UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

Colegio de Posgrados

Unveiling *Leptospira* Epidemiology in an Ecuadorian rural community: a pilot study

Tesis en torno a una hipótesis o problema de investigación y su contrastación

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Trabajo de titulación de posgrado presentado como requisito
para la obtención del título de Magíster en Microbiología

Quito, 21 de diciembre del 2023

UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

COLEGIO DE POSGRADOS

HOJA DE APROBACIÓN DE TRABAJO DE TITULACIÓN

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Quito, Diciembre 2023

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Lugar y fecha: Quito, 21 de diciembre de 2023.

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Dedicatoria

A mis abuelitos Gilberto y Lupe por cuidarme desde niña y enseñarme que los sueños solo se consiguen con trabajo y sacrificio, por seguirme acompañando en cada paso que doy para cumplir mi sueño y por siempre creer en mi.

AGRADECIMIENTOS

Quiero expresar mi profundo agradecimiento a la Universidad San Francisco de Quito, al COCIBA Grant y al Proyecto de Vinculación 1259 por el apoyo financiero para llevar a cabo este proyecto de investigación.

Al Instituto de Microbiología, por la beca de estudios que me permitió realizar mi maestría. Fue una oportunidad invaluable que me impulsó en mi formación académica y profesional.

A Verónica Barragán, mi tutora, pero sobre todo mi mentora. Su apoyo incondicional, orientación fueron fundamentales durante este proceso. Gracias por ayudarme a mejorar mis habilidades científicas, por darme voz durante toda la investigación y por confiar en mis habilidades.

A Fadya Orizco y Eduardo Díaz, agradezco enormemente sus valiosos comentarios y el respaldo logístico brindado durante el estudio.

A cada persona que formó parte de este proyecto, tanto en el campo como en el laboratorio: Carolina Sáenz, Patricia Zambrano, Julio López, Don Pedro Alvarado, Gaby Ortiz, Santy Andrade y Marcelito. Su contribución ha sido invaluable para el éxito de este trabajo.

A Patricio Rojas, agradezco su orientación constante durante mi programa de maestría, así como sus valiosos aportes a esta investigación.

Al Centro de Bioinformática del IM, especialmente a Eri, Rommelin y Dennys, pero sobre todo a Belén Prado y Mateo Carvajal por su valiosa amistad y colaboración en el secuenciamiento y análisis bioinformático. Su dedicación y apoyo fueron esenciales en este proceso.

A mi equipo Lepto: Sebas, Dieguito, Giuli, Karen y Marco, gracias por acompañarme en este viaje, siempre será mejor trabajar en equipo.

RESUMEN

La leptospirosis es una enfermedad endémica prevalente en regiones caracterizadas por climas subtropicales y tropicales. En Ecuador, específicamente en las zonas rurales de la provincia de Manabí, la leptospirosis es una enfermedad altamente endémica, de la que aún no se tiene mucho conocimiento. Por esta razón, este estudio tuvo como objetivo comprender algunos de los factores epidemiológicos que podrían influir en la alta endemicidad de *Leptospira* patógena en una comunidad rural de la costa del Ecuador. Se utilizó una metodología mixta concurrente, que combinó técnicas de investigación cualitativa tales como encuestas, entrevistas y observaciones, junto con métodos cuantitativos que incluyeron análisis de muestras de suero (MAT) y orina (ensayos Taqman) tanto en seres humanos como en animales. Los resultados obtenidos revelaron que las prácticas relacionadas con la crianza de animales, la estructura de los corrales, las interacciones entre especies animales, las fuentes de suministro de agua y la proximidad entre seres humanos y animales pueden desempeñar un papel crucial en la exposición a especies patógenas de *Leptospira*. Se identificó que el 100% de las unidades familiares estudiadas estaban expuestas a la bacteria, se detectó ADN de *Leptospira* en muestras de orina de perros, vacas y cerdos. El análisis filogenético permitió identificar la circulación de *L. interrogans*, así como la presencia de más de un genotipo dentro de la comunidad estudiada. A pesar de que las prácticas de crianza de animales en la comunidad se basan principalmente en la experiencia adquirida, se resalta la necesidad de realizar análisis más exhaustivos sobre la percepción del riesgo existente dentro de esta comunidad. Por otro lado, para robustecer los hallazgos de este estudio, se sugiere la realización de cultivos bacterianos y el análisis de muestras ambientales. Este estudio señala una elevada exposición de la comunidad estudiada a *Leptospira* se encuentra relacionada con las prácticas de cría de animales y sus vínculos afectivos establecidos con estos.

Palabras clave: *Leptospira*, Leptospirosis, rural, zoonosis, comunidad.

ABSTRACT

Leptospirosis is an endemic disease prevalent in regions characterized by subtropical and tropical climates. In Ecuador, especially in the rural areas of Manabí Province, leptospirosis is a highly endemic disease of which there is still little knowledge. For this reason, this study aimed to understand some of the epidemiologic factors that could influence the high endemicity of pathogenic *Leptospira* in a rural community on the coast of Ecuador. A concurrent mixed methodology was used, combining qualitative research techniques such as surveys, interviews and observations, together with quantitative methods that included analysis of serum (MAT) and urine samples (Taqman assays) in both humans and animals. The results showed that animal husbandry practices, pen structure, interspecies interactions, water sources, and human-animal proximity may play a critical role in exposure to pathogenic *Leptospira* species. It was found that 100% of the family units studied were exposed to the bacterium, and *Leptospira* DNA was detected in urine samples from dogs, cows and pigs. Phylogenetic analysis revealed the circulation of *L. interrogans* as well as the presence of more than one genotype within the studied community. Despite the fact that animal husbandry practices in the community are mainly based on acquired experience, the need for more exhaustive analyses on the perception of the existing risk within this community is highlighted. On the other hand, bacterial cultures and analysis of environmental samples are suggested to strengthen the results of this study. This study indicates that the high exposure of the studied community to *Leptospira* is related to animal husbandry practices and the emotional ties established with animals.

Key words: *Leptospira*, Leptospirosis, rural, zoonosis, community.

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FIGURE INDEX

PART 1: GENERAL INTRODUCTION

Leptospira and Leptospirosis

Leptospira, a bacterium classified within the spirochetes group, is the causative agent of Leptospirosis, a disease that affects humans and animals (Adler & de la Peña Moctezuma, 2010). The initial classification of this genus was based on surface lipopolysaccharides (LPS)(Adler, 2015), alowing serovars and serogroups *Leptospira* clasification (Levett, 2001a). However, over time, a genome-based classification was developed, dividing Leptospires into two clades, P and S. The P clade is divided into two subgroups: P1, which corresponds to the pathogenic group, and P2, which contains the intermediate groups. On the other hand, the S clade corresponds to the saprophytic group (Vincent et al., 2019). Currently, the genus *Leptospira* is divided into 35 species grouped into 3 clusters: 13 pathogenic, 11 saprophytic, and 11 intermediate. Of these, the pathogenic and intermediate ones are capable of causing disease, and mor than 260 serovars have been identified in this group (Goarant et al., 2021.)

