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Systemic Approach for Analyzing the Risk of Leptospirosis in France and its Application in Lebanon

Elena Harran

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**THESE de DOCTORAT DE
L'UNIVERSITE CLAUDE BERNARD LYON 1**

En cotutelle avec l'Université Saint Esprit de Kaslik Liban

**Ecole Doctorale N°205
Interdisciplinaire Sciences-Santé (EDISS)**

Discipline : Epidémiologie descriptive et santé publique

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Elena Harran

**Approche systémique d'analyse du
risque de la leptospirose en France et
son application au Liban**

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Abstract

Leptospirosis, caused by pathogenic species of *Leptospira*, is a zoonotic disease that poses a risk to public and animal health. *Leptospira* can infect multiple mammalian hosts and each host has a different role in the persistence of the pathogen in an ecosystem, which makes the epidemiology of the disease complex and restricts preventative measures. The ecological links between the multiple hosts in an ecosystem imply the existence of a maintenance community of leptospires, in favor of infecting target populations. Reducing the risk of infection in a given ecosystem implies precise knowledge of the maintenance communities of leptospires, including the key species for this persistence. The objective of this thesis was to explore populations hosts from different ecosystems to describe their relative implications in the maintenance community of leptospires and to apply and improve epidemiological investigation protocols for investigating this community to better understand the risk of infection within an ecosystem. We conducted studies in France and Lebanon using different epidemiological approaches and laboratory tools. We investigated leptospiral carriage in raccoons (*Procyon lotor*), nutrias (*Myocastor coypu*), muskrats (*Ondatra zibethicus*), Norway rats (*Rattus norvegicus*), and water voles (*Arvicola terrestris*) in France and cattle (*Bos taurus*) in Lebanon. According to our findings, raccoons were qualified as accidental hosts of *L. interrogans*, nutrias and muskrats as potential sources of human infection by *L. interrogans*, and water voles as maintenance hosts of *L. kirschneri* serogroup Grippotyphosa. Although cattle in Lebanon were found to be infected by *L. kirschneri*, their role remains unknown to date. The general findings of this thesis encourage the adaptation of adequate sampling strategies and laboratory tests to determine or clarify the role of animal hosts in the maintenance community. Additionally, they emphasize the need of adapting a One Health approach to monitor the disease and identify the threats to human and animal health and the ecosystems in order to minimize the risk of infection in France and Lebanon.

Keywords: *Leptospira*, Epidemiology, Public Health, One Health, Maintenance Community, Reservoirs, Diagnosis

Résumé

La leptospirose, causée par des espèces pathogènes de *Leptospira*, est une maladie zoonotique qui présente un risque pour la santé publique et animale. La bactérie peut infecter plusieurs hôtes mammifères et chaque hôte a un rôle différent dans la persistance de l'agent pathogène dans un écosystème, ce qui rend l'épidémiologie de la maladie complexe et limite les mesures préventives. Les liens écologiques entre les multiples hôtes d'un écosystème impliquent l'existence d'une communauté de persistance de leptospires, favorable à l'infection des populations cibles. Réduire le risque d'infection dans un écosystème donné implique une connaissance précise des communautés de persistance des leptospires, y compris des espèces clés de cette persistance. L'objectif de cette thèse était d'explorer des populations hôtes de différents écosystèmes afin de décrire leur implication relative dans la communauté de persistance des leptospires et d'appliquer et d'améliorer les protocoles d'enquête épidémiologique pour l'enquête de cette communauté afin de mieux comprendre le risque d'infection au sein d'un écosystème. Nous avons mené des études en France et au Liban en utilisant différentes approches épidémiologiques et outils de laboratoire. Nous avons étudié le portage des leptospires chez les ratons laveurs (*Procyon lotor*), les ragondins (*Myocastor coypu*), les rats musqués (*Ondatra zibethicus*), les rats surmulots (*Rattus norvegicus*) et les campagnols terrestres (*Arvicola terrestris*) en France et les bovins (*Bos taurus*) au Liban. Selon nos résultats, les ratons laveurs ont été qualifiés d'hôtes accidentels de *L. interrogans*, les ragondins et les rats musqués comme sources potentielles d'infection humaine par *L. interrogans*, et les campagnols terrestres comme hôtes de maintenance du séro groupe Grippotyphosa de *L. kirschneri*. Bien que des bovins au Liban aient été trouvés infectés par *L. kirschneri*, leur rôle reste inconnu à ce jour. Les résultats généraux de cette thèse encouragent l'adaptation de stratégies d'échantillonnage et de tests de laboratoire adéquats pour déterminer ou clarifier le rôle des populations hôtes dans la communauté de persistance. De plus, ils soulignent la nécessité d'adapter une approche « One Health » pour surveiller la maladie et identifier les menaces pour la santé humaine et animale et les écosystèmes afin de minimiser le risque d'infection en France et au Liban.

Mots clés : *Leptospira*, Épidémiologie, Santé Publique, One Health, Communauté de persistance, Réservoirs, Diagnostique

Scientific valuation of the thesis

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Harran, E.; Pinot, A.; Kodjo, A.; Djelouadji, Z.; Le Gudayer, M.; Sionfoungou Daouda, S.; Groud, K.; Lattard, V.; Ayrat, F. Identification of Pathogenic *Leptospira Kirschneri* Serogroup Grippytyphosa in Water Voles (*Arvicola Terrestris*) from Ruminant Pastures in Puy-de-Dôme, Central France. *Pathogens* **2023**, *12*, 260.

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Lekhal, L.; **Harran, E.;** Aragon, A.; Groud, K.; Le Guyader, M.; Kaidi, R.; Khelef, D.; Djelouadji, Z. First Molecular Detection of Pathogens *Leptospira* in Common Rodent Captured in North Algeria Urban Areas. *Tropical Medicine and Infectious Disease*. **2022**, *7*, 335.

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List of abbreviations

| | |
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| Centre National de Référence de la Leptospirose (CNRL)..... | 25 |
| Conventional PCR (cPCR)..... | 21 |
| Deoxyribonucleic acid (DNA)..... | 13 |
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I. Introduction to leptospirosis, thesis problematic and epidemiological objectives

1 *Leptospira* organism and microbiological characteristics

1.1 *Leptospira* (the organism)

Leptospire belong to the genus *Leptospira*, the family of *Leptospiraceae* in the phylum Spirochaetes [1]. Leptospire are long, slender, tightly coiled, flagellated, gram negative, strictly aerobes spirochetes with optimum growth at a temperature of 28 to 30°C and a pH range of 7.2 to 7.6 [2]. The form, size, and motility of the microorganism remain relatively consistent [3]. Nevertheless, freshly isolated pathogenic *Leptospira* from a mammalian host are typically shorter and more tightly coiled compared to saprophytic strains and laboratory strains that underwent sequential passage [4]. The latter (laboratory strains) can lengthen to an excessive degree which impairs cell health and decreases mobility [4]. The spectrum of temperatures at which saprophytic and pathogenic organisms will thrive is different. Although both saprophytic and pathogenic leptospire grow best at a temperature of 28 to 30°C, saprophytic leptospire solely can also grow at low temperatures (11 to 13°C) and pathogenic leptospire solely can continue to grow at high temperatures (37 °C) [3].

A genomic and a serological classification exist and both are independent and uncorrelated. The genomic classification depends on the deoxyribonucleic acid (DNA) composition of the strain and allows the repartition of *Leptospira* genus into genomic species [5]. However, a serological classification is based on cell surface antigens, allowing serotypes identification. Serovars belonging to a unique serogroup are found in distinct *Leptospira* species. Since Stimson et al. first described the genus *Leptospira* in 1907 [6], the latter has been conventionally classified into two clades—saprophytes and pathogens—based on their virulence and their genetic characteristics [2]. Although phylogenetic analysis revealed an additional *Leptospira* clade referred to as an intermediate group subsequently [7], the repartition of *Leptospira* species was recently recategorized into saprophytes and pathogens (grouping intermediates) [8]. To date, the genus *Leptospira* comprises 68 genomic species grouping 40 pathogenic species [9]. Among the latter species, *L. interrogans*, *L. kirschneri*, and *L. borgpetersenii*, are the most predominant ones circulating worldwide, with *L. interrogans* mostly retrieved in *Leptospira* cases and *L. kirschneri* being regularly associated with humans outbreaks [10–12]. *Leptospira* species are identified by serogroups that are further divided into various serovars according to the serological classification [13]. For convenience, antigenically related serovars form a unique serogroup [13,14]. Although serogroups

have no taxonomic classification, they have proven preliminary useful for serological diagnosis and epidemiological understanding at a local or regional level.

1.2 Serological classification

Both pathogenic and saprophytic *Leptospira* were classified into numerous serotypes under the traditional serological classification first proposed by Wolf and Broom in 1954 [14]. These serotypes were determined by agglutination following cross-adsorption—by the cross-agglutinin adsorption test—with homologous antigens. The word "serotype" was revised as "serovar" (serologic variation) by Kmety and Dikken in 1973 [13]. Two strains are classified as belonging to separate serovars if, at least one of the two anti-sera consistently retains 10% or more of the homologous titer in subsequent testing following cross-adsorption with sufficient quantities of heterologous antigen [15]. The list of *Leptospira* serovar exceeds 250 serovars and it is still being updated and revised as of now [9,13]. Antigenically related serovars are classified into serogroups, which are helpful for diagnostic and epidemiological purposes but have no taxonomic validity. Nevertheless, determining the serological status of an individual can be crucial as it helps understand the pathogenesis of a disease and the immunological response of the infected host. It also aids in the diagnosis process and determining the host's particular vaccine immunization.

1.3 Molecular classification

At first, the taxonomy of *Leptospira* bacteria, and that of other bacteria, was not completely determined. Techniques for measuring evolutionary differences in the structure of nucleic acids have brought about a significant shift in the taxonomy of bacteria. DNA-DNA hybridization was the first technic conceived and was referred to as the "gold standard" technic back in the time for *Leptospira* species delineation [16]. Soon after that, distinct DNA-based classification methods such as DNA guanine and cytosine content [17] or pulse-field electrophoreses [18] were undertaken and provided recent taxonomic knowledge. The next turning point was the study of small subunit ribosomal ribonucleic acid (rRNA) that redefined the taxonomy of the genus *Leptospira* [19]. The advantage of 16S rRNA analysis when compared to DNA-DNA hybridization was its simplicity and accessibility to several laboratories. Nevertheless, the 16S rRNA sequence analysis remained a limitation to taxonomic determination as it only represents 0.05% of an average 3-Mbp prokaryotic genome and cannot execute a reliable phylogenetic assessment at the species level due to the great conservation of the sequence [20]. It is therefore clear that the best way to acquire a reliable and complete phylogenetic inference, that will consequently determine the taxonomy, will be the sequence of a complete DNA sequence through Whole Genome Sequencing

(WGS) [21]. Ever since, the number of genomes that were currently available has grown tremendously and a review of the genus taxonomy was compelled through the sequencing of the DNA of 90 *Leptospira* strains [8]. To date, 68 genomic species of the genus *Leptospira* have been recognized [9] divided into two major clades: pathogens (P) and saprophytes (S), with two subclades each, P1 (species previously denoted as pathogens), P2 (species previously denoted as intermediate), S1 and S2 (saprophytic species)(Table 1) [8]. The first clade (pathogens) includes the organisms that cause illnesses in both humans and animals and the second clade (saprophytes) includes the species that are isolated from their natural habitat. However; taxonomic revision is still being undertaken today to identify further species, if available.

Table 1. List of the 68 *Leptospira* species with their corresponding subclades according to Arent et al, 2022 [9].

| Species | S* | Species | S* | Species | S* | Species | S* |
|---------------------------|----|---------------------------|----|--------------------------|----|----------------------------|----|
| <i>L. adleri</i> | P1 | <i>L. weili</i> | P1 | <i>L. saintgironsiae</i> | P2 | <i>L. kanakyensis</i> | S1 |
| <i>L. ainazelensis</i> | P1 | <i>L. yasudae</i> | P1 | <i>L. sarikeiensis</i> | P2 | <i>L. kemamanensis</i> | S1 |
| <i>L. ainlahdjerensis</i> | P1 | <i>L. andrefontaineae</i> | P2 | <i>L. selangorensis</i> | P2 | <i>L. levettii</i> | S1 |
| <i>L. alexanderi</i> | P1 | <i>L. broomii</i> | P2 | <i>L. semungkikensis</i> | P2 | <i>L. meyeri</i> | S1 |
| <i>L. alstonii</i> | P1 | <i>L. dzoumogneensis</i> | P2 | <i>L. venezuelensis</i> | P2 | <i>L. montravelensis</i> | S1 |
| <i>L. barantonii</i> | P1 | <i>L. fainei</i> | P2 | <i>L. wolffii</i> | P2 | <i>L. mtsangambouensis</i> | S1 |
| <i>L. borgpetersenii</i> | P1 | <i>L. fletcheri</i> | P2 | <i>L. abararensis</i> | S1 | <i>L. noumeaensis</i> | S1 |
| <i>L. ellisii</i> | P1 | <i>L. fluminis</i> | P2 | <i>L. bandrabouensis</i> | S1 | <i>L. perdikensis</i> | S1 |
| <i>L. gomenensis</i> | P1 | <i>L. haakeii</i> | P2 | <i>L. biflexas</i> | S1 | <i>L. terpstrae</i> | S1 |
| <i>L. interrogans</i> | P1 | <i>L. hartskeerlii</i> | P2 | <i>L. bourretii</i> | S1 | <i>L. vanthieli</i> | S1 |
| <i>L. kirschneri</i> | P1 | <i>L. inadai</i> | P2 | <i>L. bouyouniensis</i> | S1 | <i>L. wolbachii</i> | S1 |
| <i>L. kmetyi</i> | P1 | <i>L. johnsonii</i> | P2 | <i>L. brenneri</i> | S1 | <i>L. yanagawae</i> | S1 |
| <i>L. mayottensis</i> | P1 | <i>L. koniamboensis</i> | P2 | <i>L. chreensis</i> | S1 | <i>L. idonii</i> | S2 |
| <i>L. noguchii</i> | P1 | <i>L. langatensis</i> | P2 | <i>L. congkakensis</i> | S1 | <i>L. ilyithenensis</i> | S2 |
| <i>L. santarosai</i> | P1 | <i>L. licerasiae</i> | P2 | <i>L. ellinghauseni</i> | S1 | <i>L. kobayashii</i> | S2 |
| <i>L. stimsonii</i> | P1 | <i>L. neocaledonica</i> | P2 | <i>L. harrisiae</i> | S1 | <i>L. ognonensis</i> | S2 |
| <i>L. tipperaryensis</i> | P1 | <i>L. perolatii</i> | P2 | <i>L. jelokensis</i> | S1 | <i>L. ryugenii</i> | S2 |

S*, subclade

2 Pathogenesis and clinical features of leptospirosis

2.1 Pathogenesis of leptospirosis

The incubation period from the exposure to pathogenic *Leptospira* species to the onset of clinical features is generally 7 to 12 days but can be as short as 2 days or as long as 21 [22] or even 29 [23] days in the case of humans. In the case of animals, it varies slightly depending on the animal species. For instance, it ranges from 3 to 20 days in cattle [24] and from 5 to 15 days in dogs [25]. Following the latter period, hematogenous dissemination—the initial stage in the pathogenesis of both humans

and animals—occurs and pathogenic leptospires penetrate the bloodstream and persist therein throughout the leptospiremic phase of the illness. The leptospiremic phase typically starts 1 to 2 days following infection and endures for a week, when acute clinical manifestation occurs [10,26]. Leptospiremia can be diagnosed by quantitative polymerase chain reaction (PCR) and blood inoculation, and results have a greater likelihood to come in positive during the initial week of infection and can last up to 10 days before antibodies are formed and the leptospires are cleared from the bloodstream [27]. Following leptospiremia, leptospires eventually reach their target organs, which mainly include the lungs, liver, and primarily, the kidneys' proximal tubules, where leptospires can multiply and persist for several months and be voided in the urine (case of animals) during the leptospiruria [10,11]. Antibodies are detectable in blood serum through indirect tests (microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA)) in both humans and animals from 10 to 14 days following infection and reach maximum levels at around 3 to 6 weeks approximately [10,26].

2.2 Clinical features in humans

The severity of human leptospirosis relies on the host's age, health, and immunological competitiveness as well as the virulence features of the infecting strain and the infection dosage [28]. Once infected, humans have a wide variety of clinical symptoms that can fluctuate from being asymptomatic to fatal, however, most cases are subclinical or asymptomatic and do not require medical attention [29]. Several studies reported asymptomatic infections in individuals with recent leptospiral infections [30–32]. A study in Nicaragua reported 70.6% (60 out of 85) asymptomatic patients with positive immunoglobulin M (IgM) anti-*Leptospira* antibodies [30]. A second seroprevalence study in Bangladesh reported that 48% (15 out of 31) asymptomatic patients that were primordially selected as control patients turned out seropositive for leptospiral infection [31]. A third study in Seychelles reported that none of the MAT or PCR-positive control individuals expressed acute or chronic symptoms [32]. In symptomatic cases, the most recurrent symptoms that have been noted include mild fever, leptospirosis being recognized as a febrile illness, the latter symptom is generally reported in most [33] or all [34] symptomatic patients. Headache, myalgia, nausea, conjunctival suffusion, and petechiae have also been noted [26]. Some cases are more critical and involve jaundice, acute renal failure, meningitis, myocarditis, and pulmonary hemorrhage [35] and mortality rates that vary between 5% to 20% [36]. Acute renal failure and diffuse alveolar hemorrhage occur in 10% [37,38] and 3.7% [39,40] of leptospiral infection cases, respectively, and are linked to considerable morbidity and death, mainly in the case of diffuse alveolar hemorrhage

(mortality rates greater than 70%). The probability of a fatal result rises with increased age; a study showed that in comparison to patients aged 19 to 29, the rate increased by 3.7 times for patients in their 40s and 50s and by 7.3 times for those in their 60s [41]. Clinical manifestations of leptospirosis recurrently mimic several infections with febrile syndromes such as malaria [42], dengue fever [43], and hantavirus [44]. Thus, the disease is regularly misdiagnosed which causes its occurrence to be underestimated [45].

2.3 Clinical features in animals

Clinical symptoms in most animals that have been infected by host-adapted serovars are typically negligible or nonexistent [10]. However, clinical signs for infections caused by non-adapted serovars can range from moderate to severe, with severe occurrences more frequently seen in young animals [10]. Elevated body temperature, anorexia, red eyes, hematuria (and occasionally diarrhea), meningitis, hemorrhages, hemoglobinuria, and jaundice are typical symptoms observed in infected animals. For instance: hematuria, hemoglobinuria, and jaundice are commonly reported in sheep, and myalgia, anorexia, and vomiting are commonly reported in dogs [10]. Pyrexia, anorexia, jaundice, hemoglobinuria, and occasionally meningitis are commonly reported in calves, and agalactia is mainly reported in lactating cattle during the acute phase of infection [10]. Non-adapted serovars can also cause chronic infection—mainly in livestock—and result in abortion, stillbirth, and underdeveloped offspring, causing major economic impacts.

Animal renal carriage and leptospire dissemination into the environment are the focal points in the epidemiology of leptospirosis. Renal persistence and urine excretion may endure for weeks or years. For instance, *Leptospira* shedding in dogs begins on day 7–10 following infection and endures for 4–6 weeks [46], occasionally even over several years [47]. The length and amount of urinary shedding varies according to the infecting serogroups [25]. Cattle can shed leptospire for up to 40 weeks [48] and the risk of urinary shedding decreases with the increase of cattle age [49,50]. As for cats, they can shed leptospire for up to 8 months [51]. Leptospire might also have a significant affinity for organs other than kidneys, such as the genital tract, facilitating interspecies sexual transmissions [52]. Except for rare circumstances—animals with weakened immune systems, such as neonates or pregnant females in their late stages—host-maintained leptospire are ideal parasites that don't negatively impact their hosts' health [10].

3 Laboratory diagnosis

Diagnostic tests for determining *Leptospira* carriage in different specimens are numerous and can be applied to all mammalian species. Direct methods allow the determination of infection, disease prevalence, and relative risk associated with the examined species [53]. Their complementary application are satisfactory for pathogen identification as reported in a recent study [54]. Indirect serological methods that are based on antibodies detection are also commonly used for the diagnosis of leptospirosis, despite their numerous drawbacks such as the requirement of laborious work and a panel of live serovars, mainly recently-isolated only found in certain laboratories [28,55].

3.1 Indirect diagnostic

Serological tests are an important benefit in epidemiology as they allow the identification of the serogroups carried by an animal host [56]. The latter tests rely on the detection of Ig in humans and animals that share a similar pattern of reaction to IgM and IgG class antibodies [57]. During an infection, IgM antibodies appear initially (on the second day following the beginning of symptoms) and persist for a short time, contrary to IgG antibodies that appear following the IgM antibodies and persist longer [58]. Consequently, IgM antibodies detection indicates an early stage of infection whereas IgG normally indicates a residual immune response resulting from a past infection [59].

3.1.1 Microscopic agglutination test

Today, MAT continues to be the standard method for leptospirosis serological diagnosis in both people and animals [60,61]. The test is carried out on patient sera by specialized laboratories. In MATs, patient serum is titrated with live antigen suspensions, and agglutination, which is visible by dark-field microscopy, is inspected. It is routine to test different serogroups. The MAT test identifies the serogroup but is not accurate enough to detect serovars since cross-reactivity commonly occurs amongst serovars both within a particular serogroup and between different serogroups. The test requires live cultures of various serovars from precise geographic locations as controls [62]. For France, the list of the reference strains employed in the MAT antigen panel is mentioned in Table 2. MAT is extremely specific (specificity of 97% [63], 98.8%[64], and 100% [65]) for detecting antibodies in convalescent serum. However, its sensitivity is considered low on acute-phase serum, as demonstrated in a prospective study where the results of MAT in non-leptospirosis cases and in cases with isolates (*i.e.*, blood, urine, or dialysate fluid isolates) were used to assess the accuracy of the test and determined a sensitivity of 30% in the acute phase

[63]. In a different investigation, Goris and colleagues used controls having other known diseases and culture-positive individuals as leptospirosis-infected subjects (evidence of leptospirosis), to determine the accuracy of MAT and reported a sensitivity of 2.1% [65]. These findings support the notion that MAT has minor importance in diagnosing and treating the illness early on. However, the sensitivity increases in the immune phase and can reach 76% [63] or 81.7% [65]. Consequently, to boost the sensitivity of the test and solidify the diagnosis, paired serums of both acute and immune phases are required [2]. Although the test is endowed with a high specificity [63–65], one of its limitations is that it does not distinguish between antibodies that are the result of an infection or a vaccination, especially in the case of individuals that rarely show clinical signs. Consequently, it is crucial to know the individual's vaccination history, especially in animal leptospirosis diagnosis where vaccination is widely distributed, however, interpretation can be challenging in some cases. For instance, experimental studies that investigated the serological response in cattle following vaccination against serovars Hardjo and/or Pomona revealed that the vaccine-induced antibodies titers can range from undetectable to a high of 3000 and that those antibodies titers often fade quickly (don't persist for more than six months following inoculation) [66,67]. The variation in the magnitude and durability of MAT antibody titers following vaccination may be caused by the quantity and dosages of vaccinations administered as well as the age of the cattle [68]. MAT antibody titers also vary depending on the animal species and the vaccination status and can range from 1:20 (case of rats (*Rattus*) [69]) to 1:6,400 (case of dogs [70]). Therefore, MAT results interpretation should also take into consideration the sampled population(s).

Table 2. List of the reference strains employed in the MAT antigen panel of France

| Serogroup | Serovar | Strain |
|---------------------|---------------------|-----------------|
| Australis | Muenchen | München C 90 |
| Australis | Australis | Ballico |
| Australis | Bratislava | Jez Bratislava |
| Autumnalis | Autumnalis | Akiyami A |
| Autumnalis | Bim | 1051 |
| Ballum | Castellonis | Castellòn 3 |
| Bataviae | Bataviae | Van Tienen |
| Canicola | Canicola | Hond Utrecht IV |
| Grippotyphosa | Grippotyphosa | Moskva V |
| Grippotyphosa | Vanderhoedoni | Kipod 179 |
| Icterohaemorrhagiae | Icterohaemorrhagiae | ENVN |
| Icterohaemorrhagiae | Copenhageni | M 20 |
| Panama | Panama | CZ 214 K |

| | | |
|-----------|------------|-------------------|
| Panama | Mangus | TRVL/CAREC 137774 |
| Pomona | Pomona | Pomona |
| Pomona | Mozdok | 5621 |
| Pyrogenes | Pyrogenes | Salinem |
| Sejroe | Sejroe | M 84 |
| Sejroe | Saxkoebing | Mus 24 |
| Sejroe | Wolffii | 3705 |
| Sejroe | Hardjo | Hardjoprajitno |
| Tarassovi | Tarassovi | Perepelitsin |

3.1.2 Enzyme-linked immunosorbent assay

The development of ELISA test that delivers quick findings regarding leptospiral infection in humans and animals without requiring culture or MAT facilities was encouraged as an alternative to MAT known for the complexity of its performance [71,72]. In addition, ELISA test is less costly, safer, and more easily performed and provides less subjective findings than MAT. The ability to distinguish between IgM and IgG serological responses is another benefit of ELISA over MAT. Depending on the type of antibodies being tested by the ELISA, the earliest period following infection at which an antibody may be detected by that method may change [73]. Despite its advantages, a remarkable variability in the sensitivity and specificity estimates of ELISA was recognized by the performance of a commercial IgM ELISA and was reported across different geographical locations such as Hawaii [74], Thailand [75], and the United States of America [76]. Consequently, ELISA has been suggested by the World Health Organisation (WHO) as a leptospirosis diagnostic test in areas with limited access to healthcare, solely. Furthermore, the test requires validation for diverse epidemiological situations as each situation groups a different studied population and specific serogroups [61].

3.2 Direct diagnostic

3.2.1 Culture

Leptospire grow in a medium supplemented with the vitamins B1 and B12, long-chain fatty acids, and ammonium salts, however, they must first be cultivated prior to antibiotic treatment [28]. The most common medium culture for leptospirosis is the EllinghausenMcCullough-Johnson-Harris (EMJH) [77]. Typically, EMJH mediums are used to isolate leptospire from blood, urine, and fresh tissues (mainly kidneys, liver, and brain) of infected individuals [78], before being frozen [35]. They can be isolated from blood during the first week of illness, where it is unlikely to be found in urine at such a stage [1]. Thus, the culture of urine is more successful two weeks following symptoms, with the higher success rate between 14-28 days [1,35]. The culture of

leptospire from human and animal specimens may be affected by a variety of factors, such as the type of the sample and the conditions of its sampling, its survival in the primary tissue or body fluid sample that was collected, the host's immune system response, and its exposure to administered antibiotics, as antibiotic treatment can quickly remove leptospire from blood and reduce urine shedding [1,61]. Although endowed with a high specificity, diagnosis of *Leptospira* through culture is regarded as long and difficult since its sensitivity is low, nevertheless, its ability to determine the viability of a pathogen makes it important for epidemiological studies [79].

3.2.2 Polymerase Chain Reaction

Since it was initially discovered in 1990 [80], diagnostic procedure based on amplifying derived DNA by the PCR is considered more appropriate than culture for *Leptospira*-detection, in terms of velocity and sensitivity, therefore PCR is mainly employed in epidemiological studies [61,81] for a direct diagnosis of pathogenic organisms [82]. It is thought to be a reliable method for detecting *Leptospira*-derived DNA in the course of early infection and allows for the treatment of the illness and the prevention of zoonotic transmission [61,81,83]. In the PCR-based assays, a target DNA fragment of *Leptospira* strains is amplified using a specific set of primers. A particular set of primers targeting the *L. interrogans* serovar canicola rrs (16S) gene sequence (331 base pair) has undergone considerable clinical assessment by Merien et al. (1992) [84] and is now often used for the diagnosis of *Leptospira* species. The latter set of primers amplifies the DNA of both saprophytic and pathogenic *Leptospira* strains in humans and animals in numerous clinical specimens (blood, cerebrospinal fluid, urine, etc.) through a conventional PCR (cPCR) [84–86]. The sensitivity of cPCR was 95% (CI_{95%} 76% - 100%) (Low Limit of Detection (LLOD) of 1 pg by electrophoresis gel), and the specificity was 42% (CI_{95%} 23% - 63%) [84,87]. With time, a more rapid and sensitive PCR-based assay (100% (CI_{95%} 85% - 100%), LLOD was 29 copies/μL) which also targets the 16S gene, was developed to specifically detect pathogenic *Leptospira* strains, solely, and is widely used nowadays in epidemiological studies [88]. In addition to the two latter sets of primers that are extensively being used today, we can also find assays using primers aimed at additional genes. PCR methods targeting the flaB gene [89], the lipL32 gene [90], and the partial sequence of 23S rRNA gene [91] are commonly used as they allow the detection of pathogenic *Leptospira* in DNA extracted from clinical specimens during the acute phase of infection. Furthermore, PCR targeting the flaB gene [89] allows the identification of pathogenic *Leptospira* species without the need for genotyping by the flaB-PCR-Restriction Fragment Length Polymorphism system.

3.2.3 Molecular typing methods

The identification of infectious *Leptospira* strains through molecular typing methods such as PCR is crucial in epidemiological studies as it aids in understanding the cycle of leptospirosis in an ecosystem and adapting preventative measures, relatively. PCR amplified products are visualized on an electrophoresis gel and are, in some cases, subject to sequencing to determine the amplified *Leptospira* strains. Different PCR methods exist and the level of information procured by each method differs. For instance: cPCR assays that are based upon amplification of *Leptospira* rrs (16S) gene [84] are limited to identifying *Leptospira* species. However, cPCR is less likely used than real-time PCR (rtPCR) [88] since rtPCR provides rapid and more accurate results due to its unique sensitivity and specificity and its low contamination risk [92,93]. Molecular typing of leptospiral strains based on O-antigen [94] and variable-number of tandem-repeat (VNTR) [95] are rapid typing methods, giving taxonomic information without the need for sequencing, revealing the methods' relatively inexpensive cost [96]. O-antigen gene cluster-specific PCR is based on targeting and discriminating a *Leptospira* specific-serogroup genes [94]. However, VNTR is based on identifying genetic profiles related to particular serogroups and serovars of *Leptospira* called genogroup and genovars [95]. Although the benefit of the VNTR strategy lies in differentiating the genetic profile of PCR products on agarose gels according to the size of the (short) nucleotide sequence repeats, the method has certain limitations such as the need for a significant amount of high-quality DNA [97]. In addition, its results are difficult to interpret, due to a lack of discriminating power, and poor repeatability, when compared to those provided by the O-antigen PCR method, known to confer serological specificity relative to the O-antigen gene of the targeted serogroup [94,97]. Mutli-spacer Typing (MST) is a typing method that relies on the sequencing of 3 intergenic regions for which a genotype number is assigned and an MST profile is generated to identify the implicated *Leptospira* strain among the 33 strains present in the literature [98]. Multi-locus sequence typing is another typing method based on DNA sequencing and is employed to study the allelic diversity of targeted genes [99]. It is normally used to determine clusters among outbreak isolates [100]. The latter typing methods are restricted to identifying *Leptospira* strains solely as they target specific genes and can be used in leptospirosis epidemiological studies. Nevertheless, a broad visualization of circulating pathogens, including *Leptospira*, can be of a better indicator of the risk to human and animal health in an ecosystem.

3.2.4 Whole Genome Sequencing

Nowadays, WGS is replacing DNA typing methods as well as (Sanger) single gene sequencing techniques and is traditionally being used in epidemiological research and

public health investigations of infectious diseases [101–103]. Due to its maximum phylogenetic resolution for revealing transmission networks and pinpointing illness sources, WGS is regularly used for genomic surveillance in several nations [104], monitoring and the detection of pathogens and infection prevention and control [105,106], and was qualified as the reference typing approach utilized in studying outbreaks [101,107] (*e.g.*, the listeriosis and coronaviruses outbreaks [108,109]) or their early detection [110,111]. In the context of leptospirosis, WGS has aided in revising the taxonomy of the disease and the development of the pathogenicity of the genus *Leptospira*. However, to our knowledge, WGS was not utilized for epidemiological purposes (*i.e.*, studying the maintenance community, detecting the source of outbreaks in leptospirosis) due to limited access of certain laboratories to *Leptospira* strains and the need of performing culture from fresh tissues (non-frozen). Still, the method is endowed with the capacity to transfer gene sequences, enabling quick and efficient national, international, and cross-sectoral data sharing which can aid in commutating the infectious *Leptospira* strain acquired in different countries. This improves surveillance systems' ability to swiftly spot connected cases in situations when epidemiological connections lack [112] and could help in classifying animal hosts into categories (maintenance or accidental hosts). Nevertheless, the direct expenses of switching to WGS for laboratories are strenuous as it includes several obstacles such as the high cost of equipment and consumables as well as safety and training requirements, and the modifications of workflows and procedures commonly requiring staff restructuring [113]. Moreover, data management infrastructure and proper bioinformatics competence are needed to generate, analyze, visualize, and store genetic data. Furthermore, the quality of the genomic DNA [114,115], the techniques employed to prepare the libraries [116], and the various sequencing platforms may all have an impact on the clinical WGS outcomes [117].

4 Transmission pathways and epidemiology of leptospirosis

4.1 Transmission of *Leptospira*

Although exposures may occur directly through contact with body fluid of *Leptospira*-infected animals, most occur indirectly when exposed to leptospires-contaminated settings with a higher incidence in warm and humid climates (Figure 1) [118,119]. Human-to-human transmission of leptospirosis has been rarely reported [120] as the disease is usually not transmitted from one person to another [26]. The magnitude of infection risk in a particular environment depends on the prevalence of leptospiral carriage among infected hosts as well as the susceptibility of the hosts to circulating serogroups(s). Leptospirosis mostly affects people who partake in certain occupational

activities, such as mining, farming, fishing, veterinary medicine, military service, abattoir work, and sewer labor [35,121]. Furthermore, water recreational activities such as canoeing, kayaking, rafting, caving, and triathlons have also been identified as an inconspicuous risk factor for infection [122], with a high potential for large outbreaks during competitive events [123]. The typical entry ports include abrasions of the skin and exposed mucosal membranes (oral, nasal, and conjunctival) [124]. Consuming contaminated food is not believed to be a source of infection [1], but ingesting contaminated water is thought to increase your risk of infection [23]. Additionally, prolonged submersion may make the skin more permeable and enable leptospiral invasion even in the absence of skin abrasions [1].

