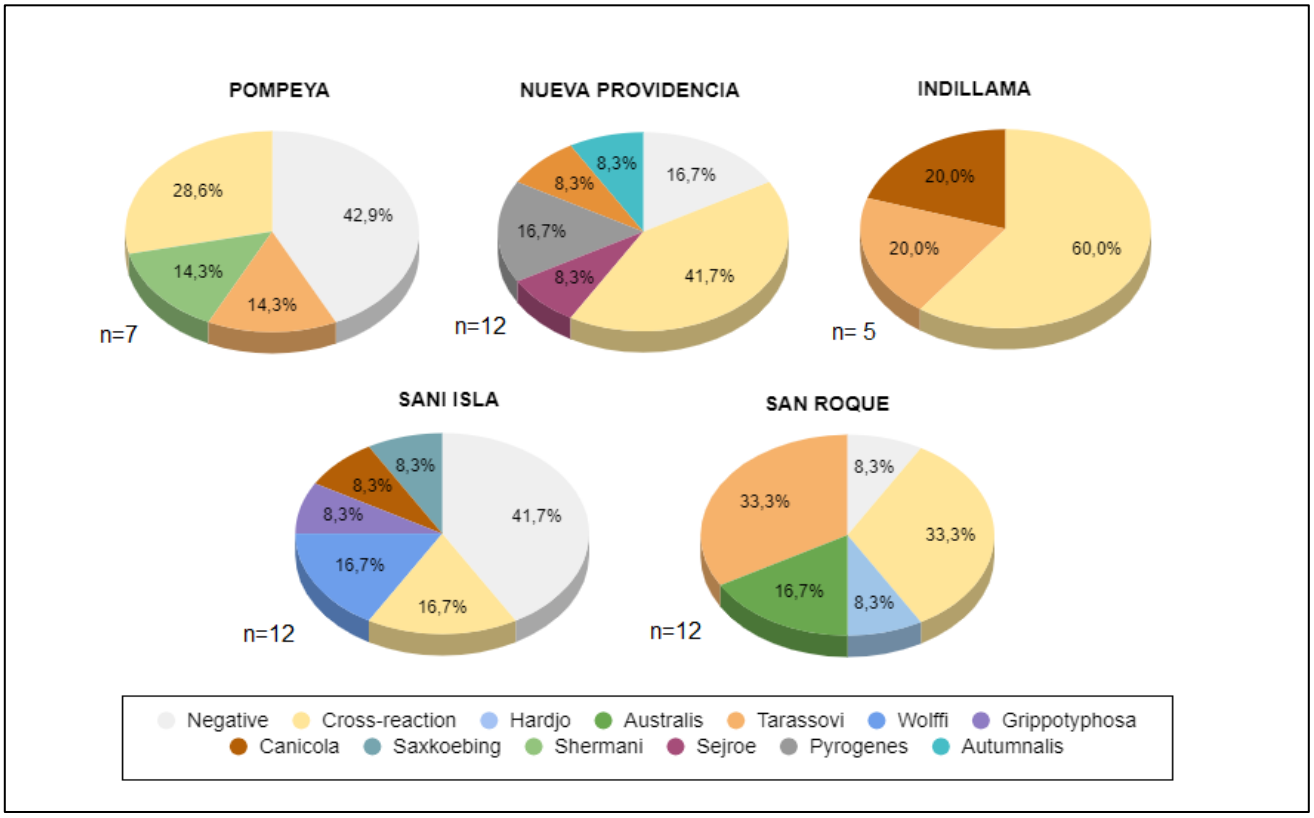

Fig 3. *Leptospira serovars detected in domestic dogs living in Kichwa communities.*

Multiple species of *Leptospira* are shed by a high percentage of dogs.

Leptospira DNA was detected in 18 out of 19 dog urine samples (94.7%). Interestingly, serum samples from 8 out of 18 dogs excreting *Leptospira* in urine registered MAT titers of 1:100 or higher (Table 1). *Leptospira* detection in dog urine was performed with two Taqman assays (*Lipl32* and SNP111) and results obtained with these assays show to be complementary, some samples that were negative for *Lipl32* were positive for SNP111 and viceversa (Table 1). The SNP111 assay allowed us to detect *Leptospira* DNA in 7 samples

that the *Lipl32* assay did not detect. Likewise, the *LipL32* assay detected *Leptospira* DNA in 8 samples that the SNP111 assay did not detect. Only 3 samples were positive for both assays.

