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Molecular and Serological approaches for the Detection and Typing of *Leptospira*

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Resumo

A leptospirose é uma zoonose emergente causada por espécies patogénicas de *Leptospira*, presente a nível mundial. As leptospiras são espiroquetas, podendo ser saprófitas, quando de vida livre na natureza, especialmente em água doce, e patogénicas, necessitando de um hospedeiro para a sua sobrevivência. Estas bactérias pertencem o género *Leptospira* e família *Leptospiraceae*. Na classificação serológica clássica de *Leptospira* spp., o grupo taxonómico de base é o sorovar. Os sorovares são agrupados em sorogrupos, com base nas suas relações antigénicas. Atualmente, a classificação sorológica é complementada pela classificação genética, na qual 21 espécies genéticas são reconhecidas, incluindo espécies patogénicas, intermédias e não-patogénicas (ou saprófitas) de *Leptospira*. Estes agentes penetram na pele (através de um corte ou abrasão) e membranas mucosas, disseminando-se por via hematogénica, o que pode resultar numa doença febril aguda. Nos humanos, a maioria das infeções são subclínicas ou apresentando gravidade ligeira. A leptospirose em animais é caracterizada pela propagação da infeção dentro de espécies ou grupos de animais de uma forma cíclica: geralmente um animal portador, sobrevivente de uma infeção aguda, infecta a sua cria. Os mamíferos, principalmente os roedores, foram os primeiros a serem reconhecidos como reservatórios de *Leptospira* e desempenham um papel importante na transmissão da doença, especialmente devido à colonização dos túbulos renais por estas bactérias, propagando-as por via urinária no ambiente. O diagnóstico laboratorial da leptospirose baseia-se principalmente na cultura e métodos serológicos, reconhecidos como métodos de referência. A especiação de *Leptospira* a partir de material clínico pode ser importante para determinar o significado clínico, a provável fonte de infeção, para distinguir os casos esporádicos de possíveis surtos e para avaliar melhor a epidemiologia da doença. Neste contexto, os estudos aqui apresentados foram desenhados visando o desenvolvimento e aplicação de abordagens baseadas no estudo do ADN, para a deteção eficaz, identificação e tipificação de estirpes de *Leptospira* em amostras clínicas, como complemento ou alternativa à cultura convencional e abordagens sorológicas. Foi

desenvolvido um sistema simples e inovador baseado na tecnologia *TaqMan* por PCR em tempo real que, através da amplificação de diferentes alvos, é capaz de detetar e diferenciar *L. interrogans*, *L. kirschneri*, *L. borgpeteresenii*, *L. noguchii*, *L. santarosai* e *L. weilii* em amostras biológicas. Esta metodologia provou ser específica e sensível, sendo mais rápida do que a cultura bacteriológica. Além disso, foi dado ênfase à implementação e aplicação da abordagem *Multilocus Sequencing Typing (MLST)*, com vista a caracterização de estirpes Portuguesas de *Leptospira*. Uma seleção de isolados foi genotipada com base em três esquemas *MLST* usando seis ou sete loci. Estes esquemas revelaram um poder discriminativo semelhante na tipificação dos isolados portugueses, permitindo a atribuição correta das espécies de *Leptospira* e, dentro de cada espécie, com alguma propensão para diferenciar isolados de acordo com seus hospedeiros. Finalmente, no decurso do trabalho desenvolvido, foi dado um enfoque especial à classificação sorológica de uma estirpe de *L. kirschneri* sorovar Mozdok tipo 2, documentada como sendo unicamente isolada em Portugal. *L. kirschneri* sorovar Mozdok pertence ao sorogrupo Pomona e compreende três tipos de estirpes Mozdok, nomeadamente, o tipo 1, o tipo 2 e o tipo 3, tendo por base os diferentes padrões obtidos a partir da aglutinação de um painel de anticorpos monoclonais (MAbs). Até à data nenhum genoma de *L. kirschneri* sorovar Mozdok tipo 2 foi sequenciado e disponível ao público. Neste trabalho, nós anunciamos a sequenciação do primeiro *draft genome* de uma estirpe de *L. kirschneri* sorovar Mozdok tipo 2 que foi isolada a partir de um cavalo com 5-10 anos de idade. Ao gerar o primeiro rascunho do genoma de uma estirpe de Mozdok tipo 2 estamos capazes de fornecer informações para uma análise genómica mais detalhada e comparativa de modo a correlacionar características entre os tipos de sorovar Mozdok, contribuindo para a compreensão da evolução dos sorovares. A principal expectativa deste trabalho é que ele possa contribuir para o avanço do conhecimento sobre a leptospirose e sobre o agente desta doença importante.