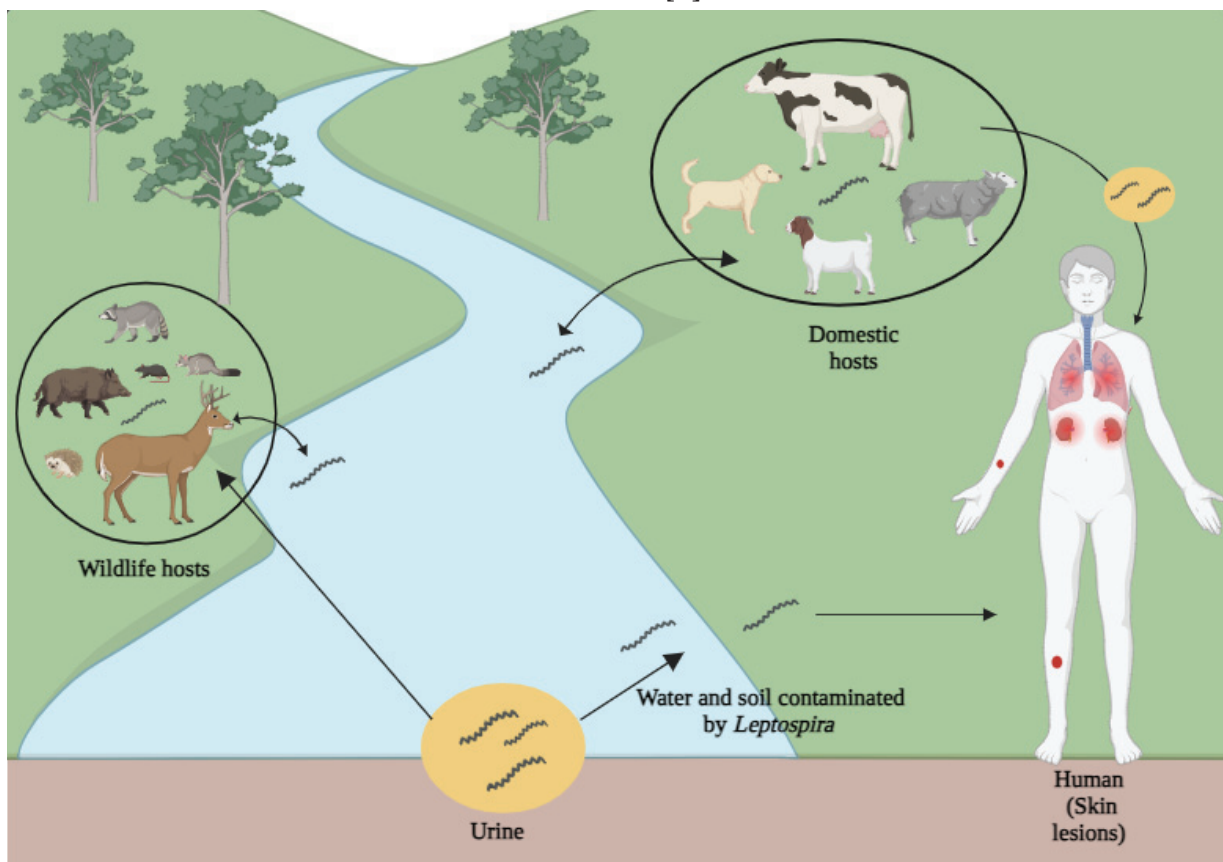


Figure 1. Life cycle of *Leptospira* and its transmission pathways. Adapted from López-Robles, 2021. Created by Harran, E., on the 1st of March 2023 using <https://BioRender.com>

4.2 Situation and burden of leptospirosis worldwide

Leptospirosis is a bacterial zoonosis that imparts a global burden, primarily affecting resource-poor populations and warm and humid settings [2,36]. Areas most prone to infection are the tropical and subtropical [1,125,126]. Human leptospirosis morbidity and mortality rates are estimated at 1.03 million (CI_{95%} 434,000 – 1,750,000) and 60 000 (CI_{95%} 23,800 - 95,900) cases based on age and gender demographic attributes of the population [36]. From the estimated annual human cases, approximately 2.90 million (UIs 1.25-4.54 million) Disability Adjusted Life Years

(DALYs) are annually lost, with DALYs representing an estimate of the disease's financial losses [36]. Such an estimate is high when compared to other zoonotic diseases such as dengue fever for which DALYs are approximately 2.31 million (UIs 2–2.62 million) [127]. Furthermore, the incidence of the illness has been estimated to range between 0.1 and 1 per 100,000 people in temperate areas, although it may be ten times higher in tropical regions [36].

The disease has a marked seasonal pattern, with an upsurge in cases linked to heat, precipitation, and extreme climatic phenomena observed in late summer and autumn seasons in temperate countries and following the rainy season in the tropics [2]. Global temperatures are expected to rise by 1.5-5.8°C by the year 2100 and to lengthen warm seasons, adapting an optimal survival, persistence, and transmission of leptospires in temperate regions [128]. However, excessive heat (>34°C) can be detrimental to *Leptospira* survival in tropical regions [129]. Nevertheless, this ongoing temperature rise is estimated to affect various ecological factors, by increasing the frequency and intensity of cyclones and floods, which increases the incidence of the disease and the magnitude of its outbreaks in humans [130]. Furthermore, flooding is assumed to aid *Leptospira* in spreading across the environment, which leads to disease transmission and infection [131].

Risk groups that are exposed to animal reservoirs or an environment contaminated by leptospires commonly involve rice/animal farmers [132–134], sewage and abattoir workers [135,136], travelers [137], and animal handlers [138,139], and those involved in recreational water activities [123,140]. The burdens attributed to leptospirosis are its life-threatening manifestation in humans and its economic impact due to livestock illness and human sickness requiring medical costs. Besides its significant burden on public health, leptospirosis affects animal health, yet, animal leptospirosis is not consistently monitored. Infected animals, mainly livestock, experience reproductive failure and reduced meat and milk production [10], clinical illness [141], and reduced growth [142], resulting in economic losses in the livestock industries. Furthermore, chronically infected animals may spread the infection to others present in the industry, posing a significant zoonotic threat to the industry workers [85,143].

Countries of relevance for this thesis included Mainland France and Lebanon. Leptospirosis is regularly diagnosed in humans and animals in Mainland France and particularly in French overseas territories (La Réunion, New Caledonia, French Polynesia, etc.) where the highest incidence rates are more than 100 times greater than on Mainland France. Mainland France is known for its expertise in leptospirosis diagnosis as it possesses a National Reference Center for leptospirosis (“Centre National de Référence de la Leptospire” (CNRL)) and a “Laboratoire des

Leptospires et Analyses Vétérinaires" (LAV) allowing leptospirosis diagnosis in humans and animals, respectively. Leptospirosis has been an underdeveloped issue with an unknown risk in Lebanon as no epidemiological investigation was carried out before this thesis due a lack of expertise in the field. To disclose leptospirosis issue in Lebanon, a partnership combining the two countries was founded through this thesis in cotutelle through the transference of knowledge and opportunity.

4.3 Particular attention to France and Lebanon

4.3.1 Leptospirosis in Mainland France

Based on age- and gender-adjusted demographic characteristics of the population, leptospirosis-related yearly morbidity (*i.e.*, disease incidence) and mortality in humans have been estimated in France to be 3.67 CI_{95%} [1.28 – 6.85] and 0.16 CI_{95%} [0.05 – 0.29] per 100,000 individuals, respectively [36]. Human leptospirosis outbreak cases are mainly associated with *L. interrogans* and *L. kirschneri*. However, human leptospirosis remains unnotified since 1987 and limits the disease diagnosis to determine the source of the disease and adapt preventative measures. Nevertheless, molecular, and serological tests (mainly PCR and ELISA) are being recently applied in abundance in specific laboratories, mainly following their registration in the nomenclature of medical biology acts and the compensation of part of the tests since 2014. The country holds a CNRL which monitors the disease towards the epidemiological surveillance of human leptospirosis. According to the CNRL, both occupational and recreational activities tend to expose people to leptospirosis in the nation. Regarding occupational exposure, more than half of the cases of leptospirosis reported to the CNRL occurred in the building industries and agricultural sectors over the past two decades [144]. Regarding recreational activities, canoeing, fishing, and swimming activities are more likely to expose humans to freshwater contaminated by leptospores, as documented in investigations previously and recently carried out in Mainland France [144–147].

Since 2014, a rise in the incidence of human leptospirosis to 1 case per 100,000 inhabitants was reported, the highest incidence ever observed in metropolitan France [148–151], and a seasonal upsurge in leptospirosis (human) cases was commonly observed between the months of July and October [152]. In 2021, according to the CNRL, an incidence higher than the national incidence (>1 case/100,000 inhabitants) was observed in various regions in Mainland France [153], supposedly due to the lack of awareness of local doctors on leptospirosis [153]. A predominance of serogroup Icterohaemorrhagiae was mainly reported among human cases (approximately one-third of the cases) diagnosed by the MAT followed by serogroups Sejroe and Canicola that presented more than 10% of the cases and other identified serogroups (*i.e.*, Grippotyphosa, Pomona, Panama and Australis) represent less than 10% of cases [153].

4.3.2 Leptospirosis in Lebanon

Based on age- and gender-adjusted demographic characteristics of the population, leptospirosis-related yearly morbidity (*i.e.*, disease incidence) and mortality in humans have been estimated in Lebanon to be 2.93 CI_{95%} [0.92-5.37] and 0.15 CI_{95%} [0.05-0.26] per 100,000 people, respectively [36]. To date, the epidemiology of leptospirosis in this country is unclear; there are no published case reports, with a unique investigation carried out in 1947 following the finding of two cases of Weil's disease [154]. In addition, molecular tests are rarely applied as the disease is not diagnosed and the expenses are not compensated. Furthermore, serological tests such as MAT are not applicable in the country as they are restricted to reference laboratories located in Foreign countries (*e.g.*, France). Although the existence of an appropriate climate (warm and wet) and of possible maintenance hosts (such as rodents) make it fitting for the persistence of the bacterium [155,156], leptospirosis preventative measures do not subsist and the disease is considered neglected in the nation [157].

To date, Icterohaemorrhagiae was the unique serogroup identified in rats in Lebanon in 1947 [154]. Thus, acquiring additional knowledge about Leptospiral infectious status and circulating serogroups in mammalian hosts is essential to understand the disease epidemiology, increase public health awareness, and serve as a preliminary point for control efforts in Lebanon.

4.4 Understanding the role of animals in the epidemiology of leptospirosis

According to reports, brown rats (*Rattus norvegicus*) are the primary source of human leptospirosis [158]. Their capacity to chronically and selectively carry *Leptospira* serogroup Icterohaemorrhagiae in various populations [158–160] due to their strong affinity with the latter *Leptospira* strain [161–163] qualifies them as maintenance hosts and selective carriers of *Leptospira* serogroup Icterohaemorrhagiae [160,164]. Such affinity may be driven by genetic compatibility between the *Leptospira* strain and the host cells as reported through experimental studies [165]. For instance, brown rats are not vulnerable to infection by *Leptospira* serogroup Icterohaemorrhagiae that is harbored in the kidneys and is disseminated in the environment through the urine where it can persist for a protracted time (>220 days) [159], qualifying them as maintenance hosts of the latter *Leptospira* strain. They are also considered reservoir hosts of human leptospirosis [22] — as humans are infected by the population of rats carriers of *L. interrogans* serogroup Icterohaemorrhagiae— following Haydon et al. (2002) [166] definition of a reservoir: “one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population”.

An extensive examination of wild animals from southwestern Indian Ocean islands regions revealed that, in addition to brown rats, a broad variety of mammals may also serve as selective carriers of particular serogroups of *Leptospira* [167]. For instance: cattle (*Bos taurus*) may serve as selective carriers of serogroup Sejroe, dogs (*Canis familiaris*) of serogroup Canicola, horses (*Equus caballus*) of serogroup Australis and swine (*Sus scrofa*) of serogroups Australis and Pomona [10]. In contrast to maintenance hosts, accidental hosts or non-maintenance hosts either recover or die as the illness progresses following infection by a non-maintenance pathogenic serogroup of leptospires. Some serogroups recognized to cause incidental infection in accidental hosts are serogroup Grippotyphosa for cattle [168,169], serogroup Pomona for sheep and goats [170,171], and serogroup Icterohaemorrhagiae for humans [22].

Although accidental hosts do not sustain the pathogen in the environment by themselves, they may (omitting humans) contribute to its maintenance in the environment and/or serve as bridge hosts in the infection of accidental and/or maintenance hosts living in proximity [172]. The association of serogroup-carrier and the ecological and environmental interactions between maintenance and accidental hosts in an ecosystem aid in the persistence of particular serogroups of *Leptospira* in an ecosystem, qualifying the latter with a specific maintenance mechanism and level of contamination. An investigation of the genetic diversity of *Leptospira* in 28 wildlife animal species was recently carried out in France [173]. It pointed out hedgehogs as maintenance hosts of *L. interrogans* serogroup Australis and reservoir of human infection by the latter strain in the studied ecosystem. Furthermore, it suggested that the remaining carnivores, infected by various genotypes including Australis, act as bridge or as the maintenance community of the latter *Leptospira* strain. The study of Ayrat et al. (2016) [173] suggests that animal groups in an ecosystem have different roles in the epidemiological cycle of leptospirosis, supporting the hypothesis of the maintenance community and that further studies are needed to better understand the role of animals in the maintenance community. According to Viana et al. (2014) [164] the maintenance community is defined as “any set of maintenance host (sub)populations that together can maintain a pathogen over the long term. A minimal maintenance community is a maintenance community of which all subsets are non-maintenance. Trivially, a maintenance population is also a (minimal) maintenance community”. Consequently, implicated mammalian hosts are considered a maintenance community and allow the infection of a target species of concern. Three models of maintenance community of leptospirosis are presented in Figure 2 and can be interpreted as follows: the first model can represent the case of rats (maintenance population) and sanitary workers (target population), the second the case of a sheep herd (non-maintenance population),

rats living in proximity (maintenance and source population) and farmers (target population), the third a complex case in an ecosystem involving different rodent species (source population), domestic animals (non-maintenance population) and humans (target model).

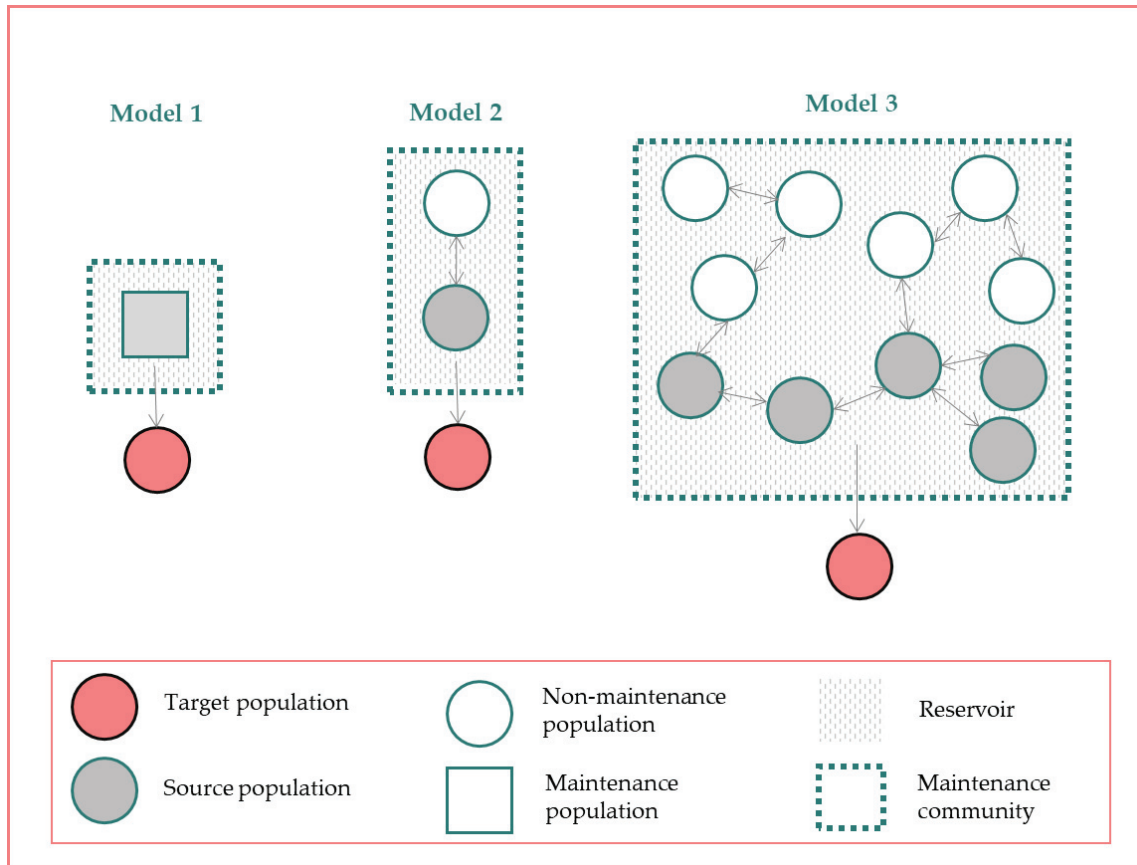


Figure 2. Illustration of reservoir-target models adapted from Viana et al. (2014)

4.5 Convenience sampling in epidemiological investigations

Epidemiological investigations are the key to classifying hosts into categories (*i.e.*, maintenance hosts for particular serogroups, accidental hosts for distinct serogroups), identifying the sources of contamination in an ecosystem (*i.e.*, mainly in outbreaks studies), and acquiring knowledge about *Leptospira* maintenance community. These investigations are based on sampling by adopting one of two categories of sampling methods: probability sampling and non-probability sampling. In a probability sample, each individual has an exactly equal chance of being selected. In comparison to non-probability sampling, probability sampling methods are typically more time- and money-consuming. This approach is the most effective at identifying the population's actual features. Sampling types used in probability include random, stratified, and systematic sampling [174]. In a non-probability sample, individuals are selected from the population in a non-random process. As a result, there is a big chance of acquiring

a non-representative sample with non-generalized conclusions. Nevertheless, non-probability sampling techniques are often more affordable and practical, and they are helpful for exploratory research and hypothesis formation [174]. Sampling types used in non-probability include convenience, judgment, quota, and snowball sampling.

The most popular kind of sampling in leptospirosis investigation is the non-probability convenience sampling which relies on gathering data from individuals who are “convenient” for the researcher to contact (*e.g.*, collaborative people who approve or are interested to participate in the proposed step included in the research) or to have access to (*e.g.*, nearby farms which with permit access) [175]. This implies that you may locate your sample at any time and any place. Consequently, convenience sampling is also referred to as grab sampling, opportunity sampling, accidental sampling, and availability sampling. An example of convenience sampling on animals is the collection of readily available sample units, such as farms near to a laboratory or animals that are presented to a veterinarian clinic. The main benefits of the method is that it is affordable, effective, and easy to use, however, its drawback is the sample's unclear generalizability [175]. Nevertheless, such a method limits our knowledge of the true prevalence of leptospirosis in mammalian hosts today and might not be adequate to clearly define the role of the host in a maintenance community.

5 The contribution of epidemiological studies for adapting preventative measures

One of the goals of epidemiological studies is to adapt control measures to reduce or eliminate the risk of accidental hosts infection [176]. Nevertheless, given the difficulty of eliminating the risk, its management should focus on its minimization so that even if the hazard exists, the exposure to it and the (health) consequences may be reduced. Risk minimization can be done by using adequate preventative measures, such as hygiene, personal protective equipment, reliable vaccination program for individuals at high risk of exposure, and animal vaccination. In some circumstances, antimicrobial prophylaxis is administrated to treat human leptospirosis as soon as the illness manifests and may also be helpful to prevent the infection of humans that have a high exposure risk. However, the most crucial preventive measures for human leptospirosis are avoiding probable infection sources such as stagnant water and livestock farm wastewater, controlling rodent populations, and safeguarding food from animal contamination.

Hand washing, the use of appropriate personal protective equipment (rubber boots, protective eyewear, and gloves) during occupational activities and the use of wound

protection during recreational activities are preventative measures that can be easily adapted to reduce or avoid the risk of leptospirosis [177]. However, vaccination is a more challenging preventative measure due to its partial immunity, its cost, and its unavailability in certain countries. However, reports showed that vaccination provided reduced renal colonization and the urinary shedding of its targeted serogroups in animals such as reported in dogs [178], sheep [179], hamsters [180], and cattle [181]. Such a step therefore seems critical to ensure the control of leptospirosis and is highly recommended in the case of cattle herds [182]. Nevertheless, it can be hardly applied to other animal species such as rats, described as the main reservoirs of human leptospirosis, due to their high proliferation, difficult trapping, and limited manipulation. Antibiotics, including doxycycline or penicillin, can be helpful to treat or prevent the disease, and their prompt treatment is recommended by WHO before the disease's fifth day of onset to deliver the best clinical effects [183]. Penicillin and doxycycline are recommended for severe and mild leptospirosis, respectively [184]. Ceftriaxone, quinolones, or aminoglycosides can also serve as alternative antibiotics [184,185]. Antibiotic administration has been seen as beneficial in many reports. Nevertheless, it is best advised for the severe type of leptospirosis, although it is debatable for the moderate form. A study was conducted in Andaman Islands, a region of Southeast Asia with a high prevalence of leptospirosis and frequent outbreaks related to flooding, to evaluate the effectiveness of antibiotics prophylaxis in preventing infection and clinical illness caused by leptospires [186]. It grouped 782 people that received either a weekly dose of antibiotics (doxycycline or placebo) for prophylaxis. It showed that antibiotic treatment significantly lowers mortality and morbidity during epidemics, providing strong protective effects [186]. A different study with 940 soldiers deployed to Panama for jungle training found that those who had received a weekly dose of doxycycline had few cases of leptospirosis [33]. Still, two reviews concluded that there was inadequate data to either advocate or oppose antibiotics in the treatment of leptospirosis and that further clinical trials are needed to better know their role in the treatment of leptospirosis [187,188].

France is one of the few European nations that acquire vaccines against certain *Leptospira* serogroups for humans, dogs, and cattle. The country has supported the use of an inactivated vaccine against the serogroup Icterohaemorrhagiae for humans (Spirolept vaccine), which requires a booster shot every two years [189,190]. The latter targets individuals with frequent exposure to rats, thus, serogroup Icterohaemorrhagiae, through their professional activities (*e.g.*, sanitary workers, laboratory workers with close contact with rodents)[191]. Additional protection for dogs against serogroup Grippotyphosa, Canicola, and Australis (Versican vaccine)

and a bovine vaccine that protects against serogroup Sejroe (Spirovac vaccine) are available today [191]. Nevertheless, prior studies have questioned the reliability of the latter vaccines as additional serogroups are found to be the cause of clinical infection in both dogs and cattle [192,193]. Consequently, acquiring knowledge about the circulating serogroups and serovars in an ecosystem where domestic animals and humans occur can be of guidance for prophylactic purposes.

To our knowledge, the human vaccine is not available in Lebanon. However, a vaccine against serogroup Canicola, Grippotyphosa, Icterohaemorrhagiae, and Pomona is available for dogs (DHPPL: Canine Distemper, Hepatitis, Parvovirus, Parainfluenza virus, *L. interrogans* serogroups Canicola and Icterohaemorrhagiae and Rabies virus) and is administered annually as a routine prophylaxis in veterinary clinics. A vaccine against (cattlemaster goldFP5) Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae, and Pomona is available for cattle, however, it is rarely administered for cattle since it is not mandatory and is not approved by farmers that are unaware of the disease and its consequence and therefore avoids additional prophylactic costs from their side. Consequently, raising awareness by professionals (e.g., veterinarians, Ministry of Agriculture) is essential — since leptospirosis is present in the nation and especially among livestock— to encourage cattle vaccination for prevention of leptospirosis, reduction of renal colonization, and optimization of both animal health and the health of humans that are in close contact with cattle.

The concept of One Health has gained considerable interest over the past decade due to the expanding severity and frequency of threats that impact the health of individuals, plants, animals, and the ecosystems. The One Health High Level Expert Panel (OHHLEP) [194], whose members represent a broad range of disciplines relevant to One Health from around the world, has issued a new definition of One Health:

« One Health is an integrated, unifying approach that aims to sustainably balance and optimize the health of humans, animals, plants and ecosystems. It recognizes the health of humans, domestic and wild animals, plants and the wider environment (including ecosystems) are closely linked and interdependent.

The approach mobilizes multiple sectors, disciplines and communities at varying levels of society to work together to foster well-being and tackle threats to health and ecosystems, while addressing the collective need for clean water, energy and air, safe and nutritious food, taking action on climate change, and contributing to sustainable development. »

The advantage of the One Health approach is its wide-range application that varies from a small community level to a global level, relying on shared and potent governance, collaboration, communication, and coordination.

Leptospirosis is a disease that affects domestic and wild animals as well as humans and has a significant worldwide impact on human mortality and morbidity [36]. Its management, therefore, requires a One Health strategy since human illness results either from direct animal contact or from exposure to contaminated environments. Poor communication and collaboration between people working in the medical and veterinary domain and environmental institutions are significant issues restricting leptospirosis management. Maximizing collaboration amongst all fields of expertise interested in leptospirosis, including International leptospirosis reference laboratories, is vital to acquire serological and molecular results, analyze the risk of leptospirosis in ecosystems, interact with governments, and ensure prophylaxis measures.

6 Thesis problematic, objectives and structure

Leptospirosis, a serious zoonotic illness caused by pathogenic *Leptospira* species, induces major health and economic challenges worldwide. All mammals are susceptible to infection, however, their dynamics and bacterial spread depend on the host's qualifications. Maintenance hosts operate as chronic selective carriers of particular serogroups of pathogenic *Leptospira*, which are maintained in the kidneys and are discharged through the urine in the ecosystem where they occur. Accidental hosts often survive or pass away following infection. However, if the latter hosts co-occur with hosts of either kind in an ecosystem, they may, omitting humans, contribute to the persistence of the bacteria. A maintenance community of leptospires arises throughout the interactions between accidental and maintenance hosts in an ecosystem and supports infecting target populations at the ecosystem level.

Each ecosystem groups a unique community of mammals and is therefore distinguished by a specific level of contamination. A suitable epidemiological design is to be conducted locally to acquire knowledge about the epidemiology of *Leptospira* infection and speculate on the relative contributions of each species of mammals to the ecosystem to recognize the level of contamination. Accordingly, effective preventative measures can be adopted, based on the level of contamination and the circulating serogroups within hosts, particularly maintenance hosts. Nevertheless, obtaining such epidemiological information depends on the ability to determine and sample the mammals of interest within an ecosystem and to acquire proper molecular and serological tools to generate the required epidemiological information.

To date, studies on the epidemiology of leptospirosis have focused on identifying the risk factors (*e.g.*, weather conditions, presence of rodents, etc.) that are responsible for leptospiral infection within the ecosystem under study without taking into account the characteristics of the maintenance community. Consequently, the general objective

of this thesis is to incorporate the notion of an ecosystem-specific maintenance community, to explore populations hosts from different ecosystems in order to describe their relative implications in the community, and to apply and improve epidemiological investigation protocols to better understand the risk of infection within an ecosystem. The specific objectives of this thesis are (1) to generate knowledge on mammalian hosts for the study of the maintenance community of leptospires in France, (2) to describe the contributions of different laboratory methods in leptospirosis epidemiological studies, and (3) to initiate the study of *Leptospira* infection risk in the livestock ecosystem in Lebanon.

A bibliographic search was first conducted in Part I of the thesis to introduce *Leptospira* organism as well as the laboratory diagnosis, the clinical features, and the epidemiology of leptospirosis. Part II of the thesis included four field studies and a literature review that have been implemented to meet the objectives. The two first field studies adapted either a mono- or a multi-host approach to potentially determine the role of the sampled hosts in leptospiral life cycle and the maintenance community. The third field study focused on different laboratory methods to assess the leptospiral carriage of the sampled hosts and determine their role in the maintenance community. The review and the fourth field study provided, respectively, an overview of the leptospirosis risk in the Middle East and the first data on *Leptospira* detection in Lebanon.

Part III includes a general discussion of our findings to highlight how this additional knowledge on *Leptospira* eco-epidemiology may change our approach to studying *Leptospira* infection risk in the future either in France or Lebanon. Supplementary materials are either included in this thesis in the appendix section (for the manuscripts) or are accessible online (for the published articles).

II. Epidemiological studies of leptospirosis in France and in Lebanon

II.1. Adapting different epidemiological approaches in France for studying maintenance hosts and communities

Knowledge about leptospirosis maintenance community is limited in France despite the numerous studies conducted in the field [160,173,195,196]. Animal hosts are classified as either maintenance or accidental hosts when infected by particular *Leptospira* serogroups. Understanding the role of animal hosts in the cycle of leptospirosis will help better comprehend *Leptospira* maintenance community and adapt adequate preventative measures to reduce the risk of *Leptospira*-infection of both humans and animals in a particular ecosystem (depending on the existing animal hosts). In order to acquire information about the role of different animal hosts species in *Leptospira* maintenance community; we conducted two epidemiological approaches. The first approach relied on sampling similar hosts species in different ecosystems and determining their leptospiral carriage in each ecosystem. This approach allows to determine the potential of animal species to act as maintenance hosts if found selective and chronic carriers of particular *Leptospira* strain(s) with high prevalences in the different sampled ecosystems. The second approach relied on sampling different hosts species in a particular ecosystem and likewise, determine their leptospiral carriage. This approach can describe the maintenance community of a particular ecosystem where different animal populations occur. The two epidemiological approaches conducted a non-probability convenience sampling in France. The first study sampled a unique population species of raccoons (*Procyon lotor*) in two different areas (NorthEast and SouthWest) in France. The second study sampled several hosts, namely, nutrias (*Myocastor coypus*), muskrats (*Ondatra zibethicus*), Norway rats (*Rattus norvegicus*) and cattle (*Bos taurus*) in a unique ecosystem in Ille-et-Vilaine in Brittany following several outbreaks of human cases in order to identify the source of human infection and the maintenance community responsible for this outbreak. The general aim of both studies was to develop our knowledge about the maintenance community through the sampled hosts.

Samples were provided for the elaboration of both studies and the detection and characterization of leptospire. The first study (article 1) was funded by the ANRT, OFB, MRRNP, DREAL of the Grand Est and the Nouvelle-Aquitaine, the Gironde Department Council, the commune of Villenave d'Ornon, the FDC51, the GREGE and the CERFE. The second study (article 2) was funded by the ARS Bretagne Pôle EVAAS (VetAgro Sup) and my PhD grant. My contribution related to both studies was the formal analysis, methodology, software, visualization, funding, technical, and the writing of the original draft.

1. **Studying a unique host species in various ecosystems: Characterization of pathogenic *Leptospira* in invasive raccoons (*Procyon lotor*) in northeastern and southwestern France.**

Article 1: Characterization of pathogenic *Leptospira* in invasive raccoons (*Procyon lotor*) in northeastern and southwestern France.

Co-authors: **Elena Harran**, Florence Ayral, Christine Fournier-Chambrillon, Manon Gautrelet, Guillaume le Loch', Céline Richomme, Océane Tourniaire, Gérald Umhang, Christian Fournier, Florence Ayral.

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Epidemiology & Infection

Short Report: Characterization of pathogenic *Leptospira* in invasive raccoons (*Procyon lotor*) in northeastern and southwestern France.

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Summary: Leptospirosis is a widespread zoonosis caused by bacteria in the genus *Leptospira*. Although crucial to mitigating disease risk, basic epidemiological information is lacking, such as the identities of *Leptospira* maintenance hosts. An invasive species in France, the raccoon (*Procyon lotor*) could pose a risk to public health if it carries pathogenic *Leptospira*. We investigated the rate and type (selective *vs* unselective) of *Leptospira* carriage in France's two main raccoon populations. Among the 141 raccoons collected, seven (5%) tested RT-PCR positive (*lfb1* gene) based on kidney, lung, and urine samples when available. Phylogenetic analysis revealed the presence of three different *L. interrogans* clusters. Our results suggest that raccoons are more likely accidental hosts that make a limited contribution to *Leptospira* maintenance.

Keywords: wildlife, invasive species, zoonosis, maintenance host, accidental host, public health

Introduction

Leptospirosis is a global and potentially fatal zoonotic disease that affects all mammals, including humans and is caused by pathogenic species of the genus *Leptospira*. The genetic polymorphism diversity of the eight pathogenic species was recently studied using the *lfb1* gene and 46 species-groups were identified [1]. *Leptospira* are mainly transmitted via soil or water contaminated by the urine of infected animals. The bacteria can survive for days in aquatic environments, which are the origin of most human cases of leptospirosis. However, the source of environmental contamination is a range of mammalian maintenance hosts: *Leptospira* colonize the kidneys, where they remain over the long term and are shed in the urine [2].

Although leptospirosis is a major public health burden, management strategies remain limited because basic epidemiological knowledge is lacking, such as the role of various animal hosts in *Leptospira* maintenance across ecosystems. To determine the ability of a given mammal species to maintain *Leptospira* and to design better disease prevention approaches, it is important to characterize *Leptospira* prevalence in target animal populations as well as any host-pathogen adaptations present (*i.e.*, whether the target animals exclusively carry a given *Leptospira* strain) [3]. The raccoon (*Procyon lotor*) is a North American species that has become invasive worldwide, notably in Europe and in different regions of mainland France [4]. Since raccoons may make different contributions to *Leptospira* epidemiology across ecosystems, it is essential to explore potential variability within the country [5,6].

The objective of our study was thus to estimate *Leptospira* prevalence in France's two main raccoon populations. In addition, we sought to determine whether selective carriage occurs in raccoons by genetically characterizing any *Leptospira* DNA retrieved.

Materials and Methods

In 2019 and 2020, we sampled raccoons using traps, and we collected dead raccoons found on roads. This work was performed in northeastern France (Grand Est region, administrative departments of Ardennes, Marne, Meurthe-et-Moselle, and Meuse) and in southwestern France (Nouvelle Aquitaine region, administrative departments of Charente and Gironde) (Figure 1). The live raccoons were euthanized in accordance with regulations regarding invasive species (French Decree of 2 September 2016) and animal welfare guidelines (Directive 2010/63/EU).

All the animals were immediately frozen following collection. They were later thawed to perform necropsies, so we could acquire samples of kidney, lung, and urine (when available) from each animal; the samples were then stored at -20°C until further analysis could occur. DNA extraction was carried out using the Nucleospin Tissue

Kit (Macherey Nagel, Hoerd, France) in accordance with the manufacturer's instructions, and DNA samples were stored at -20°C until the molecular analyses could be performed. The presence of *Leptospira* DNA in the kidney, lung, and urine samples was assessed using real-time PCR (RT-PCR) targeting the 16S gene and the AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems), as described elsewhere [7]. DNA specimens with a cycle threshold (Ct) of less than 40 were considered to be positive and were further amplified by conventional PCR (cPCR) targeting the *lfb1* gene, as described by Merien *et al.* [8]. The amplified products were verified utilizing 1% agarose gel electrophoresis and subject to Sanger sequencing (Genoscreen, Lille, France). A nucleotide BLAST search was conducted (NCBI: <http://blast.ncbi.nlm.nih.gov>) to identify the *Leptospira* species-groups present. A phylogenetic tree was then generated using the *Leptospira spp. lfb1* partial gene polymorphism in raccoon specimens and reference strains provided elsewhere [1].