Leptospirosis is an emerging disease, especially in developing countries, and is considered a growing public health problem (Evangelista & Coburn, 2010). Symptoms can range from mild to severe, with Weil's disease being its severe manifestation (Van Thiel, 1948). In the past, people thought that specific serovars caused the disease; however, now we know that there are two forms of Leptospirosis: the anicteric form presents subclinical manifestations or mild disease, and icteric Leptospirosis that is characterized as more severe and progressive, with a clinical manifestation that can lead to mortality (Levett, 2001b)

Transmission cycle of *Leptospira*

The main route of transmission of *Leptospira* is through direct contact with urine of infected animals or by exposure to water contaminated with urine. Rodents, such as

Rattus norvegicus and *Mus musculus*, are the main reservoirs of this bacterium (Strand et al., 2023). These rodents harbor the bacteria in their kidneys without showing signs of disease, there is evidence that the bacteria develop biofilms in their renal tubules, facilitating persistent infection (Santos et al., 2021).

Reservoirs release the bacteria into the environment through urine. If humans or animals come in contact with contaminated water, either through mucous membranes or cuts in the skin, the bacteria can infect and multiply (Sykes, Reagan, et al., 2022a). Both humans and animals can be exposed to the pathogen by direct or indirect contact with a contaminated environment (Boey et al., 2019). The ability of *Leptospira* to persist for long periods in the environment is remarkable; during times of heavy rainfall, the bacterium can become suspended on soil with the water and ascend to the surface (Fig. 1), exposing humans and animals (Bierque et al., 2020).

Weather plays a crucial role in the transmission of Leptospirosis since it is known that the presence of the bacterium is favored in tropical and subtropical climates (Ciceroni et al., 2000). Rainy periods and the combination of water, warm and humid soils create optimal conditions for *Leptospira* proliferation (Sivakumar, 2022)

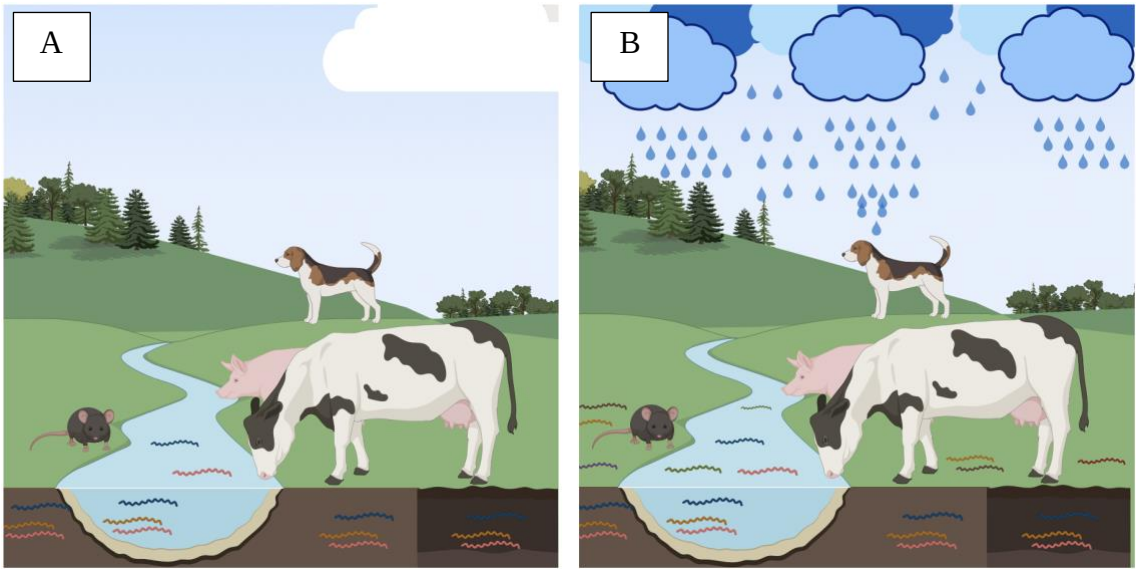

Figure 1. Illustration shows the increase of *Leptospira* load in the environment as a result of humidity on soil. Adapted from (Bierque et al., 2020). (A*) Leptospira* is found deep in the soil in the dry season and very few on the surface. (B) *Leptospira* is more abundant on the surface due to rainfall.

Poverty is closely associated with infection, as rats, the main reservoirs of *Leptospira*, are more prevalent in areas with low sources (Richardson et al., 2019).

In rural areas located in tropical areas, several risk factors are associated with the presence of the pathogen. These factors include agricultural work, animal husbandry, barefoot walking, the presence of rodents, and unhygienic slaughterhouses. One of the most relevant factors is the proximity to farm animals. Although its importance is recognized, wild animals' role in transmitting the bacterium must still be fully understood (Levett, 2001c).

Cattle excrete an average of $3.7x10^4$ ($3x10^2$-$3.7x10^4$), units of *Leptospira* per mL while rats excrete $5.7x10^6$ (– $8.5x10^8$) units per mL, dogs $1.4x10^2$ ($3.5x10^1$-$1.3x10^6$), and humans $7.9x10^2$ ($3.2x10^1$-$8.5x10^6$) units per mL of urine. Even though rats show more bacteria per mL of urine, cattle release more bacteria into the environment by excreting a larger volume of urine (Barragan et al., 2017). Also, rural areas are characterized by the accumulation of stagnant water during flooding due to the absence of filtration systems,

which creates an environment conducive to bacterial proliferation. In addition, these areas are often home to many farm animals, such as pigs and cows, that are in direct contact with moist soils where bacteria may be present. These animals are usually in close contact with humans (Muñoz-Zanzi et al., 2014). The high exposure in rural environments has led to the observation of the presence of *Leptospira* in hosts, such as backyard animals (cows, pigs) and pets (dogs) (Cilia et al., 2021; Orlando et al., 2020).

Leptospirosis and its transmission to humans

People living in rural areas have an increased susceptibility to Leptospirosis due to three main factors: epidemiological conditions, host susceptibility, and pathogen virulence, as shown in Figure 2. Nevertheless, in highly endemic areas, infections caused by *Leptospira* in humans can often be mild or asymptomatic (Haake & Levett, 2015)

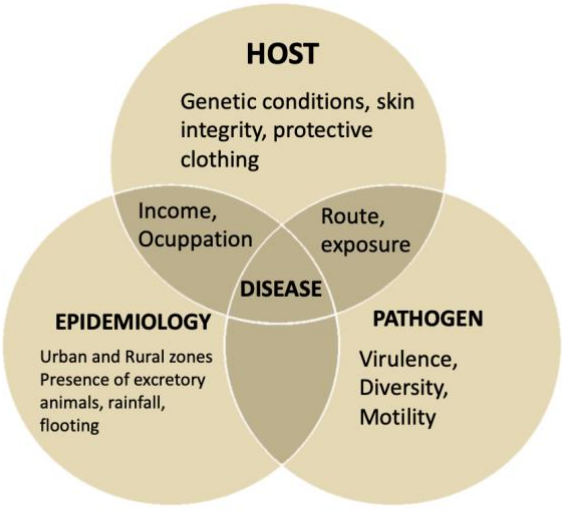

Figure 2. Factors contributing to Leptospirosis in humans include rainfall levels, flooding, occupational occupation, age, type of housing, clothing, and the pathogen's virulence. Adapted from (Haake & Levett, 2015)

Leptospira enters the body primarily through cuts, abrasions, and mucous membranes (Haake & Levett, 2015), ; in rural areas, studies suggest that people are exposed to the pathogen by participating in recreational activities such as swimming in contaminated

water (Stern et al., 2010). Unlike other spirochetes, *Leptospira* disseminates through the blood stream during leptospiremia, a phase of illness that is responsable for the first eight days of fever (Katz, 2012). Humans are considered incidental hosts, which means that the infection could be severe in them because the human receptor TLR4 receptor cannot recognize *Leptospira* LPS (Lipopolysaccharide), unlike mice, which can generate an innate immune response (Nahori et al., 2005). The incubation phase is usually 7 to 12 days after exposure, although there are cases where symptoms appear within three days or up to a month later (Morgan et al., 2002). If the infection progresses it might manifests with severe symptoms such as hemolytic uremic syndrome. There are approximately 1,300,000 cases of Leptospirosis annually worldwide, of which 58,900 correspond to deaths Most of these cases occur in tropical regions and mainly affect men between 20 and 49 years of age (Costa et al., 2015).