Summary

Leptospirosis is an emerging and underestimated zoonotic disease caused by pathogenic species of *Leptospira* found all over the world. Leptospires are spirochetes, some saprophyte, free-living in nature, particularly in freshwater, and others pathogenic, requiring a host for survival. They comprise the genus *Leptospira*, family *Leptospiraceae.* In the classical serological classification of *Leptospira* spp., the basic taxon is the serovar. Serovars are grouped into serogroups, based on their antigenic relatedness. The serological classification system is complemented by a genotypic one, in which 21 genetic species are currently recognized, including pathogenic, intermediate and non-pathogenic (or saprophytic) species of *Leptospira*. These agents penetrate skin (via a cut or abrasion) and mucous membranes, making their way into an hematogenous dissemination, which may result in an acute febrile illness. In humans, the majority of infections are subclinical or presenting a mild severity. Leptospirosis in animals is characterized by the spread of infection within a species or groups of animals in a cyclical fashion: usually a carrier animal, survivor of an acute infection, infects its young. Mammals, primarily rodents, were the first to be recognized as reservoirs of leptospires and are playing an important role in the transmission of the disease, especially due to the colonization of renal tubules by leptospires and its urinary shedding in the environment. The diagnosis of leptospirosis relied on culture and serological techniques, which have been the gold standard methods. The speciation of infecting *Leptospira* from clinical material may be important for determining the clinical significance, the probable source of infection, to distinguish sporadic cases from possible outbreaks and to better access the epidemiology of the disease. It is within this context that the present studies were designed, aiming the development and application of DNA-based approaches for the efficient detection, identification and typing of *Leptospira* strains in clinical specimens as a complement or alternative to the conventional culture and serological approaches. A novel and simple *TaqMan*®-based multi-gene targeted real-time PCR approach was developed able to detect and differentiate *L. interrogans*, *L. kirschneri*, *L. borgpeteresenii*, *L. noguchii*, *L. santarosai* and *L. weilii* in biological samples. The assays proved to

be specific and sensitive, and much faster than the bacteriological culture. Additionally, a major effort was placed on the implementation and applying of *Multilocus Sequencing Typing* (MLST) approach, focusing on the characterization of Portuguese *Leptospira* strains. A selection of isolates was genotyped with three MLST schemes using six or seven loci. These schemes revealed a similar discriminatory power for typing the Portuguese isolates, allowing the correct assignment of *Leptospira* species and, within each species, with some propensity to differentiate isolates according to their hosts. Finally, within the work carried out, particular emphasis was given to the serological assignment of specific *L. kirschneri* serovar Mozdok type 2 strain, documented as being only isolated in Portugal. *L. kirschneri* serovar Mozdok belongs to Pomona serogroup and comprises three types of Mozdok strains, i.e., type 1, type 2 and type 3, based on the different patterns obtained from the agglutination of a panel of monoclonal antibodies (MAbs). To date, no genome sequences of serovar Mozdok type 2 strains are publicly available. In this work we announce the first draft genome sequence of *L. kirschneri* serovar Mozdok type 2 strain, which was isolated from a 5–10 years old horse. By generating the first draft genome of a serovar Mozdok type 2 strain, we are able to provide insights for a more detailed and comparative analysis to correlate serovar Mozdok´s types characteristics and genomic sequences, contributing to the understanding of the serovars evolution. The main expectation of this work is that it may contribute to the advance of the knowledge about leptospirosis and about the agent of this important disease.