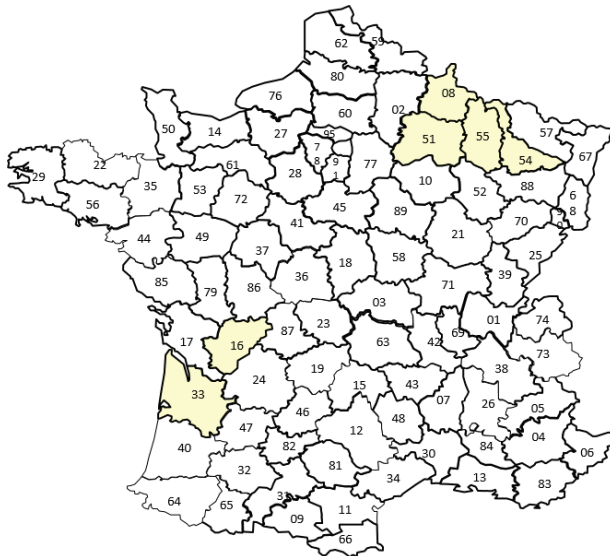


Figure 1. Map indicating the departments of northeastern and southwestern France included in the study.

Results

We collected a total of 141 raccoons (99 and 42 from northeastern and southwestern France, respectively). Seven raccoons were RT-PCR positive based on kidney and/or urine samples. All the lung samples were RT-PCR negative. Overall prevalence was 5% (CI_{95%} [2%, 10%]). Regional prevalence was 5% (CI_{95%} [1.7%, 11.4%]) in northeastern France and 4.8% (CI_{95%} [0.6%, 16.2%]) in southwestern France (Figure 2). *L. interrogans* was detected in 6 samples coming from 5 of the 7 RT-PCR-positive raccoons. The phylogenetic analysis identified three different *lfb1* species-groups: the first (three amplicons coming from three raccoons) was described in reference strains belonging to the serogroups Autumnalis, Bataviae and Australis; the second (one amplicon) was

described in a reference strain belonging to the serogroup Djasiman; and the third (two amplicons) was described in reference strains belonging to the serogroup Icterohaemorrhagiae (Figure 3).

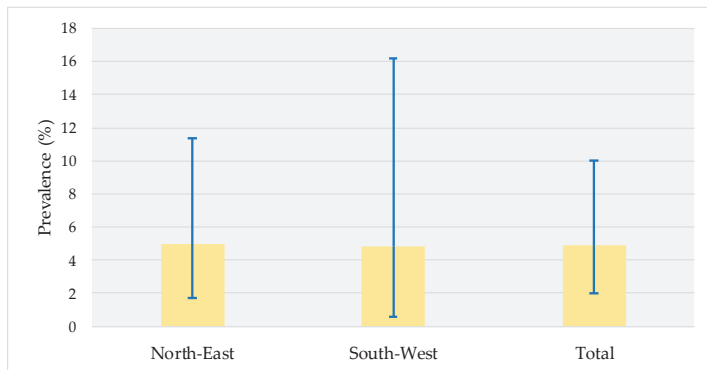


Figure 2. Distribution of *Leptospira* prevalence with 95% confidence interval among raccoons

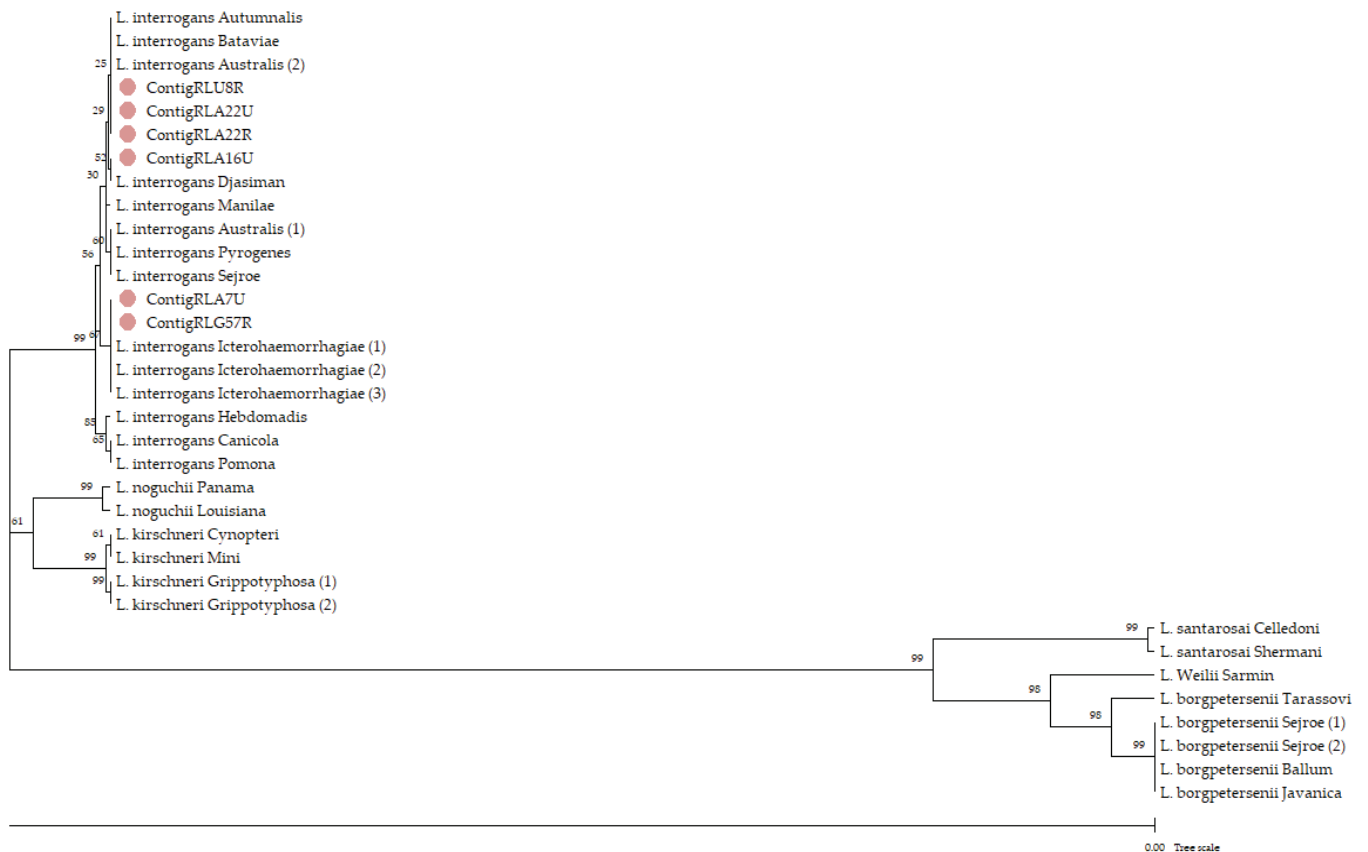


Figure 3. Phylogenetic tree of *Leptospira* DNA extracted from raccoons and characterized based on the *lfb1* gene sequences (bootstrap analysis involving 1,000 replicates and 34 nucleotide sequences). The evolutionary distances (unit = base substitutions per site) were computed using the maximum composite likelihood method [9] and MEGA11 software. The pink circles represent the amplicons from the samples. The contigs are identified using a coding system with the format “RL NN M”: where NN indicates sample geographical origin (northeastern France = A and U, southwestern France = G) plus ID number and M indicates sample tissue type (urine = U, kidney = R).

Discussion

Our results suggest that raccoons could potentially spread pathogenic *Leptospira* given that *Leptospira* DNA was found in the kidney and urine samples. However, raccoons seem more likely to serve as non-maintenance or accidental hosts (*i.e.*, short-term infection and shedding) as opposed to maintenance hosts (*i.e.*, long-term infection and shedding). Indeed, maintenance hosts, namely *Rattus* species, display prevalence values that exceed 20% in France [10] and in other countries as a result of chronic kidney colonization [2]. Thus, populations with lower prevalence values are unlikely to maintain *Leptospira* over extended periods. In this study, *Leptospira* prevalence was 5% (CI_{95%} [2%, 10%]), which is lower than that seen in rats. While this result could be an underestimate because sample thawing could reduce the likelihood of detecting *Leptospira* DNA [3], the pathogen's prevalence in rats in France was estimated under the same methodological conditions, which suggests our comparison is reliable.

In addition, we identified three species-groups of *Leptospira* within the infected raccoons, a result that is consistent with the hypothesis that they are non-maintenance hosts. Indeed, *Leptospira* maintenance hosts appear to selectively carry specific strains; for example, rats are the primary hosts for the *L. interrogans* serogroup Icterohaemorrhagiae [2]. The diversity of *Leptospira* species-groups we found in raccoons supports the idea that carriage is not selective, as also hypothesized elsewhere [6]. In other words, raccoons may sporadically become infected by strains present in the environment but are unable to maintain any given strain for prolonged periods. However, only one specimen (RLG57R) coming from the southwestern's population could be typed. Thus, additional raccoons should be analysed to further clarify species-group diversity in this sub-population.

Lastly, *Leptospira* prevalence were similar in raccoon populations in northeastern and southwestern France, suggesting that raccoons make similar epidemiological contributions in both regions and ecosystems.

Conclusion

In conclusion, our results suggest that, in contrast to rats, raccoons are unlikely to maintain *Leptospira*, although they might disseminate them somewhat throughout the environment. That said, some raccoon populations are presently in peri-urban areas however, in close proximity with humans and dogs, and locally the risk of transmission may not be negligible, especially if raccoon densities are very high, as in the south-east in Gironde.

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2. Studying a unique ecosystem grouping various hosts species: Tracking potential *Leptospira* sources following human cases of leptospirosis: A One Health approach applied to an ecosystem in Brittany, France

Article 2: Tracking potential *Leptospira* sources following human cases of leptospirosis: A One Health approach applied to an ecosystem in Brittany, France

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Tracking potential *Leptospira* sources following human cases of leptospirosis: A One Health approach applied to an ecosystem in Brittany, France

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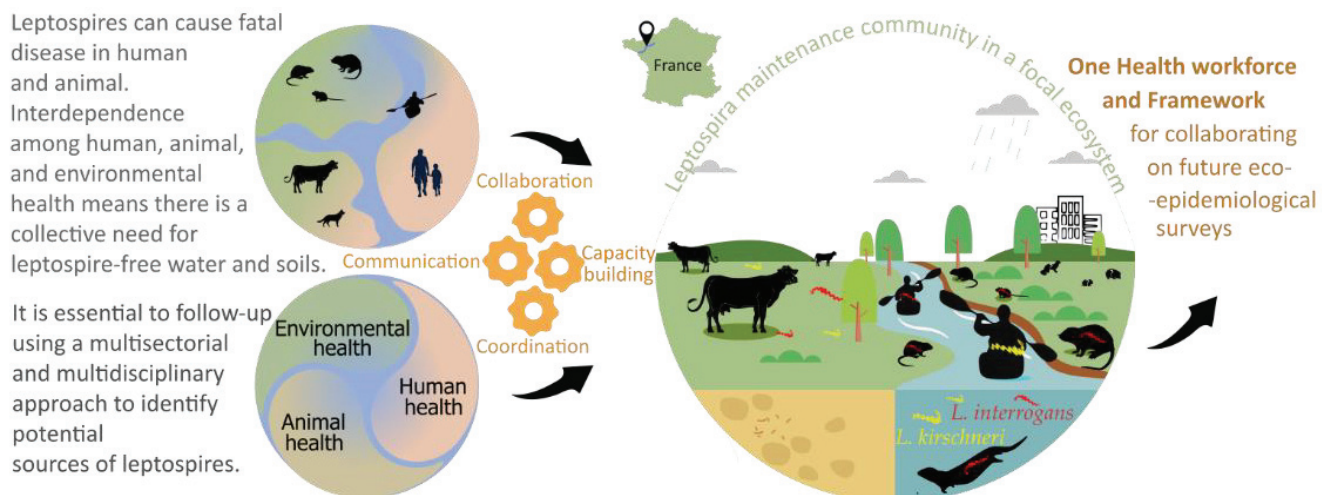
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Graphic abstract



Abstract

Pathogenic *Leptospira* can cause leptospirosis: a widespread, potentially fatal bacterial zoonosis whose risk is strongly mediated by ecohydrological patterns and animal host distributions. When human cases of leptospirosis occur, it is challenging to track down their source because ecosystem-level epidemiological knowledge on *Leptospira* is needed. As a first step in this process, we investigated leptospiral carriage in the main animal hosts found in a focal ecosystem in Brittany, France. Between 2016 and 2019, the region's human population experienced an outbreak and successive cases of leptospirosis attributable to *L. kirschneri* and *L. interrogans*. We sampled 143 nutrias, 17 muskrats, and 10 Norway rats. DNA was extracted from their kidneys, lungs, and urine and subject to real-time PCR (RT-PCR) targeting the *Leptospira* 16S rDNA and *lfb1* genes. We also sampled serum from 439 cattle and used a microscopic agglutination test to detect the presence of antibodies against *Leptospira*. Urine samples were concomitantly obtained from 145 cattle and were used in two analyses: RT-PCR targeting the *Leptospira* 16S rDNA gene and *Leptospira* culturing. We found that rodents were the most likely source of the *L. interrogans* behind the human cases. The cattle tested negative for *Leptospira* DNA but positive for antibodies against the serogroups implicated in the human cases. We failed to identify the potential source of the *L. kirschneri* responsible for several human cases of leptospirosis. Our results call for further clarification of the *Leptospira* maintenance community, which may comprise known maintenance hosts, such as rodents, as well as taxa not commonly considered to be maintenance hosts but that can still spread *Leptospira*. This study was carried out using the One Health approach, as described in international health guidelines. The resulting research network will collaboratively conduct future ecoepidemiological surveys to illuminate the leptospirosis risks faced by humans and animals within ecosystems.

Keywords: Maintenance community, Cattle, Rodents, Reservoirs, Zoonosis, Rivers

1. Introduction

Leptospirosis is a widespread bacterial zoonosis whose occurrence is strongly influenced by ecohydrological dynamics, giving rise to endemic and epidemic patterns of infection; the disease can result in systemic organ failure and death in both animals and humans (Bharti et al., 2003; Levett, 2015). The genus *Leptospira* is currently subdivided into 68 genomic species, including saprophytic or pathogenic bacteria. Infections in humans and animals are caused by eight pathogenic species (Vincent et al., 2019), which are divided up into at least 26 serogroups (Nieves et al., 2023). Annually, members of the genus, predominantly *L. kirschneri* and *L. interrogans*, cause an estimated 1 million cases of disease and 60,000 deaths in human populations worldwide (Costa et al., 2015). Several leptospirosis outbreaks in Western Europe have been traced back to *L. kirschneri* (Fiecek et al., 2017; Guillois et al., 2018; Picardeau, 2017), and both bacterial species commonly occur in wild and domestic animals (Ayril et al., 2016a; Fischer et al., 2018; Harran et al., 2023; Obiegala et al., 2016).

Humans mainly become infected through exposure to soil or water contaminated by the urine of infected animals (Levett, 2015). Water sports and occupational activities have been identified as risk factors, but outbreak occurrence remains otherwise unpredictable across space and time (Munoz-Zanzi et al., 2020). Over the last two decades, Western Europe has experienced leptospirosis outbreaks related to water sports, but the animal sources potentially responsible were not described (Brockmann et al., 2010; Guillois et al., 2018) or described with weak supporting evidences (Perra et al., 2002). While all mammals can be infected by pathogenic *Leptospira*, a given host's relative contribution to environmental contamination depends on the host-pathogen relationship as well as on how animal populations interact with each other and the environment. Thus, the specific reservoirs causing leptospirosis infections in humans depend on ecosystem characteristics, which makes it challenging to implement standardized preventive measures (Roberts and Heesterbeek, 2020). Water sports typically take place in ecosystems containing high levels of mammalian biodiversity and, therefore, a variety of potential *Leptospira* hosts, which contrasts with the low-diversity situation in urban ecosystems, where murines usually serve as reservoir hosts (Blasdell et al., 2021; Vinetz et al., 1996). Several mammals can serve as accidental or maintenance hosts for different serovars (Ellis, 2015). Consequently, the sum of an ecosystem's *Leptospira* hosts can be thought of as a maintenance community, promoting environmental contamination and causing *Leptospira* infections in other animals and humans (Viana et al., 2014). It is thus crucial to identify the potential hosts that make up maintenance communities to comprehensively assess human and animal health risks.

With the advent of climate change, aquatic recreational activities will increase, bacteria will persist for longer in the environment, and there may be variation in the dynamics of wild animal populations. Consequently, we are likely to see more leptospirosis outbreaks in the coming decades (Velardo et al., 2022). At present, they occur sporadically, and their drivers remain poorly described (Schneider et al., 2013). Collaborative multisectorial and multidisciplinary ecoepidemiological surveys are needed if we wish to fully characterize the patterns of leptospirosis outbreaks. Although international organizations recommend using the collaborative multisectorial and multidisciplinary approach, namely the One Health approach, for epidemiological surveys related to leptospirosis (Munoz-Zanzi et al., 2020; Pereira et al., 2018), there remains a lack of local-level frameworks and examples for putting One Health into practice.

As a first step, we investigated *Leptospira* carriage in various animal populations found in an ecosystem where a leptospirosis outbreak occurred among kayakers in September 2016 (n = 14 cases, of which 8 were confirmed by serological tests or polymerase chain reaction [PCR]). Additional human cases of leptospirosis appeared in May–June 2018 (n = 3 confirmed clustered cases and 5 probable cases) and in November 2019 (n = 1 confirmed case and 1 probable case). In 8 of the 12 confirmed cases, typing or serological profiling identified the causative bacteria as *L. kirschneri* (n = 3), *L. interrogans* (n = 1), or a member of the serogroup Grippotyphosa (n = 4) (Anonymous, 2023; Guillois et al., 2018), to which both *L. kirschneri* and *L. interrogans* belong (Levett, 2001).

In this study, our goal was two-fold. First, we sought to clarify *Leptospira* ecoepidemiology within a focal ecosystem in Brittany, France, that has given rise to recurrent human cases of leptospirosis. Second, we wished to use our findings to inform future ecoepidemiological work looking at outbreaks of multihost zoonoses and to encourage the adoption of a One Health approach, as described in international health guidelines (Anonymous, 2022, 2019).

2. Materials and Methods

2.1 Ethics statement regarding animal sampling

Samples were collected from cattle in accordance with the procedure approved on September 19, 2018, by VetAgro Sup Ethics Committee (agreement n°1813). The rodents sampled in the study were killed during pest control campaigns by professionals in the field and certified trappers. As a result, we did not perform any lethal sampling ourselves, which meant that no approval was needed from the ethics

committee. The pest control campaigns use strategies that adhere to French and European legislation on the treatment and usage of animals (Directive 2010/63/EC and French Administrative Decision 2007/04/06).

2.2 Cattle sampling

Ten cattle farms occur along a stretch of the Vilaine River located upstream from Rennes, Brittany’s capital city. Eight of these farms—six dairy and two beef—made their herds available to us. Approximately 30 individuals per farm were sampled over the course of two sessions (Fig. 1); the same cattle were sampled both times when possible. The latter effective (30) was based on the minimum effective required to detect at least one cattle with urinary excreting in a batch of approximately 60 individuals, infected with a limit prevalence of 5%. Likewise, the effective of 30 was taken into account during blood sampling to increase the specificity of the PCR by the MAT, in the scenario where sequencing could not be carried out (*e.g.*, insufficient amounts of DNA). As a result, blood and urine sampling were conducted on similar cattle. The first session took place from March to May in 2019, which was the pre-grazing period; both serum and urine were collected. The second session took place from November 2019 to January 2020, which was the post-grazing period; only serum was collected. Sampling was performed by the farmers’ veterinarians and a local farmer’s association for herd disease control (*Groupement de Défense Sanitaire*). To collect the urine sample, an animal’s vulva was cleaned and disinfected before introducing a catheter directly into the bladder via the urethral canal. Samples were also collected when spontaneous urination occurred. Fresh urine specimens were used in our attempt to culture *Leptospira* and were kept at 16–22°C pending further analysis. The serum samples and other urine samples were stored at -20°C until the molecular and serological analyses could be performed.

| Session 1: March to May 2019 | | | Session 2: November 2019 to January 2020 | | |
|------------------------------|---------------|--------------------------|--|---------------|--------------------------|
| Herd ID | Sampling date | Number of sampled cattle | Herd ID | Sampling date | Number of sampled cattle |
| 1 | 19/03/2019 | 28 | 1 | 01/01/2020 | 35 |
| 2 | 19/03/2019 | 30 | 2 | 09/12/2020 | 30 |
| 3 | 19/03/2019 | 14 | 3 | 01/01/2020 | 17 |
| 4 | 21/03/2019 | 29 | 4 | 22/11/2020 | 29 |
| 5 | 04/04/2019 | 30 | 5 | 17/12/2020 | 30 |
| 6 | 03/04/2019 | 29 | 6 | 23/12/2020 | 30 |
| 7 | 07/05/2019 | 30 | 7 | 07/01/2020 | 34 |
| 8 | 26/03/2019 | 22 | 8 | 01/01/2020 | 22 |
| Total | | 212 | Total | | 227 |

Figure 1. Sampling date and number of sampled cattle during Session 1 and Session 2.

2.3 Rodent sampling

Three rodent species—Norway rats (*Rattus norvegicus*), nutrias (*Myocastor coypus*), and muskrats (*Ondatra zibethicus*)—are all likely excreting leptospire into the Vilaine River. They are also the predominant mammals in the region (survey conducted on July 31, 2018; Supplemental Data 1). Representatives of each species were gathered between March and November 2019, during pest control campaigns conducted in the proximity of two nautical bases (sites A and B, respectively). These areas are located 8 km apart and near where human cases of leptospirosis were reported in 2016 and 2018. The animals' bodies were stored at -20°C until tissue sampling could occur. At that time, samples were taken of the kidneys, lungs, and urine (when available) and subsequently stored at -20 °C until the molecular analyses were conducted. Furthermore, individuals were classified as male (presence of testes) or female (presence of a genital tract) and as sexually immature (absence of seminal vesicles for males and a developed uterus for females) or sexually mature (presence of these structures).

2.4 Urine cultures

We attempted to culture leptospire from the cattle urine samples using Ellinghausen–McCullough–Johnson–Harris (EJMH) medium and EJMH STAFF medium, which were prepared under sterile conditions as described elsewhere (Chakraborty et al., 2011). Briefly, 0.1 ml of urine was added to a first tube containing 5 ml of EJMH STAFF medium. Next, 0.1 ml of this dilution was transferred to a second tube containing 5 ml of EJMH medium, yielding a further dilution (1:50). This second set of tubes was incubated at 30 °C over a two-month period. Their contents were regularly examined using dark-field microscopy to ascertain whether leptospire were present (Picardeau, 2013).

2.5 DNA extraction and *Leptospira* detection

DNA was isolated from rodent kidney and lung tissue and from rodent and cattle urine using the QIAamp DNA Mini Kit (Qiagen), in accordance with the manufacturer's instructions. To detect *Leptospira* DNA, we performed real-time PCR (rt-PCR; TaqMan method). We employed the β -actin endogenous housekeeping gene as an internal control for target gene expression, DNA extraction efficiency, and the absence of inhibitors in the samples (Toussaint et al., 2007). We specifically targeted pathogenic *Leptospira* using the TaqVet PathoLept Kit (LSI, France). Samples with C_T values of less than 40 were considered to be *Leptospira* positive, and a given animal was considered to be infected with *Leptospira* if at least one of its tissues or fluid (kidney, lung, or urine) tested positive.

2.6 Genotyping

Positive samples were then subject to conventional PCR (cPCR). One analysis targeted the 16S rDNA gene (as described elsewhere: (Merien et al., 1992) and used a DreamTaq Green PCR Master Mix (2X) Kit (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. A second analysis targeted the *lfb1* gene (as described elsewhere: (Merien et al., 2005) and used a HotStarTaq DNA Polymerase Kit (Qiagen, Courtaboeuf, France) in accordance with the manufacturer's instructions. The amplified products were visualized using electrophoresis (1% agarose gel). Then, samples yielding clearly visible, high-intensity bands underwent Sanger sequencing, which was carried out by a service provider (Genoscreen, Lille, France). The *Leptospira* species present were identified through a Nucleotide BLAST search (<http://blast.ncbi.nlm.nih.gov>).

2.7 Microagglutination testing

A microscopic agglutination test (MAT) was performed using a panel of live isolated leptospires. They represented 12 serogroups (serovars in parentheses): Australis (Australis, Bratislava, Munchen), Autumnalis (Autumnalis, Bim), Ballum (Castellonis), Bataviae (Bataviae), Canicola (Canicola), Grippotyphosa (Grippotyphosa, Vanderhoedoni), Icterohaemorrhagiae (Icterohaemorrhagiae, Copenhageni), Panama (Panama, Mangus), Pomona (Pomona, Mozdok), Pyrogenes (Pyrogenes), Sejroe (Sejroe, Saxkoebing, Hardjo, Wolffi), and Tarassovi (Tarassovi). A titer of 1:100 was used as the cut-off threshold for seropositivity, as per the guidelines issued by the World Organization for Animal Health (WOAH) (Anonymous, 2018). Particular care was taken when interpreting cross-reactions (Levett, 2001; Miller et al., 2011). A serogroup-level analysis of the MAT results was performed as described elsewhere (Ayrat et al., 2014; Naudet et al., 2022). When a high titer against a particular serogroup was observed, that single serogroup was assumed to be responsible for the antibodies in the sample. When we obtained titers against several serovars from the same serogroup, the serovar with the highest titer was assigned to the serogroup. When a sample displayed titers against two or more serogroups, we used the following approach. If one serogroup clearly predominated (*i.e.*, its titers were at least threefold higher than those of the next most prominent serogroup), then this serogroup was assigned to the sample. If the difference in titers among serogroups was not as clear cut (*i.e.*, there was less than a threefold difference between the highest titer and the next highest titer), then both serogroups were assigned to the sample as they were equally likely to be the predominant serogroup.

2.8 Statistical analysis

The data were analyzed in Rstudio (v. 1.3.1093, Apricot Nasturtium). The function *binom.test* was used to estimate *Leptospira* prevalence and its 95% confidence intervals. For the nutrias, univariate Poisson regression models were used to examine the factors influencing infection status; the independent variables were sex (male *vs* female), sexual maturity (immature *vs* mature), sampling site (A *vs* B), and sampling season (spring = March to May, summer = June to August, and autumn = September to November). An α -level of 0.05 was used.

2.9 Mapping

We mapped the spatial distributions of the animals sampled, their infection status, and their exposure status for sites A and B using R Core Team (v.4.2.1). The background map came from IGN GEOFLA®.

3. Results

3.1 *Leptospira* exposure in cattle

The MAT results are summarized in Figure 2. In the first sampling session, 11 of the 212 serum samples were MAT positive and came from 5 of the 8 farms where sampling occurred. The serological profiles showed that cattle on 4 farms had been exposed to the serogroup Grippotyphosa and cattle on 1 farm had been exposed to the serogroup Sejroe. In the second sampling session, 27 of the 227 serum samples were MAT positive and came from all 8 farms. The serological profiles showed that cattle on 3 additional farms (4, 6, and 7) had been exposed to the serogroup Grippotyphosa and that cattle on 6 farms had been exposed to the serogroup Icterohaemorrhagiae. The latter had not been detected during session 1. Sampling took place at each farm across both sessions, and repeated measures were obtained for 5 to 17 cattle per farm (the only exception being farm 3). These data showed that at least one animal had seroconverted (*i.e.*, was negative during session 1 and positive during session 2). Lastly, neither live *Leptospira* nor *Leptospira* DNA was detected in the urine samples (n = 145). While the cultures were positive for other bacterial groups (*i.e.*, sample bacterial contamination), they were negative for *Leptospira*, even when the cattle showed other evidence of *Leptospira* exposure. Additional details on the MAT, PCR, and culture results are available in Supplemental Data 2.

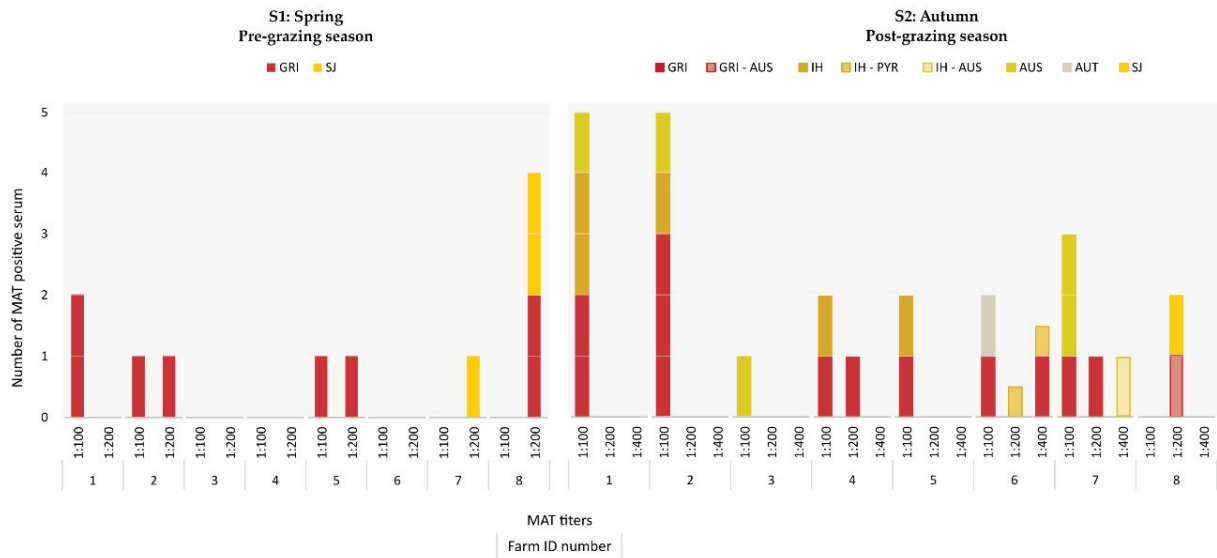


Figure 2. Number of MAT-positive cattle in session 1 (S1) and session 2 (S2) across the 8 farms, ranked according to titer (1:100 to 1:200 or 1:400).

The different putative serogroups are color coded. The mixed serogroups GRI-AUS and IH-AUS are cases in which the serological profile showed equal titers for two serogroups. On Farm 6 during session 2, one cattle displayed titers against IH (1:200) and PYR (1:400), which resulted in the mixed serogroups IH-PYR inclusion in the two titer categories.

Abbreviations. GRI: Grippotyphosa; SJ: Sejroe; AUS: Australis; PYR: Pyrogenes; AUT: Autumnalis; IH: Icterohaemorrhagiae.

3.2 Demographic characteristics of the nutrias, muskrats, and Norway rats

The demographic characteristics of the nutrias, muskrats, and Norway rats collected during each sampling season are summarized in Figure 3. The total sample size was 170 rodents, which was made up of 143 nutrias, 17 muskrats, and 10 Norway rats. Overall, there were more males than females for the nutrias (males: 59%, n=84/143; females: 41%, n=59/143) and for the muskrats (males: 76%, n=13/17; females: 24%, n=4/17). Additionally, there were sexually mature than sexually immature nutrias (sexually mature: 63%, n=91/143, sexually immature: 37%, n=52/143) and muskrats (sexually mature: 59%, n=10/17; sexually immature: 41%, n=7/17). For the Norway rats, the two ratios were equal. Sample size varied across seasons. No Norway rats were captured in the spring, one muskrat was captured in the summer, and fewer nutrias

were collected in the autumn (n = 36) than in the spring (n = 52) or summer (n = 56). Nutrias and muskrats' males outnumbered females in all three seasons.

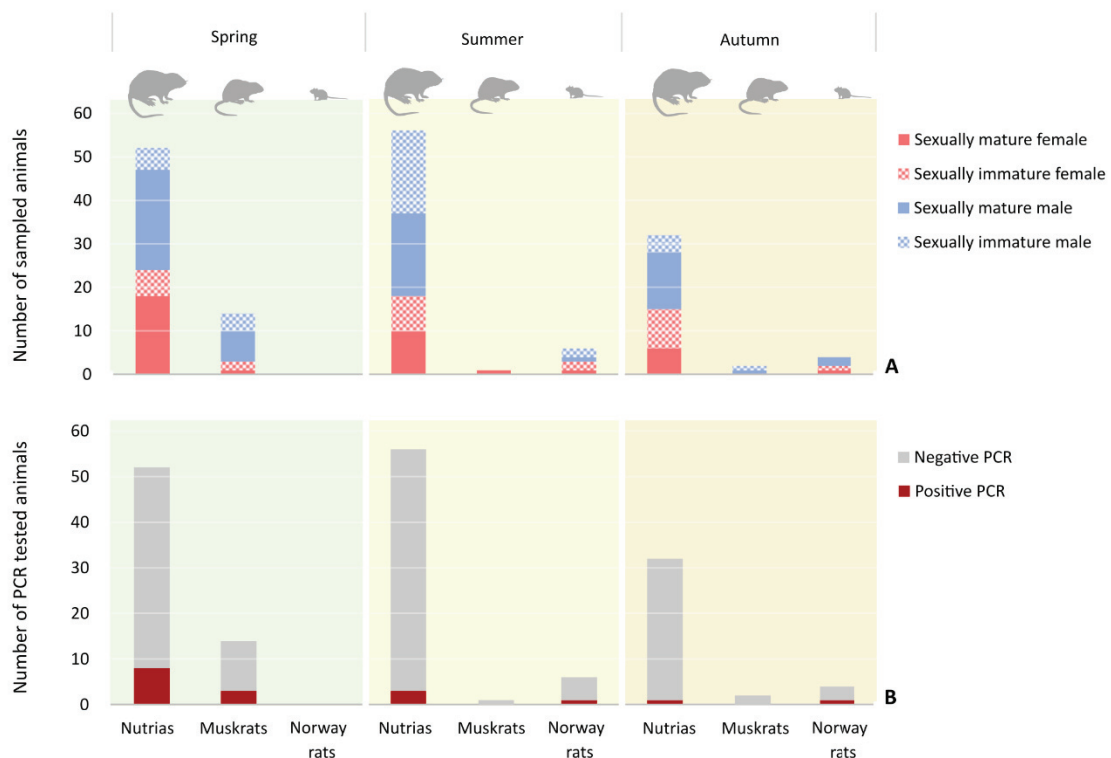


Figure 3. (A) Demographics for each rodent species across seasons. The season in which three nutrias were collected (one sexually mature female, one sexually immature female, and one sexually mature male) was not recorded and therefore does not appear above. (B) Number of rodents in each species testing rt-PCR positive and negative across seasons. The three nutrias not shown were rt-PCR negative.