Leptospira and Leptospirosis in animals

Leptospira can infect a wide variety of animals. In endemic countries, a limited number of serovars have been identified in maintenance hosts, species where the infection is chronic and is maintained in the renal tubules (Hathaway & Little, 1981; Levett, 2001c). On the other hand, incidental (severe infection) hosts often arise in unsanitary environments where rodent populations are not controlled and cohabitation with other animals is common (William A. Ellis, 2015). Maintenance hosts have been identified, such as rats (Icterohaemorrhagiae), cattle and sheep (Hardjo-Canicola), dogs (Hardjo-Canicola), and pigs (Bratislava). However, it is essential to note that this information may vary according to geographic location and climatic conditions (Hathaway & Blackmore, 1981). A distinctive feature of maintenance hosts is that infection does not usually progress to the manifestation of clinical signs; in these animals, the bacterium may persist

in the kidneys, be excreted through the urine for long periods, and affect other tissues (W. A. Ellis, 1994).

The bacterium enters the animals through the mucous membranes or genital tract. In the case of carnivores, infection can occur orally by ingesting contaminated animals. When infection progresses, symptoms usually appear 1 to 2 days after exposure, and the bacterium can be detected during the bacteremia phase, which lasts 10 to 14 days (Hathaway et al., 1983). One of the main virulence factors leading to disease in animals is the presence of hemolysin, that are powerful inducer of proinflammatory cytokines (Wang et al., 2012); it could be on serogroups are characteristic producers of this enzyme (Bolt & Marshall, 1995). *Leptospires* can be localized near the renal tubules and multiply to be excreted through urine (William A. Ellis, 2015ª).

The serovar Hardjo has been identified as the main serovar found in cattle, such as cows and pigs (William A. Ellis, 2015ª). The main source of exposure for these animals is consuming contaminated water or mating with infected animals (Rinehart et al., 2012). In severe cases of infection, symptoms such as fever, hemolytic anemia, hemoglobinuria, meningitis, and even death may occur. Lactating cows are more susceptible to incidental infections (William A. Ellis, 2015ª). In case of infection, they may experience a decrease in milk production, with yellowish coloration and clots; they may not present fever, but the symptoms resemble those of mastitis. This phenomenon usually lasts 10 to 14 days, resulting in significant economic losses (Higgins et al., 1980). . However, it has been observed that economic losses decrease when cattle are exposed to the bacterium early, especially in highly endemic areas (Sanhueza et al., 2013). Dogs have been the subject of great interest in the study of *Leptospira* due to their proximity to humans and the bonds they establish as pets. Worldwide, serovar Canicola is considered maintenance in these animals, in addition to serovars Icterohaemorrhagiae, Australis, and Grippotyphosa; for

this reason, the vaccine intended for these animals is tetravalent. It includes these known serovars, although it is recognized that other serovars may also be pathogenic.. (Grippi et al., 2023; Klaasen et al., 2013). Nevertheless, in progressive cases, symptoms such as anicteric disease, fever, bacteremia 3-4 days after infection, photophobia, myalgia, anorexia, nausea, vomiting, and prostration may occur (Tangeman & Littman, 2013).

In the case of cattle and dogs, it can be observed that there are cases ranging from mild to severe, which implies that there are cases in which, although they are infected, there is no disease (Levett, 2001c)

Diagnostic and detection of *Leptospira*

Diagnostics must consider that after infection, *Leptospira* needs an incubation period in the host. In the case of humans, the first few weeks between seven and 14 days of infection are critical, after this time antibodies can be detected, and the it is also possible to detect the bacteria in the urine. After this initial period, levels of antibodies tend to decrease. However, it has been observed in some animals that excretion of the pathogen through urine can persist for years (Haake & Levett, 2015).

In animals, for diagnostics it is essential to consider the time of exposure to the pathogen to accurately detect *Leptospira*. For example, the period between exposure and sign manifestation in cattle is shorter than in humans. In addition, unlike humans, many animals can act as maintenance hosts, leading to variations in antibody titers. *Leptospira* detection in animals, can be performed in urine, blood, serum, kidneys, and genital tract. Detection can also be done post-mortem (William A. Ellis, 2015).

Serological Test.

The standard test for Leptospirosis diagnosis is the Microagglutination Test (MAT), which requires the culture of representative *Leptospira* serogroups. Preferably, local

isolates are used to improve the accuracy of this test. However, this test has limitations, such as cross-reactivity, which can lead to false positives. This serological diagnostic test should be taken at the onset of symptoms. Antibodies have been detected in the animal or human body after infection; this can be a good tool for detecting exposed patients or animals, for diagnostic samples must be taken twice, 7 and 10 days apart. MAT indicates exposure to the pathogen and is not suitable for evaluating chronic infections. Animals are considered exposed at titers up to 100 (Hamond et al., 2012).

The performance of the test involves combining a *Leptospira* culture with the serum of the species to be evaluated and observing the agglutination through microscopy to determine the titers. MAT is time consuming and requires expertize to be performed and analized (Beran, 1994). Nonetheless, one of the limitations is its serogroup specificity, which implies that if the serogroup affecting a specimen is not in the detection panel, the test may not detect it (Smythe et al., 2009). The result is observed by darkfield microscopy, considering the highest serum dilution that produces 50% agglutination (Feigin et al., 1975).

Despite being the most recommended test for epidemiological studies, as it considers exposure from titers of 100, it is only conclusive to verify the serovar with the support of bacterial culture (Peters et al., 2017).

Another serological test used is ELISA, which detects the production of antibodies against *Leptospira*. However, its limitation lies in the impossibility of identifying the specific serovar the individual was exposed to; it only indicates exposure to the bacterium (Wilson-Welder et al., 2021)

Molecular Detection.

PCR assays for the molecular detection of *Leptospira* have been been used in the last decades. The most sensitive test is real-time PCR, which detects the *Lipl32* and *16S rRNA*

genes, which have been useful for detecting bacterial genetic material in clinical samples(A. Ahmed et al., 2009; Barragan et al., 2016). Two categories of genes that are commonly used for genotyping are: housekeeping genes, such as *rrs, gyrB, or secY*, and pathogen strain-specific genes, such as *lipl32, lig, and lfb1* (A. A. Ahmed & Grobusch, 2012).

qPCR is more sensitive and faster than culture, hovever despite its advantages, Microagglutination Test (MAT) remains the standard test because it can detect a more significant number of cases (Thaipadunpanit et al., 2011; Truccolo et al., 2001). A significant limitation of PCR is that it does not allow for serovar identification, a crucial aspect of epidemiological and public health analyses (Haake & Levett, 2015).