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List of abbreviations

BLAST	Basic Local Alignment Search Tool
CAAT	Cross-Agglutinin Absorption Test
CF	complement fixation test
CSF	Cerebrospinal fluid
C_T	Threshold Cycle
DFM	Dark-field microscopy
DNA	Deoxyribonucleic Acid
dsDNA	Double Stranded DNA
e.g.	*exemplī grātiā*
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EMJH	Ellinghausen-McCullough-Johnson-Harris
FAO	Food AND Agriculture Organization of the United Nations
FlaA	Flagellar protein A
FlaB	Flagellar protein B
GC	Guanine and Cytosine
HL	Haemolysin test
i.e.	*id est*
IHMT/UNL	Instituto de Higiene e Medicina Tropical/Universidade Nova de Lisboa
IFA	Indirect Immunofluorescent Antibody
Ig	Imunoglobulins
INIAV	Instituto Nacional de Investigação Agrária e Veterinária
I.P.	Instituto Público
IS	Insertion Sequence
KIT	Royal Tropical Institute
LAMP	Loop-Mediated Isothermal Amplification
ligB	Leptospiral Immunoglobulin-like gene
LNIV	Laboratório Nacional de Investigação Veterinária
LOD	Limit of Detection
LPS	Lipopolysaccharide

List of abbreviations (cont.)

LSSP-PCR	Low Stringency Single Primer PCR
LSU	Large subunit
LUX	Light Upon eXtension technology
MALDI-TOF-MS	Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry
MAbs	Monoclonal Antibodies
MAT	Microscopic Agglutination Test
MCAT	Microcapsule Agglutination Test
MEE	Multilocus Enzyme Electrophoresis
MEGA	Molecular Evolutionary Genetics Analysis
MLPA	Multiplex Ligation-dependent Probe Amplification
MLST	Multilocus Sequencing Typing
MLVA	Multi-locus Variable Number of Tandem Repeats
NASBA	Nucleic Acid Sequence-Based Amplification
NCBI	National Center for Biotechnology Information
OIE	World Organisation for Animal Health
PAUP	Phylogenetic Analysis Using Parsimony
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field gel electrophoresis
PGM	Personal Genome Machine
REA	restriction-endonuclease DNA analysis
RefSeq	Reference Sequence
RFLP	Restriction Fragment Length Polymorphism
RFU	Relative Fluorescence Units
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
SAM	Sequence Alignment/Map
SNPs	Single Nucleotide Polymorphisms
SSCP	Single Strand Conformation Polymorphism
SSU	Small Subunit

List of abbreviations (cont.)

ST	Sequence Type
UTAD	Universidade de Trás-os-Montes e Alto Douro
VNTR	Variable Number Tandem Repeats
WGS	Whole Genome Sequencing
WHO	World Health Organization

List of units

µM	micromolar
bp	base pair
°C	Degrees Celsius
GE	genome equivalents
kb	kilobase
Mb	megabase
µg	microgram
µl	microliter
mg	miligram
ml	milliliter
min	minute
nM	nanomolar
s	second
%	percent

CHAPTER 1

General Introduction

1.1. General description of *Leptospira* and Leptospirosis

Leptospirosis is an emerging and underestimated zoonotic disease [1] caused by pathogenic species of *Leptospira*. Overall, leptospires are spiral-shaped spirochetes, long (6-20 µm), thin (0.1-0.2 µm) and highly motile [2] (Figure 1.1.). They are helically coiled gram negative bacteria with one or two distinctive hooked ends and two endoflagella (or periplasmic flagella), each arising at one end of the bacterium. FlaA and FlaB constitute two distinct classes of flagellar sheath and core proteins, respectively. The rigidity, shape and strength of leptospires derives from the peptidoglycan layer closely adhering to the cytoplasmic membrane [3]. One peculiar characteristic of the genus is in the surface where lipopolysaccharide (LPS) exists, within an outer membrane, that constitutes the basis for the sero-identification of *Leptospira*, as the target antigen, and plays an important role in its virulence [2]. Among other proteins, lipoproteins are in abundance on the cell surface, such as LipL32 > LipL21 L > LipL41 [4] and the porin OmpL1, as integral membrane protein, is also located in the outer membrane of *Leptospira*. Leptospires are aerobic and microaerophilic bacteria with an optimum in vitro growth temperature of 28 to 30°C and optimum pH of 7.2-7.6 [2]. *Leptospira* uses ammonium salts as a nitrogen source, long-chain unsaturated fatty acids as a carbon source, and purines [5, 6].

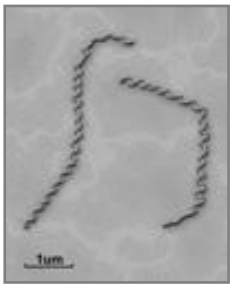

Figure 1.1. *Leptospira* by a computerized electron microscope. (available in www.leptospirosis.org).