3.3 *Leptospira* infection in the nutrias, muskrats, and Norway rats

3.3.1 Presence of *Leptospira* DNA

Of the 170 rodents collected, 12 nutrias, 3 muskrats, and 2 Norway rats were PCR positive for *Leptospira*. Despite these results, no morphological abnormalities were seen in the animals' tissues upon dissection. The seasonal pattern in the PCR results is displayed in Figure 3.B.

Prevalence varied between sites A and B and across rodent species, although not significantly so. It was 18% in muskrats (n = 3/17, CI_{95%} [4%, 43%]), 20% in Norway rats (n = 2/10, CI_{95%} [2%, 56%]), and 8% in nutrias (n = 12/143, CI_{95%} [4%, 14%]).

3.3.2 Characterization of *Leptospira* DNA

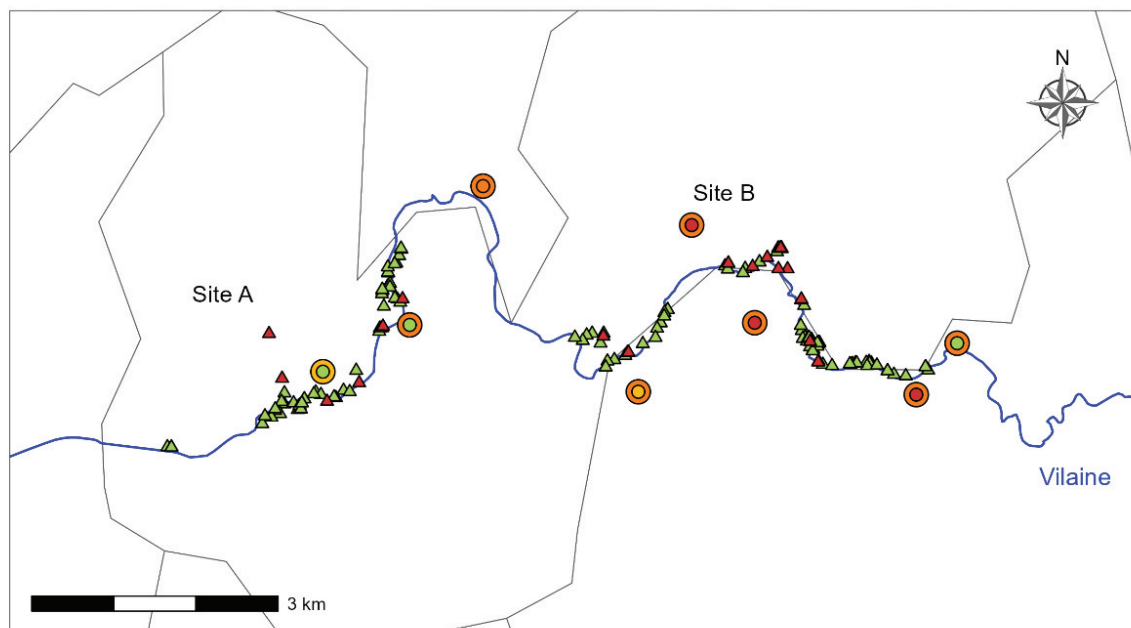
For 11 of the 17 PCR-positive rodents, a high-intensity band emerged during cPCR. Subsequent sequencing revealed the presence of *L. interrogans* in 7 nutrias (n = 7/12), 2 muskrats (n = 2/3), and 2 Norway rats (n = 2/2).

3.3.3 Characteristics of the infected rodents

For the nutrias, *Leptospira* prevalence was uninfluenced by sex, sexual maturity, site, or season (p -value > 0.05). That said, there was a marginally insignificant trend ($p = 0.1$) whereby *Leptospira* prevalence seemed to be higher in the spring than in the autumn (PR = 4.9, CI_{95%} [0.9, 91]) and in males versus females (PR = 3.4, CI_{95%} [0.9, 22]).

3.4 Spatial distribution of rodents and cattle farms

The individuals testing positive for *Leptospira* (via PCR or MAT) occurred all along the Vilaine River and across both sites (Fig. 4).



Tests results according to sampling sites

- Cattle MAT s1: GRI
- Cattle MAT s1: others
- Cattle MAT s2: GRI-others
- ▲ Rodents PCR negative
- Cattle MAT s1: GRI-others
- Cattle MAT s1: seronegative
- Cattle MAT s2: others
- ▲ Rodents PCR positive

Figure 4. Spatial distribution of the rodents and cattle farms tested for *Leptospira*. For the cattle, samples were collected during session 1 (S1) and session 2 (S2). There were farms where cattle were exposed to serogroup Grippotyphosa exclusively (GRI); farms where cattle were exposed to various serogroups, including Grippotyphosa (GRI – others); and farms where cattle were exposed to various serogroups other than Grippotyphosa (others). Data are not shown for 10 uninfected rodents (1 from site A and 9 from site B).

4. Discussion

Our results revealed that substantial numbers of rodents were shedding *L. interrogans* into the environment across seasons, while the cattle appeared to be experiencing incidental infections, similar to humans. However, our investigation failed to identify the potential source of *L. kirschneri*, which has also caused human cases of leptospirosis. Below, we discuss the potential animal sources of pathogenic *Leptospira* and the challenges inherent to comprehensively describing the reservoirs responsible for human cases of leptospirosis. We also propose a partial multisectorial framework

for implementing future ecoepidemiological surveys following cases of leptospirosis associated with aquatic recreational activities.

4.1 Potential animal sources of *Leptospira*

4.1.1 Cattle were unlikely to be a source of pathogenic *Leptospira*

Despite our meticulous sampling procedure on the farms, no *Leptospira* were cultured from any of the 145 urine samples and medium were contaminated. This outcome might have been the result of culture contamination, which could have limited our ability to detect leptospirae *via* microscopic observations, and/or the result of culture sensitivity, since the technique is far less sensitive than PCR, as highlighted by numerous studies (Fornazari et al., 2012; Wagenaar et al., 2000). However, the urine samples were also PCR negative for *Leptospira* DNA, confirming the taxon's absence. Taken together, these results strongly suggest that none of the 145 cattle sampled were infected at the time of sampling, which implies that the cattle were not contributing to environmental contamination. In addition, the serological results indicate that the cattle were exposed to the serogroups Grippityphosa and Icterohaemorrhagiae. These serogroups are rarely seen in cattle from France (Ayrál et al., 2014), which suggests an unusual epidemiological situation underlies their circulation at both sites. In conclusion, cattle were not a significant source of *Leptospira* but rather appeared to have been incidentally infected *via* exposure to *Leptospira* in the environment, as is the case for humans.

4.1.2 Rodents were likely a source of pathogenic *Leptospira*

The pathogenic species *L. interrogans* was detected in nutrias, muskrats, and Norway rats at the two sampling sites along the Vilaine River, suggesting that infection with this pathogen is common among local rodent populations.

Norway rat. Although few Norway rats were captured due to weather conditions (*i.e.*, persistent flooding) and our estimates of prevalence were imprecise (20%, CI_{95%} [2%, 56%]), the level of prevalence within the population was comparable to that within other populations in previous studies (Ayrál et al., 2015a; Himsworth et al., 2013). In addition, the Norway rats were carrying *L. interrogans*, which was not unexpected, given that they are maintenance hosts for *L. interrogans* serogroup Icterohaemorrhagiae (Thiermann, 1981). However, we were unable to genetically determine the serogroup present because the *Leptospira* DNA was poor in quality and quantity. Despite the caveats mentioned above, our results suggest that, like elsewhere, Norway rats could potentially be reservoirs of *L. interrogans* (Ayrál et al., 2015b; Thiermann, 1981) and thus could have been responsible for the human case of leptospirosis in 2018.

Nutrias. Like Norway rats, nutrias seem to be able to carry *Leptospira* without experiencing significant health consequences (Athanasio et al., 2008; Waitkins et al., 1985), which is consistent with the absence of any organ lesions in this study. *Leptospira* prevalence was lower in nutrias than in Norway rats, a finding that is consistent with previous epidemiological surveys of nutrias in France (Aviat et al., 2009; Ayral et al., 2020). As has occurred previously, *L. interrogans* was detected in nutrias but could not be genotyped to determine serogroup identity (Mazzotta et al., 2023; Vein, 2013). Further efforts should be made to characterize the *Leptospira* present and, eventually, confirm whether the serogroups Australis and Icterohaemorrhagiae predominate, as suggested by past MAT results (Ayral et al., 2020). Compared to Norway rats, nutrias likely contribute less to the maintenance of *L. interrogans*. That said, in this ecosystem, nutrias density can increase drastically, and the relative abundance of a host species is an important consideration when estimating the risk of leptospirosis faced by humans.

Muskrats. The prevalence values observed here and elsewhere (Aviat et al., 2009; Ayral et al., 2020) suggest that muskrats could play a more important role than nutrias as *Leptospira* carriers. However, muskrats were scarce among our field specimens and have a low level of occurrence in the focal ecosystem, which means that they likely make a limited contribution to environmental contamination by *Leptospira*. We found that two of the three infected muskrats were carrying *L. interrogans*. This finding is inconsistent with a previous serological study suggesting that muskrats might be predominantly infected by *Leptospira* serogroup Grippotyphosa, which is frequently associated with *L. kirschneri* (Ayral et al., 2020; Salaün et al., 2006). To our knowledge, this is the first study to describe *Leptospira* species in muskrats. Thus, additional research should be performed to flesh out these preliminary findings and further clarify *Leptospira* carriage in muskrats.

4.2 Source of several human cases of leptospirosis remains unidentified

4.2.1 *Leptospira* circulating in humans and animals

Epidemiological source tracking compares the identity of the *Leptospira* found in target versus potential source populations utilizing preferably DNA or serological data. Uncertainty can arise if data on *Leptospira* circulation dynamics are missing. Although the outbreak reported by Guillois et al. (2018) was attributed to *L. kirschneri* serogroup Grippotyphosa, only 5 of the 14 cases were typed, either *via* serology (4 *Leptospira* serogroup Grippotyphosa) or molecular testing (1 *L. kirschneri*). During the study period (2018 and 2019), there were two cases of leptospirosis in humans that were attributed to *L. kirschneri*, suggesting that this species recurrently occurs in the environment. However, it is possible that some of the remaining cases were caused by other *Leptospira* species, such as the case related to *L. interrogans* in 2018. At least two

Leptospira strains are responsible for human infections, with *L. kirschneri* being the most frequently detected.

Surprisingly, *L. kirschneri* was not detected in the animal samples although it has been responsible for many human infections in the focal ecosystem. Recent research looking at *Leptospira* infection has suggested that placing the rodents' whole bodies in cold storage prior to tissue sampling could lead to alterations in DNA structural integrity and, thus, to underestimates of prevalence (Harran et al., 2023). As we employed this cold-storage method, it is likely that we underestimated the true number of infected rodents. Furthermore, we were only able to identify *Leptospira* species for 11 of the 17 PCR-positive rodents. Our cold-storage method could have been responsible for the poor quality of the DNA and the inability to perform genotyping, despite the sensitivity of cPCR testing (Merien et al., 1992). This issue could have limited the likelihood of detecting *L. kirschneri*, if present. Like for human cases, it is essential to comprehensively describe the identity and distribution patterns of host-maintained *Leptospira* to reliably characterize the reservoirs behind leptospirosis outbreaks. Crucial to this goal are the methodologies used to sample animal specimens and tissues.

4.2.2 Animal sampling

The source of the human cases caused by *L. kirschneri* could be a species yet to be sampled. Although we collected the main reported carriers of *Leptospira* in the focal ecosystem, it could be that another animal species was responsible for the above cases. In Western Europe, *L. kirschneri* has also been detected in other small mammals known to occur at sites A and B, such as the wood mouse (*Apodemus sylvaticus*), bank vole (*Myodes glareolus*), greater white-toothed shrew (*Crocidura russula*), crowned shrew (*Sorex coronatus*), and common vole (*Microtus arvalis*) (Fischer et al., 2018; Mayer-Scholl et al., 2014; Obiegala et al., 2016). Furthermore, in a previous study, a single wood mouse at site A was found to have antibodies against *Leptospira* serogroup Grippotyphosa (Guillois et al., 2018), and the common vole is thought to have caused a leptospirosis outbreak in humans (Desai et al., 2009). Taken together, all these findings indicate that *L. kirschneri* is likely circulating among small mammals. Considering that *L. kirschneri* serogroup Grippotyphosa can survive 72 hours at best under the study conditions (Nau et al., 2019), it seems probable that *Leptospira* persists in local water resources because hosts are continually shedding bacteria. It is important to investigate the ecology of the small mammals mentioned above and whether or not they could contaminate water resources directly or indirectly, *via* bridge hosts (Caron et al., 2015). However, access to samples from wild species is limited, and sampling is extremely restricted in the case of many taxa, such as endangered species, given the need to preserve animal populations, health, and welfare. Some animals, such as

mustelids (*e.g.*, species in the genera *Martes* and *Mustela*) could serve as sentinels and be used to characterize the *Leptospira* genotypes circulating among their prey, which are mainly small rodents or other small mammals (Ayrat et al., 2016b).

4.3 Putting the One Health concept into practice

4.3.1 From a collaborative study to a One Health workforce

In our study, we adopted a One Health approach (OHHLEP et al., 2022) because the recurrence of leptospirosis in kayakers using the Vilaine River is a complex health issue involving a strong interdependence among human, animal, and environmental health. Humans are not the only ones facing health risks because pathogenic *Leptospira* in the environment could cause infections in other susceptible mammals, such as livestock, dogs, and even endangered species (*e.g.*, Eurasian beaver [*Castor fiber*], European mink [*Mustela lutreola*]) (Marreros et al., 2018; Moinet et al., 2010). Furthermore, control efforts are frequently applied to rodent populations to mitigate public health risks but can elicit animal welfare concerns when evidence for their role as disease reservoirs is weak (Mankad et al., 2019).

Leptospira infections can have severe consequences for human and animal health, underscoring the collective need for leptospire-free water resources and soils, an objective that has brought together multiple sectors, disciplines, and communities. Here, the result has been a multisectorial approach that has exploited human and financial resources provided by institutions focused on human health, animal health, and environmental protection. In addition, data sampling, submission, and analysis have been carried out by various stakeholders and communities (*e.g.*, veterinarians, farmers, kayak professionals, professionals in the domain of health and environmental safety from public and private organizations, academics, and laboratories) and required expertise from various disciplines (*e.g.*, epidemiology, microbiology, pathology, wildlife ecology, population management, and public health). In this regard, our multisectorial and multidisciplinary ecoepidemiological research corresponds to Action Tracks 1.2 and 2.1 in the One Health Joint Plan of Action, which focuses on methods for facilitating One Health work and seeks to understand the drivers behind the emergence of zoonotic pathogens, respectively (Anonymous, 2022). Lastly, the collaboration has produced a One Health workforce, as required by tri- and quadripartite guidelines for building and implementing a One Health framework (Anonymous, 2022, 2019). This workforce can be deployed during future collaborative ecoepidemiological surveys.

4.3.2 From a One Health workforce to a One Health framework

Identifying relevant stakeholders is a task required by the tri- and quadripartite guidelines for building and implementing a One Health framework (Anonymous, 2022, 2019). This work was a focal point in our study. More specifically, we have identified the stakeholders that could serve as potential contributors and the actions that they could undertake (Supplemental Data 3). This list will help pinpoint who should be invited to participate in future ecoepidemiological surveys and the specific role they could play. Compared to national or regional stakeholders, local stakeholders may make highly diverse, informal contributions. For example, farmers could report the presence of rodents in their pastures, and everyday citizens could report on any ecosystem changes observed during their recreational activities. Thus, identifying stakeholders is a crucial step that should be taken with care and caution. The support of social scientists can also be sought, which we did not do here.

It is important to fine-tune this One Health framework so that it can better support leptospirosis management and outbreak preparedness. Additionally, the framework must include One Health coordination modalities, action type and timing, task allocation, and instruments for monitoring framework progress. In France, leptospirosis in humans recently became a notifiable disease (Article D-31138 of the Public Health Code modified by the “Décret” n°2023-716). Thus, this One Health framework will be developed against a backdrop of administrative shifts that will facilitate outbreak reporting in the near future.

5. Conclusion

This study revealed that it can be challenging to track down the source of *Leptospira* outbreaks because of limited access to high-quality samples that can be used for diagnostic testing and DNA sequencing, as well as because of gaps in knowledge about the maintenance community. Future ecoepidemiological research must be collaborative and multisectorial if we are to progress in describing reservoir communities and the associated risk of leptospirosis within ecosystems. The collaboration that has emerged from this study has revealed the complementary areas of expertise and converging interests within the partnership. It spurred the creation of a One Health framework, so that sustainable actions could continue into the future. Our collaborative research has therefore given rise to a workforce whose purview extends beyond this study and that will help manage leptospirosis cases in the future.

Appendices:

Appendix 1: Descriptions of sites A and B.

Appendix 2: Detailed MAT, PCR, and culture results for the cattle samples.

Appendix 3: Stakeholders to be identified and contacted about collaborations, noting roles and responsibilities.

Appendix 4: Preliminary analysis of the environmental situation related to a leptospirosis outbreak

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3. Conclusion and future perspectives

Recognizing each animal host's role in leptospirosis epidemiology can be difficult and calls for the use of specific epidemiological approaches. According to the results of the single-host approach adapted, we could not clearly identify the role of raccoons in the maintenance community as the prevalence reported in the latter animal species in both ecosystems in France was low and different genetic profiles were acquired. According to the results of the multi-hosts approach adapted, the role of the sampled animals was not clear and the source of human infection could not be determined due to limited sampled animal species. Results of both studies supported the need to adapt more suitable approaches when studying the role of hosts in *Leptospira* maintenance community. A more suitable epidemiological approach was applied in a unique study that investigated leptospiral carriage in three different populations of water voles (*Arvicola terrestris*) in Puy-de-Dôme department in France. This investigation was valorized in a research article present in Part II.2.(2) of the thesis.

II.2. Requirement of laboratory methods for describing the status of sampled hosts in a maintenance community

1. Introduction of laboratory methods for *Leptospira*-detection in epidemiological studies

In the field of infectious disease epidemiology, suitable laboratory tests are essential for the study of outbreaks, diagnosis and surveillance [197]. Their complementary application—despite the drawback(s) that each method can present—is satisfactory for the description of the biological characteristics of the pathogen and increases the accuracy of the bacteria's detection in order to better understand the disease pathogenicity and transmissibility [54]. Consequently, these tests can support and improve the design of epidemiologic studies and the knowledge about the maintenance community. In this context, different molecular tests were applied on different specimens of water voles in three ecosystems in Puy-de-Dôme, Central France. The main objective of this study was to determine the role of water voles in the maintenance community. The more specific objectives were to provide a reliable combination of molecular tests and processing measures to be considered in future epidemiological investigations when studying a maintenance community and to determine whether further methods are required to have more reliable and complete information.

2. Identification of Pathogenic *Leptospira kirschneri* Serogroup Grippytyphosa in Water Voles (*Arvicola terrestris*) from Ruminant Pastures in Puy-de-Dôme, Central France

Article 3: Identification of Pathogenic *Leptospira kirschneri* Serogroup Grippytyphosa in Water Voles (*Arvicola terrestris*) from Ruminant Pastures in Puy-de-Dôme, Central France.

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Article

Identification of Pathogenic *Leptospira kirschneri* Serogroup Grippytyphosa in Water Voles (*Arvicola terrestris*) from Ruminant Pastures in Puy-de-Dôme, Central France

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Abstract: Rodents are the primary reservoirs for pathogenic *Leptospira* species, which cause leptospirosis. Among the key potential carriers are water voles, whose population outbreaks can consequently pose a major threat to human and animal health. We studied the prevalence, prominence, and epidemiology of pathogenic *Leptospira* species in water voles in central France. First, 46 voles were captured, and DNA was extracted from kidney, lung, liver, blood, and urine and tested for the presence of *Leptospira* using three molecular methods: PCR, O-antigen typing, and variable number tandem repeat (VNTR) typing. We also attempted to culture leptospire from kidney and urine samples. In addition, we investigated leptospiral antibodies in serum samples from 60 sheep using microscopic agglutination testing. These animals co-occurred with the voles, so we sought to assess their degree of exposure and involvement in pathogen dynamics. The overall prevalence of infection was 76.1% (CI_{95%} [61.2%, 87.4%]). The only strain found was *L. kirschneri* serogroup Grippytyphosa and a similar VNTR profile was acquired. Leptospire were successfully cultured from kidney and urine samples for four voles. Three sheep had low antibody titers against the *Leptospira* serogroup Grippytyphosa. Taken together, our results suggest the exclusive carriage of *L. kirschneri* serogroup Grippytyphosa among water voles in central France. Nevertheless, their ability to act as reservoir hosts that transmit the pathogen to co-occurring livestock remains unclear and merits further research.

Keywords: leptospirosis; water voles; maintenance hosts; reservoir; diagnosis; epidemiology



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1. Introduction

Leptospirosis is considered to be the world's most widespread bacterial zoonosis and is responsible for 1.03 million cases of illness and 60,000 deaths annually [1,2]. The clinical manifestations of the disease depend on the host. For example, infections may be asymptomatic and highly persistent in rodents, lead to subclinical and chronic disease in domestic ruminants, or cause severe, potentially fatal illness in humans [2–6]. Transmission in humans and animals is mainly indirect, and incidence is higher in warm and humid climatic zones [7,8]. Global climate change is likely behind the recent increase in leptospirosis incidence and outbreaks [9,10], a trend that has been observed in the human population of mainland France since 2014 [11]. Awareness is thus growing that leptospirosis represents a significant threat to public health [12–14]. That said, little is known about many fundamental aspects of leptospirosis epidemiology, such as pathogen ecology and pathogen dynamics in wildlife hosts. Consequently, we have a limited understanding of disease drivers and effective prevention strategies.

The genus *Leptospira* currently comprises 68 species of highly diverse bacteria that form two clades—the pathogens and the saprophytes—that each, in turn, contain two subclades (P1 and P2 vs. S1 and S2); these groups differ in their virulence and genetic characteristics [15]. Among the pathogenic species, *L. interrogans*, *L. kirschneri*, and *L. borgpetersenii* predominate and circulate worldwide; *L. interrogans* is most commonly seen in individual cases of leptospirosis, while *L. kirschneri* is regularly associated with outbreaks in humans [5,16,17]. *Leptospira* species are categorized into serogroups that are further divided into serovars [18]. In epidemiological studies conducted in nature, it is essential to reliably characterize *Leptospira* serogroups via standardized laboratory tests [1] because there are strong associations between certain serogroups and hosts [5,19].

Pathogenic *Leptospira* species can infect all mammal species, but their dynamics and dissemination are host specific. For example, some mammals may act as maintenance hosts for particular serogroups, while others act as accidental hosts. In the latter group, the outcome of infection is either recovery or death. The pathogen does not naturally persist in accidental hosts; however, except in the case of humans, accidental hosts can contribute to ecosystem-level pathogen persistence if they co-occur with hosts of either type [20]. Conversely, maintenance hosts tend to be infected by particular serogroups that colonize the kidneys and are shed in the urine over long periods of time [21]. They may act as chronic selective carriers of particular *Leptospira* serogroups in a range of ecosystems and possibly transmit the pathogen to accidental hosts [1]. For example: brown rats (*Rattus norvegicus*) are thought to be selective carriers of the serogroup Icterohaemorrhagiae and cause accidental infections in humans [22,23]. Cattle (*Bos taurus*) are selective carriers of the serogroup Sejroe [24]; dogs (*Canis familiaris*) of the serogroup Canicola [25]; and swine (*Sus scrofa*) of the serogroups Australis and Pomona [5,26–28]. Although rats, cattle, dogs, and pigs might serve as maintenance hosts for other *Leptospira* serogroups, experimental research has yet to explore this question. Particular *Leptospira* serogroups persist within ecosystems thanks to specific host–pathogen relationships and ecological interactions, which means that ecosystems each possess their own set of maintenance mechanisms and levels of environmental contamination [20,29]. The hosts making the greatest contributions to these dynamics form the maintenance community and can end up infecting target species of concern [20,21]. Over the past 50 years, mainland France has witnessed dramatic shifts in land cover as a result of agricultural development, leading to decreased biodiversity [30]. In the current pasture ecosystems of central France, water voles (*Arvicola terrestris*) and domestic ruminants are the dominant fauna [31]. They co-occur and may share a number of epidemiological connections.

Outbreaks of water vole populations occur every 6 years on average, with densities peaking at 500–1000 individuals/hectare for 1–3 years [32,33]. If a vole population is headed toward an outbreak and is hosting a pathogenic *Leptospira* species, humans, livestock, and other animals in the same environment may be at risk [34]. Recently in Europe, the presence of *Leptospira* species has been reported in a variety of voles, such as common voles (*Microtus arvalis*), field voles (*Microtus agretis*), water voles (*Arvicola terrestris*), and bank voles (*Myodes glareolus*). These studies were mainly conducted in Germany and Spain and specifically identified *L. kirschneri*, *L. borgpetersenii*, and *L. interrogans* [35–38]. A handful of more detailed studies have been carried out on European water vole populations, including two in eastern France. One showed that water voles were carrying *Leptospira* species but did not identify any genetic profile [39]. The second found *L. kirschneri* to be the only species present [40]. However, research has yet to address the prevalence of *Leptospira* species in water voles in central France while also examining the voles' potential role in transmitting leptospires to hosts such as domestic ruminants. This issue is particularly important to address given the regular occurrence of vole outbreaks.

Our study's major aim was to investigate the potential role of water voles and sheep in *Leptospira* epidemiology in pasture ecosystems in central France. To this end, our specific objectives were as follows: (1) characterize *Leptospira* prevalence in vole populations during outbreaks; (2) determine whether leptospires were present in vole tissues to clarify

potential infection type (chronic vs. acute) and transmission pathways; (3) obtain complete genetic profiles, namely serogroup identity, and assess whether selective carriage could be occurring; and (4) ascertain whether water voles might transmit leptospire to co-occurring sheep.

2. Materials and Methods

2.1. Ethics Statement on Vole Sampling

The authors confirm that all the research described herein complied with national and institutional regulations related to the care and use of animals (APAFiS, no. 37713, project authorization no. 2238).

2.2. Provision of Sheep Blood Samples

Sheep blood samples collected for annual herd prophylaxis program were used in this study after obtaining owner consent.

2.3. Sampling Sites

We trapped water voles in three different livestock breeding sites over a single day in November 2021. The pastures were separated by 1.4 to 1.6 km and were located in the French administrative department of Puy-de-Dôme. This department is in central France and nearly 50% of its surface area (400,000 ha) is dedicated to agriculture, mainly in the form of grasslands [41]. Vole outbreaks are common in Puy-de-Dôme; population densities can exceed 500 voles/ha [42,43]. We selected these three study sites because they had large vole populations and displayed specific environmental characteristics, notably agricultural lands with crops organized into plot systems (Figure 1).

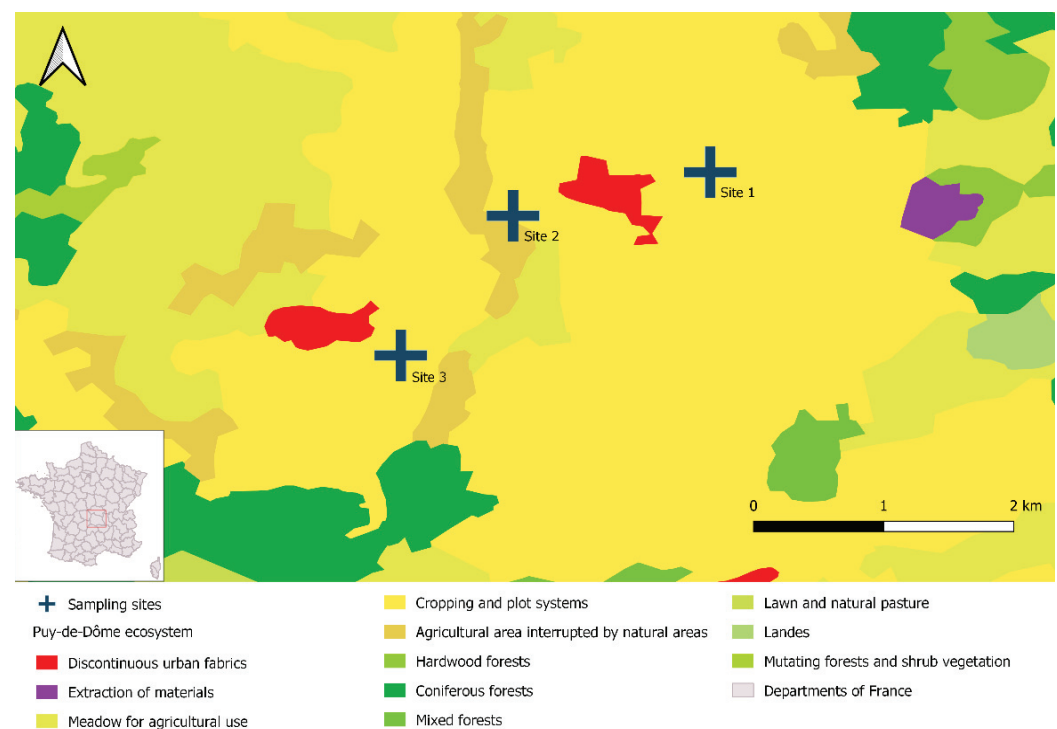


Figure 1. Location of the three study sites in Puy-de-Dôme, France. The map was created with DIVA-GIS (v. 7.5) and data from Corine Land Cover (2018 Edition, mainland France); it was designed with QGIS 3.16.1 Hannover.

2.4. Vole Trapping and Sampling

Voies were live captured using tube traps and lethally captured using Topcat traps. The 26 living voles were immediately anaesthetized using isoflurane and euthanized via

cardiac puncture and cervical dislocation. They were quickly dissected to take tissue samples, which were then stored at $-20\text{ }^{\circ}\text{C}$ for 4 weeks, at which point the molecular analyses were conducted. These animals are hereafter referred to as the euthanized voles. Twenty voles were lethally captured. Their entire bodies were immediately frozen and then stored at $-20\text{ }^{\circ}\text{C}$. Dissection and tissue sampling occurred 10 weeks later and were immediately preceded by a 24-h thawing period. These animals are hereafter referred to as the cold-stored voles. For each vole, we noted the following: sex (presence of genital tract = females, presence of testes/penis = male), length (body = from nose to anus and overall = from nose to tail), and state of sexual maturity (developed uterus for females, presence of seminal vesicles for males).

For both groups, dissection and tissue sampling took place as follows. Three sets of dissecting instruments were alternated during these processes. To prevent cross-contamination, the instruments were regularly cleaned and disinfected, notably between tissue sampling within animals and between dissections across animals. When possible, samples of kidney, lung, liver, blood, and urine were collected for each animal. Kidney and lung samples were obtained to evaluate evidence of renal colonization and pulmonary carriage, respectively; the latter has been described in rats [44]. Blood and liver samples were obtained to assess the occurrence of acute septicemic infection, and urine was obtained to evaluate the possibility of leptospire excretion. All the samples were stored individually in 2-mL Eppendorf tubes at $-20\text{ }^{\circ}\text{C}$ until the molecular analyses could be carried out.

2.5. Sheep Sampling

Serological diagnosis is considered adequate to define *Leptospira*-status among sheep herds and determine circulating leptospiral antibodies (with antibodies persisting several months) by the World Organization for Animal Health (WOAH) [45]. At least 10% of the herd was sampled, in order to gather relevant data at the herd level as recommended by the WOAH [45].

Site 3 hosted a herd of nearly 200 sheep. Consequently, samples were taken from 2 batches of 30 sheep at 2 time points: 6 months after the grazing period and 6 months after the initial sampling period, which was 2 weeks immediately after the next grazing period.

2.6. Culturing *Leptospira* from Urine and Renal Tissue

We attempted to culture *Leptospira* bacteria from urine and kidney samples using Ellinghausen–McCullough–Johnson–Harris (EMJH) medium. First, EJM and EJM STAFF media were prepared under sterile conditions, as described elsewhere [46]. Briefly, a volume of 1 mL of urine was collected and a piece of fresh kidney tissue of about 1 cm^3 was crushed inside the tube of a sterile syringe of 5 mL using the plunger. A volume of 1 mL of both preparations was independently added to a first tube containing EJM STAFF medium. The tubes were vortexed for a few seconds, yielding a 1/10 dilution. Next, 1 mL of the diluted solutions was transferred to a second set of tubes that contained EJM medium. These tubes were vortexed for a few seconds, yielding a 1/100 dilution. The tubes were then incubated at $30\text{ }^{\circ}\text{C}$. Over a two-month period, we regularly assessed the presence or potential growth of leptospira on the medium via dark-field microscopy (DFM) [47]. If no leptospira were observed during that period, culture was considered negative.

2.7. Extraction and Detection of *Leptospira* DNA

DNA was isolated from kidney, lung, liver, blood and urine using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and from successful cultures (containing at least 5×10^6 cells) using a QIAamp DNA Mini Kit (Qiagen, Courtaboeuf, France). In both cases, we followed the manufacturer's instructions. The quantitation of the β -actin endogenous housekeeping gene using real time PCR (RT-PCR) was used to assess the efficiency of DNA extraction and the absence of inhibitors for each sample and served as an internal control for the target gene expression [48]. RT-PCR targeting the 16S rRNA gene was performed using AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems) and specific primers

previously described to detect for pathogenic *Leptospira* species [49]. The PCR conditions were as follows: 10 min at 95 °C as the denaturation step, and 40 cycles of (a) 15 s at 95 °C as the amplification step and (b) 1 min at 60 °C as the annealing step. Each run included a negative control (the PCR mix without the target DNA) and a positive control (DNA from the *L. interrogans* serovar Icterohaemorrhagiae ENVN strain). When a sample had a cycle threshold (C_T) that was equal to or less than 40, it was considered to be positive for *Leptospira*.

2.8. Genetic Characterization of *Leptospira*

2.8.1. Conventional PCR Targeting the 16S rDNA Gene

Samples found to be positive for *Leptospira* via RT-PCR were then subject to conventional PCR (cPCR) targeting the 16S rDNA gene, using specific primers described elsewhere [50]. We used a HotStarTaq DNA Polymerase Kit (Qiagen, Courtaboeuf, France) and the following cPCR conditions: 15 min at 95 °C as the first denaturation step; 40 cycles of (a) 15 s at 95 °C for the second denaturation step, (b) 30 s at 57 °C as the annealing step, and (c) 1 min at 72 °C as the initial elongation step; and 10 min at 72 °C as the final elongation step. Each run included a negative control (the PCR mix without the target DNA) and a positive control (DNA from the *L. interrogans* serovar Icterohaemorrhagiae ENVN strain). The amplified products were verified utilizing electrophoresis on 1% agarose gel (30 min at 100 v). Under ultraviolet light (UV) conditions, the products' molecular weights were assessed via comparisons with a 100-bp ladder (Invitrogen) and the positive control. The products were then subject to Sanger sequencing (performed by Genoscreen, Lille, France). ChromasPro (v. 2.6.6) was used to assemble the sequences, creating contigs. We then identified the *Leptospira* species present using an NCBI Nucleotide BLAST search (<http://blast.ncbi.nlm.nih.gov>, (accessed on 1 November 2022)).