Sequencing methods to identify *Leptospira*.

Next-generation sequencing is an innovative technology that significantly expands knowledge of previously unknown genomes. This technology offers several ways to analyze genomes, such as whole genome sequencing when culture is available, metagenome analysis to assess the abundance of a sample, and DNA fragment sequencing, which can be performed with long or short reads (Reis-Filho, 2009). In this work, the Oxford Nanopore technology will be specifically addressed.

Oxford Nanopore technology identifies DNA bases by measuring the changes in electrical conductivity generated by the nucleotides that make up the DNA strand as they pass through a biological pore. One of its main features is its portability, as the sequencing kit is compact and can be easily transported anywhere. In addition, data production is performed in real-time (Lu et al., 2016).

In the specific case of *Leptospira*, some studies used the target sequencing technique. This methodology involves obtaining amplicons, initially from housekeeping genes such as *secY*, which has a length of 410bp and sufficient variability to differentiate between

species, as well as being conserved among them. For this purpose, a two-step Nested PCR that previous studies described to recover more fragments corresponding to the pathogen (Grillová et al., 2020). Sequencing can be performed from these fragments obtained with the amplification of specific housekeeping genes or MLST genes using NGS technologies, comparison with database in authorized web pages, and identification with phylogenetic analysis.

PART 2: SCIENTIFIC RESEARCH

Unveiling *Leptospira* Epidemiology in an Ecuadorian rural community: a pilot study

Introduction

Leptospirosis, a disease caused by the bacteria *Leptospira*, is a threat to both humans and animals. Previous studies have suggested the possibility of interspecies transmission; among these factors there's the presence of reservoir animals, climatic conditions and human-animal interactions (Sykes et al., 2022). In rural communities worldwide, exposure to *Leptospira* is influenced by climatic variations such as El Niño-Southern Oscillation (ENSO), and occupational activities, particularly in regions where the economy is based on activities such as livestock (Kembhavi et al., 2021; Quintero-Vélez et al., 2021).

In rural communities, exposure to this pathogen occurs mainly through contact with soil or water contaminated with urine from infected animals or through direct contact with these animals. Recent results from Argentina confirmed a higher exposure to *Leptospira* in rural environments rather than in urban areas (Rivero et al., 2022). Similarly, studies of disease transmission in a rural community in Thailand (Narkkul et al., 2021a), showed an increased risk among people working near Leptospira-positive water sources and those in close contact with animals.

In Ecuador, leptospirosis has been under-diagnosed. It is often confused with other febrile diseases that are given more importance, such as dengue, chikungunya and others. One of the latest epidemiological official report estimated an average of 1 case per 100,000 inhabitants, with the province of Manabí recording 14 cases, mainly in people aged between 20 and 49 (Ministerio de Salud Pública, 2021). However, in 2023, when 54 cases

were reported in 2 different outbreaks, the increase in cases was clear (Vigilancia En Salud Pública, 2023).

During the present study, we wanted to understand some of the epidemiological factors that might have an impact on the high endemicity of pathogenic *Leptospira* in a rural community in the coastal region of Ecuador.

Methods

Study site.

This study was conducted in eight households within Rocafuerte-Manabí, a rural community in the coast of Ecuador. Specific sites are shown in Figure 5: Guarango (site 1): 0°53’31.9’’S, 80°23’54.4’’W, Guarango (site 2): 0°53’32.6’’S, 80°23’47.7’’W, Cardon (site 3): 0°54’39.6’’S, 80°24’05.3’’W, La seca (site 4): 0°54’42.1’’S, 80°23’13.8’’W, Frutillo (site 5): 0°53’02.3’’S, 80°29’38.6’’W, Frutillo (site 6): 0°53’02.0’’S, 80°29’36.9’’W, Frutillo (site7) : 0°52’43.0’’S, 80°29’37.3’’W, Resbalon (site 8): 0°57’40.7’’S, 80°26’27.6’’W. All the houses included in this study had in common that they had at least one type of animal, such as cows, pigs or dogs.

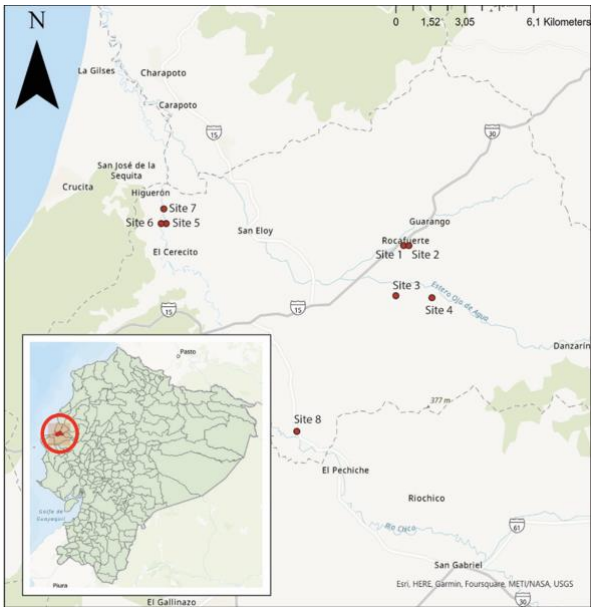

Figure 3. The study site is on Ecuador’s coast, Manabí Province, Rocafuerte, in eight specific family units of a rural community.

Sample and Data collection.

For sample collection, we asked the largest local pig feed distributor to refer us to families with backyard pigs. Nineteen family units were identified. We visited them and asked to the household if he/she would be willing to participate in the study. Eight of them agreed. Sampling was performed during two different dates the first one on October 2021 and the second one on May 2022.

The study employed a concurrent mixed method approach, utilizing both quantitative and qualitative methods for data collection. The data was collected through surveys, group interviews, participant and nonparticipant observations, and laboratory analysis of urine samples.

Data collection for qualitative analysis.

Individual surveys were applied to households from eight family units, which were multiple-choice and divided into five categories: environment (floods, contact with rivers, walking barefoot), contact with animals, type of sanitation, water consumption, and food storage. Group interviews were conducted in six out of the eight family units with questions divided into five categories: Feeding practices (females during lactation, young and adult pigs, source of drinking water), closeness and emotional attachment to animals (names, hugs from family members, distance from pen to house), pen structure and cleanliness (materials used, frequency of cleaning, footwear used), veterinary guidance (care at birth, in cases of illness or assistance required, routine visits), and social aspects (role of women in agriculture, handling of money related to agriculture, marketing of pigs). Participatory and non-participatory observations were concurrently conducted (Ethical approval: CEISH 2023-066IN).

After data collection, two researchers conducted a cualitative microanalysis. Each one of them did a preliminary analysis, extracted concepts, and collated them. Categories were established by open coding, followed by axial coding, afterwards they counted properties from each category according to their presence or absence in each analysis unit. Based on the method described by Kuckartz and Rädiker, 2019.

Quantitative data collection.

Blood sample collection for serological analysis.

Blood samples were collected from dogs, cows, pigs, and humans. Dogs samples were obtained using the jugular vein method, cows from tail vein, pigs from the marginal ear vein, and humans through venous puncture (Michigan, n.d.) (Ethics committee permit issue 2021-010). Serum from samples was obtained by centrifugation at 3000 rpm for 10 minutes.