Leptospirosis transmission requires circulation of agents among animal reservoirs, such as cattle, rats and other small mammals, where pathogenic *Leptospira* establish persistent renal carriage with urinary shedding [7, 8]. This infection occurs in humans and animals worldwide, causing economic losses in cattle and sheep industries, fundamentally due to reproductive wastage and decreased milk production [5, 7, 8].

Humans are incidental hosts, acquiring a systemic infection by direct or indirect contact with infected material. The most common sources of infection are water or

soil contaminated with infected urine of maintenance or accidental hosts, the infected urine itself or tissues from infected animals [5, 9, 10].

Pathogenic leptospires penetrate skin (via a cut or abrasion) and mucous membranes, making their way into an hematogenous dissemination, which may result in an acute febrile illness [9, 11]. The incubation phase is 7–12 days, with a range of 3 days to 30 days [9]. The acute or leptospiremic phase lasts for approximately 1 week and it is prior to the immune phase, characterized by antibody production (Figure 1.2.).

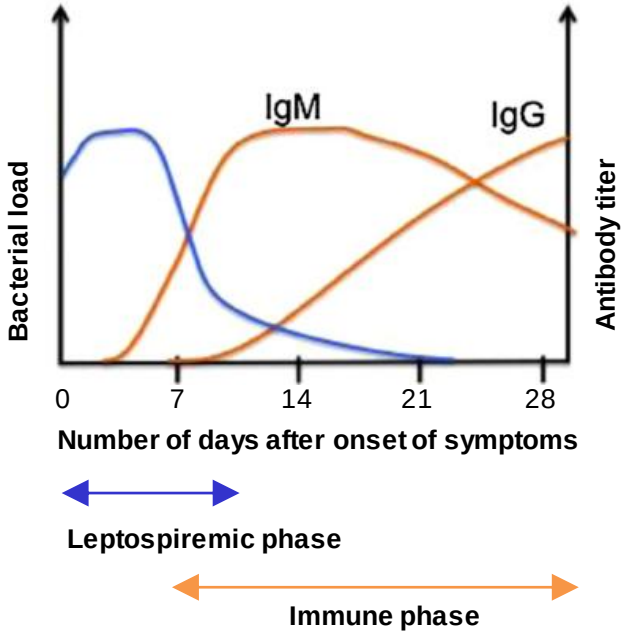

Figure 1.2. Dynamic of leptospirosis in blood (adapted from Picardeau [12]). After exposure, the infection is divided in two phases: (i) the leptospiremic phase in the first week (lasts from 3 to 10 days) and (ii) the immune phase, during the second week (lasts from 4 to 30 days). Antileptospiral IgM production precedes the production of IgG antibodies. The increased of antibody titer is correlated to the migration of leptospires from blood to the organs.

In humans, the majority of infections are subclinical or presenting a mild severity [13]. However, patients can exhibit moderate to severe symptoms. Typical symptoms are a sudden onset of fever, chills, and headache. These signs and symptoms are nonspecific and can occur due to other causes of acute febrile syndrome, depending on the setting, like influenza, dengue fever, or malaria. The headache is often severe and has been described as a bitemporal, frontal throbbing headache accompanied by retro-orbital pain and photophobia. Myalgia, abdominal pain, conjunctival suffusion, rash and nonproductive cough are other clinical presentations that may occur in leptospirosis patients [9, 13]. A subset of patients can develop more severe manifestations, such as organ dysfunction, known as Weil's disease, which may be fatal [9]. The emergence of leptospirosis is usually associated with certain groups, considered at risk due to their activities, namely farmers, veterinarians, abattoir workers, scientists, sewer workers, military personnel, etc. Recreational exposures, especially water-based sports, also increase the chance of acquiring the disease [5, 14].

1.2. Cycle of infection and clinical aspects of animal leptospirosis

Leptospirosis in animals is characterized by the spread of infection within a species or groups of animals in a cyclical fashion: usually a carrier animal, survivor of an acute infection, infects its young. Alternatively, the urine from a carrier contaminates moist soil and the areas around the animal's habitat. Young animals of the same and other species, within the same area, also become infected [10]. Between rodents or other small mammals and farm animals a cycle of infection is commonly observed, in which the carriers, especially rats, infect particularly cattle and pigs when housed indoors. Carrier small mammals can also contaminate water or soil, originating a source of infection for pigs, cattle or sheep, which in turn, become carriers and excretors, thus infecting other rodents or more animals of their own species [10, 15].