2.8.2. Molecular Typing of *Leptospira* DNA Based on O-Antigen and Variable Number Tandem Repeat (VNTR) Methods

To identify the *Leptospira* strains present, we used two forms of molecular typing, one using O-antigen and one using VNTR. O-antigen typing was performed on positive samples ($C_T \leq 40$) obtained from renal tissue and successful cultures, using primers conceived in previous article [51]. The cPCR conditions were as follows: 15 min at 95 °C as the first denaturation step; 30 cycles of (a) 30 s at 94 °C as the second denaturation step, (b) 30 s at 60 °C, and (c) 1 min at 72 °C as the initial extension step; and 10 min at 72 °C as the final extension step. Each run included a negative and a positive control. The PCR mix without the target DNA was the negative control. DNA from the *L. kirschneri* serovar Grippotyphosa Moskva V strain and from the *L. interrogans* serovar Icterohaemorrhagiae ENVN strain were the positive controls; they allowed serogroup differentiation. The amplified products were verified utilizing electrophoresis on 1% agarose gel (30 min at 100 v). Under UV conditions, the products' molecular weights were assessed via comparisons with a 100-bp ladder (Invitrogen) and the positive control.

VNTR typing was performed on positive samples ($C_T < 35$) obtained from renal tissue and successful cultures. VNTR-4, VNTR-7, and VNTR-10 loci were amplified using primers described elsewhere [52]. The cPCR conditions were as follows: 15 min at 95 °C as the first denaturation step; 40 cycles of (a) 30 s at 95 °C as the second denaturation step, (b) 30 s at 54 °C (VNTR-4/VNTR-7) or 52 °C (VNTR-10) as the annealing step, (c) 1 min at 72 °C as the initial extension step; and 10 min at 72 °C as the final extension step. Each run included a negative control as well as VNTR-specific positive controls: there were 4 controls for VNTR-4 (copies 0-1-2-3), 2 controls for VNTR-7 (copies 1 and 2), and 4 controls for VNTR-10 (copies 4-7-11-12). The amplified products were verified utilizing electrophoresis on 1% agarose gel (30 min at 100 v). Under UV conditions, the products' molecular weights were assessed via comparisons with a 100-bp ladder (Invitrogen) and the positive controls. Band sizes were used to deduce the copy number of the repeats relative to each VNTR and, thus, establish a VNTR profile for each sample.

2.9. Microagglutination Testing

Microscopic agglutination test (MATs) were carried out on the sheep's blood samples using a panel of live isolated leptospire. We considered a series of 12 serogroups (and their associated serovars): Australis (Australis, Bratislava, Munchen), Autumnalis (Autumnalis, Bim), Ballum (Castellonis), Bataviae (Bataviae), Canicola (Canicola), Grippotyphosa (Grippotyphosa, Vanderhoedoni), Icterohaemorrhagiae (Icterohaemorrhagiae, Copenhageni), Panama (Panama, Mangus), Pomona (Pomona, Mozdok), Pyrogenes (Pyrogenes), Sejroe (Sejroe, Saxkoebing, Hardjo, Wolffi), and Tarassovi (Tarassovi) (Table S1). Leptospire agglutination was assessed using DFM. Any samples with an agglutination level of at least 50% were further diluted to establish the titer endpoint for each of the 22 serovars tested. A titer of 1:100 was used as the cut-off threshold for seropositivity, as per WOAH guidelines [45]. MAT results were analyzed at the serogroup level [53]. The putative serogroup responsible for an infection was identified when a titer was obtained against one or more serovars in a given serogroup or when the maximum titer against a given serogroup was at least three-fold higher than those against any other serogroups. We classified samples as displaying equal dominance when they reacted to two or more serogroups but there was less than a threefold difference in titers. In such instances, the result was deemed to be inconclusive; such is a frequent outcome given cross reactions.

2.10. Data Analysis

A vole was considered to be infected with *Leptospira* if at least one specimen was tested positive. The characteristics of infected and uninfected voles were compared using Rstudio (v. 1.3.1093, Apricot Nasturtium). An ANOVA (t.test function) was performed to evaluate the influence of length on infection status. A Chi-square test (chisq.test function) was used to assess the influences of sex, sexual maturity, category, and study site on infection status, including the estimate of *p*-value to consider small populations. The 95% confidence intervals for prevalence were calculated using the binom.test function.

3. Results

3.1. Vole Characteristics

Among the voles sampled, there were more females than males (56.5% [26/46] vs. 43.5% [20/46], respectively). Over half had reached sexual maturity (56.5% [26/46]). Their average body length (nose to anus) was 15 cm, and average overall length (nose to tail) was 20.5 cm. Vole characteristics differed among the three study sites (Figure 2).

Most voles displayed an absence of morphological abnormalities in their tissues. Just three euthanized voles were found to have liver cysts. Additional information is available in Table S2.

3.2. Vole Infection Status

3.2.1. Cultures

Leptospire were successfully cultured from the tissues ($n = 4$ kidney samples, $n = 1$ urine sample) of four euthanized voles.

3.2.2. Characteristics of Infected Voles

Based on the RT-PCR results for the kidney samples, infection status was unrelated to vole sex, sexual maturity status, or site of origin. However, euthanized voles were more likely than cold-stored voles to be infected; the same was true for voles with greater overall lengths (Table 1).



Figure 2. Differences in vole characteristics across the three Puy-de-Dôme study sites. (**Top left**): Sex. (**Top right**): Sexual maturity status. (**Bottom left**): Overall average length. (**Bottom right**): Sampling category.

Table 1. Relationship between infection status (positive vs. negative) and vole characteristics based on the RT-PCR results for kidney samples.

| Characteristics | Subcategory | Total (%) (n = 46) | Positive (%) (n = 35) | Negative (%) (n = 11) | p-Value * |
|---------------------|----------------|-----------------------|--------------------------|--------------------------|-----------|
| Sex | Male | 20 (43.5) | 15 (42.9) | 5 (45.4) | 1 |
| | Female | 26 (56.5) | 20 (57.1) | 6 (54.5) | |
| Sexual maturity | Mature | 26 (56.5) | 22 (62.9) | 4 (36.4) | 0.2 |
| | Immature | 20 (43.5) | 13 (37.1) | 7 (63.6) | |
| Sampling category | Euthanized | 26 (56.5) | 25 (71.4) | 1 (9.1) | 0.001 |
| | Cold-stored | 20 (43.5) | 10 (28.6) | 10 (90.9) | |
| Site | Site 1 | 16 (34.8) | 15 (42.9) | 1 (9.1) | - |
| | Site 2 | 9 (19.6) | 8 (22.9) | 1 (9.1) | 1 |
| | Site 3 | 21 (45.6) | 12 (34.2) | 9 (81.8) | 0.3 |
| Overall length (cm) | Median (Q1–Q3) | 21 (18.25–22.6) | 21 (19.5–22.8) | 17 (16.5–21.8) | 0.03 |

* determined using the most appropriate test (Chi-squared test or Anova exact test).

3.2.3. Detecting *Leptospira* Infection in Voles

Based on the RT-PCR results, 35 of the 46 voles were positive for *Leptospira* DNA, resulting in an overall prevalence of 76% (CI_{95%} [61%, 87%]). Although the differences were not statistically significant, prevalence tended to vary among the study site: site 1 had a prevalence of 93.7% (CI_{95%} [69.7%, 99.8%], n = 16); site 2 had a prevalence of 88.9% (CI_{95%} [51.7%, 99.7%], n = 9); and site 3 had a prevalence of 57.1% (CI_{95%} [34%, 78.2%], n = 21) (Figure 3).

All the voles that tested positive had positive kidney samples. In most cases, their other specimens (urine, lung, liver, and blood) also tested positive (Figure 4). Among the 25 positive euthanized voles, all but one had multiple infected specimens. Among the 10 positive cold-stored voles, infections were detected solely in the kidney specimens (9/10) or in both the kidney and urine specimens (1/10).

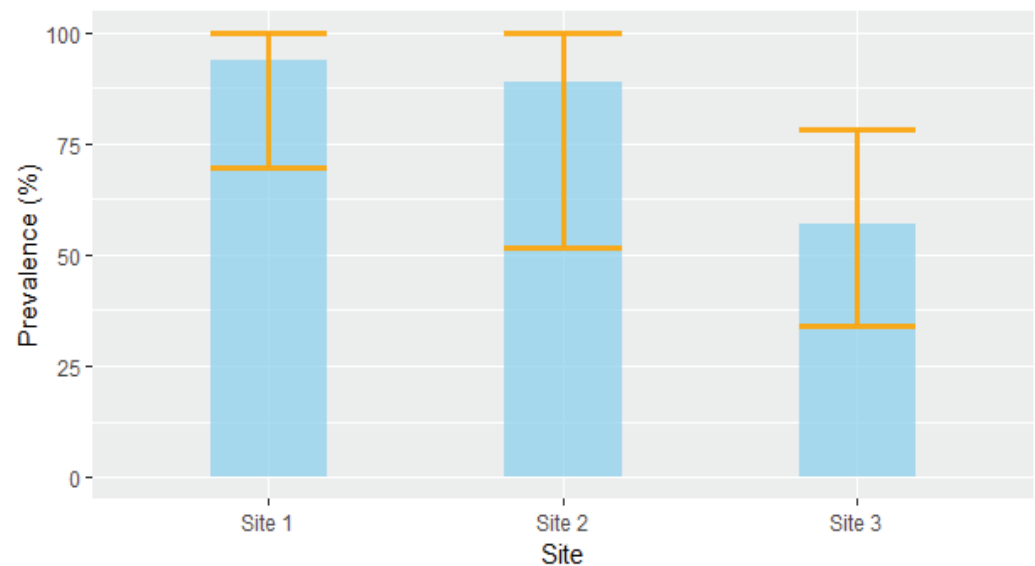


Figure 3. Prevalence (\pm CI_{95%}) of *Leptospira* infection in voles across the three study sites.

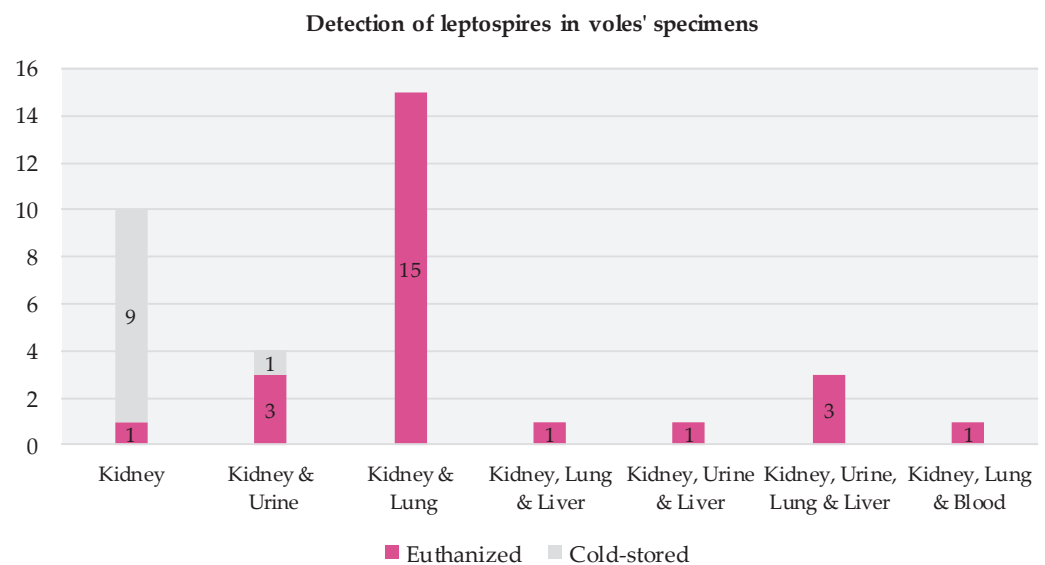


Figure 4. Presence of infection across specimens in voles testing positive for *Leptospira* via RT-PCR.

3.2.4. Molecular Typing of *Leptospira* Strains in Voles

We obtained the predicted 330-bp fragments when we conducted cPCR on *Leptospira* DNA obtained from the vole specimens (i.e., kidney, lung, liver, blood, and urine) and successful cultures that had tested positive via RT-PCR ($C_T \leq 40$); the positive controls confirmed result reliability [54]. In the BLAST search, all the sequences displayed high nucleotide affinity (97–100%) with *L. kirschneri* (GenBank accession number MK726123.1).

O-antigen typing was applied to DNA obtained from kidney samples ($n = 19$ samples) and successful cultures ($n = 4$) that had tested positive via RT-PCR ($C_T \leq 40$); the positive controls confirmed result reliability. Evidence was found for the presence of the *Leptospira* serogroup Grippityphosa (GRIP) but not for the *Leptospira* serogroup Icterohaemorrhagiae (IH).

Two VNTR profiles were observed in the DNA obtained from the kidney samples. The 1-2-11 profile was complete and had 1 TR copy at the VNTR 4 locus, 2 TR copies at the VNTR 7 locus, and 11 TR copies at the VNTR 10 locus. The 1-2-X profile was incomplete and had 1 TR copy at the VNTR 4 locus, 2 TR copies at the VNTR 7 locus, and no observable amplification at the VNTR 10 locus. The first profile was seen in 9 of the 14 samples, and the second profile was seen in 5 of the 14 samples. The first VNTR profile was also seen

in all four of the DNA samples from the successful cultures. A summary of the molecular typing results for the DNA obtained from the kidney samples is provided in Table 2.

Table 2. Molecular results for *Leptospira* DNA extracted from renal tissue.

| Voles | RT-PCR Positive | Presence of <i>L. kirschneri</i> | O-Antigen—GRIP | VNTR Profile 1-2-11 | VNTR Profile 1-2-X |
|--------|-----------------|----------------------------------|----------------|---------------------|--------------------|
| Nb/Nbt | 35/46 | 35/35 | 19/35 | 9/14 [†] | 5/14 [†] |

Nb, number of voles testing positive; Nbt, total number of voles tested; RT-PCR, real time PCR; GRIP, Grippotyphosa; VNTR, variable number of tandem repeat; [†], positivity threshold: C_T < 35.

3.3. *Leptospira* Seroprevalence in Sheep

Microagglutination Testing

In the first sampling round, four sheep were seropositive (MAT titers \geq 1:100). Their reactions to the *Leptospira* serogroups were as follows: two reacted to IH (titer values = 1:100); one reacted to IH and GRIP (both titer values = 1:100); and one reacted to Sejroe (SJ) (titer value = 1:200).

In the second sampling round, two sheep were seropositive (MAT titers = 1:100), and both reacted to GRIP.

4. Discussion

This study shows the important and potential exclusive carriage of *L. kirschneri* serogroup Grippotyphosa in water vole populations across three Puy-de-Dôme pastures, as well as the seroconversion of sympatric sheep likely exposed to *Leptospira* serogroup Grippotyphosa following their grazing on site 3.

Leptospira infections in water voles

Across all three water vole populations, the prevalence of *Leptospira* infection was 76% (CI_{95%} [61%, 87%]). In infected animals, pathogenic *Leptospira* DNA was consistently found in the kidneys, which is unsurprising since these organs are frequently colonized by leptospires [1,55,56]. What is surprising is that this prevalence is markedly higher than that seen in *R. norvegicus* in France (26%, CI_{95%} [20%, 33%]) [57], an intriguing result because *R. norvegicus* is considered to be the primary reservoir host for the pathogen causing human leptospirosis [23]. Such findings underscore that *Leptospira* prevalence can be extremely high in water vole populations, which highlights the need to explore their role in the epidemiology of leptospirosis.

If anything, it seems likely that 76% was an underestimate given that 20 of the 46 voles making up our sample belonged to the cold-stored group, which was less likely than the euthanized group to test positive for infection. This pattern may stem from methodological differences. The euthanized voles immediately underwent dissection and tissue sampling, and the molecular analyses occurred after just four weeks of sample cold storage. In contrast, the cold-stored voles went about ten weeks before dissection and tissue sampling were conducted. DNA concentrations start to decline after such lengths of time [58] because freezing affects bacterial richness and abundance; consequently, the likelihood of detecting any *Leptospira* DNA probably fell [59–61]. In addition, compared to the euthanized voles, the cold-stored voles experienced one additional cycle of freezing/thawing prior to the molecular analyses. It is known that the number of freezing/thawing cycles can cause DNA degradation and reduce amplification success, leading to a higher probability of false negatives [62–64]. Finally, the thawing period experienced by the cold-stored voles could have allowed the intestinal microbiota to trickle through the permeable intestinal tissue. If leptospires were present, their concentrations could have been diluted by this other source of bacteria, reducing the detectability of *Leptospira* DNA. That said, no such phenomenon has ever been reported in the literature.

In past research, certain rodent characteristics have been associated with infection status. Indeed, females are at greater risk of *Leptospira* infections than are males [65–67]. Furthermore, sexually mature rodents have a higher probability than juveniles of acquiring

the pathogen because they can experience direct transmission during copulation and they spend more time exploring the outer environment [68]. Our study's small sample size ($n = 46$) might be inadequate for testing whether sex and sexual maturity influence infection status in water voles.

Evidence of chronic *Leptospira* infection in water voles

To better understand how water voles contribute to *Leptospira* epidemiology in pasture ecosystems, it is necessary to determine whether they experience acute or chronic infection. Indeed, if the infection is chronic and has a limited impact on host health, the bacteria could more easily be maintained in the kidneys and shed over prolonged periods of time, such as occurs in rats [22]. The tissue infection patterns we observed provide insight into this question. None of the voles we captured displayed morphological abnormalities (e.g., nephritis or hemorrhaging) that could be attributed to *Leptospira* [69,70]. At the same time, leptospires were clearly present in one or more of the voles' tissues. Out of the 35 infected voles, 14 had positive kidney or kidney and urine samples but negative lung, liver, and blood samples. Such results suggest that bacterial presence is long lasting and stable in the kidneys. This pattern is seen in chronic infections in rats: leptospires remain in the kidneys long after they have been cleared from other organs (~one week post infection) [71]. In the other infected voles, *Leptospira* was simultaneously present in multiple tissues. The latter was unlikely to be evidence of an acute infection because leptospires were absent from the blood in all but one case.

Potential modes of *Leptospira* transmission in water voles

To assess whether voles could shed *Leptospira* in their urine, it is necessary to evaluate whether leptospires in the urine are viable, which can be assessed by culturing bacteria from urine samples [72,73]. In the case of one infected animal (euthanized vole 10), we obtained a successful culture from both urine and kidney samples. This finding provides evidence of leptospire viability and suggests that the other voles with positive urine samples could have been shedding viable *Leptospira*. Overall, samples from 4 of the 26 euthanized voles gave rise to successful cultures, even though *Leptospira* bacteria are notoriously challenging to isolate because of their slow growth [74]. One reason that successful cultures were not obtained for the other infected voles could relate to pathogen concentrations. The greater the pathogen concentration in a sample, the lower the C_T value and the higher the probability of obtaining a successful culture [75,76]. Indeed, kidney and urine samples with C_T values exceeding 22 never resulted in successful cultures. These results indicate that culturing is a far less sensitive technique than is PCR, as highlighted by numerous studies [77,78].

Observations supporting selective *Leptospira* carriage by water voles

In our study, genotype related to serogroup GRIP was reported for most of the infected voles (except for those for which $C_T \geq 34$) through O-antigen typing, a finding that suggests selective carriage is occurring.

The VNTR results provide additional support for this idea. Nine of the 14 voles with low C_T values (≤ 28) were found to carry *Leptospira* with the same genetic profile: 1 TR copy at VNTR4; 2 TR copies at VNTR7; and 11 TR copies at VNTR10. Five voles with higher C_T values (range: 29.8–34.4) carried *Leptospira* with a similar but incomplete profile: 1 TR copy at VNTR4; 2 TR copies at VNTR7; and X TR copies at VNTR10. Voles are territorial and live in colonies whose members display restricted movements. Therefore, a given population is likely to maintain a given *Leptospira* strain. We hypothesize that the incomplete second profile is actually the same as the first. Our second profile is the same as an incomplete *L. kirschneri* profile obtained from voles in eastern France [40]; as such, it might also be identical to our complete genetic profile. These findings provide support for the notion that even voles in distant ecosystems selectively carry *Leptospira* with similar genetic profiles. The complete profile we obtained does not match up with any of the profiles described previously [52], which may suggest it is an unreported profile for the serogroup GRIP. Future research should use next-generation sequencing to completely characterize the

genomes of these strains. The results should reveal the regions that best differentiate the strains, allowing for easier identification.

Role of water voles in *Leptospira* epidemiology

O-antigen and VNTR typing both yielded support for the hypothesis that voles selectively carry and maintain *Leptospira* related to the serogroup GRIP. Thus, the pathogen is likely present in large concentrations in pasture soils. The MATs revealed that 3 of the 60 sheep tested had antibodies (titer = 1:100) against the serogroup GRIP, suggesting they had previously been infected by the latter serogroup. Previous research using MATs found similarly low antibody titers in sheep [79–81]. We might have expected higher seropositivity in the sheep given the markedly high pathogen prevalence in the water voles. Furthermore, Puy-de-Dôme experiences climatic conditions (e.g., regular rainfall and stable relative humidity) that should promote the survival of any leptospires present in diluted animal urine [82,83]. However, it may be that sheep rarely come in contact with water vole urine. In grasslands, most voles remain underground in burrows that are permanently sealed, although they may occasionally open up during dry periods; as a result, the animals rarely come up to the soil surface [84]. It is also possible that sheep display a low rate of seroconversion following infection [5]. Consequently, the frequency of transmission between water voles and sheep remains unclear, as does the degree to which sheep may be accidental hosts. Beyond understanding that water voles are maintenance hosts for *L. kirschneri* serogroup GRIP, we know little about the role played by these rodents in leptospirosis epidemiology.

Studying the water vole's predators could be instructive. Research in mainland France characterized the *Leptospira* bacteria behind renal infections in various wildlife species and discovered that genetic profile diversity was greater in species that prey upon rodents [85]. Notably, *L. kirschneri* serogroup GRIP was detected in certain mustelids, while foxes (*Vulpes vulpes*) tended to harbor *L. interrogans*. In contrast, no wolves (*Canis lupus*) were found to be infected. Furthermore, other studies found that canines were seropositive for the serogroup GRIP in France [86], as were wolves in Italy [87]. It is evident that further research is needed to clarify how the water vole's predators might contribute to *Leptospira* transmission.

Over recent years, several leptospirosis outbreaks in human populations in France have been linked to *L. kirschneri* associated to the serogroup GRIP [14,88]. In addition, these same *Leptospira* strain have been found to cause accidental infections in domestic animals [89–91]. To date, a single study has sought to characterize the genetic profiles of pathogenic *Leptospira* serogroups in wild mammals in France: none of the animal species tested appeared to be a maintenance host for the serogroup GRIP [85]. Consequently, none of them seem to be responsible for cases of leptospirosis provoked by this *Leptospira* serogroup. Given that our results strongly support the water vole's role as a maintenance host of *L. kirschneri* serogroups GRIP, they can inform future work tracking the sources of *Leptospira* infections in humans and domestic animals.

5. Conclusions

This study found a high prevalence of *Leptospira* infection in water vole populations in central France. Across infections and sites, a single profile emerged: *L. kirschneri* serogroup GRIP. This finding suggests that water voles selectively carry this *Leptospira* strain, an idea supported by results from RT-PCR, cPCR, O-antigen typing, and VNTR typing. At the same time, the water vole's contribution to infections in domestic animals remains undetermined given the low seroprevalence observed in sheep co-occurring in the same habitat. It is important to take this research further because the water vole is the first species to be identified as a selective carrier of *L. kirschneri* serogroup GRIP. To clarify the epidemiology of leptospirosis in France, additional studies should be conducted on voles as well as on the wild and domestic animals that share the same habitat.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/pathogens12020260/s1>—Table S1: List of the reference strains employed in the MAT antigen panel; Table S2: Vole characteristics across the study sites and the RT-PCR results; and Table S3: Antibody titers against different *Leptospira* serovars in sheep.

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3. Conclusion and future perspectives

Our results showed an important and exclusive carriage of *L. kirschneri* serogroup Grippotyphosa and a similar VNTR profile 1-2-11 in water voles in the three ecosystems of Puy-de- Dôme, suggesting water voles as maintenance hosts of the latter *Leptospira* strain. It also revealed the benefit of adapting complementary laboratory tests and their application on different specimens to determine the infectious *Leptospira* strain and the infectious status of the sampling animals, respectively. It also supported the testing of euthanized animals for which the extracted DNA has not been cold-stored for a prolonged period of time in order to obtain more accurate molecular tests and prevalence results. These findings suggest that such strategy can be conducted in future epidemiological studies when sampling rodent species. Nevertheless, a complete genetic profile of the infectious *Leptospira* strain could not be acquired as the acquired VNTR profile was not unknown. Consequently, WGS is required in complementarity to the traditional methods as it identifies novel sequences. It will better qualify the infectious *Leptospira* strain and thus, the role of water voles in the maintenance community.

II.3. First time epidemiological study in the Middle East and application of a first time epidemiological approach in Lebanon

1. General context

To date, knowledge about the epidemiology of leptospirosis lacks in different countries. In order to acquire such knowledge, epidemiological investigations should be adapted and screening of *Leptospira* infection should be performed on different animal populations in different ecosystems. Consequently, information about the circulating serogroups in the different screened animal populations and about the maintenance hosts and community will be provided. Leptospirosis' risk is unknown in Lebanon since no epidemiological study relative to the disease were conducted. Thus, the disease is considered neglected in the country. In order to acquire such information, we began by analyzing leptospirosis risk in neighboring countries which provided epidemiological knowledge about the disease through a literature review. Our results testified a non-negligible risk in the Middle East region that includes Lebanon. Consequently, we initiated a first-time epidemiological study in Lebanon by investigating the risk in a livestock ecosystem, particularly, in the population of cattle farms in Mount Lebanon governorate and in imported cattle, both in close contact with people. The study was first challenging since laboratory tests specified for the identification of *Leptospira* strains or the circulating serogroups lack in the nation. Therefore, the partnership between France and Lebanon was a key that allowed the shipment of the collected specimens to France and their analysis at the LAV with basic molecular and serological tests for the identification of *Leptospira* (through PCR) or its relative serogroups (through MAT). Conducting the latter step relied on the financial support provided by VAS to ensure the shipment of the samples and the provision of diagnostic tools, extraction kits, and (*Leptospira*) culture mediums.

2. Epidemiology of leptospirosis: The First Literature review of the Neglected Disease in the Middle East

Article 4: Epidemiology of Leptospirosis: The First Literature Review of the Neglected Disease in the Middle East.

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Review

Epidemiology of Leptospirosis: The First Literature Review of the Neglected Disease in the Middle East

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Abstract: Leptospirosis is a major zoonotic disease that has emerged worldwide, and numerous studies performed in affected countries have provided epidemiological knowledge of the disease. However, currently, there is inadequate knowledge of leptospirosis in the Middle East. Therefore, we grouped publications from various Middle Eastern countries to acquire a general knowledge of the epidemiological situation of leptospirosis and provide an initial description of the leptospiral relative risk and circulating serogroups. We conducted a detailed literature search of existing studies describing *Leptospira* prevalence and seroprevalence in Middle Eastern countries. The search was performed using online PubMed and ScienceDirect databases. One hundred and one articles were included in this review. Some countries, including Iran, Turkey, and Egypt, reported more publications compared to others, such as Lebanon, Kuwait, and Saudi Arabia. Frequently, the seroprevalence of leptospirosis varied considerably between and within countries. The prevalence of leptospirosis was comparable in most Middle Eastern countries; however, it varied between some countries. The methods of detection also varied among studies, with the microscopic agglutination test used most commonly. Some hosts were more recurrent compared with others. This review summarizes the epidemiological situation of *Leptospira* infection in the Middle East, reporting predominant serogroups—Sejroe, Grippotyphosa, Icterohaemorrhagiae, Autumnalis, and Pomona—that were identified in the most commonly tested hosts. Our findings emphasize the need to develop a deeper understanding of the epidemiology of *Leptospira* spp. and prioritize the disease as a public health problem in this region. To achieve this goal, increased awareness is critical, and more publications related to the topic and following a standardized approach are needed.

Keywords: *Leptospira*; one health; diagnosis; epidemiology; middle eastern countries



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1. Introduction

Leptospirosis is a zoonosis that is prevalent worldwide and has major impacts on both humans and animals [1,2]. The disease is caused by species of *Leptospira*, a spirochaete bacterium with increasing genetic diversity [3,4]. To date, 38 species of pathogenic *Leptospira* have been described, and new species are continually being discovered [5]. The morbidity and mortality rates of leptospirosis in humans are estimated at 1 million and 60,000 cases, respectively [6]. Of the reported cases, 2.90 million disability-adjusted life years are estimated to be lost per annum [7]. Various human-acquired syndromes, ranging from flu-like to life-threatening hepatorenal syndromes, have been described to be associated with leptospirosis in the literature [8–10]. In severe cases, mortality rates vary between 5% and 20% [6]. Humans, as well as wild and domestic animals, can be infected either directly following contact with the body fluid of *Leptospira*-infected animals or indirectly when exposed to environments contaminated by leptospirosis [11–14]. Animal *Leptospira* infection presents not only as acute clinical manifestations observed in humans but also as a chronic infection that can lead to major economic depletion due to reproductive failure [2].

Following several human leptospirosis outbreaks reported worldwide [15], this infectious disease has been categorized as a (re-) emerging disease and is still qualified as such by the World Health Organization (WHO) [16].

The limit of understanding of the natural history of *Leptospira* infection and the under-recognition of its burden are due to insufficient diagnosis, largely as a result of the two stages experienced by the host [17,18]. The first stage of leptospirosis is the septicemic or acute stage, which occurs in the first week of infection, wherein the host generally shows symptoms of *Leptospira* circulating in the bloodstream [1,2,17,19]. The second stage of the disease is the immune stage, which generally occurs in the second week of infection, wherein the host starts to acquire and show anti-*Leptospira* antibodies in the serum [1,2,17,19]. Currently, the most accurate test to detect the acute phase of infection is the polymerase chain reaction (PCR) [18]; this technique is highly sensitive and can rapidly detect the *Leptospira* species [17,20]. Treatment following a positive PCR result at this stage might be effective, unlike the culture and microscopic agglutination test (MAT), which is less advantageous for early diagnosis. Culture is time-consuming and known for its difficulty in isolating *Leptospira*, whereas the MAT only detects antibodies indicating a past or current infection [8,21]. Nonetheless, MAT is considered the immunological reference standard method for leptospirosis experimental diagnosis by the World Organization for Animal Health (WOAH) [22] and the WHO [23]. Another supportive immunological test for the detection of antibodies is enzyme-linked immunosorbent assay (ELISA) [24]. Although the diagnostic accuracy of ELISA has not been completely established [25], the facilities of performing ELISA (manipulation with killed antigens) rather than MAT (live antigens) shows a promising alternative to several laboratories in tropical countries which reported its high sensitivity and specificity [26,27].

In recent years, cases of human and animal leptospirosis have been reported in numerous countries in the Middle East through direct and/or indirect diagnostic techniques. Human cases commonly involve farmers, rice field workers [28–36], travelers [37], and plumbers [38]. Leptospirosis cases in children and/or adults in contact with infected livestock or contaminated water have also been reported [28,39–41]. However, cases without a clear history of pathogenicity [42] and those without obvious occupational activities known to be risk factors for leptospirosis transmission have also been noted in some Middle Eastern countries and are qualified as “inner-city related”. Moreover, cases of co-infection with both dengue fever and leptospirosis have been described in the Middle East [43]. Animal case reports normally include numerous species but mainly involve livestock. Furthermore, the direct detection of leptospirosis in water resources has been reported [44]. Preventive measures to reduce health and economic consequences following *Leptospira* infection in the community relies on a deep understanding of the epidemiology, public awareness, and vaccination of domestic animals and populations at risk. The knowledge of the predominant serogroup of a host is an important guide for an effective vaccination since the latter strategy only bestows a protective immunity restricted to homologous or closely associated serovars [45].

This review aimed to acquire general knowledge about the epidemiological situation of leptospirosis in the Middle East and provide an initial description of the leptospiral relative risk and circulating serogroups in this region to develop and adopt prophylactic strategies if necessary. This study focused on collecting and revising the available data on the prevalence of *Leptospira* in humans and animals in the Middle East. We expect to determine the variability in *Leptospira* spp. prevalence and seroprevalence in different Middle Eastern countries according to variations in related records/publications. Variability was expected because leptospirosis outbreaks are related to local factors, such as environmental and meteorological factors, and because it is commonly reported in reviews from different geographical areas, such as Africa, the Pacific Islands, and China. For instance, Africa reported a variability in the seroprevalence between 2.3% and 19.8% in hospital patients having febrile illness [46]; in Pacific Islands (y = number of islands), the seroprevalence varied between 19.6% and 45.0% in cattle (y = 4) and between 10% and 88% in humans

($y = 7$) [47], and in China, human case studies reported a seroprevalence between 8.2% and 56.7% [48]. In addition, we expected to find similar genetic profiles or circulating serogroups in particular hosts, such as humans, cattle, and rodents of the Middle East, as has been reported in other areas. For instance, we expect to find the predominance of the serogroups *Icterohaemorrhagiae* (ICT) in humans and rodents [49,50] and Sejroe (SJ) in cattle [51], as has been shown in previous literature. However, for some other hosts, no specific serogroups were expected.

2. Materials and Methods

2.1. Literature Search

We conducted a detailed literature search of the existing studies describing the *Leptospira* seroprevalence and prevalence in Middle Eastern countries. In our review, we included the following countries; Bahrain, Cyprus, Egypt, Iran, Iraq, Jordan, Kuwait, Lebanon, Oman, Palestine, Qatar, Saudi Arabia, the Syrian Arab Republic, Turkey, the United Arab Emirates, and Yemen.

The search was conducted using PubMed and ScienceDirect online databases. Search terms were used to manually find relevant articles and included “((Leptospira) OR (Leptospirosis)) AND (Middle East) AND (Prevalence)”, “((Leptospira) OR (Leptospirosis)) AND (Middle East country name) AND (Prevalence)”, “((Leptospira) OR (Leptospirosis)) AND (Middle East) AND (Seroprevalence)”, “((Leptospira) OR (Leptospirosis)) AND (Middle East country name) AND (Seroprevalence)”, “((Leptospira) OR (Leptospirosis)) AND (Middle East) AND (Human)”, “((Leptospira) OR (Leptospirosis)) AND (Middle East) AND (Animals)” without any stipulation or precondition on publication date. Similar research terms were also used in Google Scholar to extract relevant articles.