Table 1. *Leptospira* seropositivity, and detection and identification of pathogenic Leptospira DNA in dog urine samples.

Individials	**Origin**	***Lipl32* gene**	**SNP 111**	**MAT[1]**	**Serovar**	***Leptospira spp.* identified**
M02	Pompeya	neg	Pos		-	*L. noguchii*
M03	Pompeya	neg	Pos	100	Tarassovi	*L. interrogans*
M08	Pompeya	neg	Pos		-	*L. noguchii*
M10	Pompeya	pos	Pos		-	*L. noguchii*
M14	San Roque	pos	Neg	400	Australis	*L. noguchii*
M18	San Roque	pos	Neg	neg	neg	-
M19	San Roque	pos	Neg	100	Pyrogenes	*L. santarosai*
M21	San Roque	pos	Neg	100	Cross-reaction[2]	*L. santarosai*
M23	Sani Isla	pos	Neg	400	Wolffi	*L. santarosai*
M27	Sani Isla	neg	Pos	neg	neg	*L. noguchii*
M28	Sani Isla	pos	Neg	200	Canicola	*L. noguchii*
M31	Sani Isla	pos	Neg	100	Cross-reaction	-
M34	Sani Isla	neg	Pos	100	Saxkoebing	*L. santarosai*
M38	Nueva Providencia	pos	Pos	100	Cross-reaction	*L. santarosai*
M44	Nueva Providencia	neg	Pos	200	Tarassovi	*L. santarosai*
M47	Indillama	neg	Neg	neg	neg	-
M49	Indillama	neg	Pos	100	Cross-reaction	*L. noguchii*
M51	Indillama	pos	Neg	100	Canicola	-
M53	Pompeya	pos	Pos	200	Shermani	*L. santarosai*

[1] When multiple serovars were reactive, only the one with the highest titers was reported.
[2] When multiple serovars with same titers were reactive, it was reported as "Cross-reaction"

Three species of *Leptospira* were identified from 15 urine samples by sequencing and phylogenetic analyzing the 541 and 202 bp fragments of the *16S rDNA* and *SecY* genes respectively. *Leptospira noguchii* (7 samples) was present in all sampled communities except for Nueva Providencia, *Leptospira santarosai* (7 samples) was detected in all communities except for Indillama, and *Leptospira interrogans* was detected in a single sample from Pompeya (Fig 4).