MAT analysis on serum samples were performed in the National Reference Laboratory AGROCALIDAD and Instituto Nacional de Investigación en Salud Pública (INSPI). All samples were tested against 23 different serovars, including Bratislava, Autumnalis, Icterohaemorrhagiae, Canicola, Hardjo, Grippotyphosa, Wolffi, Saxkoebing, Shermani, Celledonis, Javanica, Tarassovi, Pyrogenes, Australis, Bataviae, Andamana, Castellonis, Sejroe, Copenhagen, Pomona, Hebdomadis, and Djasiman. Samples with titers equal to or greater than 1:100 were considered positive for agglutination and indicate exposure to *Leptospira*. In cases where multiple serovars had the same titer, we named them “cross-reaction.”

Urine and tissue sample collection for Leptospira DNA detection.

During this study urine samples from dogs, cows, pigs, and humans were collected. Dog samples were collected using transurethral catheterization; stimulated cows to urinate

using bulbar stimulation; pig samples through spontaneous urination; and for humans, containers for urine collection were provided (2023-066IN permit).

Samples were collected from at least one type of animal and at least two family members within each household. To avoid degradation of the genetic material, we used 2x DNA/RNA Shield® (Zymo, USA). Samples were transported to the Microbiology Institute of Universidad San Francisco de Quito at room temperature.

Rat kidney samples were collected using life traps and a bait made of a mixture of peanut butter, sardine, and oatmeal. When a rat was captured in the trap, it was humanely sacrificed following the Inhalant Euthanasia protocol with CO_2 under the permission 2021-010 (Guidelines for Rodent Euthanasia,n.d.), After being sacrificed, the animals were dissected, and their kidneys were carefully removed and preserved in 1x DNA/RNA Shield® (Zymo, USA). For both the October and December sampling, we placed 3 traps in each family unit and checked them for 5 consecutive days. There were two rat captures in October 2021 and two rat captures in May 2022.

Molecular detection of pathogenic *Leptospira* species.

Urine samples were centrifugated at 4500 xg for 30 min at 4°C. We removed the supernatant and saved 200 µL of the pellet. DNA extraction was performed using the Dneasy Blood and Tissue kit from Qiagen Company (Qiagen, Hilden, Germany); DNA was stored at -20°C for future use.

To discard false negative samples caused by inhibitory PCR compounds, we amplified a fragment of the β actin gene according to the protocol described by Hopwood et al. (1999). Pathogenic *Leptospira* DNA was detected using TaqMan assay targeting the Lipl32 gene (Stoddard et al., 2009) and another assay for 16S rDNA (Barragan et al.,

2016), both of which were designed to detect *Leptospira spp*. A sample was classified as positive if *Leptospira* DNA was detected in either one or both assays .

Identification of pathogenic *Leptospira* species using Oxford Nanopore MinION technology and Sequence analysis.

Leptospira identification was carried out using NESTED PCR. In the first PCR, we used the primers described by Ahmed et al. (2011), and the second one was made using primers Mod Picardeu (unpublished) and Picardeu (Medeiros et al., 2020). Both PCR were performed using the Q5® High-Fidelity DNA Polymerase.

The protocol for the first PCR consisted of an initial step at 98°C for 30 s, followed by 15 cycles at 98°C for 10 s, 55°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 2 min. For the second PCR, we used Picardeu (Medeiros et al., 2020). primers with a cycling program of 98°C for 30 s, followed by 30 cycles of 98°C for 10 s, 52°C for 30 s, 72°C for 20 s, and a final extension at 72°C for 2 min. The same program was used for the Mod Picardeu primers with an annealing temperature of 55°C for 32 s.

The amplicons were purified using AMPure XP magnetic beads and quantified using a QubitTM 1X dsDNA high-sensitivity kit. Samples were normalized to a concentration of 20 ng/μL, and sequenced using Oxford Nanopore Technology with a Ligation Kit. The final library concentration was 50 ng/μL, loaded into a MinION flow cell (FLO-MIN 106), where the highest number of reads were obtained after 24 h of sequencing.

The sequences were demultiplexed using the software Guppy (version 3.4.5) and Porechop (version 0.2.4). The resulting fastq pass files were analyzed using the amplicon sorter protocol through the command line, following the method outlined by Vierstraete and Braeckman in 2022. Subsequently, this consensus sequence was compared with the representative genomes accessible in the GenBank database. A phylogenetic tree was

constructed using the Maximum Likelihood method and Tamura Nei statistical model using MEGA X.

Results

Pathogenic *Leptospira* and characteristics of the peridomestic environment.

Urine samples of 70 animals were analized with a Taqman assay, where 48.57% (n=34/70) had *Leptospira* DNA. For details, see figure 4.

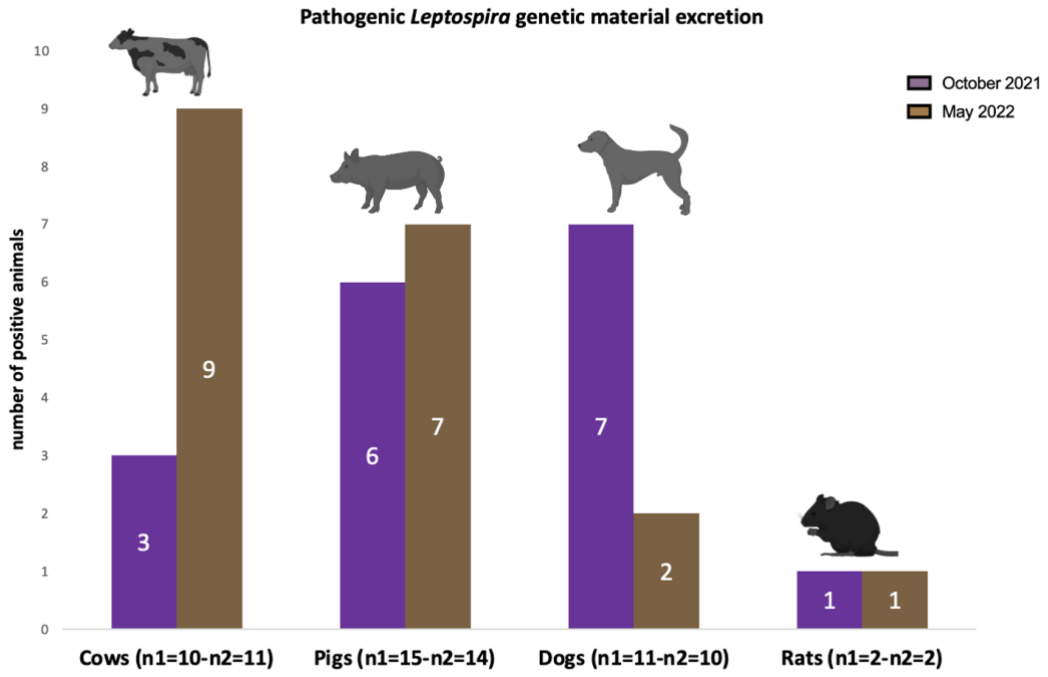

Figure 4. Comparison of number of animals excreting *Leptospira* DNA in urine between in October 2021 (purple) and May 2022 (brown).