2.2. Inclusion and Exclusion Criteria

Both fully accessible publications and abstracts describing the seroprevalence and prevalence of leptospirosis in humans or animals were included in this review, regardless of their publication date. Publications that did not describe this topic were excluded. In addition, publications written in a language other than English were excluded; nevertheless, if the latter publication contained an English abstract with relevant data for the review, it was included.

2.3. Data Extraction

The data extracted from the retrieved publications, abstracts, or reviews included the author, year of publication, and geographical location. Additional information on the chosen hosts, their effectiveness, prevalence, seroprevalence, method of detection [culture, PCR, MAT, ELISA, serology for serogroup identification (SSI), or direct method], reference serovars, and identified serogroups were also extracted. Serogroups referring to any host in the Middle East were reported, regardless of the titers obtained in the SSI. The latter methods included the MAT, indirect immunofluorescent antibody test (IFAT), complement fixation test, lysis-agglutination test (LAT), immunoglobulin test, silver staining (SS), latex agglutination test, macroscopic plate agglutination test (MPAT), microtube agglutination analysis, and agglutination test.

2.4. Distribution of Publications

Information regarding the methods of detection applied and the serogroups identified were retrieved from both research papers and case studies because the objective was to dispose of all the available information regarding the circulating *Leptospira* in the Middle East. Nonetheless, information regarding the prevalence and seroprevalence of *Leptospira* was only retrieved from research papers. However, the cartography demonstrating the geographical distribution of the Middle East publications included all retrieved publications (research papers, case studies, and reviews).

3. Results

3.1. Literature Search

A database search using the previously cited queries retrieved 7169 articles (458 articles from PubMed and 6711 from Science Direct), of which 7058 were excluded. The exclusion criteria were based on the absence of adequate information about leptospirosis in humans or animals or those that were not published in English. The review yielded 111 articles (104 research papers and case studies and 7 reviews), with publication dates varying between 1947 and 2021. Seventeen of the latter articles were published prior to 2000; however, the remaining 94 articles were published after a remarkable increase in publication throughout the last decade.

3.2. Distribution of *Leptospira* Seroprevalence and Prevalence in Middle Eastern Countries

The reported publications comprised research papers, case studies, and review articles that included 14 countries (Figure 1). Publications related to Oman were included but did not record any seroprevalence or prevalence, as they only involved case studies [37,40,43]. Detailed data on prevalence, seroprevalence, and serogroup/serovar information related to each publication can be found in Table S1.

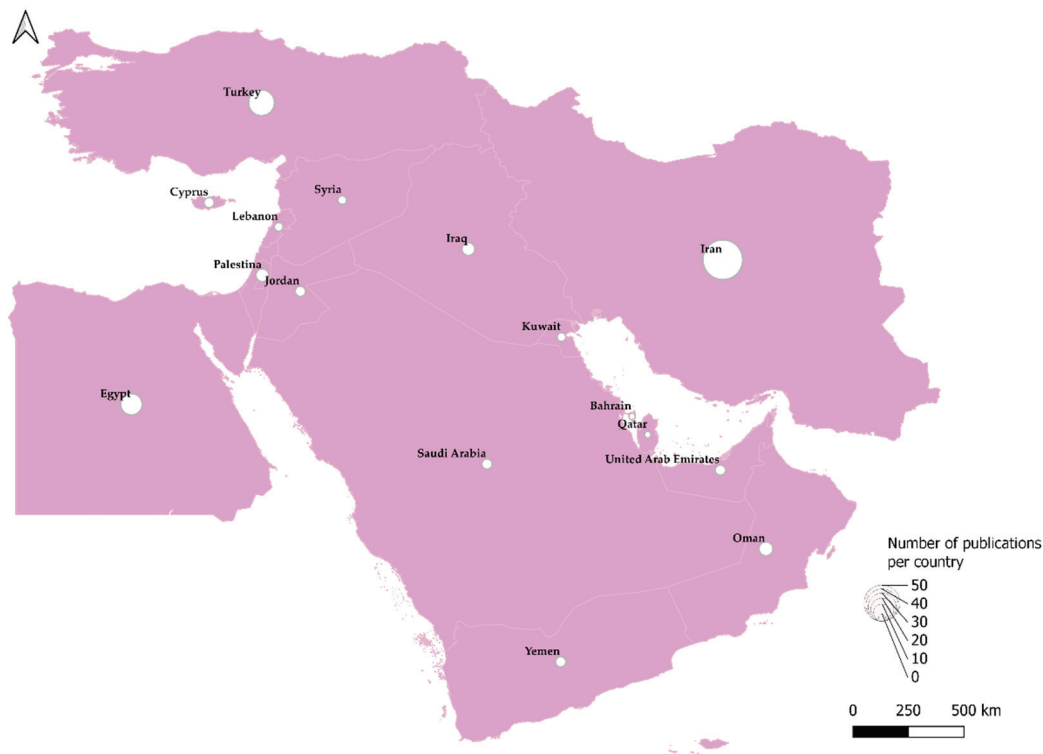


Figure 1. Geographical distribution of the Middle Eastern publications. Map created with DIVA-GIS version 7.5 and designed with QGIS 3.16.1 Hannover.

Some countries tested a high number of hosts but recorded a low seroprevalence and contrariwise. For example, Palestine tested 2018 rodents and Cyprus tested 261 cattle, 195 goats, and 507 sheep, and both the countries reported a seroprevalence of 0% [52–54] by SSI. However, Egypt tested two cats and two weasels, and one of each host showed positive antibody titers [55].

Studies from other countries have focused on determining the seroprevalence in particular hosts. For example, two studies from Jordan reported a seroprevalence of 49.7% and 92.3% in cattle only by SSI [56,57]. Among studies from the United Arab Emirates, one showed a seroprevalence of 4.1% [58] in camels, and another showed a seroprevalence of 1.7% in cattle [59]. In Yemen, a seroprevalence of 41.3% [60] and 42% [61] by ELISA

was reported in humans, and a seroprevalence of 6.7% by SSI was reported in camels in Saudi Arabia [62].

The Syrian Arab Republic has one of the lowest rates of publications on this topic, with only one publication in 1984. However, the seroprevalence by SSI was reported as 2.9%, 11.9%, and 33.9% in sheep, goats, and cattle, respectively [63]. In contrast, Iran, Turkey, and Egypt were the most reported countries in the Middle East, with 50, 21, and 16 publications, respectively, with both low and high seroprevalence reported. A seroprevalence of 1.1% [64] and 0.05% [65] was reported by SSI in humans in Iran and Turkey, respectively. Again, using SSI, a seroprevalence of 0% [44,55,66,67] was reported in camels, cattle, donkeys, dogs, horses, rodents, and sheep in Egypt. A seroprevalence of 71% [68] in dogs, 82.1% [69] in humans, and 78.4% [70] in cattle were reported by SSI in Iran, Turkey, and Egypt, respectively.

Iran, Turkey, and Egypt also reported studies that estimated the prevalence of the *Leptospira* infection in various hosts, although many of these reported prevalence values were low. For example, a prevalence of 0% was reported in Iran [71] in goats, and a prevalence of 0% was reported in Egypt [44,55,66] in humans, buffaloes, camels, cattle, donkeys, horses, sheep, water buffaloes, and weasels by PCR and/or culture. Moreover, a prevalence of 1.4% [72] in cattle has been reported in Turkey. Only one remarkably high prevalence of 74.4% [69] was reported in humans in Turkey by dark field microscopy (DFM). The highest prevalence in the Middle East was reported in Iraq (94.3%) by DFM and direct microscopic examination (DFE) [73]. The detailed data on *Leptospira* seroprevalence and prevalence in Middle Eastern countries are shown in Tables 1 and 2, respectively.

Table 1. Summary of *Leptospira* seroprevalence in the Middle East according to the test performed (SSI or ELISA) and the country from which the sampling originated.

| Country | Number of Articles | Publication Year | Host | Total Number of Host Tested | Seroprevalence Range SSI | Total Number of Host Tested | Seroprevalence Range Elisa | References |
|---------|--------------------|------------------|-----------------|-----------------------------|--------------------------|-----------------------------|----------------------------|------------------------|
| Cyprus | 1 | 2000 | Cattle | 261 | 0% | - | - | [54] |
| | | | Goats | 195 | 0% | - | - | [54] |
| | | | Sheep | 507 | 0% | - | - | [54] |
| Egypt | 14 | 1957–2018 | Human | 4334 | 0.5–49.7% | 1278 | 16% | [44,66,67,74–78] |
| | | | Buffaloes | 409 | 15.4–30% | 97 | 20% | [44,55,75,79] |
| | | | Cats | 2 | 50% | - | - | [55] |
| | | | Camels | 82 | 0–50% | - | - | [44,75,79,80] |
| | | | Cattle | 1093 | 0–78.4% | - | - | [44,55,66,67,70,75,79] |
| | | | Donkeys | 73 | 0–29% | - | - | [44,55,67,75] |
| | | | Dogs | 394 | 0–58.3% | - | - | [44,55,66,67,81] |
| | | | Goats | 317 | 1.5–42.1% | - | - | [66,67,75] |
| | | | Horses | 14 | 0% | - | - | [44] |
| | | | Pigs | 158 | 14.3–43.8% | - | - | [67,75] |
| | | | Rodents | 1087 | 0–75.9% | - | - | [44,55,66,67] |
| | | | Sheep | 736 | 0–45.4% | - | - | [44,66,67,75,79,82] |
| | | | Water buffaloes | 99 | 14.1% | - | - | [66] |
| | | | Weasels | 2 | 50.00% | - | - | [55] |
| Iran | 36 | 2003–2021 | Human | 1782 | 1.1–61.6% | 3983 | 10.4–64.7% | [34,64,83–97] |
| | | | Buffaloes | 130 | 25.40% | - | - | [98] |
| | | | Caspian seal | 164 | 5.3–25.8% | - | - | [99,100] |
| | | | Cats | 213 | 4.9–27.03% | - | - | [101,102] |
| | | | Cattle | 1178 | 17.4–37.8% | - | - | [103–107] |
| | | | Dogs | 48 | 71% | - | - | [68] |
| | | | Donkeys | 170 | 40–41.2% | - | - | [108,109] |
| | | | Goats | 210 | 8.5–11.7% | - | - | [110–112] |
| | | | Horses | 541 | 7.8–39.2% | - | - | [108,109,113] |
| | | | Mules | 20 | 39.2% | - | - | [109] |
| | | | Rodents | 187 | 21.2–33% | - | - | [114,115] |
| | | | Sheep | 412 | 10.9–19.3% | - | - | [105,110] |
| | | | Cattle | 334 | 57.3–85% | 96 | 7.3% | [73,116,117] |
| | | | Dogs | 218 | 17% | - | - | [118] |
| | | | Goats | 153 | 22.4–57.9% | - | - | [116] |
| | | | Sheep | 199 | 24.6–42.9% | - | - | [116] |

Table 1. Cont.

| Country | Number of Articles | Publication Year | Host | Total Number of Host Tested | Seroprevalence Range SSI | Total Number of Host Tested | Seroprevalence Range Elisa | References |
|------------------------|--------------------|------------------|----------------------|-----------------------------|--------------------------|-----------------------------|----------------------------|------------------|
| Jordan | 2 | 1992–2019 | Cattle | 448 | 49.7% | 240 | 92.3% | [56,57] |
| Palestine | 2 | 1948 | Human | 207 | 1.9% | - | - | [52] |
| | | | Cattle | 1665 | 8.5–9.5% | - | - | [52,53] |
| | | | Rodents | 2018 | 0% | - | - | [52,53] |
| Saudi Arabia | 1 | 2009 | Camels | 90 | 6.7% | - | - | [62] |
| Syrian Arabic Republic | 1 | 1984 | Human | 407 | 0% | - | - | [63] |
| | | | Cattle | 1894 | 33.9% | - | - | [63] |
| Turkey | 14 | 1999–2016 | Sheep | 1735 | 2.9% | - | - | [63] |
| | | | Goats | 1203 | 11.9% | - | - | [63] |
| | | | Horses | 258 | 0% | - | - | [63] |
| | | | Human | 1834 | 0.05–62.8% | 192 | 42.8–82.1% | [65,69,119–122] |
| | | | Rodents | 59 | 8.5% | - | - | [123] |
| | | | Cattle | 21722 | 3.4–45% | 4560 | 14–38.6% | [72,119,124–127] |
| | | | Dogs | 116 | 44% | - | - | [128] |
| | | | Sheep | 200 | 8% | - | - | [119] |
| | | | Buffaloes | 93 | 32.3% | - | - | [129] |
| | | | United Arab Emirates | 2 | 1994–2020 | Camels | 73 | 4.1% |
| | | | Cattle | - | - | 350 | 1.7% | [59] |
| Yemen | 2 | 2015–2018 | Human | - | - | 467 | 41.3–42% | [60,61] |

Table 2. Summary of *Leptospira* prevalence in the Middle East according to the test performed (PCR or culture) and the country from which the sampling originated.

| Country | Number of Articles | Publication Year | Host | Total Number of Host Tested | Prevalence Range PCR | Total Number Of Host Tested | Prevalence Range Culture | References |
|-----------------|--------------------|------------------|---------------|-----------------------------|----------------------|-----------------------------|--------------------------|--------------|
| Egypt | 5 | 1957–2015 | Human | 175 | 0% | 175 | 0% | [44] |
| | | | Buffaloes | 38 | 0% | 38 | 0% | [44,55] |
| | | | Cats | 2 | 50% | 2 | 50% | [55] |
| | | | Camels | 22 | 0% | 22 | 0% | [44] |
| | | | Cattle | 634 | 0–1.1% | 634 | 0–1.1% | [44,55] |
| | | | Donkeys | 30 | 0% | 30 | 0% | [44,55] |
| | | | Dogs | 193 | 11.3–12% | 261 | 0–11.3% | [44,55,81] |
| | | | Horses | 14 | 0% | 14 | 0% | [44] |
| | | | Rodents | 370 | 24–26% | 1461 | 0.37–7% | [44,55,66] |
| | | | Sheep | 124 | 0% | 189 | 0% | [44,55,82] |
| Water buffaloes | 12 | 0% | 12 | 0% | [66] | | | |
| Weasels | 2 | 0% | 2 | 0% | [55] | | | |
| Iran | 9 | 2010–2018 | Human | 119 | 50.4% | - | - | [97] |
| | | | Camels | 130 | 14.6% | - | - | [130] |
| | | | Caspian Seals | 128 | 18.7–20.3% | - | - | [100] |
| | | | Cats | 132 | 21.2% | - | - | [131] |
| | | | Cattle | 238 | 14.3–43% | 98 | 1.0% | [71,103,132] |
| | | | Goats | 26 | 0% | - | - | [71] |
| | | | Rodents | 151 | 3.3–11.3% | 151 | 0% | [115] |
| | | | Sheep | 110 | 4.5% | - | - | [71] |
| | | | Dogs | 37 | 13.5% | - | - | [118] |
| | | | Rodents | - | - | 49 | 16.3% | [133] |
| Lebanon | 1 | 1947 | Rodents | - | - | 70 | 5.70% | [134] |
| | | | Rodents | - | - | - | - | - |
| Turkey | 6 | 2006–2015 | Human | - | - | 192 | 14–45.7% | [69,120] |
| | | | Cattle | 499 | 0–9.4% | - | - | [72,135] |
| | | | Rodents | 102 | 16.9–46.5% | - | - | [123,136] |
| Saudi Arabia | 1 | 2009 | Camels | - | - | 36 | 0% | [62] |
| | | | Camels | - | - | - | - | - |

3.3. Surveillance Methods Used in the Middle East

Various direct or indirect methods of detection (MOD) have been reported in 104 publications, as shown in Table 3. The indirect methods—MAT or ELISA—were most commonly used, either solely (n = 52) with MAT in addition (n = 38), or in parallel with other methods (n = 40). Culture or direct PCR MODs were also commonly used either solely (n = 8) or in parallel with others (n = 26) but in fewer numbers of publications. Some of the reported MODs are LAT [66], IFAT [97], and MPAT [116] for SSI and immunoperoxidase (IP) [137], DFM [73], DFI [134], and DFE [120] for direct diagnosis. The PCR target genes varied according to each publication, and the list of genes is shown in Table 4.

Table 3. Methods of detection performed in 104 publications (research articles and case studies) in the Middle East.

| Major Tests Performed | Number of Articles | Supplementary Information |
|------------------------------------|--------------------|-------------------------------|
| MAT only | 38 | |
| ELISA only | 14 | |
| PCR only | 7 | mPCR, qPCR, nPCR |
| Culture only | 2 | |
| MAT, ELISA * | 8 | |
| MAT, culture * † | 3 | |
| MAT, PCR * † | 2 | |
| ELISA, PCR * † | 1 | |
| PCR, culture † | 1 | |
| ELISA, culture * † | 4 | |
| MAT, ELISA, PCR * † | 1 | |
| MAT and others * | 3 | DFE, IFAT, MPAT |
| Culture and others * † | 3 | DFE, CFT, LAT, DFI, IFT, IGT |
| PCR and others † | 1 | IFAT, S |
| Culture, PCR and others * † | 1 | IFAT |
| MAT, culture and PCR * † | 2 | |
| MAT, culture, PCR and others * † | 1 | PGE |
| MAT, culture and others * † | 4 | IFAT, DFM, SS, DFE, LAG, MPAT |
| Culture, ELISA, and others * † | 1 | DFM, DFE |
| MAT, ELISA, culture and others * † | 1 | MAA, DFM |
| Others * | 5 | AT, LAT, IFA |
| Others | 1 | IP |
| | 104 | |

mPCR, multiplex PCR; qPCR: quantitative PCR; nPCR, nested PCR; DFE, dark field examination; IFAT, indirect immunofluorescent antibody test; CFT, complement fixation test; LAT, lysis-agglutination test; DFI, dark field illumination; IFT, Immunofluorescent technique; IGT, immunoglobulin test; S, sequencing; PGE, Pulsed-gel electrophoresis; DFM, dark field microscopy; SS, silver staining; LAG, latex agglutination test; MPAT, macroscopic plate agglutination test; MAA, Microtube agglutination analysis; AT, Agglutination Test; IP, immunoperoxidase; *, MAT and ELISA in parallel with other techniques; †, PCR and culture in parallel with other technics.

Table 4. PCR target genes used by 15 studies.

| PCR Target Genes | Number of Articles |
|-------------------------------------|--------------------|
| rrs (16s rRNA) | 6 |
| lipl32 | 1 |
| rrs (16s rRNA) and lipl32 | 1 |
| g1/g2 primers | 2 |
| lig1/lig2 primers | 1 |
| g1/g2 primers and lig1/lig2 primers | 1 |
| flaB | 1 |
| hap1 | 1 |
| NM | 1 |
| | 15 |

NM, not mentioned.

3.4. Geographical Distribution of Serogroups in the Middle East

We relied on all SSI tests (such as MAT, LAT, IFAT, and MPAT) to demonstrate the serovars/serogroups circulating in most Middle Eastern countries. Reference serovars were also determined to evaluate the results in a dependent manner, as presented in Table 5. A total of 51 reference serovars, each referring to a specific serogroup, were reported in the Middle East. Twenty serogroups were identified, each with its own occurrence in the Middle East; however, only those related to the predominant hosts in this region are presented (Figure 2). The top five reported hosts in the Middle East were cattle, humans, rodents, sheep, and goats, with a population density of 28,595, 8564, 3351, 3789, and 2078, respectively. The predominant serogroups identified in the previously cited hosts were SJ (n = 3200), Grippytyphosa (GRIP) (n = 938), ICT (n = 515), Autumnalis (AUT) (n = 404), and Pomona (POM) (n = 102). Other serogroups such as Cynopteri (n = 12), Celledoni (n = 7), and Mini (n = 5) were also identified but had low numbers. However, each host had a predominant serogroup, including AUT for goats; SJ for cattle; and ICT for humans, sheep, and rodents. All serogroups presented in Table 5, except for Andamana, were identified in Egypt, Turkey, Iran, Iraq, Palestine, Jordan, and the Syrian Arab Republic. Other countries also reported serogroup identification in other hosts; however, they were not presented because the number of hosts was relatively low.

Table 5. Reference serogroups and serovars in the Middle East.

| S * | S | S * | S | S * | S | S * | S | S * | S |
|-----|-------------|-----|---------------|------|---------------------|-----|------------|-----|-----------|
| AND | Andamana | BAT | Bataviae | | Icterohaemorrhagiae | | Pyrogenes | | Wolffi |
| | Australis | CAN | Benjamin | | Mankarso | SEM | Patoc | TAR | Hyos |
| AUS | Bratislava | | Canicola | | Javanica | | Semaranga | | Tarassovi |
| | Jalna | CEL | Celledoni | JAV | Poi | | Balcanica | | |
| | Autumnalis | CYN | Cynopteri | | Sorexjalna | | Bovis* | | |
| | Bulgarica | DJA | Djasiman | | Georgia | | Burgas | | |
| AUT | Butembo | | Sentot | MINI | Mini | | Hardjo | | |
| | Rachmat | GRI | Grippytyphosa | | Swajizak | SJ | Istrica | | |
| | Arborea | HEB | Borincana | PAN | Panama | | Polonica | | |
| BAL | Ballum | | Hebdomadis * | POM | Pomona | | Saxkoebing | | |
| | Castellonis | ICT | Copenhageni | PYR | Alexi | | Sejroe | | |

AND, Andamana; AUS, Australis; AUT, Autumnalis; BAL, Ballum; BAT, Bataviae; CAN, Canicola; CEL, Celledoni; CYN, Cynopteri; DJA, Djasiman; GRI, Grippytyphosa; HEB, Hebdomadis; ICT, Icterohaemorrhagiae; JAV, Javanica; PAN, Panama; PYR, Pyrogenes; SEM, Semaranga; SJ, Sejroe; TAR, Tarassovi; S *, serogroup, S, Serovar, *: Bovine strain of Leptospira.

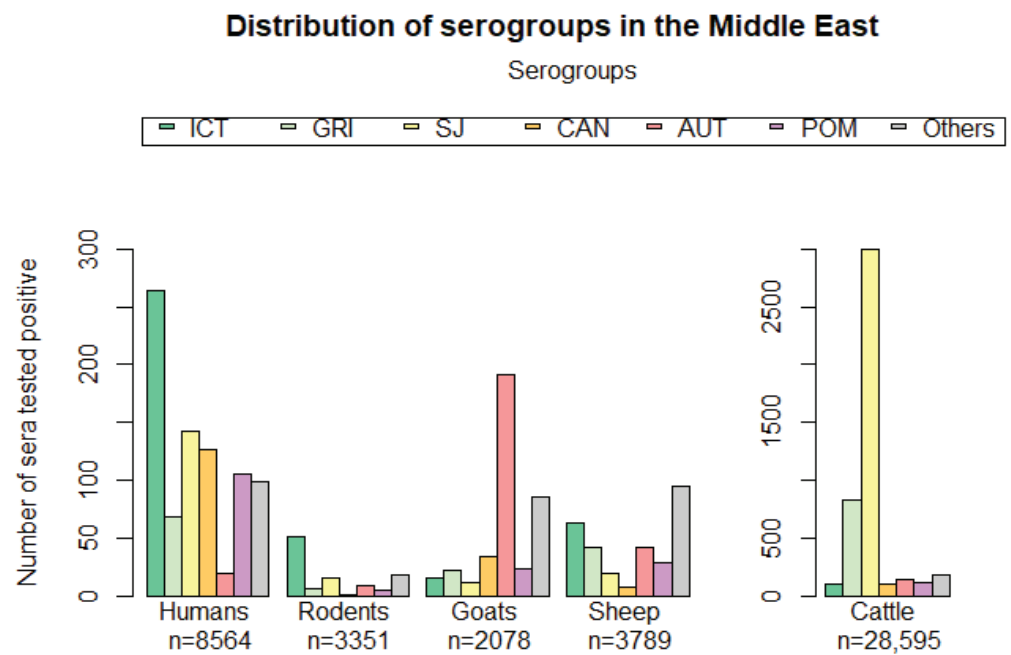


Figure 2. Distribution of serogroups in the five major hosts in the Middle East. Plot created by RStudio (RStudio Team (2021), RStudio: Integrated Development Environment for R; RStudio, PBC, Boston, MA, USA; URL <http://www.rstudio.com/>, accessed on 22 June 2022).

4. Discussion

To the best of our knowledge, this is the first review to summarize the prevalence and seroprevalence of leptospirosis and *Leptospira* infection in the Middle East. The disease, which continues to inflict a high burden worldwide, has been neglected in this particular region. From a broad perspective, almost all Middle Eastern regions reported information on the prevalence and/or seroprevalence of leptospirosis and *Leptospira* circulating serogroups. However, the prevalence values and serogroup distribution differed according to hosts and countries.

The prevalence and seroprevalence of leptospirosis in humans appear important in the Middle East, especially when compared with the prevalence and seroprevalence in other regions worldwide. Many leptospirosis outbreaks have been described in tropical and subtropical regions, including Latin America, Northern America, Southern Asia, and Africa, with some having incidence rates reaching 100 per 100,000 inhabitants per year [46,138,139]. However, to the best of our knowledge, a seroprevalence and prevalence of >41% have not been reported in these regions, despite having an adequate climate to support *Leptospira* survival and favorable human exposure. Indeed, the highest seroprevalence rates reported recently in Latin America were 40.2%, 23.6%, 8.8%, and 7.2% in Brazil, Peru, Colombia, and Ecuador, respectively [140]. In addition, the highest prevalence reported in Africa is 19.8% [46]. These results may be due to the sampling design being subject to selection bias by only reporting severe/laboratory-confirmed cases and/or hospital patients with acute febrile illness. Such a research design cannot provide an accurate presentation of leptospirosis cases because the disease is only known to cause severe complications in 5% to 10% of cases [141]. The reported prevalence and seroprevalence rates in the Middle East are higher (>42%) when either applying the same or different selection bias. This region not only reported acute cases but also asymptomatic cases relevant to the controlled group and recorded higher seroprevalence than the previously cited regions [84]. For instance, Iran tested seroprevalence in both healthy and hospitalized patients and recorded values of 48.5% and 64.7%, respectively [84,95]. Therefore, the risk factors for acquiring the disease in the Middle East may be more important than those in other regions.

SSI combined with MAT was reported in our review as the indirect method of serogroup identification. The tests related have the advantage of identifying a particular serogroup using available reference strains for manipulation. Therefore, the host serum is exposed to a panel of serovars, and the results vary in a dependent manner. Various serogroups have been reported in the Middle East; however, these serogroups are unlikely to be exhaustive, given that they could have been expanded with the use of additional reference serovars. In the top five hosts (humans, rodents, goats, sheep, and cattle) of this region, the predominant serogroup was SJ in cattle; AUT in goats; and ICT in humans, sheep, and rodents. The predominance of each serogroup in each of these hosts has been commonly reported in the literature, except in the case of goats. Serogroup SJ has been the most commonly reported serogroup in cattle in different countries worldwide, including the United States of America, France, Ireland, the Netherlands, Belgium, and others [51,142–145]. The serogroup ICT is commonly reported in rats worldwide [50,146] and is known to be the major causative agent of leptospirosis in humans [147,148]. In sheep, the predominance of the latter (ICT) serogroup supports previous observations in other countries [149]. In the case of goats, the predominance of the serogroup AUT has not been reported regularly in other countries worldwide [150–152] but has been commonly reported in related publications in the Middle East. However, this result appears robust given that the number of MAT positive cases associated with AUT ($n = 191$) in goats was considerably higher than the expected serogroup SJ ($n = 12$), which is considered predominant in goats in other countries [153,154]. Nonetheless, local variation may have led to such results and, as this is the first epidemiological study in the Middle East, it may be an indication of the most predominant serogroup in goats in this region. However, this assumption should be consolidated or proven by further studies. Moreover, the preponderance of the serogroup ICT in sheep in the Middle East can be explained by the high probability of their infection by rodents (carrying ICT) or their capacity for selective carriage of some ICT strains, as described for other hosts (pigs) in the literature [2]. The predominance of the serogroup ICT in humans in the Middle East may be due to infection by cattle, sheep, and rodent carriers of this serogroup, during their occupational work (farmers, rice farmers), travel, or contact with contaminated water. It may also be due to the importance of pathogenicity caused by the latter (ICT) serogroup leading to leptospirosis susceptibility and, therefore, its diagnostic examination [1]. Despite the various biases within the collected data, data regarding the serogroups in the Middle East remain informative because the objective of this review was to describe the circulating serogroups regardless of their titers and MODs used. In addition, no records of human or animal vaccination were mentioned in the selected publications; therefore, the serogroups detected in the Middle East were not concluded according to antibodies developed by vaccination but by infection. Such results help orient the type of vaccine that will be regarded as effective to each host. For instance, protection against *Leptospira* serovar ICT and SJ through vaccination should reduce the risk of leptospirosis in humans and cattle, respectively. Nonetheless, the adequacy of the serogroup repartition in the Middle East may be questioned because of the possibility of cross-reactions, which may lead to the consideration of serogroups that are not actually present. However, the distribution of the serogroups was analyzed at the whole population scale to minimize the effects of cross-reaction in our results, as has been performed in previous studies [144,155].

As expected, the reported seroprevalence and prevalence differed according to studies, likely due to variability in the MODs of *Leptospira* spp. Some diagnostic methods, such as PCR or any other direct method, can only detect nucleic acids in the first week of the host infection, known as the bacteremia phase of infection [1]. However, other MODs, such as MAT, ELISA, or any other SSI, can detect antibodies days after the onset of the disease and for a much longer duration [27]. The difference in the time margin between the persistence of the bacteria and the antibodies in host tissues lessens the chances of prevalence reports, in contrast to the chances of seroprevalence reports.

Several studies have reported null prevalence and seroprevalence. For some studies, this can be due to the small sampling size (<30 samples), 26 goats [71], 22 camels and 14 horses [44], five sheep [67], two weasels [55], one cattle, and one dog [67], which may not indicate the true distribution of the infection in the geographic location. Moreover, the sampling size may not be sufficient to detect an infected or exposed host if present in the population; indeed, a sampling size of 26 individuals allows the detection of a minimum prevalence or seroprevalence of 11% [156]. In the case of Cyprus, the country did not state human leptospirosis cases for several consecutive years [157], except for the year 2003 when 0.3 cases of 100,000 habitants were infected [158]. However, the case definition may not respond to sensitive detection of the *Leptospira* infection given that many asymptomatic or moderate cases could be experienced by the host, including humans. The European Center for Disease Prevention and Control reports were sent in accordance with the case definitions established by the European Union that included Cyprus. The general principles for the application of the case definitions are to only report laboratory-confirmed symptomatic cases, while suspected cases were only regarded as cases if they revealed a clear clinical picture with a judicious laboratory diagnosis [157,158]. In addition, the 2008 case definition was restricted to pathogenic *Leptospira* spp., namely *L. interrogans*, whereas, starting in 2012, all pathogenic *Leptospira* spp. were considered in the detection panel [157]. Such pathogenic *Leptospira* species restrictions may lead to an underestimation of the incidence [158]. Moreover, a null seroprevalence was reported in cattle, goats, and sheep in Cyprus, and, to the best of our knowledge, only a few imported calves tested positive in 1983 [54]. However, one publication is insufficient to determine the seroprevalence of ruminants in Cyprus but could explain the possibility of acquiring such seroprevalence. Surprisingly, studies in Palestine reported a null seroprevalence in rodents, even with a high sampling number. This may have been due to the antibody response of the *Leptospira*-infected rodent, which is frequently found to be under the threshold of positivity [159,160]. It may also be due to the remarkable variation in prevalence and/or seroprevalence from one rat colony to another starting from 0%, as reported in previous studies [161,162]. Thus, the apparent seroprevalence in Palestine could be underestimated compared with the true seroprevalence of the tested rodents because the sampling concerns a limited number of colonies ($n = 2$). Although the seroprevalence is null in rodents, infections have been detected in Palestine in both humans (seroprevalence of 1.9%) [52] and cattle (seroprevalence of 8.5% and 9.5%) [52,53]. The apparent seroprevalence in both hosts was elucidated by co-authors, who suggested that numerous outbreaks of leptospirosis in hundreds of cattle were the cause of human infection, such as those reported a few years prior to sampling [163,164].

Some countries have reported a remarkable seroprevalence range in the human population. The greater the number of studies combining various factors, such as the time interval between the two studies, the spatial variation, and the design of the study adapted in different publications related to the same country, the greater the seroprevalence variability.

An important time interval in the same country could lead to variations in disease epidemiology. For example, in Egypt, a seroprevalence of 0.5% was reported in 1957 [63], whereas a seroprevalence of 49.7% was reported in 2015 [44]. The risk factors for acquiring the disease depend on the environmental features and animal carrier abundance, which differ with the spatial variation, explaining such variability [165,166]. Therefore, spatial variability in the same country with a large surface area and an important distribution of studies throughout those areas, such as Iran, Turkey, and Egypt, could lead to seroprevalence variability. In addition, the lack of comparable design studies in the same host may lead to variable seroprevalence. The design of the study is specific to each publication because the sampling criteria were unique; some groups were chosen because of their tendency to be infected due to their occupations or professional activities, some were chosen randomly as controlled groups [65,66], and others were sampled for a differential diagnosis (cases of hepatitis, acute febrile illness for humans, and brucellosis for cattle) [74,76,127]. However, when the same type of group was chosen, low seroprevalence variability was

observed. For instance, in the case of humans in Yemen, when only two publications targeting similar types of groups (people at risk) were reported, the seroprevalence ranged between 41.3% [60] and 42% [61]. The sampling period may also have had a major impact on the incidence of leptospirosis throughout the year because of the seasonal pattern of the disease and its recrudescence in specific seasons, in which the highest incidence occurs mainly in summer or/and fall in temperate countries and in rainy seasons in warm-climate regions [8]. Therefore, the seroprevalence can differ throughout the year in a particular country and within the same population. For instance, a study in the north of Iran (temperate region) demonstrated a higher prevalence of leptospirosis in individuals in autumn and summer compared with that in spring [83]. In summary, the greater the number of studies combining the latter factors, the greater the seroprevalence variability. Moreover, the more these factors vary between publications, the lower the comparability of the prevalence or seroprevalence.