Between farm animals, there are three means of transmission: (i) By a congenital or neo-natal infection, followed by recovery and a continuing carrier state; (ii) By spreading of infection by direct or indirect contact with infected urine of carriers,

which can contaminate floors, muddy ground, or sources of drinking water; (iii) By venereal transmission [10, 15].

Symptoms of acute leptospirosis in animals can include: (i) Sudden onset of agalactia (milk drop syndrome) in adult cattle and sheep; (ii) Subclinical agalactia in cattle; (iii) Jaundice and haemoglobinuria particularly in young animals, meningitis, acute nephritis, particularly in dogs [10, 16]. Sudden death in young animals, particularly bovines is often observed, especially with Pomona group infections [10, 17]. A diagnosis of chronic leptospirosis in animals should be considered in cases of: abortion, stillbirth, birth of weak (premature) offspring, infertility and, in horses, periodic ophtalmia [10, 16].

Incidental infections, caused by non-host-adapted serovars, are usually in the origin of acute leptospirosis, while mild or sub-clinical disease usually occurs with host-adapted strains. Chronic disease generally results from infection with host-adapted strains, such as Hardjo in bovines and Bratislava in swine (Table 1.1.). When acute infections occur with host-adapted strains, it results in mild clinical symptoms. Infections with host-adapted strains may also occur without any clinical signs. Serovar Hardjo causes outbreaks of mastitis and abortion and was found in aborted foetuses and in premature calves. In addition, Hardjo has been isolated from normal foetuses, the genital tracts of pregnant cattle, vaginal discharge after calving, and the genital tract and urinary tract of cows and bulls [10, 16].

1.3. Epidemiological settings

Over the last decade, an increasing trend of outbreaks during sporting events [18, 19], adventure tourism, urban slums and rainfalls has contributed to the emergence of human leptospirosis, becoming a public health problem worldwide [1, 9, 11]. Leptospirosis has been found all over, though it is often under- or misdiagnosed, due to the similarity of its symptoms with various other diseases (such as dengue or malaria) [1] and to a high percentage of asymptomatic infections. Yearly, the incidence of severe human leptospirosis is reported with ranges from 0.1 to 1 per 100,000 inhabitants in temperate climates and 10 per 100,000 inhabitants in tropical regions [12] with case fatality rates exceeding 10% [1].

In Portugal, the average incidence of human leptospirosis is reported at 57 cases per year with a much higher rate in the Azores islands (11.1 per 100 000 population) [20]. Most cases are diagnosed in the central mainland and São Miguel and Terceira islands [20]. Overall, in human leptospirosis, a predominance of serogroups Icterohaemorrhagiae and Ballum was observed in Portugal (including islands), with emphasis in the Pomona serogroup, which is reported as been only identified in mainland Portugal [20].

The study of animal leptospirosis in Portugal was neglected for several decades after the early studies done by Azevedo and Palmeiro between 1963 and 1972, which reported low prevalences and reduced pathogenicity of the infecting serovars [10] until 1985, when a new leptospirosis department was set at the *Instituto Nacional de Investigação Agrária e Veterinária*, I.P. (INIAV, I.P., then named LNIV). Since then, extensive studies have been made in several animal species which evidenced, contrarily to those early studies, a widespread occurrence and a high prevalence of animal leptospirosis in Portugal, particularly in cattle and swine [10, 17]. Serogroups Sejroe (sv Hardjo), Pomona, Australis and Icterohaemorrhagiae were those most commonly found, with Pomona group infections, including serovar Mozdok, exhibiting high pathogenicity particularly in cattle and swine [10, 17, 21, 22]. In accordance to the results later obtained in humans [20], leptospirosis in cattle in the Azores also exhibited particular high prevalence, over 27% [10, 17], having been reported for the first time in Terceira Island in 1987 when leptospiral tires in animals, namely Icterohaemorrhagiae, were detected for the first time in the Azores region [23].

Worldwide, animal leptospirosis has been found everywhere, with the exclusion of the polar regions [8]. The venereal transmission, which may occur in animals, facilitates the spread of the infection even in dry regions. *Leptospira* infection is described as having a strong association between serovars and specific maintenance hosts (Table 1.1.), with variations depending on the regions [7].