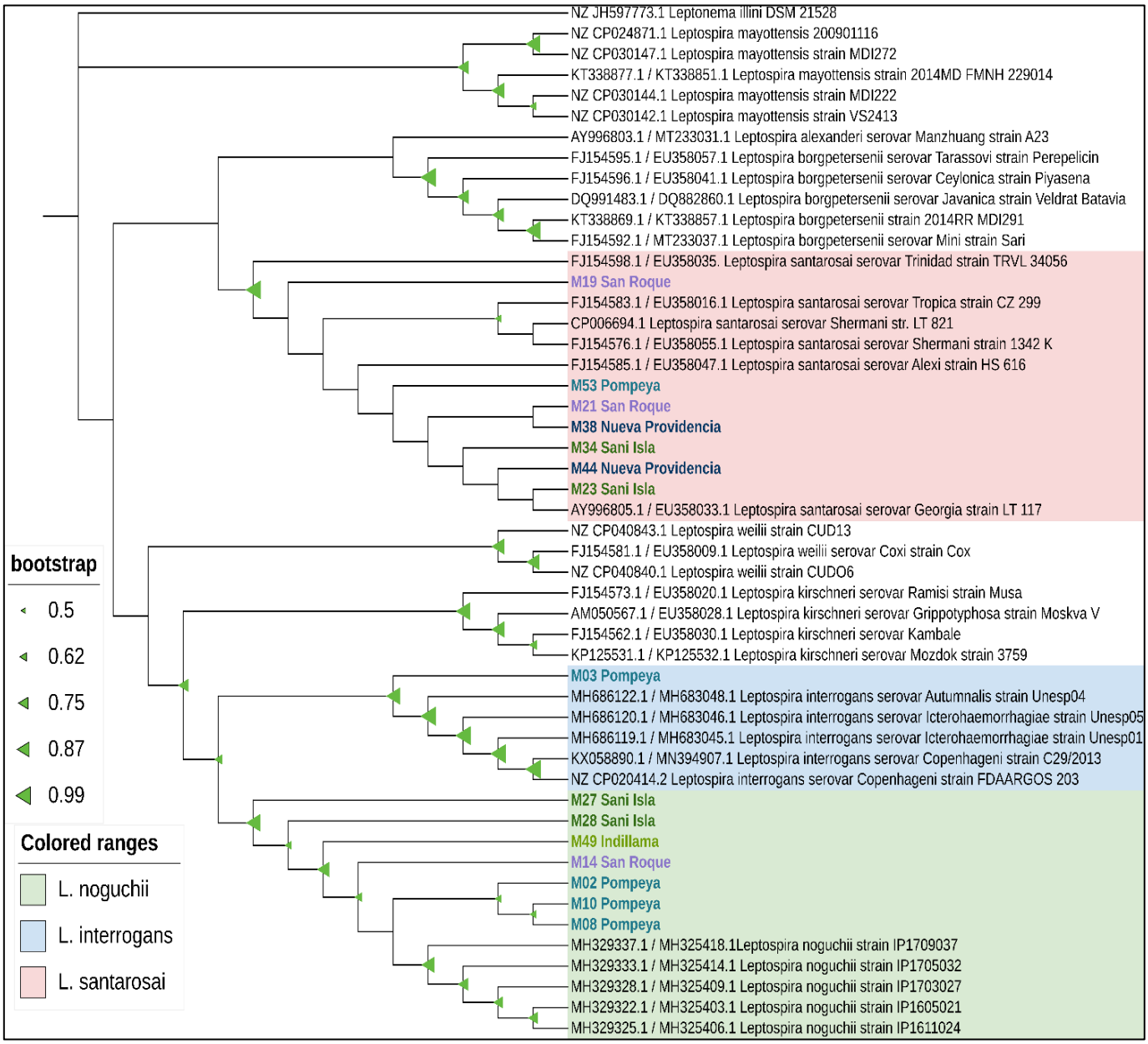

Fig 4. Phylogenetic analysis based on the Leptospira 16S rDNA and SecY genes. *Bootstrap values (500 replicates) are displayed for most important internal nodes. The tree was rooted with sequences from Leptonema illini DSM 21528.*

Discussion

The Amazon basin is an ideal scenario for the circulation of pathogenic leptospira species due to its tropical climate, year-round rainfall and the high diversity of wildlife that can act as reservoirs of the pathogen. However, it is surprising that very few cases of human leptospirosis are reported annually in the Ecuadorian Amazon basin, thus, between 2015 and 2019 an average of only 35 cases were reported and this number decreased to an average of 10 cases in 2020 and 2021 (Ministerio de Salud Pública, 2021). Although the decrease in the number of cases in recent years could be due to the fact that the public health system directed all its efforts to the diagnosis and control of COVID-19 cases during the pandemic, the neglect of leptospirosis in this region has occurred for decades (Ellwanger et al., 2020).

Human interaction with infected animals is an important risk factor for leptospirosis. One animal that is affectively and spatially close to humans is the dog, and at the same time interacts with wild animals in the forest as well as with the forest environment. Dogs living in Kichwa communities roam freely in search of food and water, which might expose them to leptospirosis and other zoonotic diseases circulating among wild animals. MAT results from dog samples, show a high seropositivity (75%, n=53) with titers that suggest that most dogs have been exposed to leptospirosis. The serovar Canicola was present in only 2 dogs, which was not expected because this is the most common in dogs and one of the serovar responsible for infections transmitted from dogs to humans (Benitez et al., 2021; Blanco et al., 2016; Ellis, 2015; Martins et al., 2012; Suepaul et al., 2010). Serovars Pyrogenes, Australis and Tarassovi were present in most reactive sera, these serovars have been

previously found in dogs from Colombia, Argentina, Nicaragua, and Brazil (Abreu et al., 2019; Calderón et al., 2013; de Castro et al., 2011; Flores et al., 2017a; Fonzar & Langoni, 2012; Rubel et al., 1997). Moreover, all these serovars are not included in dog leptospiral vaccines locally available, highlighting the emerging need to produce new and multivalent vaccines to protect not only severe disease in dogs but also shedding of the bacteria in dog urine (Abreu et al., 2019; Calderón et al., 2013; de Castro et al., 2011; Flores et al., 2017b; Fonzar & Langoni, 2012; Rubel et al., 1997; Van De Maele et al., 2008). Additionally, a high percentage of samples cross-reacted with multiple serovars (44.4%), what is commonly seen in samples from dogs in acute phase or due to the presence of common leptospiral antigens (B. Adler & Faine, 1978; Sykes et al., 2011). This situation is usually overcome by using local isolates that have been previously characterized and whose cross-reactivity is known (World Health Organization, 2003). Unfortunately, local isolates are not available in Ecuador, which limits the understanding of serology results, especially if cross-reactivity is present, and impacts directly in the understanding of the epidemiology of leptospirosis.