The epidemiological knowledge of leptospirosis is unclear for some countries in the Middle East because of the type and content of publications. For instance, studies in Oman only reported case studies [37,40,43]; therefore, the magnitude of leptospirosis in Oman remains unknown. However, a high prevalence is expected due to the globalization of travel and trade, occupational activities, and the temperate climate of the country [167]. Other countries, such as Saudi Arabia and the United Arab Emirates, only reported a prevalence and/or a seroprevalence in a few hosts (camels and cattle) due to a greater interest in describing the health status of the mammals that are largely present in these countries. Epidemiological knowledge is also lacking in some countries in the Middle East due to the absence of studies. For instance, in the twenty-first century, Lebanon, Kuwait, and the Syrian Arab Republic did not renew their interest in studying *Leptospira* infection. This suggests that leptospirosis is not within the public health policy priorities and/or that its burden is underestimated; this is in contrast to that noted in other Middle Eastern countries, such as Iran, which continuously show their interest in studying the disease by attempting to revise and authenticate its detection methods [168,169].

The time interval between the reported publications, the difference in the spatial environment, the particular design of the study adapted, and the number of publications related to each country led to the cognizance of leptospirosis variable epidemiology in the Middle East. Therefore, heterogeneous strategies applied in each country and between different Middle Eastern countries should be limited as much as possible, and a harmonized strategy should be adapted for better comparison of epidemiological studies relating to the seroprevalence of leptospiral infection.

- For the detection methods, PCR and culture should be prioritized for direct detection and MAT and ELISA for indirect detection. These methods can be applied in parallel when sampling particular hosts, whereas their efficacy can be limited to others. For instance, direct methods should be prioritized in the case of rodents because they have a low antibody response to leptospiral infection [159].
- As MAT remains the reference detection method, a minimum and common panel of serovars from selected serogroups should be included in all Middle Eastern countries that require shared reference strains. The minimum number of serogroups that should be tested are ICT, GRIP, SJ, CAN, AUT, and POM.
- A common human case definition should be a reference to all Middle Eastern countries to report the maximum, confirmed, and suspected number of clinical cases of *Leptospira*. Random sampling could be performed to describe the epidemiological situation in humans more comprehensively, considering asymptomatic or moderate cases.
- For the surveillance of *Leptospira* infection in domestic animals, an analysis of data on a continuous basis following diagnostic examinations in veterinary laboratories should be considered to determine the distribution of *Leptospira*. Such data should be communicated to the organizations and the public, indicating the applied diagnostic method of examination. Moreover, the sampling modalities should be stated by the

community of veterinary practitioners in order for them to be interpreted at the Middle East region level.

- Veterinary practitioners should be encouraged to provide all available information on animals and herds to enable a good diagnosis and improve epidemiological analyses. Information on the reason for examination, and the farms, herds (size and type), and animals (age, sex, clinical status) at diagnostic testing will facilitate improved epidemiological analysis and the ability to suggest risk factors to move toward more efficient risk-based surveillance in the future.

5. Conclusions

This review summarizes the epidemiological situation of *Leptospira* infection in the Middle East. Leptospirosis was found to be endemic in the countries of the Middle East for which data were available, excluding Cyprus. Variability in the prevalence and seroprevalence of *Leptospira* spp. has been reported in these countries. Furthermore, several serogroups have been reported in hosts in the Middle East, including ICT, GRIP, SJ, CAN, AUT, and POM. Some serogroups might be considered for certain hosts, whereas others are commonly reported in hosts worldwide. Therefore, a deeper understanding of the epidemiology of *Leptospira* spp. is required. In addition, leptospirosis should be prioritized as a public health problem in this region, for which increased awareness is critical. Therefore, more publications following a harmonized and appropriate study design, while also prioritizing particular seasons leading to leptospirosis recrudescence, and specific spatial environment or risk factors favorable for bacterial existence and survival are needed to achieve this goal.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/tropicalmed7100260/s1>, Table S1: Summary of *Leptospira* prevalence, seroprevalence and serovars distribution in the Middle East.

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3. **Molecular and serological identification of pathogenic *Leptospira* species and serovars in local and imported cattle of Lebanon**

Article 5: Molecular and Serological Identification of Pathogenic *Leptospira* in Local and Imported Cattle from Lebanon

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Research Article

Molecular and Serological Identification of Pathogenic *Leptospira* in Local and Imported Cattle from Lebanon

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Leptospirosis is a worldwide bacterial zoonosis for which the risk increases in warm and wet climates. Despite the suitability of the local climate for the bacteria's persistence, Lebanon lacks a formal system of prophylaxis for the prevention of *Leptospira* infection in both humans and animals, and the disease's epidemiology is unknown so far. As a preliminary step, we focused on *Leptospira* infection in cattle, which is of public health and economic concern. We conducted a descriptive study in cattle from the governorate of Mount Lebanon (ML) and in imported cattle. A total of 187 blood and 135 serum samples were provided. Among the 187 blood samples, 135 were from randomly selected animals from 14 herds, while the remaining 52 were from imported livestock. Serum specimens ($n = 135$) were obtained exclusively from cattle in the ML governorate. DNA was extracted from all blood samples and subjected to real-time PCR targeting 16S rRNA. All *Leptospira*-positive DNA samples were then amplified using conventional PCR (cPCR), and *Leptospira* species were identified via Sanger sequencing. A microscopic agglutination test (MAT) was performed on the 135 serum samples from local cattle. The real-time PCR revealed *Leptospira* infection in 7 of 135 local animals and 1 of 52 imported animals. DNA from six of the seven local animals and the one imported animal were amplified by cPCR and successfully sequenced, identifying the pathogen as the species *L. kirschneri*. Seven animals located in five out of the 14 tested herds had MAT titers $\geq 1:100$. Serogroup Grippotyphosa was predominant. This is the first study to provide epidemiological data on bovine *Leptospira* infection in Lebanon. Pathogenic *Leptospira* species and serogroups were identified in both local and imported cattle. These findings highlight the cattle associated risk of *Leptospira* infection in Lebanon, in the contexts of farming and trade. **Summary.** Leptospirosis is a severe zoonotic disease that can have critical consequences for people and animals. Within the country of Lebanon, this disease has been reported, but its epidemiology is unknown so far. The present study thus provides the first description of the existence of *Leptospira* in cattle in Lebanon (local and imported). It also highlights the existence of different pathogenic serogroups of *Leptospira* in local cattle. Our results should raise public health awareness of the threat posed by this underdiagnosed disease and serve as a starting point for control efforts in Lebanon.

1. Introduction

Worldwide, leptospirosis is an important emerging zoonosis caused by pathogenic spirochaetes in the genus *Leptospira* [1]. Tropical and subtropical regions are the most vulnerable to infection [2]. In Lebanon, annual morbidity (i.e., disease incidence) and mortality in humans due to leptospirosis have been estimated at 2.93 IC_{95%} [0.92–5.37] and 0.15 IC_{95%}

[0.05–0.26] per 100,000 individuals, respectively, based on age- and gender-adjusted demographic attributes of the human population [3]. However, the epidemiology of leptospirosis in this country remains unknown; no case reports have been published, with the sole exception of a 1947 inquiry launched following the discovery of two cases of Weil's disease [4]. Lebanon lacks a formal system of prophylaxis for leptospirosis despite the suitability of its climate

(warm and wet) for the bacteria's persistence and the presence of potential maintenance hosts such as rodents [5, 6]. Furthermore, several domesticated animals that are known to be susceptible to leptospirosis, primarily local and imported cattle (*Bos taurus taurus*), are raised in abundance [7], but their *Leptospira* infection status remains unknown.

Once infected, cattle experience a period of bacteremia that can persist for up to a week [8]. Nonmaintenance pathogenic *Leptospira* spp., such as the serogroup Grippotyphosa [9, 10], cause incidental infections in cattle and are recognized as a leading cause of reproductive failure, icteric abortions, stillbirth, economic loss, and occasionally, meningitis and death [8, 11]. In addition, cattle are well known as maintenance hosts for serogroup Sejroe (mainly serovar Hardjo). The acute phase of such infection is mainly subclinical and often goes unobserved, except in lactating cattle, which might develop agalactia [8]. However, chronic infection associated with the Hardjo bovine-adapted serovar can also lead to reproductive failure, stillbirth, and perinatal death [12, 13]. Regardless of the infection type, cattle may shed pathogenic bacteria in their urine—for up to 40 weeks for Hardjo infection [14]—that can expose humans to the bacteria either directly during the milking process or indirectly following exposure to urine-contaminated water or soil [8, 15, 16].

Leptospira infection can be screened through several different routes (blood, serum, urine, and renal tissue) in which *Leptospira* or leptospiral antibodies can be detected [17]. In cattle, blood and serum are typically the most easily accessible, compared to the sampling of urine on farms or renal tissue in slaughterhouses [18, 19], and both blood and serum can be used to perform several leptospiral diagnostic techniques [20]. However, *Leptospira* and/or leptospiral antibody detection can be hindered by differences between the two phases (septicemic and immune) experienced by the host, which leads to an underestimation of its impact [21, 22]. The diagnosis of pathogenic organisms such as *Leptospira* increasingly relies on molecular methods based on the polymerase chain reaction (PCR) [23], which can accurately detect *Leptospira*-derived DNA in the early stages of infection [20, 24, 25]. The serology-based microscopic agglutination test (MAT) is less advantageous for early diagnosis since it only detects antibodies from a past or current infection. The results of the latter test are reliable at the level of the serogroup but cannot be used to detect infectious serovars [26]. In addition, MAT results are interpreted subjectively, which can lead to repeatability issues and variability in the identification of the putative infecting serogroup [27, 28]. Despite these drawbacks, MAT is considered the immunological reference method for the experimental diagnosis of leptospirosis by the World Organization for Animal Health (WOAH) [29] and the World Health Organization (WHO) [30], and is recommended by the former for use in herd *Leptospira* screening [31]. Overall, a complementary approach that combines both PCR and MAT is the best option for improving early detection of biphasic leptospirosis [32].

Within the larger region of the Middle East, attempts have been made to survey cattle for *Leptospira* infection [33].

Prevalence ranged from 0% to 43%, and seroprevalence ranged from 0% to 85%, with Sejroe and Grippotyphosa as the predominant pathogenic serogroups in seropositive cattle. With the exception of a single publication from 1947 [4], though, no epidemiological studies have been carried out in Lebanon to describe the occurrence of *Leptospira* and the risk linked to infection. To fill this gap, this preliminary study aimed to provide an initial characterization of the threat posed by *Leptospira* infection in cattle in Lebanon. Specifically, our goal was to describe the *Leptospira* infection status and circulating serogroups within cattle herds in Lebanon, starting with the governorate of Mount Lebanon (ML), which is an important operational area for raising cattle [34]. Furthermore, we investigated the occurrence of *Leptospira* in imported cattle upon their arrival to Lebanese soil, with the goal of potentially differentiating between the genetic profiles of autochthonous and exotic *Leptospira* DNA.

2. Materials and Methods

2.1. Provision of Blood Samples. Cattle blood collected during regular professional consultations by a commercial veterinary company—that provides services related to animal importation and development—for their private annual prophylaxis program was used later on in this study for further investigation of *Leptospira* infection in Lebanon. Nonetheless, all herd owners were contacted by the company and agreed to provide cattle specimens for leptospirosis research purposes.

2.2. Description of Cattle of Interest. Cattle, particularly of the Holstein breed, are raised in abundance in Lebanon for dairy milk production [35]. The veterinary company that provided blood samples is dedicated to the development of cattle production in this country and therefore imports breeding dairy cattle from Europe, mainly France and Germany. It conducts consultations on dairy herds in all of Lebanon's governorates, but the majority of inspections are carried out in ML, where there are 50 dairy herds of interest to the company. The cattle in the ML herds are mainly of the Holstein breed, and their numbers range between 5 and 80 heads per farm. The leptospirosis vaccine had not been administered to local cattle.

2.3. Sampling Design. This study was carried out in Lebanon, in the ML and Beirut governorates (port of Beirut), for local and imported cattle, respectively. Animals were conveniently selected, regardless of their reproductive performance or clinical picture, from 14 of the 50 farms followed by the veterinary company in the ML governorate; this was carried out during the end of the dry season in 2021, in the months of October and November. This amount of sampling would allow the detection of at least one seropositive herd given a minimum between-herd seroprevalence of 20% and an uncertainty of 5% [36]. In addition, a one-time sampling campaign was performed by the company in the same period on a subset of a group of approximately 400 imported cattle

upon their arrival at the port of Beirut. Tests were performed at an individual level, however, the serological results were interpreted at the herd level, as recommended by the WOAHP manual for terrestrial animals [29]. In order to obtain relevant information at the herd level, samples were provided from 10% of the imported and local herd populations, with a minimum of 10 heads per farm or the whole herd if the total was less than 10, and a 10-cow sample being appropriate to reveal the presence or absence of an infection in a herd [29].

2.4. Repartition of Blood Samples. A total of 187 blood and 135 serum samples were provided from cattle. Of the 187 blood specimens, 135 were from arbitrarily chosen animals in herds located in the governorate of ML, while the remaining 52 were from imported livestock. Serum specimens ($n = 135$) were acquired solely from cattle in the ML governorate; they could not be collected from imported livestock due to the stress experienced by the animals, resulting in serum hemolysis following centrifugation. No animal showed clinical signs of illness.

2.5. *Leptospira* Microagglutination Testing. Microscopic agglutination tests were performed based on the standard methodology [29] using a panel of live leptospire. In total, twelve *Leptospira* serogroups, with related serovars in parentheses, were used: Australis (Bratislava, australis, muenchen), Autumnalis (autumnalis, bim), Ballum (Castellonis), Bataviae (bataviae), Canicola (canicola), Grippytyphosa (grippytyphosa, vanderheidon), Icterohaemorrhagiae (icterohaemorrhagiae, copenhageni), Panama (panama, mangus), Pomona (pomona, mozdok), Pyrogenes (pyrogenes), Sejroe (sejroe, saxkoebing, hardjo, and wolffii), and Tarassovi (tarassovi). Information on the serogroups, serovars, and strains used is available in Table 1. To avoid biases in interpretation, all MAT reactions were analyzed by a single technician. As recommended in the WOAHP manual, a 1:100 titer was used as the cut-off point for seropositive samples [29]. MAT results were interpreted at a global level, and the epidemiological unit considered was the herd. A herd was considered currently or recently infected at the herd level when at least one animal showed a positive MAT result. However, given the high specificity of MAT, serum samples were primarily tested using a 1:50 titer as evidence of previous exposure to *Leptospira*, as suggested by WOAHP [29]. Seropositive reactions were analyzed as follows:

- (1) If a serum specimen demonstrated reactivity to only one serogroup, that serogroup was designated dominant.
- (2) If a serum specimen reacted to two or more serogroups, but with a difference of threefold or more between the highest and the next highest titer, the former was designated the dominant serogroup.
- (3) If a serum specimen reacted to two or more serogroups with less than a threefold difference between the highest and the next highest titer, the serogroups were designated equally dominant. This

TABLE 1: List of the reference strains employed in the MAT antigen panel.

| Serogroup | Serovar | Strain |
|---------------------|---------------------|----------------------|
| Australis | Muenchen | München C 90 |
| Australis | Australis | Ballico |
| Australis | Bratislava | Jez Bratislava |
| Autumnalis | Autumnalis | Akiyami A |
| Autumnalis | Bim | 1051 |
| Ballum | Castellonis | Castellón 3 |
| Bataviae | Bataviae | Van Tienen |
| Canicola | Canicola | Hond Utrecht IV |
| Grippytyphosa | Grippytyphosa | Moskva V |
| Grippytyphosa | Vanderheidon | Kipod 179 |
| Icterohaemorrhagiae | Icterohaemorrhagiae | ENVN |
| Icterohaemorrhagiae | Copenhageni | M 20 |
| Panama | Panama | CZ 214K |
| Panama | Mangus | TRVL/CAREC 137774 |
| Pomona | Pomona | Pomona |
| Pomona | Mozdok | 5621 |
| Pyrogenes | Pyrogenes | Salinem |
| Sejroe | Sejroe | M 84 |
| Sejroe | Saxkoebing | Mus 24 |
| Sejroe | Wolffii | 3705 |
| Sejroe | Hardjo | Hardjoprajitno |
| Tarassovi | Tarassovi | Perepelitsin |

most often occurs as a result of cross reactions [37, 38], and the result was considered inconclusive in this case.

3. Molecular Detection of *Leptospira* DNA

3.1. DNA Extraction and Purification. DNA was extracted from cattle whole blood samples using the Quick-DNA Miniprep Kit, Cat. No. D4068 (Zymo Research, USA), following the manufacturer's instructions for liquid tissues. In addition, the OneStep PCR Inhibitor Removal Kit, Cat. No. D6030 (50 spin columns/purifications) (Zymo Research, USA), was used directly on the extracted DNA following the manufacturer's instructions, in order to efficiently remove contaminants that might inhibit downstream PCR reactions.

3.2. Real-Time PCR Targeting the 16S rRNA Gene. As an initial step, the efficiency of DNA extraction and the absence of inhibitors were tested for each sample by the amplification of the β -actin endogenous housekeeping gene. The β -actin primers were $5'$ CAGCACAATGAAGATCAAGATCATC $3'$ (forward) and $5'$ CGGACTCATCGTACTCCTGCTT $3'$ (reverse), as described in Toussaint et al. [39], and the sequence of the β -actin probe was $5'$ FAMTCGCTGTCCACCTTCCAGCAGATGTT $3'$ TAMRA. Real-time PCR reactions were performed on a Stratagene-Agilent Mx3000P qPCR system.

β -actin gene expression also served as an internal control for expression of the 16S rRNA target gene. This gene sequence was amplified from all purified DNA using AgPath-IDOne-StepReal-Time PCR Reagents (Applied Biosystems). Real-Time PCR reactions contained 12.5 μ L 2X Real-Time

PCR Buffer, 2.5 μ L of probe and each primer, 1 μ L of 25X Real-Time PCR Enzyme Mix, and 4 μ L of DNA in a final volume of 25 μ L. Amplification was performed using primers targeting a region of the *Leptospira* rrs (16S) gene that were designed in a previous study, with a slight modification of the cycling protocol [40]. The 16Spatho primers were 5'CGGGAGGCAGCAGTTAAGAA3' (forward) and 5'AACAACGCTTGACCATACG3' (reverse). The sequence of the 16Spatho probe was 5'^{FAM}GCAATGTGATGATGGTACCTGCCT^{BHQ1}3', as described in Waggoner et al. [40]. Real-Time PCR cycling was performed on a Stratagene-Agilent Mx3000P qPCR system using the following parameters: 95°C for 10 min, followed by 40 cycles of (1) 95°C for 15 s and (2) 60°C for 1 min. Fluorescence was provided by TaqMan probes (based on reporter and quencher fluorochromes) which continuously detected and reported DNA amplification; a C_T was automatically set for each DNA sample, and any exponential curve that reached a C_T prior to cycle 40 was considered a positive result [41]. A no-template mix and a positive control were added in each run of the real-time PCR.

3.3. Conventional PCR Targeting leptA and leptB Primers. For samples with positive results in the real-time PCR, conventional PCR (cPCR) was performed with HotStarTaq DNA polymerase (250 U), using primers targeting the *Leptospira* rrs (16S) gene that were designed in previous studies, with a slight modification of the cycling protocol [42]. cPCR reaction mixes contained 5 μ L 10x PCR Buffer, 2 μ L MgCl₂ (25 mM), 1 μ L leptA primer (10 μ M), 1 μ L leptB primer (10 μ M), 1 μ L of dNTPs (10 mM), 0.5 μ L Taq polymerase (5 μ / μ l), 34.5 μ L nuclease-free water, and 5 μ L of DNA in a final volume of 50 μ L. The leptA forward primer (5'GGCGGCGCGTCTTAAACATG3') and leptB reverse primer (5'TTCCCCCATTGAGCAAGATT3'), specific for the genus *Leptospira*, were used, as described in a previous study [42]. cPCR cycling was performed on an Eppendorf Mastercycler Nexus Gradient Thermal Cycler as follows: 95°C for 15 min, 40 cycles of (1) 95°C for 15 s, (2) 57°C for 30 s, and (3) 72°C for 1 min, and a final elongation step at 72°C for 10 min. A no-template mix and a positive control were added in each run of the cPCR. PCR products were confirmed in duplicate using 1% gel electrophoresis for 30 min at 100 volts, and the size of the amplified DNA fragment was checked under ultraviolet light.

3.4. Sanger Sequencing. Samples that were cPCR-amplified and visualized on a 1% agarose gel under ultraviolet light were then Sanger sequenced by a service provider (Genoscreen, Lille, France) using the same primers employed in the cPCR. ChromasPro (version 2.6.6) was used to assemble nucleotide sequences that were at least 330 bp in length and compatible with the genus *Leptospira*. Each contig was queried using the nucleotide Basic Local Alignment Search Tool (BLAST) in the NCBI database (<https://blast.ncbi.nlm.nih.gov/>) to determine *Leptospira* species assignment. A

phylogenetic tree was then generated based on the partial 16S gene rDNA sequences obtained from our blood sample amplicons and reference *Leptospira* DNA sequences provided by the "Laboratoire des Leptospires" [42]. The tree was constructed using Muscle version 5 [43] with IQ-TREE 2.2.0.3 [44], using the maximum likelihood method (log-likelihood -991.593) and the best-fit model TPM3 + G4, chosen based on values of the Bayesian information criterion (BIC). A bootstrap analysis was performed with 1000 replicates (S1 Fig.).

3.5. Agreement between PCR and MAT. Compliance between PCR and MAT results at the herd level was determined using Cohen's Kappa coefficient, calculated using Rstudio (version 1.3.1093, "Apricot Nasturtium") with the formula $K = \frac{\Pr(a) - \Pr(e)}{1 - \Pr(e)}$, where $\Pr(a)$ is the observed percentage of agreement and $\Pr(e)$ the expected percentage of agreement. This comparison was repeated for two different MAT dilutions: PCR and MAT (titer 1 : 50) and PCR and MAT (titer 1 : 100).

4. Results

4.1. Molecular Analysis. β -actin gene expression was reported by Real-Time PCR from all 187 DNA samples that were extracted from whole blood. The 16S rRNA gene sequence of *Leptospira* was detected by Real-Time PCR in 7 of 135 local cattle (representing 5 of the 14 herds) and in 1 of 52 imported cattle. For six of the seven Real-TimePCR-positive local animals (representing the same five herds) and the Real-TimePCR-positive imported animal, cPCR amplification revealed a 330-bp fragment compatible with the genus *Leptospira* [45]. All seven sequences demonstrated 100% nucleotide affinity with a published sequence corresponding to *Leptospira kirschneri* (GenBank accession number MK726123.1). The five PCR-positive herds were geographically distributed as follows: two were located in the north, one in central ML, and two in the south (Figure 1).

4.2. Leptospira Microagglutination Testing in Cattle in Mount Lebanon. Five herds (three in the center and two in the south of ML) contained cattle with MAT titers $\geq 1:100$ (Figure 1). Herds F, J, and M each contained a single serogroup—Sejroe (1 : 400), Canicola (1 : 100), and Grippotyphosa (1 : 200), respectively—even though the herds had multiple seropositive animals. The remaining seropositive herds—herds N and G—contained two serogroups each, with neither appearing to be dominant as the reported MAT titers—CAN (1 : 200) and GRI (1 : 200) in herd G, and GRI (1 : 200) and SJ (1 : 100) in herd N—had less than a threefold difference. In herd G, a single seropositive individual demonstrated equal antibody titers against serogroups Canicola and Grippotyphosa, hindering the identification of a single dominant serogroup. In herd N, the two seropositive animals had distinct MAT profiles, with Grippotyphosa as the putative serogroup for the first and Sejroe for the second. For each seropositive animal, the reactive antibody titers for

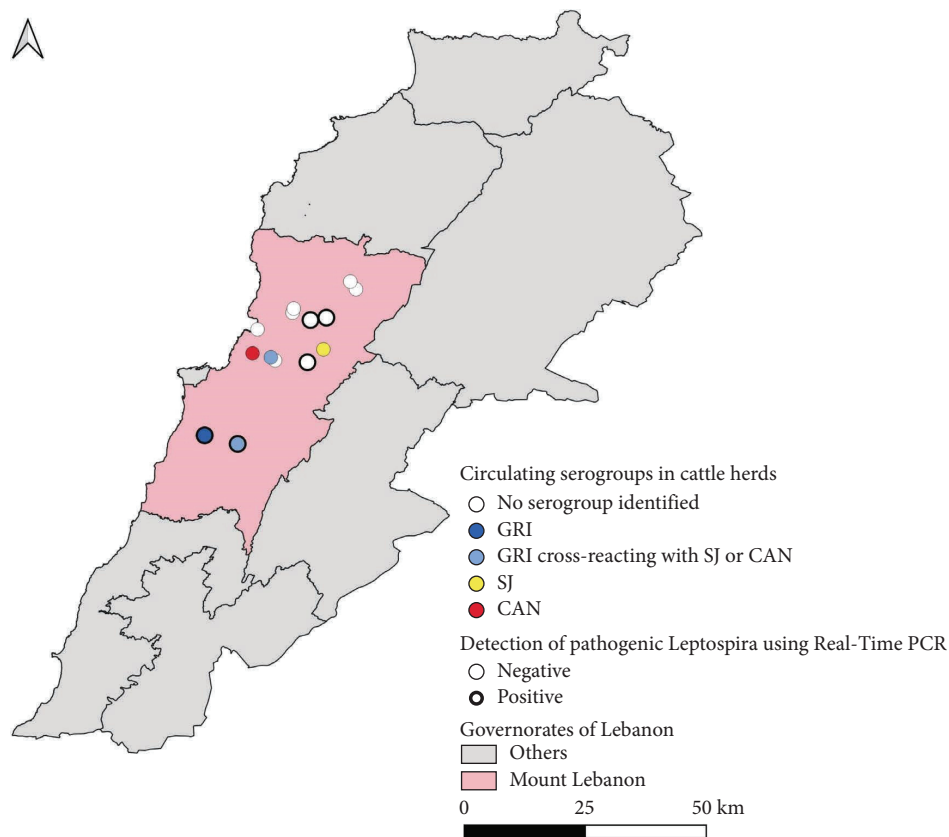


FIGURE 1: Geographical distribution of circulating serogroups and real-time PCR-positive herds in Mount Lebanon. Map created with DIVA-GIS version 7.5 and designed with QGIS 3.16.14 hannover. GRI, Grippotyphosa; SJ, Sejroe; CAN, Canicola.

each tested serovar/serogroup in MAT are displayed in Table 2.

4.3. Combining Molecular and Serological Results Among Cattle Herds in Mount Lebanon. When we examined both PCR results and MAT results at titers $\geq 1:100$, evidence for *Leptospira* or antileptospira antibodies was found in 8 of the 14 tested herds. Cohen's Kappa coefficient was 0.07 with a p value of 0.8. When we examined both PCR results and MAT results at titers $\geq 1:50$, the total number of positive herds remained the same (8 of 14), but the agreement between the two methods changed. The resulting Cohen's Kappa coefficient was 0.43, with a p value of 0.06. The serogroups and species of *Leptospira* found in each herd are presented in Table 2 and Figure 1.

5. Discussion

To the best of our knowledge, this is the first study to use molecular and serological methods to characterize bovine *Leptospira* infection in Lebanon, particularly in Mont Lebanon (ML). It also applied the same molecular approach to evaluate *Leptospira* infection among imported cattle upon their arrival to Lebanese soil (port of Beirut) prior to their distribution to Lebanese herds. The identification of pathogenic *Leptospira* species and circulating serogroups in cattle highlights an unaddressed threat to public health in Lebanon, particularly

for people working with cattle. In addition, the detection of pathogenic *Leptospira* in imported cattle suggests that animal importation may be one of the means by which pathogenic bacteria like *Leptospira* are introduced to this country.

Pathogenic *L. kirschneri* was the only species detected in all positive cattle, local or imported. This species is known worldwide as an agent of human leptospirosis [16, 46, 47] and can have notable clinical manifestations in some patients, as reported in France [48] and Malaysia [49]. Furthermore, it has been suggested that infection by *L. kirschneri* can also have an impact on herd production output, but the clinical manifestations of such infection are ambiguous [50]. *L. kirschneri* has not been described in studies conducted in neighboring countries, which to date have been limited to reports of molecular positivity in cattle and have not identified the infecting bacterial species. However, this species has been described in cattle in countries in North and South America (Brazil, Uruguay, and Mexico) [50–52]. Our finding of *L. kirschneri* in local herds is consistent with our serological finding of the predominant serogroup Grippotyphosa since the related serovars Grippotyphosa and Vanderheiden, tested in our study, belong to the *L. kirschneri* species [53]. In addition, the fact that *L. kirschneri* was also identified among imported cattle likely destined for introduction into Lebanese herds suggests the potential introduction or maintenance of pathogenic *Leptospira* through global trade.

TABLE 2: Serovars and species of *Leptospira* identified in seroreacting and PCR-positive cattle, respectively.

| Herds ID (number of individual cattle tested for each herd) | ID of cattle testing positive by MAT and/or PCR | Distribution of antibody titers among serum samples reacting for each tested serovar in MAT | | | | | | | | | | 16S PCR <i>Leptospira</i> spp. identified by sequencing | | | |
|---|---|---|-----|-----|-----|-----|----|-----|----|------|-----|--|---|---|---|
| | | AUT | CAN | COP | ICT | GRI | SJ | SAX | HJ | WOLF | NP* | | | | |
| A (10) | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| B (10) | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| C (10) | C1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| | C2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| | C3 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| D (10) | D1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| | D2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| E (10) | E1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| F (10) | F1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| | F2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| G (10) | G1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| H (10) | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| I (10) | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| J (10) | J1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| | J2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| K (10) | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| L (5) | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| M (10) | M1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| | M2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| | M3 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| | M4 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| | M5 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| | M6 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| N (10) | N1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| | N2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| | N3 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| | N4 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| PORT (52) | P1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| | NP | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| | NP | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| | NP | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| | NP | — | — | — | — | — | — | — | — | — | — | — | — | — | — |

AUT, autumnalis; CAN, canicola; COP, copenhageni; ICT, icterohaemorrhagiae; GRI, grippityphosa; SJ, sejroe; SAX, saxkoebing; HJ, hardjo; WOLF, wolffii; NP, not performed; NP*, refers to animals for which real-time PCR was positive but cPCR was negative and therefore sanger sequencing was not performed; P1, refers to imported cattle for which only molecular tests (real-time PCR 16S and cPCR leptoS and B) were performed.

The number of *Leptospira* infections detected in our study is more likely an underestimation of the genuine occurrence in this population due to the time of sampling as well as the clinical specimen chosen for analysis. The illness has a distinct seasonal pattern and is closely associated with climatic factors [54]. In subtropical countries such as Lebanon, most cases of leptospirosis in both humans and cattle naturally occur following rainfall and flooding [55–58]. In general, flooding is thought to help *Leptospira* disseminate in the environment [59], resulting in leptospirosis transmission and infection, as it has been described in cattle in other subtropical countries [57, 60]. In our study, sampling was performed in October and November, a period with relatively little rainfall, which might have reduced our chances of detecting *Leptospira* through Real-Time PCR. In addition, *Leptospira* bacteria are found in relatively low numbers in the bloodstream, which can impede the identification of infected cattle despite the sensitivity of the 16S Real-Time PCR (7.0 to 2.0 log₁₀ copies/μL) [40] and the optimization of primer sequences and annealing temperatures [61]. Moreover, leptospires can only be found in blood during the first week of illness in both humans and animals, mainly from the second to the fourth day of infection [8, 15, 62]. This likely explains the low number of *Leptospira*-infected cattle detected here as well as in two studies conducted in Egypt (country with similar meteorological circumstances and herd management techniques) that also used blood as a sampling matrix [63, 64] and detected *Leptospira* DNA in seven out of 625 cattle blood samples in one study, solely [64]. The use of sample specimens other than cattle blood, such as urine, where leptospires are retrieved for a longer period of time, could have led to a higher incidence report, as obtained in other studies that tested cattle urine and blood samples by Real-Time PCR and only detected *Leptospira* DNA in urine samples [65]. In the population used in our study, whole blood was the only available sample that remained appropriate for bacteremia detection and *Leptospira* characterization, although the detection period was limited to the acute phase of infection. Overall, despite its drawbacks, our approach enabled us to identify *Leptospira* and reveal its presence in domestic cattle with ML, but it was not inappropriate to assess the true prevalence of *Leptospira* infection.

A low degree of concordance between PCR and MAT was observed using a cut-off titer of $\geq 1:100$. All cattle which tested positive by Real-Time PCR still had not produced antibodies and were categorized as negative by MAT. The latter finding supports the biphasic nature of leptospirosis, as seroconversion mainly occurs 10 to 14 days following infection [8]. However, some cases of (*Leptospira* serovar Hardjo) infected cattle who survived the bacteremic phase but did not develop agglutinating antibody titers above the 1:100 threshold have also been documented in the literature [66, 67]. Still, our work is consistent with previous studies demonstrating that the use of Real-Time PCR in conjunction with MAT increases the sensitivity of *Leptospira* detection, mainly in the early stages of infection [32, 68–70]. Here, the use of both molecular and serological methods within the same herd enabled us to acquire more information about the

status of *Leptospira* infection in cattle herds from ML than either method could have revealed by itself.

Although the methodology used most likely underestimates the occurrence of *Leptospira* infection in cattle, it provides some initial data, namely, that *Leptospira kirschneri* and serogroup Grippotyphosa are the predominant species and serogroup, respectively. These results are useful in developing further research studies, therefore, we make the following recommendations. Despite its weakness regarding sensitivity, the methodology used in this paper could be extended in space and time to assess the relative leptospiral risk throughout the remaining governorates of Lebanon and across seasons, assuming that in each case the degree of underestimation would be relatively constant. To improve the precision of prevalence estimates and thus assessments of the level of risk for people in contact with cattle, the sensitivity of detection should be improved by sampling specimens other than blood, such as kidneys (in abattoirs) or urine (in farms and abattoirs), where leptospires persist longer [71].

In Lebanon, cattle carriers of pathogenic *L. kirschneri* can spread the bacteria through their urine and potentially act as a reservoir for humans—particularly farmers in close proximity—and domestic and wild animals [72], as well as a source of water and soil contamination in which the bacteria can remain viable for months in optimal condition [73]. Cattle being an interface between wildlife and humans, managing *Leptospira* infection in cattle herds (e.g., by following an appropriate vaccination plan) can not only reduce the cattle's health impact but also prevent leptospirosis in humans and other animals besides cattle. The finding of *L. kirschneri* in cattle in ML raises a One Health concern for leptospirosis control in Lebanon in order to sustainably ensure the health of the ecosystem, including humans and animals [74]. Consequently, there is a need to implement a response according to the quadripartite One Health concept definition [75] that includes intersectorial mobilization and communication related to the presence of *L. kirschneri* and leptospirosis risk management among veterinarians, farmers, general physicians, and workers in wild mammal associations practicing in Mont Lebanon. In addition, services related to zoonosis management at the ministries of agriculture, public health, and environment should be aware of the leptospirosis risk and be able to support future efforts on intersectorial and collaborative epidemiological surveillance, disease control, and research [76].