Positive pigs were identified in four out of the six family units. Through group interviews, surveys, and observations, it was found that in all family units (6/6), the pens were located between 1 and 5 meters from the houses. The pens for these animals were built with wood in 2/6 family units, which facilitated the entry of small animals such as rodents, as shown in Figure 5B. Additionally, baby pigs were observed to interact with other animals, such

as dogs (Figure 5A). Furthermore, in these two family units with wooden pens, rats were found to carry *Leptospira* DNA in their kidneys.

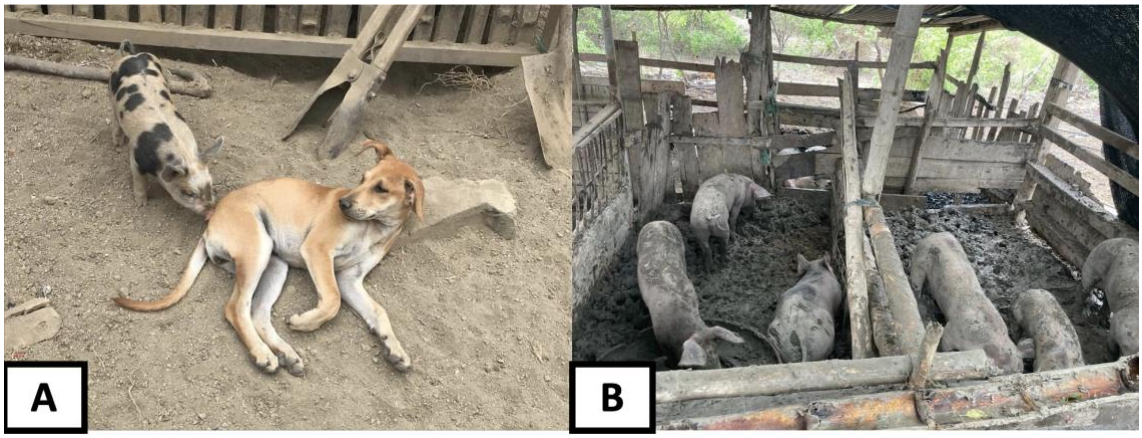

Figure 5. (A.) Interaction between animals, (B.) infrastructure of the corrals using wood.

Three out of eight family units confirmed the presence of rats in the surroundings. *Leptospira* DNA was found in rats collected from the unit with a wooden corral and grain storage. In contrast, dogs in four out of six family units were excreting the bacteria's genetic material, and in the two family units with cows, both of them tested positive.

As observed in figure 6 the drain out of the pens were open and, therefore, discharged directly into the surrounding environment.

Figure 6. Drainage of pig pens in one of the family units. The water that is coming out of the barn can be seen to be puddled outside and the formation of sludge can be seen.

Pathogenic *Leptospira* in humans and breeding practices.

Leptospira DNA was found in human urine: 15/23 (65.22%; CI 9.08-20.92) in October 2021 and 6/18 (33.33%; 95% CI 3.47-8.53) in May 2022.

The observed behavior in the family units and confirmed through group interviews, depicted people wearing open-toed shoes or walking barefoot when cleaning the pens or having contact with animals, as depicted in Figure 7.

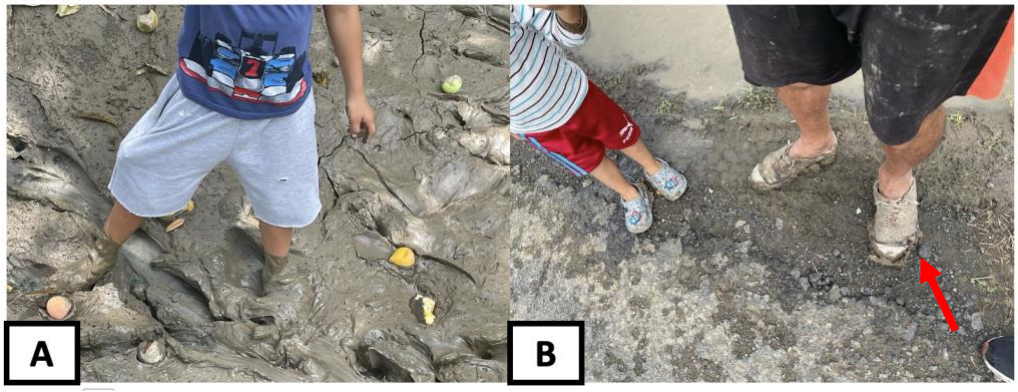

Figura 7. (A.) Walking barefoot arround home peridomestic environment, (B.) Wearing open shoes.

Pens were cleaned with untreated water in all family units (8/8). The water for animal consumption also came from the same source, while only humans consume bottled water. Breeding animals is considered as a family activity, with all the members participating, from children to adults. Through observations, it was noted that men are responsible for slaughtering the animals, but they do not use any protective clothing. In one of the family units, a slaughter was observed without wearing any footwear (see Figure 8) Even though, as previously mentioned in group interviews, it was stated that boots or closed footwear should be worn as a safety practice.

Figure 8. Man performing the slaughter of a pig without footwear.

Breeding animals is often a family activity. In 4 out of 6 cases, the individuals expressed a close relationship with the animals, even allowing children to hug pigs. In 2 out of 6 cases, the animals were given names. Group interviews revealed that women played a significant role in farming and caretaking practices. However, quantitative analysis showed no significant differences in the levels of *Leptospira* DNA excretion in urine between males and females (see Figure 9). Only in 1 out of 6 cases women played an important role in breeding, selling animals, and handling money.

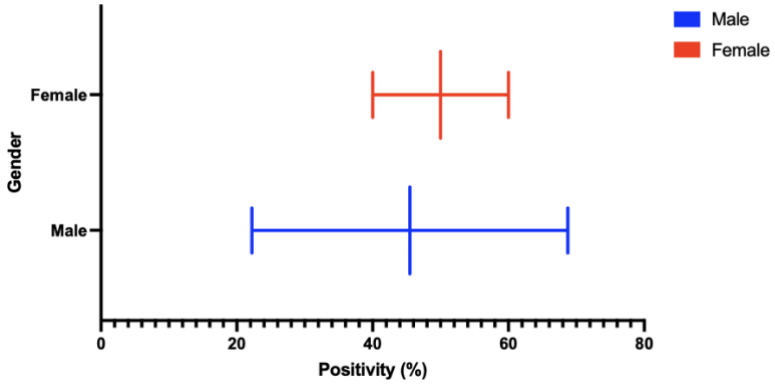

Figure 9. Analysis of *Leptospira* DNA excretion in human urine, paired t-test was used as statistical analysis. (No significant differences were found.)

According to group interviews, in 5 out of 6 family units veterinary assistance is provided exclusively at the time of the animal's birth. In 4 out of 6 family units, veterinary assistance is only provided when the animal is seriously ill, without a regular use of the service (see Table S3). This pattern is also observed in feeding, as only balanced feed is given to females after birth. Otherwise, young and adult animals are fed food waste.

High *Leptospira* exposure in all family units.

A total of 79 serum samples from Animals (56) and humans (23) were collected and tested using MAT; samples collected during October 2021 showed significantly higher seropositivity (86,07%, n= 68/79; 95% IC 53,12-82,88).