According to Little [24], maintenance hosts are defined as: (i) high susceptible to specific infection; (ii) relatively low pathogenecity of the organism to the host; (iii) long-term kidney infection and (iv) natural transmission within the host species. Concomitantly, as with humans, incidental infection is common, presenting symptoms depending on the infecting serovars and the affected species [8].

Table 1.1. Some *Leptospira* serovars and their known maintenance and incidental hosts (based on Ellis [8]).

Serovar	Maintenance Host	Incidental Host
Bratislava	Rats; swine, dogs, horses; hedgehogs	Humans, cattle; sheep; horses
Canicola	Pigs; dogs	Cattle; sheep; swine; horses
Itcterohaemorrhagiae	Brown rat;	Humans; cattle; sheep; swine; horses; dogs
Hardjo	Cattle; sheep;	Humans; cattle; sheep; horses; dogs
Kennewick	Cattle*; swine*, skunks*	Horses*; young pigs.

*depending on geographical distribution

Mammals, primarily rodents, were the first to be recognized as reservoirs of leptospires [13, 25, 26] and are playing an important role in the transmission of the disease, especially due to the colonization of renal tubules by leptospires and its urinary shedding in the environment. Notwithstanding, all mammal species are potential carriers and shedders of pathogenic leptospires [8, 13], although small mammals are the most important reservoirs, with large herbivores as additional significant sources of infection. Pathogenic *Leptospira* species have been isolated from hundreds of mammalian species, including bats and pinnipeds [27-29]. In addition, leptospires have been recovered from poikilothermic animals such as frogs and toads [30-32], and it is possible that these animals play a role in the circulation of leptospirosis in the environment, although they may not be significant reservoirs of human infection. Only a few studies have reported isolation of leptospires from amphibians, however, the results justify further attempts to understand the role of amphibians in maintaining leptospires in nature [33].

On the other hand, leptospires are able to survive up to weeks or months in warm, moist soil and water [34], showing sensitivity to drying and to acid pH [13].

The diagnostic techniques have their influence in the understanding of the epidemiology. Although the isolation of the bacterium is imperative, mainly to know and characterize the local strains, cumbersome serological approaches are commonly used for the diagnosis of leptospirosis, which has lately been changing

with the expansion of the molecular genetics studies. Nevertheless, both are far from answering questions such as those related to the epidemiological understanding of the circulating strains, both in humans and in animals. Moreover, bacteriological results do not always corroborate the serological results [7]. This emphasizes the importance of effective tests, for a more accurate knowledge of the disease, which can have a wider and more successful use in routine clinical laboratories, including laboratories in the developing countries, where leptospirosis is endemic and still a huge scourge, as the Caribbean, Central and South America, Southeast Asia and Oceania [13]. The sequencing tools provide a promising approach for the epidemiological scenario since they can produce electronically transferrable data, with online databases, enabling the easy access for data insertion and comparison. Identifying infecting leptospires is of utmost importance in animal and human health fields, not only to improve the disease diagnosis but also to make way for new insights on epidemiology that would lead to the definition of adequate intervention strategies, particularly in the prevention of the disease.

1.4. Molecular Biology

Leptospires are phylogenetically related to other spirochetes. In 2003 and 2004, two *Leptospira* genomes were the first to be sequenced, namely *L. interrogans* serovars Lai [35] and Copenhageni [36], whose strains belonged to the serogroup Icterohaemorrhagiae. Both genomes share 95% of genetic homology at the nucleotide level, comprising a large circular chromosome (4,277 kb, 35 mol% GC) and a smaller replicon (350 kb, 35 mol% GC). Other complete genome sequences are currently published, such as the saprophyte *L. biflexa* [37], the intermediate *L. licerasiae* [38] and further pathogenic species, *L. borgpeterseni* [39] and *L. santarosai* [40]. Comparison with other spirochetes (*Treponema pallidum*, *Treponema denticola*, and *Borrelia burgdorferi*) revealed a wide diversity despite of some similarity in the genes responsible for morphological features.

All *Leptospira* species have at least two circular replicons, with the exception of *L. biflexa* that possesses a third circular replicon of 74 kb, designated p74, and which is not present in the pathogenic species [41]. Naturally occurring plasmids have

not been reported in *Leptospira* and the mechanisms of gene transfer are largely undiscovered. The chromosome of *Leptospira* is characterized by a G + C content of 35–41 mol%, depending on species (Table 1.2.) and presents a genome size of 3.9–4.7Mb.