One of the findings of this study is the detection of pathogenic *Leptospira* in imported cattle, which highlights the risk related to importation. WOAHA recommends the application of a reference test to 10% of each batch of imported cattle [29]. This step cannot ensure a disease-free herd, but it can minimize the potential risk of infection to herds in the importing country. Another approach to improving the sensitivity of the detection of infected herds could be the use of simultaneous direct and indirect detection, but further studies are necessary to assess the efficiency of such a measure.

6. Conclusion

Pathogenic *Leptospira* was detected in both local and imported cattle in Lebanon. As a result, leptospirosis risk should be addressed as a public and animal health concern in this country, raising the need to follow a “One Health” approach. Enhancing public awareness is essential, particularly among veterinarians and general physicians, so they can detect and report clinical forms of leptospirosis and consequently, maximize the health of humans, and animals. Additional studies on *Leptospira*-infected populations (e.g., rodents and dogs) should be conducted in Lebanon in order to characterize potential maintenance hosts and have a more thorough understanding of leptospirosis epidemiology to design effective disease control strategies.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Original Draft Preparation was written by EH; investigation was performed by EH; visualization was provided by EH; methodology was done by EH and FA; Writing–review & editing were performed by FA; EH, FA, AAR, and CH conceptualized the study; Formal analysis was carried out by EH and SAP; resources was done by FA and VL; supervision was performed by FA and KG.

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Supplementary Materials

S1 Fig. Phylogenetic tree based on partial 16S rDNA gene sequence obtained from blood samples amplicons. The phylogenetic tree was constructed using Muscle version 5 with IQ-TREE 2.2.0.3 using the maximum likelihood

method and the best-fit model TPM3 + G4. A bootstrap analysis of 1000 replicates was performed. Samples colored in red correspond to the *L. kirschneri* cluster. (*Supplementary Materials*)

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4. Conclusion

The most predominant pathogenic serogroups circulating in the Middle East region were Sejroe, Grippotyphosa, Icterohaemorrhagiae, Autumnalis and Pomona, highlighting the disease as a public health threat in the region, the non-negligible risk in Lebanon. Pathogenic *Leptospira* species (*L. kirschneri*) and serogroups (mainly Grippotyphosa and Sejroe) were revealed in local and imported cattle. Such finding revealed leptospirosis epidemiological situation in livestock population in Lebanon for the first time and indicate the likely presence of a maintenance community in the nation. Consequently, it supports the need of acquiring information about the maintenance community by sampling additional animal species in different ecosystems in Lebanon and determine their infectious status. However, performing this step relies on the development of adequate laboratory methods in Lebanon (as far as possible) through the adaptation of a multi-sectoral and multi-disciplinary One Health approach to build mechanisms of communication, coordination and collaboration in the nation, specially since the shipment of clinical samples for the performance of laboratory tests in foreign countries is challenging. The findings of the literature review of the Middle East can also be a guidance in determining the role of the hosts in the maintenance community, however, the information provided is limited since molecular tests did not determine a complete genetic profile. Consequently, further studies should also be adapted in the Middle East in order to clarify the role of hosts and adapt preventative measures accordingly.

III. General discussion and conclusion of the thesis

The general findings of this thesis revealed that the studied populations have different roles in the maintenance community of *Leptospira* according to their characteristics and possibly, the ecosystem. These findings encourage the adaptation of adequate sampling strategies and the elaboration of multilayer network models to determine or predict the role of animal hosts in the maintenance community. In addition, they guide us to choose adequate laboratory tests in epidemiological studies to determine the leptospiral carriage of the sampled hosts. Furthermore, they highlight the need to raise a One Health approach awareness to minimize the risk of infection in the studied ecosystems of France and Lebanon. The latter statements are discussed here through a thoughtful critique of the research epidemiological methods of investigations and recommendations for future epidemiological investigations, mainly if carried out in France and Lebanon. The main drawback of the thesis was the sequencing sensitivity due to factors involved in the sampling of animal hosts and the preservation of their specimens and/or their DNA, limiting our knowledge about the role of the hosts in the maintenance community.

Adapting an adequate sampling approach to determine the role of animal hosts in the maintenance community

A mono-host approach to determine the role of a host in the maintenance community

The mono-host approach adopted on raccoons could not reveal their role as maintenance hosts (high *Leptospira* infection by particular *Leptospira* strain(s) and shedding for a prolonged period) of a particular *Leptospira* strain due to the low prevalence and the different genetic profiles acquired. This suggests their qualification as accidental hosts (*Leptospira* infection and shedding for short period). Consequently, when tracking the source of human leptospirosis in France, raccoons may not be as significant as other hosts with higher *Leptospira* prevalence such as Norway rats, described as the primary maintenance hosts of *L. interrogans* and reservoir hosts for human leptospirosis [160], or voles' invasive species, described as selective carriers of *L. kirschneri* [198–201]. However, since *Leptospira* ecology includes interactions with raccoons, the latter species may contribute to a limited extent to the maintenance of *Leptospira* in a given ecosystem as they may act as bridge hosts given their propensity for thriving in semi-aquatic and grassland habitats (where people and domestic animals co-exist), particularly in the case of male raccoons (home ranges (mean \pm Standard Error) of males racoons reported as 266 ± 14 ha) [202]. In addition, a study in Colorado suggested that raccoons may serve as a bridge host for zoonotic pathogens [203]. According to the definition provided by Caron et al. (2015) [204], a bridge host

performs the transmission role but not the maintenance role. To be eligible to serve as a bridge host, a host must fulfill two primary requirements. The first requirement is that the host must be receptive to infection and be able to excrete it without having the ability to maintain it on its own, (unlike the maintenance host), or the host must be able to mechanically transmit the pathogen [205,206]. The second requirement is that the host comes into contact with the maintenance and target populations, mutually [204]. Raccoons most likely meet the requirements to serve as bridge hosts in a multi-host system, still, additional epidemiological studies on the latter species are needed to help us confirm this statement and understand raccoon's role in the maintenance community of *Leptospira*.

A multi-host approach to identify the source of human infection following an outbreak

Adapting a mono-host approach can be useful to speculate the role of the host(s) of interest in the maintenance community, however, is insufficient to determine the source of *Leptospira* infection in an ecosystem, a step that requires the adaptation of a multi-host approach. Based on our multi-host approach elaborated following the Vilaine outbreak [207], we were able to determine three rodent species—nutrias, muskrats, and Norway rats—as sources of *L. interrogans*. The serological tests revealed cattle exposure to Grippotyphosa, a serogroup belonging to *L. kirschneri* [95], which underlined the circulation of *L. kirschneri* in the Vilaine ecosystem. The failure in identifying the source of *L. kirschneri* is probably due to the limited panel of species sampled. Although four animal species—nutrias, muskrats, Norway rats, and cattle—were sampled in the Vilaine area, the context of the ecosystem was not priorly clarified to consider additional animal species that may serve as a source of *L. kirschneri*. A possible route of investigation would have been to sample additional rodent species in Brittany, particularly those that have been previously identified as *L. kirschneri* carriers. Voles, for instance, might be a species of interest because they have recently been identified as *L. kirschneri* serogroup Grippotyphosa carriers in Central France [198]. Additionally, a 2009 epidemic of *L. kirschneri* serogroup Grippotyphosa in Germany led to a research that suggested voles were a reservoir for people [208]. The striped field mouse (*Apodemus agrarius*), least weasel (*Mustela nivalis*), and crowned shrews (*Sorex coronatus*) might be species of interest as well as they showed high prevalences for *L. kirschneri* (42.86% CI_{95%} [15.75% – 75.02%]; 50% CI_{95%} [9.5% – 37.5% and 90.5%], respectively) in Germany [200,209]. Another possible route of investigation would have been to sample semi-aquatic mammals that might have served as maintenance hosts of *L. kirschneri* in the Vilaine. Nutrias and muskrats would have served as the bridge hosts in that scenario. Unlike small mammals that tend to

reduce their home ranges (mainly with increasing population density and resource), semi-aquatic mammals have a larger home range as they exploit food- and building resources both in water and on land to meet their requirements, if not impacted by ecological factors (e.g., food plant density, predator activity, and human disturbance) [210–212]. Consequently, semi-aquatic mammals (e.g., voles) (maintenance hosts) could have been the population source that contaminated the water environment which then contaminated nutrias and muskrats (bridge hosts). Nutrias and muskrats in their turn contaminated the pasture and cattle (target hosts) as well as humans (target hosts). Another scenario is that semi-aquatic mammals (maintenance hosts) present near the pastures infected cattle, nutrias, and muskrats (bridge hosts), which in turn contaminated the water of and the kayakers (target hosts). Although the bridge host concept may not be applicable in all multi-host systems, it contributes to structuring investigations into the ecology of emerging diseases, such as leptospirosis. Knowing and understanding the multi-host system and its transmission pathways might help inhibit transmission routes from bridge hosts to humans. A notable example of a situation where paying attention to bridge hosts might improve our comprehension of pathogen dynamics in multi-host systems is the avian influenza pandemic. Another example is the Hendra virus between bats and humans, considered as maintenance and target hosts, respectively, whereas domestic horses are thought of as bridge hosts [213].

Adequate sampling strategies to understand the maintenance community and pinpoint the source of infection

A large sampling size can accurately reflect the characteristics of the population, leading to a smaller prevalence confidence interval when it comes to disease diagnosis and more reliable results [214]. Nevertheless, using an excessively large sample can be unethical and/or impractical. An alternative can be the analysis of environmental samples to identify the presence of *Leptospira* strain(s) as performed elsewhere [215–217], as the environment is the main pathway that may contaminate humans and animals in an ecosystem. This strategy may not determine the direct source of human and animal infection in an ecosystem but can help us determine the circulating species and speculate the risk of infection in the ecosystem.

The panel of species sampled when tracking the source of infection following outbreaks is a key step that will either allow or forbid the determination of the population source responsible for the infection of an accidental host of concern. Nevertheless, adopting a suitable sampling process can be hindered when it comes to particular rodent species (e.g., field voles (*Microtus agrestis*) and water voles) due to ethical and practical limitations and should require other ways of proceeding. An

alternative can be the sampling of species that may serve as bridge hosts, mainly species that do not require ethical approval for sampling (*e.g.*, raccoons, nutrias and muskrats) being invasive in France, therefore legally trappable and regularly controllable by authorized persons [173]. The analysis of scats of the latter species would help to determine their diet as performed elsewhere [218–222] and identify the prey species that were fed on and potentially infected by. This will allow us to better understand the ecosystem ecology and speculate for which animal species they are bridge hosts for. Furthermore, sampling can be limited or even avoided for certain species for which the prevalence and the leptospiral status are known according to the literature. For instance, rats sampling can be avoided as they are difficult to trap and are known as maintenance hosts of pathogenic *L. interrogans* serogroup Icterohaemorrhagiae with an estimated prevalence of 26% CI_{95%} [20% - 33%] [160]. We can refer to the hypothesis of rats being maintenance hosts of *L. interrogans* serogroup Icterohaemorrhagiae when studying the maintenance community of *Leptospira* and adopt a limited sampling that confirms the latter assumption. As an alternative, we can completely omit sampling.

Knowledge regarding the existing animal species in an ecosystem, their status of *Leptospira* infection, and the interactions that they have with other species is essential as it pinpoints the source of infection and helps predict the risk of outbreaks in an ecosystem. However, acquiring such information is not always possible as epidemiological research for understanding the maintenance community of *Leptospira* lack in the literature. In this case, the establishment of multilayer networks models can be helpful to better understand the interactions between the different animal species present in the same ecosystem and the level of involvement of each species in the maintenance of specific serogroup(s) of leptospires.

Elaborating multilayer network models to predict the role of animal hosts in the maintenance community

The non-exhaustive multi-host approach provides partial knowledge about the role of animal hosts in the maintenance community. Filling up this gap can include taking the ecology into account, meaning, considering the relationship between organisms and their environment [223]. All species in an ecosystem—both those that are hosts to a specific bacteria and those that are not hosts—will have an impact on the dynamics of a pathogen as a result of their intricate interactions. In parallel, the features and mechanisms of each ecosystem have an impact on the pathogen's capacity to invade an ecosystem and the dynamics of transmission. As a result, the interactions between host species and non-host species and their environment are noteworthy as they have a significant impact on epidemiological outcomes regarding the

maintenance/reservoir host(s) and/or the maintenance community. However, if the ecology is not specified, epidemiological outcomes may be erroneous considering that a similar host species may serve as a maintenance host for a particular pathogen in one ecosystem but not in another that differs only marginally (*e.g.*, where one host species is removed from or added to the system). Recognizing the environmental context and adapting multilayer networks accordingly could help us better understand the context of an ecosystem. Both steps are important to predict the transmission of a pathogen in a host population and to study the characteristics of mixed species interaction networks, which can give vital insights into multi-host pathogens, such as *Leptospira* [224].

Multilayer network models to understand bacterial transmission and predict outbreaks

In disease ecology, some multilayer network models concentrate on the scenario where a source species infects a target species [164,225]. These models imply very little to no transmission from the target species back to the source [164,225]. Other multilayer network models deal with diseases that can be maintained in several host species, especially at the livestock-wildlife interface and the insights offered are really valuable for veterinary epidemiology. These models may give a clearer picture of how pathogens transmission occurs and may spot the source of transmission, as described by Pilosof et al. (2017) [226] and Silk et al. (2018) [227]. Pilosof et al. (2017) [226] offered an adaptable approach to simulate the dynamics of disease transmission in various hosts while considering the within- and between-host interactions. The latter study measured the risk of outbreak probability through the elaboration of a multilayer network model where layers presented populations of two distinct hosts, and intralayer and interlayer edges presented within- and between-host interactions, respectively. Silk et al. (2018) [227] relied on empirical data of direct and indirect interactions between domestic cattle and European badgers (*Meles meles*) to present a multilayer network model and detect the chances for between-hosts transmission of pathogens, particularly *Mycobacterium bovis* (bovine tuberculosis) [227]. In the latter study, two layers represented the social network of either cattle or badgers, and one additional layer represented (badger) latrines (*i.e.*, the environment) following their location within the site of study. The intralayer edges presented the contact between badger-badger or cattle-cattle and interlayer edges represented the contact between badgers-latrines or cattle-latrines. He provided evidence of the importance of indirect interactions in presenting chances for *Mycobacterium bovis* (as well as other diseases) transmission between badgers and cattle. As bovine tuberculosis and leptospirosis are both multi-host bacterial diseases that survive in the environment in favorable

conditions [228,229], we can turn to the process of Silk [227] to elaborate multilayer network models for understanding *Leptospira* maintenance community.

Multilayer network models in the context of leptospirosis

When elaborating a multilayer network model in the context of leptospirosis, each species (*e.g.*, cattle and rats) can represent a layer, and intralayer and interlayer interactions can represent the interactions between the same species (*i.e.*, cattle-cattle, rats-rats) and between each species and the environment contaminated by the urine of rats, respectively. It is important to note that a critical point when studying *Leptospira* transmission is that different serogroups of *Leptospira* may circulate in an ecosystem and infect numerous animal species, leaving the interpretation of the multilayer model more complex. Consequently, models should be elaborated considering a unique *Leptospira* species to determine the source of infection and/or the maintenance community of the specific *Leptospira* strain. This would be ideal to implement this type of model to better designate the maintenance host that requires primarily attention. However, such information requires a deeper understanding of *Leptospira* strain-host adaptability, notably the length of the carriage duration of the particular *Leptospira* strain and the strength of the spread of this *Leptospira* strain by the specific host species.

Mono-host sampling and experimental studies to determine leptospiral carriage

Epidemiological investigations following a suitable approach and experimental studies are required to speculate the role of additional animal species in the maintenance community of *Leptospira* and can help elaborate multilayer networks in the future. The epidemiological investigations carried out on raccoons (Part II.1(1)) and water voles (Part II.2(2)) [198] suggest that adapting a mono-host approach in different ecosystems in France and performing suitable sampling processes and laboratory tests are suitable ways of proceeding to determine a host Leptospiral carriage status. Another suitable way of proceeding can be the application of experimental studies on animal hosts through the inoculation of specific *Leptospira* strains to understand the capability of an animal to carry or exclude the given strains, as reported elsewhere [165]. Examples of experimental studies are the studies performed on brown rats that allowed to witness persistent renal colonization for several months (> 7 months for *Icterohaemorrhagiae*) [159] and urinary shedding that began at day 9 following infection and persisted up to 40 days for *Grippotyphosa* and 220 days for *Icterohaemorrhagiae* [230]. The health status of the host infected is also crucial as it can indicate the efficacy of a host to maintain and transmit the pathogen in an ecosystem. For instance, brown rats are an effective reservoir of human leptospirosis as they remain asymptomatic following inoculation by *L. interrogans* as it was described during experimental studies [158,231]. Nevertheless, elaborating and performing

experimental studies can be challenging as they are restricted to certain animal species (e.g., rats, guinea pigs (*Cavia porcellus*), and golden hamsters (*Mesocricetus auratus*)) and necessitate specific infrastructure and expertise. If other rodent species need to be targeted, ethical approval shall be requested. Still, the approval can be difficult to acquire owing to the requirement for authorization which necessitates a rigorous administrative process and/or the potential influence that the sampling, particularly large sampling, can have on the ecosystem. Furthermore, preserving *Leptospira* strains is fundamental for performing experimental studies on animals, a step that might be critical as research on the effectivity of culture medium is few and demonstrate that while certain serovars may respond well to a particular culture medium, other serovars may not [232].

Still, even locally, one can never fully account for the dynamics of an ecosystem as all trophic levels of species constantly impact and respond to one another as well as to changes in the (abiotic) environment. The mono- and multi-host approaches are thus both required to improve our knowledge of *Leptospira* maintenance communities.

Performing and combining adequate laboratory tests in epidemiological studies

Application of complementary traditional laboratory tests

Several traditional laboratory tests (i.e., PCR) were conducted on DNA extracted from water voles specimens [198]. Although the level of information procured by each PCR method differed, their common application increased the accuracy of *Leptospira* diagnosis and allowed the determination of a unique genetic profile (i.e., *L. kirschneri* serogroup Grippytyphosa and a unique VNTR profile). Thus, combining traditional laboratory tests can be useful in epidemiological studies as it may help determine the leptospiral carriage of animal species when performed correctly. However, even when adapting critical steps to ensure adequate sampling and processing (e.g., experimenting on euthanized animals for which the extracted DNA was not subject to cycles of freezing and thawing), traditional laboratory tests do not always provide us with a complete genetic profile, especially when the acquired genetic profiles are unknown according to the online database. In addition, the application of complementary tests for the identification of a specific *Leptospira* strain is challenging, especially when having low-concentrated DNA with C_T (above 35). It also requires excessive work time and is only limited to determining the specific pathogen of interest.

Application of the Whole Genome Sequencing to identify sources of *Leptospira* infection

Today, WGS can be used as either an alternative or a complementary method to traditional PCR tests and DNA Sanger typing methods. On a large scale, it provides

us with a broad vision of the circulating pathogens in the DNA of the sampled hosts, procures knowledge about the risk of infection in a specific ecosystem, and determines novel or poorly characterized genomes [233]. In a study that is now being undertaken, the WGS was conducted on the (euthanized) water voles' DNA samples from our previous investigation in Auvergne [198]. The WGS was conducted on pools of DNA extracted from kidneys, lungs, livers, and spleens of 26 water voles and revealed the presence of Tula orthohanta-, Hepaci- and Picobirnavirus, *Picornavirus*-, *Leviviridae* and *Virgaviridae* families, and *Leptospira* and *Bartonella* bacteria among others. In the context of *Leptospira*, the isolation of the strain of interest is required for the sequencing of the whole genome. Although the isolation is challenging (was only successful in our case for kidneys for which the obtained C_T ranged between 19 and 23), WGS of a culture-extracted DNA can provide us with a complete genetic profile of *Leptospira* strain through a unique application, unlike traditional typing methods that might not provide us with the latter information. Its application was revealed efficient in the context of revealing sources of *Leptospira spp.* transmission. For instance, a study [234] was conducted to isolate and sequence the whole genome of *Leptospira spp.* from small mammals in the urban area of Bogor, Indonesia following important records of human hospitalization due to leptospirosis in Indonesia [235]. It revealed that sampled rats carried a significant rate of *Leptospira spp.* and may be a source of human leptospirosis, according to genetically similar strains of *L. interrogans* serogroup Bataviae and *L. borgpetersenii* serogroup Javanica between human and rat isolates. Another study conducted in New Caledonia isolated *Leptospira* from soils suspected to be contamination sources [236]. Studying *Leptospira* in the soils (river soils and moist soils) of New Caledonia confirmed the significance of soils in the infection cycle by virulent *Leptospira* strains, indicating that ecosystems that support the survival of virulent leptospires should be thought of as passive, yet significant source in the epidemiology of the disease. In light of these studies, carrying WGS on animal species (mainly rodents) and environmental samples (soil or water) in the context of outbreaks can help pinpoint the source of infection. Nevertheless, WGS might not confer the true prevalence of leptospirosis as it is less sensitive when it comes to targeting a gene sequence corresponding to a specific pathogen, unlike traditional PCR with good coverage even when having low pathogen load as the primers are designed to target a specific gene [233]. Furthermore, the application of the WGS is of high cost and does not fit the budget of all laboratories. It also requires high-quality DNA sequences that are sometimes difficult to obtain during field sampling [114,115].

One Health approach to raise awareness and minimize the risk in Lebanon

Despite leptospirosis significant human morbidity and mortality estimated globally [36], the disease remains neglected and underdiagnosed in various countries due to a lack of awareness and funding, non-specific clinical symptoms, and its competence for resources with more serious issues such as malaria and human immunodeficiency virus [237]. The diagnosis of leptospirosis in people is important, but it is just as important to diagnose the disease in animals to minimize the infection of both humans and animals and the contamination of the environment. The concept of a One Health approach for leptospirosis surveillance can help adopt effective preparedness to minimize the risk of *Leptospira* infection in an ecosystem. According to the definition of One Health by the OHHLEP, multiple sectors, disciplines, and communities should be mobilized to look after the well-being and address the threats to human and animal health and ecosystem [194]. The latter concept is being developed in France, mainly in ecosystems with high infection risk or where outbreaks tend to occur regularly. For instance, following the outbreak reported in the Vilaine, awareness was spread and an adaptation of a One Health approach was suggested [207]. Nevertheless, the advantages of using a One Health strategy to address leptospirosis health threats at the interface of humans, animals, and the environment lack in certain countries, including Lebanon.

According to the results of our literature review, we revealed that cattle, humans, rodents, sheep, and goats were the main tested host in the region and identified Sejroe and Grippotyphosa as predominant serogroups in cattle, Autumnalis in goats, and Icterohaemorrhagiae in humans, rodents and sheep [238]. After conducting a first-time epidemiological investigation on cattle in Lebanon, we identified pathogenic *L. kirschneri* by PCR and Sejroe and Grippotyphosa (predominant) serogroups by MAT, a result that correlates with the findings of the literature review [239]. Most probably, the serogroups that circulate in humans and animals in the Middle East tend to circulate in Lebanon. It is necessary to conduct further epidemiological research and develop a suitable surveillance system in Lebanon to confirm the latter assumption. However, to our knowledge, no leptospirosis surveillance by veterinarians is conducted in Lebanon and there is poor coordination and communication between individuals working in the medical and veterinary field despite few reports of leptospirosis suspected cases in both humans (Dr. Georgio Medawar (medical doctor), personal communication, September 2, 2020) and animals (Dr. Jirji Maasri (veterinarian), personal communication, June 20, 2020). There is also a lack of awareness of the likely presence of the disease by the Ministry of Public Health and the Ministry of Agriculture, causing a major gap in the control of disease spread. These

drawbacks highlight the need for the country to adopt a One Health approach in the nation. Primarily, the ministries of Public Health, Agriculture, and Environment should communicate the results of our survey [239] that detected the presence of pathogenic *Leptospira* (*L. kirschneri* species and Grippotyphosa and Sejroe serogroups) in Mont Lebanon to individuals with a high risk of infection, mainly, farmers and veterinarians. The collaboration of veterinarians and medical doctors is also needed to ensure the direct diagnosis of leptospirosis, mainly in cattle, goats, sheep, rodents, and humans showing symptoms suggestive of leptospirosis, with collaboration with efficient veterinarians, hospitals, and public laboratories. To ensure the latter step, PCR tests for diagnosing leptospirosis in humans and animals need to be established in the nation and primers targeting the DNA of pathogenic *Leptospira* need to be ordered for PCR runs, at a minimal level. These tests should be conducted according to known and certified laboratory protocols (such as the protocols conducted in the LAV). Still, culture and MAT cannot be conducted as a first step as these methods are fastidious and require specific competencies. Being the reference test for the diagnosis of leptospirosis, MAT should be considered in the medium term with the financial support of the Ministries of Public Health, Agriculture, and Environment and could be elaborated in neighborhood countries (*e.g.*, Iran that carried out MAT at the country's laboratories [240–242]).

Prophylaxis measures in France and in Lebanon

Setting up efficient leptospirosis preventive and control measures necessitates a thorough understanding of the eco-epidemiological context, the transmission pathways, and the cultural characteristics of a community that faces leptospirosis economic and health threats [243]. Once the latter information is provided, it is feasible to significantly reduce the risk of infection by implementing pre- or post-exposure prophylaxis measures and adapting initiatives to limit exposure to *Leptospira*. For the successful accomplishment of these steps at the community or national level, prophylactic measures need to be addressed by specialized collaborators.

Prophylaxis approach in France

In Part II.1(1) and Part II.1(2) of the thesis, we could not determine the leptospiral carriage of raccoons and of nutrias and muskrats, respectively. However, the serological profiles obtained for cattle in Part II.1(2) primarily suggested exposure to Grippotyphosa, a *Leptospira* serogroup for which the related serovars (Grippotyphosa and Vanderheiden) belong to *L. kirschneri* [95]. Grippotyphosa was also found to be one of the predominant serogroups among cattle in France, according to a retrospective study that described the distribution pattern of *Leptospira* serogroups in cattle in France [191]. Cattle can pose a threat on human health due to their close

contact. To date, the available bovine vaccine in France protects cattle against infection by serogroup Sejroe (Spirovac vaccine), solely. Thus, serogroup Grippytyphosa should also be included in cattle vaccine to manage *Leptospira* infection in herds, reduce its impact on their health, reduce renal colonization and urinary shedding of leptospires, as demonstrated elsewhere [181]. Consequently, cattle vaccination will not only reduce the impact on their health but on the health of humans and other animals in close contact as well.

In Part II.2(2) of the thesis, we identified water voles as selective carriers of pathogenic *L. kirschneri* serogroup Grippytyphosa [198], a strain associated with human leptospirosis outbreaks in France [207]. Although vaccination (against the serogroup Icterohaemorrhagiae for humans (Spirolept vaccine)) is carried out for people with high infection risk (*i.e.*, mainly sewers, miners, farmers, and recently kayakers), their immunization is only limited against serogroup Icterohaemorrhagiae [189,190]. However, the question of considering a new vaccine against serogroup Grippytyphosa is being debated to date, as human infection by *L. kirschneri* serogroups Grippytyphosa, seems to have limited impact on human health when compared to infection by *L. interrogans* serogroup Icterohaemorrhagiae. Despite this, leptospirosis outbreak reporting is increasing as more individuals are engaging in water-related leisure activities as a result of climate change [244,245]. Leptospirosis-related health problems may so become more significant in the years to come.

Prophylaxis approach in Lebanon

The field study conducted in Lebanon (Part II.3(3)) in this thesis highlighted the presence of pathogenic *L. kirschneri*, mainly serogroup Grippytyphosa, in local and imported cattle for the first time [239]. Preventative measures to reduce *Leptospira* infection in humans and animals are hardly adopted in Lebanon due to the negligence of the presence of the disease in the nation. Today, a simple preventive rodent control strategy is adopted regularly by municipalities of a few regions of Lebanon. It is based on a mixture of dicoumarol rodenticide and wheat grain seeds, resulting in hemorrhage. Nevertheless, adapting additional preventative measures for leptospirosis prevention to take into account other potential sources such as cattle remains crucial to ensure public and animal health safety.

Even though cattlemaster goldFP5 vaccine (against Canicola, Grippytyphosa, Hardjo, Icterohaemorrhagiae, and Pomona) is available in Lebanon, it is not administrated to cattle herds due to the lack of recommendations by veterinarians. Furthermore, only a few farmers might afford its cost. Therefore, compensations should be considered by the Ministry of Agriculture and awareness should be spread to farmers to ensure cattle vaccination program. Cattlemaster goldFP5 vaccine may be

effective as it protects against several targeted serogroups, including Grippityphosa. Furthermore, cattle vaccination status must be verified before shipping and a booster or a vaccination shot must be administered to cattle on their arrival at the Lebanese soil. To reduce the risk of herd infection in the nation of import (Lebanon), MAT should be administered to 10% of each batch of cattle as recommended by the WOAHA [60]. The risk of infection of abattoir employees in Lebanon should also be considered; any direct infection by the bacteria in urines should be avoided [135,246]. This step may be challenging as Lebanon's infrastructure for ensuring food safety is substandard and needs significant improvement [247,248]. Finally, further epidemiological research should be conducted, mainly to acquire information on the prevalence of leptospirosis in all Lebanese regions.

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Appendices

Appendix 1 - Supplemental data 1 of Article 2

Site A and Site B descriptions.

Presence and abundance indices collected on July 2018, prior to animal screening.



Figure 1. Nutrias (*Myocastor coypu*) feces detected in various places around the kayak club. The white arrow points one specimen.



Figure 2. Opened bivalves in various places around the kayak club suggesting the presence of muskrats (*Ondatra zibethicus*). The white arrow points one specimen.



Figure 3. Nutrias (*Myocastor coypu*) and muskrats (*Ondatra zibethicus*) gallery entrances, pointed by the white arrows.



Figure 4. Norway rat (*Rattus norvegicus*) gallery entrance, pointed by the white arrow.



Figure 5. Cattle grazing close to the Vilaine river.

Appendix 2 - Supplemental data 2 of Article 2

Cattle culture, PCR, MAT result details

Table 1. Laboratory test results observed for the eight farms during Session 1 and Session 2.

| Farm ID | Session 1: March to May 2019 | | | | | | | Session 2: November 2019 to January 2020 | | | | | | | |
|--------------|------------------------------|-----------------|----------------------------|--------------|------------|-----------------|------------|--|------------|-----------------|---|----------------------------------|------------------------|--------------------|----------------------|
| | Date | No MAT positive | No sero-reacting serogroup | Titers | No PCR | No positive PCR | No culture | No positive culture | Date | No MAT positive | No Sero reacting serogroup | Titers | No paired serum tested | No sero-conversion | No persistent titers |
| 1 | 2019/03/19 | 28 | 2 GRI | 1:100 | 27 | 0 | 27 | 0 | 2020/01/01 | 35 | 5 2 GRI, 2 IH, 1 AUS | 1:100 | 17 | 3 | 1 |
| 2 | 2019/03/19 | 30 | 2 GRI | 1:100; 1:200 | 30 | 0 | 30 | 0 | 2019/12/09 | 30 | 5 3 GRI, 1 IH, 1 AUS | 1:100 | 16 | 3 | 1 |
| 3 | 2019/03/19 | 14 | - | - | 12 | 0 | 12 | 0 | 2020/01/01 | 17 | 1 1 AUS | 1:100 | 11 | 0 | 0 |
| 4 | 2019/03/21 | 29 | 0 | - | 15 | 0 | 15 | 0 | 2019/11/22 | 29 | 3 2 GRI, 1 IH | 1:100; 1:200 | 21 | 1 | - |
| 5 | 2019/04/04 | 30 | 2 GRI | 1:100; 1:200 | 30 | 0 | 30 | 0 | 2019/12/17 | 30 | 2 1 GRI, 1 IH | 1:100 | 17 | 1 | 1 |
| 6 | 2019/04/03 | 29 | 0 | - | 31 | 0 | 31 | 0 | 2019/12/23 | 30 | 4 2 GRI, 1 IH, 1 PYR [‡] | 1:100; 1:200; 1:400 [‡] | 5 | 1 | - |
| 7 | 2019/05/07 | 30 | 1 1 SJ | 1:200 | 0 | 0 | 0 | 0 | 2020/01/07 | 34 | 5 2 GRI, 3 AUS [‡] , 2 IH [‡] | 1:100; 1:400 [‡] | 7 | 2 | 0 |
| 8 | 2019/03/26 | 22 | 4 2 SJ / 2 GRI | 1:200 | 0 | 0 | 0 | 0 | 2020/01/01 | 22 | 2 1 GRI, 1 AUS, 1 SJ | 1:100; 1:200 | 8 | 1 | - |
| Total | | 212 | 11 8 GRI, 3 SJ | 0 | 145 | 0 | 145 | 0 | | 227 | 27 13 GRI, 8 IH, 7 AUS | | 102 | 12 | 3 |

For each of the eight farms, number of cattle MAT performed (No MAT), number of positive MAT (No positive MAT), number of sero reacting serogroups (No sero reacting serogroup), maximum titer among the seroreacting serovars belonging to the same serogroup (Titers), number of PCR performed on urine (No PCR), number of positive PCR (No positive PCR), number of culture performed on urine (No positive culture), number of cattle tested during session 1 and session 2 (No paired serum tested), number of MAT negative cattle from Session 1 that become MAT positive during Session 2 (No sero-conversion) and number of MAT positive cattle from Session 1 that remained MAT positive during Session 2.

[‡]indicates the serogroups that was associated to a titer of 1:400

Appendix 4 - Supplemental data 4 of Article 2

Preliminary analysis of the environmental situation related to a leptospirosis outbreak

Aims:

To study,

- The presence/absence of risk factors for leptospirosis persistence in the environment, as described in the literature (increased humidity in a given area, heavy rainfall in the 2 to 3 weeks preceding the first case, accumulation of runoff water [249] muddy areas)
- Environmental features that may promote exposure.
- Environmental changes that may promote the movement of animal populations.

Institutions / resource teams that can provide information on these points:

OFB – Local Services, xxx@ofb.gouv.fr / telephone
Local Water Agency

Preliminary survey

General environmental features

- Is there a climatic context in the area that would have favored runoff and therefore displacement of leptospires from the soil: heavy rains, storms, flooded areas? Delineate the watershed for the river concerned, if applicable.
- What are the characteristics of the watercourse concerned, if any: low, strong or variable low water in recent months, change in flow, change in water quality?

Environmental characteristics related to human exposure

- Is there an increase in tourist use during the period in question vs. previous years: development of new outdoor or freshwater activities, change in location of freshwater activities?
- Are the areas where people enter the water or wait for freshwater activities muddy?

Environmental characteristics related to animal populations

- In the watershed, were there events that could have favored the movement of animal populations? These are events that occurred or increased in the 3 to 6 months preceding the epidemiological situation:
 - low/high water levels, flooded areas, modification of banks, modification of crops, vegetation cover, major works, human activities such as hunting or involving a modification of habitats or their use...