Multiple serovars were detected in the different sampled animal and human species. Interestingly, 84% of pigs (n=27/32), 75% of cows (n=9/12), 83.83% of dogs (n=10/12), and 13.04% of humans (n=3/23) co-reacted with multiple serovars. The predominant serovars in pigs were Icterohaemorrhagiae 1:400-1:2000, Grippotyphosa 1:400, Saxkoebing 1:200. In cows Canicola 1:400, Saxkoebing 1:400, Icterohaemorrhagiae 1:200. In dogs Autumnalis 1:400, Grippotyphosa 1:400. In humans Australis 1:200, Bratislava 1:200, Saxkoebing 1:200 (Table S1). Some serovars were found in multiple animal species: Icterohaemorrhagiae (cows and pigs), Saxkoebing (cows, pigs and humans), and Grippotyphosa (pigs and dogs).

***Leptospira* species detected in the family units.**

A total of 55 urine samples tested positive for TaqMan assays. In October 2021, 14 secY sequences were obtained, as well as 10 sequences in May 2022, where all of them showed 100% identity with *L. interrogans*. Phylogenetic analysis of secY gene sequences revealed distinct clusters related to *L. interrogans* in both October 2021 and May 2022. Alignment detected three single-nucleotide polymorphisms (SNPs), indicating the

presence of two different bacterial strains during each period. Notably, the secY sequences from different animals did not exhibit significant distance differences (see Figure 10).

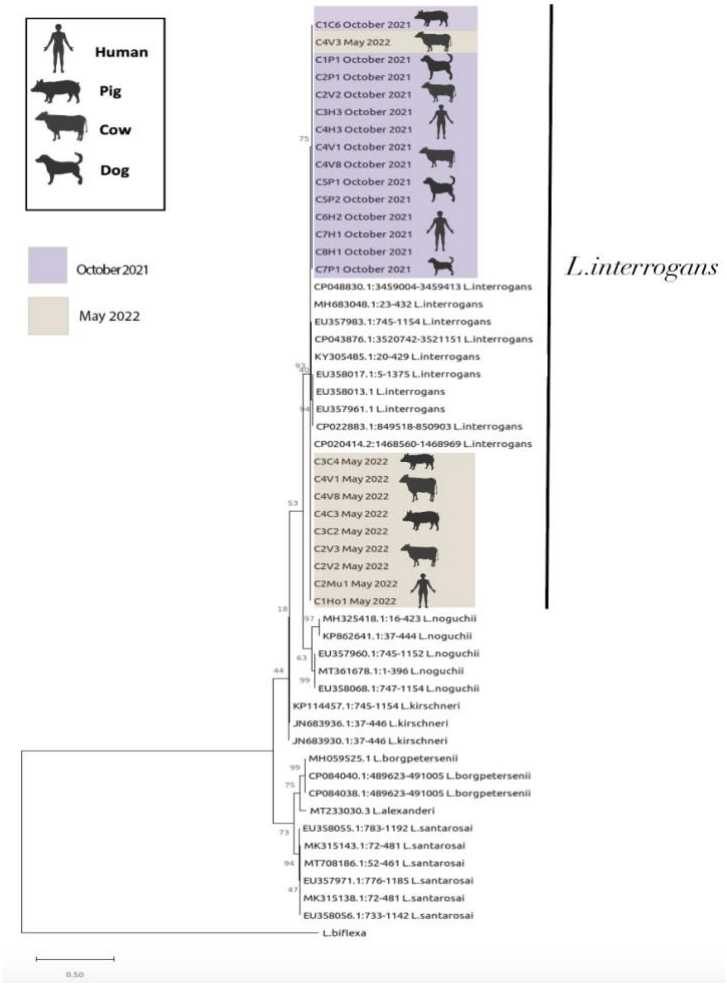

Figure 10. The phylogenetic analysis compares *Leptospira* species: samples from October 2021 and May 2022 were depicted in purple and brown, respectively. The analysis was conducted with Maximum Likelihood and Tamura Nei statistical model with a bootstrap value of 1000, using *L. biflexa* as an outgroup.

Discussion

Most rural communities in Ecuador rely on agriculture and livestock activities, such as cow and pig breeding. According to a study by Barragan et al. (2016) in rural areas near the Ecuadorian coast, pigs and backyard animals, in addition to rodents, can serve as reservoirs of multiple pathogens, increasing the risk of infection diseases.

The same research group conducted an additional unpublished previous study using 40 pig kidney samples from the Rocafuerte community collected between 2018 and 2019. The study found 31 sequences corresponding to pathogenic *Leptospira*. Based on this information and considering the high endemicity of pathogenic *Leptospira* in Manabí province, this study aimed to understand particularities of human behaviour that could be related to *Leptospira* infection.

Our results revealed similarities between the socio-environmental conditions and behaviors observed in Rocafuerte-Manabí and other regions of the world. This suggests that the findings of this study have applicability and relevance beyond the specific geographic area of study.

In this study, we identified a high exposure to *Leptospira*. It is important to note that none of the animals, including humans, exhibited symptoms related to leptospirosis at the time of sampling. All animals had been exposed, and some shared serovars. For instance, serovars Icterohaemorrhagiae were detected in cows and pigs, Saxkoebing in cows, pigs, and humans, and Grippotyphosa in pigs and dogs. This pattern is typical when evaluating populations as a whole (Dreyfus et al., 2021).

No significant differences were found in the excretion of bacterial DNA through urine in both periods. In all family units (8/8), at least one animal excreted *Leptospira* DNA. The probability of becoming infected with this bacteria increases in rural communities due to demographic and socio-environmental factors. (Taylor et al., 2022).

The study identified significant environmental factors in the analyzed family units, such as the construction of the pens. Two of the family units had pens made of wood, which had openings that allowed rats to enter. We observed several behaviors in the animals that confirmed the interaction between them. For example, a dog was observed entering the cow's pens and walking through their excrement. In this particular house, the drains carried water to peridomestic environments located very close to the houses.

In some cases, the proximity of the animal pens is due to the lack of land for expansion, which obligates farmers to keep them close (Ebata et al., 2020). However, in one of the family units we observed, they had extense land. Nevertheless, for the convenience of animal interaction and care, they preferred to have the corral close to the house.

Understanding *Leptospira* transmission requires three main components: humans, animals, and the environment (Narkkul et al., 2021b). Although environmental samples were not analyzed in this study, it was found that animals of different species, such as dogs, cows, and pigs, can coexist in the same physical space and defecate and urinate in the same location. When this happens, the drainage system that carries animal waste-contaminated water is directed towards the residential area, putting the residents at risk.

The study identified various factors that could contribute to the spread of *Leptospira* infection in the family units under investigation. Some of the practices that can lead to contamination include: people's attachment to the animals, inappropriate footwear during slaughtering or pen cleaning activities, and the use of untreated well water for animal feeding and pen cleaning.

Animal husbandry, particularly with pigs and cows, is often viewed as means of reducing poverty. However, the social impact of this practice and the significance of animal welfare are frequently disregarded. (Alary et al., 2011; Dietze, 2012). Animal husbandry communities face significant challenges, including lack of access to financial capital, low

profits due to production scale, animal diseases, and limited medical care for both animals and owners (Ebata et al., 2020). In the family units of this study most of the producers are small, so it is important to mention that in the time span between the first and the second visit one family unit enhanced the living conditions of their animals. Economic circumstances may also play a crucial role in this improvement.

Livestock activity is a fundamental form of subsistence in our study area, with the raising and sale of pigs being one of the main sources of income for most of the family units studied. Additionally, fewer family units raise cows for milk production.