Table 1.2. General points about *Leptospira* species complete genomes (adapted from Picardeau [41]).

***Leptospira* spp. (strain)**	**Source**	**Replicons**	**Genome Size (Mbp)**	**G+C (%)**	**References**
***L. borgpetersenii* (L550)**	Human (Austrália)	2	~3.9	40.2	[39]
***L. borgpetersenii* (JB197)**	Cattle (USA)	2	~3.9	40.2	[39]
***L. biflexa* (Paris)**	Water (Italy)	3	~4.0	38.9	[37]
***L. interrogans* (Fiocruz L1-130)**	Human (Brazil)	2	~4.6	35.1	[36]
***L. interrogans* (Lai 56601)**	Human (China)	2	~4.7	35.1	[35]

Adler and de la Pena Moctezuma [5] illustrated 2052 genes in common between the two pathogenic (*L. borgpetersenii* and *L. interrogans*) and one saprophytic species (*L. biflexa*). Comparative genomics might allow the recognition of pathogen-specific genes and as far as we know, 893 pathogen-specific genes were identified wherein 1,547 proteins are common for *Leptospira* genus [41, 42]. The genomes of *L. borgpetersenii* and *L. interrogans* can be distinguished from each other by substantial molecular differences and organization, such as pseudogenes or insertion sequences (IS). However, a large part of the genes discovered encode hypothetical proteins or proteins of unknown function [5].

The analysis of whole genome sequences allowed findings of significant structural differences, such as the large chromosomal inversion and the distribution of several insertion sequences [36, 43, 44]. Furthermore, lateral DNA transfer has been reported, corroborating the concept of genome plasticity as suggested by

several studies [36, 39, 45]. Complete genome sequences will be necessary to enable detailed studies on *Leptospira* evolution and distribution and to identify features that are unique to pathogenic species. Concomitantly, lack of adequate and efficient genetic tools for the manipulation of pathogenic strains of *Leptospira*, such as the extrachromosomal cloning vectors, impeded further analysis in the identification and characterization of genes in pathogenic *Leptospira*. Picardeau [41] also attributes the complexity of the culture media for *Leptospira*, their slow growth and the loss of virulence after several in vitro passages as factors that hamper the genetic analysis.

However, in the future, things are likely to change with the sequencing of several genomes, already completed but not yet analyzed, achieved through the ongoing *Leptospira* Genomics and Human Health Project (http://gcid.jcvi.org/projects/gsc/leptospira/) that can provide valuable insights in the characterization and understanding of the biology of this genus. In the same way but at the postgenomic level, analysis in *Leptospira* has also undergone progress, concerning the transcriptomics and proteomics; however it has been slow and difficult.

1.5. Classification of *Leptospira*: systematic review

Leptospires are spirochetes, some saprophyte, free-living in nature, particularly in freshwater, and others pathogenic, requiring a host for survival [46]. They comprise the genus *Leptospira*, family *Leptospiraceae* [47]. Over time these bacteria have been reported, firstly, as *Spirocheta biflexa* [48], before the isolation of the first pathogenic leptospires, followed by *Spirochaeta icterohaemorrhagica japonica,* that later changed to *Spirochaeta icterohaemorrhagiae* [49]. Noguchi [26] proposed *Leptospira* as a genus and, subsequently, in 1982, saprophytic and pathogenic leptospires were designated in two species, *Leptospira biflexa* and *Leptospira interrogans* respectively (presently referred to as *sensu lato*, due to the existence of genomospecies with the same name, that are referred to as *sensu stricto*) [50]. *L. biflexa* was differentiated from *L. interrogans* by phenotypic characteristics, namely its capability to growth at 13°C and in the presence of 8-azaguanine [6, 51].

In the classical serological classification of *Leptospira* spp., the basic taxon is the serovar. Each of the two above mentioned species, comprise numerous serovars, defined by cross-agglutinin absorption tests (CAAT- described later in this review). Serovars are grouped into serogroups, based on their antigenic relatedness [52]. While serogroups have no taxonomic standing, they have proved useful for epidemiological understanding of infection [6].