Infected dogs shed pathogenic leptospira in their urine during 7 to 10 days after infection, and excretion can persist for weeks or even years (Bal et al., 1994; Greenlee et al., 2005b). Additionally asymptomatic shedders have been found in 0.2 to 48.8% of dogs from different parts of the world (R. S. da Costa et al., 2021; Delaude et al., 2017) . To our surprise, the results of this study show that 94,7 % of the domestic asymptomatic dogs were excreting leptospira, the highest percentage recorded so far in asymptomatic dogs. Three pathogenic leptospira species were identified in dog urine, *L. santarosai*, *L. noguchii* and *L. interrogans.* These species have been previously identified in South America (Nalam et al., 2010), and all are responsible of severe disease in humans and dogs (Chou et al., 2019; R. S. da Costa et

al., 2021; Miotto et al., 2018; Naotunna et al., 2016; Silva et al., 2009; Valverde et al., 2013; C. Yang et al., 2001; C. W. Yang, 2007). *L. santarosai* and *L. noguchii* have been previously reported in asymptomatic wildlife and dogs, and it is also responsible of severe disease in humans (Costa et al., 2021; Medeiros et al., 2020; Miotto et al., 2016, 2018; Silva et al., 2007, 2009). While *L. interrogans* is the most common and widely distributed pathogenic specie, it is transmitted by rodents and small mammals and identified as the main human-infecting leptospira specie (Azhari et al., 2018; Kakita et al., 2020). In addition, these species of pathogenic leptospira have been previously found in urine from of asymptomatic dogs (Costa et al., 2021; Mackintosh et al., 2011; Miotto et al., 2016; Silva et al., 2009), indicating that these animals are important reservoirs for leptospires and an source of infection for humans. In our study, *L. interrogans* was found only in a community located very close to the city (Pompeya), while *L. noguchii* and *L. santarosai* were found in remote communities, which might suggest that these species came from wildlife, however further research is needed to test this hypothesis. Additionally, due to the close interaction of the dog with the environment and with other animals, our results suggest that the prevalence of leptospira may be high in forest wildlife. Moreover, high positivity in dogs is strong evidence supporting that interaction of people with dogs may be an important risk factor for human leptospirosis, probably more than in other settings (Altheimer et al., 2020; Benitez et al., 2021; Jansen et al., 2005; Castellanos et al., 2003)

We believe that the methods used to preserve and transport the urine samples, as well as the assays used to detect leptospira DNA in the laboratory, may have influenced the success of pathogen detection. Recovering leptospira DNA from urine samples is complicated due to the urine pH, degradation of leptospiral DNA at room temperature or after freezing and

thawing of samples (Branger et al., 2005). In addition, when samples are taken in remote locations, such as in the Amazon basin, where the availability of freezers is limited due to lack of power, sample preservation becomes a challenge. We were able to overcome this difficulty by using RNA/DNA shield (Zymo) to preserve DNA integrity at room temperature. Also, we believe that we were able to increase the sensitivity of leptospira DNA detection by combining the results of two Taqman assays that have proven to be very sensitive detecting 1×10^{1} copies/µL (Barragan et al., 2016; Stoddard et al., 2009). In our hands, the use of both PCRs increases the sensitivity in the detection of pathogenic leptospira DNA from urine samples, this strategy can be useful for the identification and follow-up of carrier animals and to prevent human and other animal infection.

Understanding leptospirosis within the framework of the One Health concept (Degeling & Rock, 2020), considering the particularities of the epidemiology of the disease in different settings is essential, especially in places with high heterogeneity of leptospira and animal species, such as in many rural areas of the world (Barragan et al., 2016; Kurilung et al., 2017; Castellanos et al., 2003; Muñoz-Zanzi et al., 2014). Knowing details about the diversity and density of animal reservoirs, the environment surrounding them and the interaction of reservoirs with other animals and humans is relevant information for implementing effective prevention and control plans for leptospirosis. In the Amazon rainforest animals such as capuchin monkey, coati, nine-banded armadillo, opossum, porcupine, snakes, reptiles, primates, wild boar, etc., have been found to be exposed to the disease or to excrete leptospira in their urine (Bauso et al., 2020; Fornazari et al., 2017; Medeiros et al., 2020; Vengust et al., 2008; Vieira et al., 2018), the results from our study adds important information to this knowledge and suggest that dogs might not only be an important risk factor for human

leptospirosis but also might be contributing to the sylvatic transmission cycle of the disease. However, much remains to be learned about the epidemiology of leptospirosis in a megadiverse place like the South American Amazon basin. Undoubtedly, one of the most difficult challenges is the sampling of wild animals, which is not only logistically but also methodologically complex, but indispensable for understanding the complex epidemiology of leptospirosis in one of the most diverse ecosystems on the planet.