In our study we observed that humans not only live near animal pens, but there is a sentimental attachment with animals. Although dogs are also part of the epidemiology of *Leptospira*, there is a significant difference in the level of affection or attachment that people show towards pigs and cows compared to dogs. In some cases, pigs receive names and even generate a higher level of affection from people, while most of dogs didn't have a name.

In the family units, it is common to use open shoes or even walking barefoot when working with animals: slaughtering animals or cleaning pens. These practices should be taken into consideration when implementing health interventions to prevent zoonotic diseases, always taking into account the behaviors of the community.

An good example is described in a study conducted in the United Kingdom (Garforth, 2015) emphasizes the significance of collaborating with farmers to comprehend why they don't follow the suggestions of veterinarians and scientists. It mentioned that farmers frequently base their practices on their personal experiences, whether positive or negative. If any recommendation or advice provided by veterinary health professionals has negatively impacted their profits or the welfare of their animals, they are less likely to repeat that action (Garforth, 2015).

Future research should focus on understanding the community's perception of the risk of contracting zoonotic diseases. Although this was a pilot study with only eight family units observed, it was found that is a discrepancy in the perception of risks associated with disease exposure during animal interaction or pen management. This can be accomplished through activities with the community, such as developing specific group interviews that help us to comprehend how individuals perceive risks and which factors they consider relevant to disease acquisition.

In some cases, farmers rely on their empirical knowledge and don't regularly consult veterinarians, unlike other places with similar characteristics (Garforth, 2015). This perspective may happen because farmers value the ancestral knowledge in animal care. Additionally, economic considerations are crucial, as expensive treatments and lengthy recovery times could result in significant financial losses.

Human exposure had been described principally in males (Afandi et al., 2023; Moinet et al., 2021). In this case, it is observed that both males and females excrete *Leptospira* DNA, this might be due that breeding activities are performed by all family members which includes the participation of both genders.

Although the bacteria's genetic material was found in urine in this study, a direct relationship with exposure could not be established because the bacterium could not be cultured with urine from these animals. To understand the epidemiology of *Leptospira*, a bacterial culture is essential as it is the definitive test to analyze transmissibility between humans and animals. (Sykes, Reagan, et al., 2022b).

In all family units we identified *L. interrogans* in both periods, which is consistent with previous research of the same group (Unpublished), which found a higher prevalence of *L. interrogans* in pig kidneys from the same community. However, this time we found a

variation in the *secY* sequence that suggests a change between time periods, which needs to be confirmed by whole genome sequencing.

This study examines the complex relationship between human behavior, breeding practices, and high exposure to *Leptospira* in a rural community on the coast of Ecuador. The emotional bond between people and their animals leads to the excretion of *Leptospira* DNA, which raises unresolved questions. Although the possible presence of different strains at different times is considered, it is crucial to confirm this hypothesis through culture and future epidemiological studies. In future studies, it will be important to confirm that these are live Leptospires that are capable of being transmitted. Despite the limited sample size, the study was strengthened by a mixed approach that integrated qualitative and quantitative variables, consolidating the ideas presented in this manuscript. This project is a pilot for the current NIH RO1 Project. The focus is on expanding sampling efforts to obtain *Leptospira* whole genomes and native cultures. This will strengthen not only the epidemiological understanding but also the diagnostic process through the MAT test.

Conclusion

This study shows that *Leptospira* is prevalent in all tested family units, with at least one animal in their peridomestic environment excreting *Leptospira* DNA during sampling. This indicates a consistent pattern of excretion over time, which may be linked to animal husbandry practices and peridomestic environments, such as the material used to construct corrals. Wooden corrals, in particular, have gaps that can allow animals, including rodents, to enter, facilitating interaction between them. One notable characteristic is the presence of water drains that flow into areas near homes or crowded places, which increases the risk of exposure to *Leptospira* for people. Additionally, the emotional bond between people and animals is significant, as animal care is often

considered a family task that involves people of all ages and genders. Furthermore, it has been observed that individuals engaging in activities such as slaughtering or cleaning corrals do not wear appropriate footwear. Additionally, there is an underdiagnosis due to the lack of veterinary care and the use of untreated water sources in animal-related activities. Moreover, it has been observed that different strains of the same species may circulate within the same population at different times, indicating that climatic, social, or environmental factors may influence the change of strains.

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SUPPLEMENTARY MATERIAL INDEX

Table S1. Detail of seropositivity in October 2021.

Animal	Serovar	Exposure percentage
Dog (n=12)	Cross reaction 1:200	83.83% (n=10/12)
	Autumnalis 1:400	8.33% (n=1/12)
	*Grippotyphosa 1:400	8.33% (n=1/12)
Cows (n=12)	Cross reaction 1:200	75% (n=9/12)
	Canicola 1:400	8.33% (n=1/12)
	*Saxkoebing 1:400	8.33% (n=1/12)
	*Icterohaemorrhagiae 1:200	8.33% (n=1/12)
Pigs (n=32)	Cross reaction 1:200	84% (n=27/32)
	Icterohaemorrhagiae 1:400	3.12% (n=1/32)
	*Grippotyphosa 1:400	3.12%(n=1/32)
	*Icterohaemorrhagiae 1:200	6.25% (n=2/32)
	*Saxkoebing 1:200	3.12% (n=1/32)
Humans (n=23)	Negative	52.17% (n=12/23)
	Cross reaction 1:200	13.04% (n=3/23)
	Australis 1:200	8.69% (n=2/23)
	Bratislava 1:200	21.73% (n=5/23)
	*Saxkoebing 1:200	4.34% (n=1/23)

*Serovars that appeared in more than one animal.

Table S2. Tabulation of the results obtained from the indivirual survey applied to hoseholds from the eight family units from the studied rural community.

Characteristics	Rural community n= 8 units
Flooding in the last month	0
Contact with rivers or ponds in the last month	1
Walking barefoot in river or lagoon	1
Animals near pond or river	1
Rats around house	3
Animals in backyard	8
Barefoot around house	1
Boil water	1
Store grains or products such as peanuts near home	4
Type of toilet in the house	
Septic tank	5
Connected to public sewage system	2
Latrine	1
Where the water comes from in the house	
Bottle for consumption	8
Public network for all other activities	8

Table S3. People behaviors and farming practices

Number of pigs per house	1-10 (4), more than 10 (2)
Feeding practice	
Female during breastfeeding	Balance mix and food waste (4), food waste and plants (2)
Juvenile and adult pigs	Balance mix and food waste (2), food waste and plants (4)
source of drink water	Untreated well water (2), untreated tap water (4)
Nearness and emotional bond with animals	
naming	yes (2), no (4)
hugs from family members	yes (4), no (2)
distance of pen from house	1-5 meters (5), more than meters (1)
Pen structure and cleaning	
Materials used for pens	wood (2), concrete (4)
Water used for cleaning	river or untreated well water (6)
frecuency of pen cleaning	daily (5), weekly (1)
tyoe of footwear	closed shoes (4), open shoes (2)
Veterinary guidance	
when animals are born	professional care (5), owner (1)
when sick or need assistance	professional care (2), owner (2), professional recommendations (2)
routine visits	professional veterinary care (0), owner (6)
Social	
Woman's role in farming	none (2), feeding and cleaning (4)
handling of money related to farming	woman (1), man (5)
Comercialization of pig	meat sold to neighbors and family (5), alive (1)

Note: in parentheses, the number of houses meeting the parameter