Conclusions

The shedding of pathogenic *Leptospira* spp. in dog urines suggests that these animals may play an important role for human leptospirosis. We were able to detect 3 species of leptospira *L. interrogans, L. noguchii* and *L. santarosai*. Interestingly, *L. interrogans* was found only in the most populated community that is located very close to the city, while *L. noguchii* and *L. santarosai* were found in the more remote communities. It is possible that the latter two are circulating in wildlife and transmitted to dogs due to their interaction with the forest, however, studies on wild animals in the area are needed to confirm our hypothesis. This research is an important contribution to the understanding of the epidemiology of leptospirosis in remote rural areas of the Ecuadorian Amazon. It also indicates that human leptospirosis in this area could be much more prevalent than reported by health authorities.

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SUPPLEMENTARY MATERIAL INDEX

TABLE S1: SEROLOGICAL ANALYSIS OF 48 DOGS BY MAT

Individuals	Origin	MAT	Serovars															
			AND	AUS	AUT	BAT	BRA	CAN	DJA	GRI	HAR	HEB	POM	PYR	SAX	SER	SHE	TAR	WOL
M01	Pompeya	neg																	
M03	Pompeya	pos																100	
M06	Pompeya	neg																	
M07	Pompeya	neg																	
M09	Pompeya	pos															100	100	
M11	San Roque	pos		100			100	100		100				100					
M12	San Roque	pos		100										100					
M13	San Roque	pos		100			100			100	400					100			100
M14	San Roque	pos		400				100			100								
M15	San Roque	pos														100	100	200	
M16	San Roque	pos		400														200	
M17	San Roque	pos																100	
M18	San Roque	neg																	
M19	San Roque	pos												100					
M20	San Roque	pos													100				
M21	San Roque	pos						100									100		
M22	San Roque	pos						100		100					100	100	100	100	100
M23	Sani Isla	pos													100	100	100	100	400
M24	Sani Isla	neg																	
M25	Sani Isla	pos																	100
M26	Sani Isla	pos								100									
M27	Sani Isla	neg																	

M28	Sani Isla	pos				200		100									
M29	Sani Isla	neg															
M30	Sani Isla	neg															
M31	Sani Isla	Pos					100	100									
M32	Sani Isla	Pos							100				100				100
M33	Sani Isla	Neg															
M34	Sani Isla	Pos											100				
M35	Nueva Providencia	Pos												100			
M36	Nueva Providencia	Pos	100			100											
M37	Nueva Providencia	Pos									100						
M38	Nueva Providencia	Pos					100			100	100						
M39	Nueva Providencia	Pos									100						
M40	Nueva Providencia	Neg															
M41	Nueva Providencia	Pos	100				200						200		100	100	
M42	Nueva Providencia	Pos													100	100	
M43	Nueva Providencia	Neg															
M44	Nueva Providencia	Pos	100	100					100				100	100		200	100
M45	Nueva Providencia	Pos												100			100
M46	Nueva Providencia	Pos	100														
M47	Indillama	Neg															
M48	Indillama	Pos	100													200	

M49	Indillama	Pos	100					100					100	100				100	100
M50	Indillama	Pos	100	200		100		200		100	100							100	
M51	Indillama	Pos						100											
M52	Pompeya	Pos		100	100	100	100	200	100	200	200						100		100	
M53	Pompeya	Pos				100												200		

MAT titers against more than one serovar were detected in 15/36 MAT-positive dogs.
AND= Andamana; AUS=Australis; AUT=Autumnalis; BAT=Bataviae; BRA= Bratislava; CAN=Canicola; DJA=Djasiman; GRI=Grippotyphosa; HAR= Hardjo; HEB=Hebdomadis; POM=Pomona; PYR=Pyrogenes; SAX= Saxkoebing; SEJ=Sejroe; SHE=Shermani; TAR=Tarassovi; WOL= Wolffi.