Concomitantly, the phenotypic serological classification of leptospires has been replaced by a genotypic one, in which a number of genomospecies include all serovars of both (sensu lato) *L. interrogans* and *L. biflexa* [6]. The genotypic classification presently recognizes 21 species of *Leptospira*, categorizing them in three groups according to their 16S rRNA gene sequences and pathogenicity (Table 1.3.) [46]. In fact, the groups clustered for *Leptospira* are the result of a phylogenetic analysis provided not only by the analyses based on the *rrs* gene [53], but also by *Multilocus Sequencing Typing* (MLST) data [54], spc-α locus analysis [55], *ligB* analysis [56] and DNA homology [57, 58]. Similar phylogenies are gathered by other genes including *rpoB* [59] and gyrB [60].

However, the molecular and serological classifications of leptospires show little correlation, as serovars of the same serogroup can be found in a single or more different species. For example, serovars of the Bataviae serogroup can be found in *L. interrogans sensu stricto*, *L. santarosai* and *L. kirschneri*, *L. noguchii* and *L. borgpetersenii* [6].

The reclassification of leptospires on genotypic grounds is taxonomically correct and provides a strong foundation for future classifications. However, the molecular classification is problematic for the clinical microbiologist, because it is clearly incompatible with the system of serogroups which has served clinicians and epidemiologists well for many years. In addition, the retention of *L. interrogans* and *L. biflexa* as specific names in the genomic classification also allows nomenclatural confusion [6]. Thus, to date, both clinical laboratories and scientists still retain the serological classification of pathogenic leptospires.

Table 1.3. Genomospecies of *Leptospira*.(adapted from Levett [46]).

Group (status)	Species	References
Pathogenic	*L. alexanderi*	[57]
	L. alstonii	[61]
	L. borgpetersenii	[58]
	L. interrogans	[50]
	L. kirschneri	[62]
	L. kmetyi	[63]
	L. noguchii	[58]
	L. santarosai	[58]
	L. weilii	[58]
Intermediate	*L. broomii*	[64]
	L. fainei	[65]
	L. inadai	[58]
	L. licerasiae	[66]
	L. wolffii	[67]
Non-pathogenic	*L. biflexa*	[50]
	L. idonii	[68]
	L. meyeri	[58]
	L. terpstrae	[61]
	L. vanthielii	[61]
	L. yanagawae	[61]
	L. wolbachii	[58]

1.6. Current strategies for the detection and characterization of *Leptospira*

1.6.1. Diagnosis

Human and animal leptospirosis diagnosis is based either on direct detection of the organism in the clinical specimens, isolating the bacterium, detecting anti-leptospiral antibodies by serological tests or detecting leptospiral DNA by molecular methods (Figure 1.3.). The observation of symptoms in association with suggestive laboratory test results can corroborate the diagnosis of clinical disease. The use, interpretation, and value of laboratory diagnostic procedures for animal leptospirosis vary with the clinical history of the animal or herd, the duration of infection, and the infecting serovars [69]. There are many diagnostic tests for leptospirosis and their assortment depends on the purposes (mainly detection or

identification) and intended objective such as: (i) the evaluation of the infection status, e.g. herd control or eradication program; or (ii) the individual assessment of the animal immune status, e.g. international trade goals or its introduction into and uninfected herd [8]. The test methods available for diagnosis of animal leptospirosis according to their purpose are summarized in Table 1.4.

Specimens and timing of collection, as well as the duration of symptoms, are also important factors to be considered for a higher diagnostic accuracy [9]. The probable stage of infection should be considered in the choice of the tests to be done, as well as in their interpretation (Figure 1.3.).

Table 1.4. Test methods available for diagnosis of leptospirosis and their purposes [69].

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribution to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification						
Isolation and identification	-	+++	-	+++	-	-
PCR	-	++	-	++	-	-
Detection of immune response						
MAT	-	+++	-	++	+++	-
ELISA	+++	-	+++	+++	++	+++

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose. Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable. PCR = polymerase chain reaction; MAT = microscopic agglutination test; ELISA = enzyme-linked immunosorbent assay.

1.6.2. Specimen collection and direct detection of leptospires

Leptospiral infection can be divided into two stages (Figure 1.3.). The first stage is the leptospiremia (or acute phase), when the bacterium is found in the

bloodstream in decreasing numbers up to 15 days [70]. To detect leptospires in blood, samples must be collected in this stage and before the start of antibiotic therapy. The second stage of infection is marked by the migration of leptospires from the blood to the tissues (and urine) and the corresponding increase of IgM antibodies, commencing during the second week. By the same time, *Leptospira* can be detected and isolated in the urine of infected animals (Figure 1.3.).

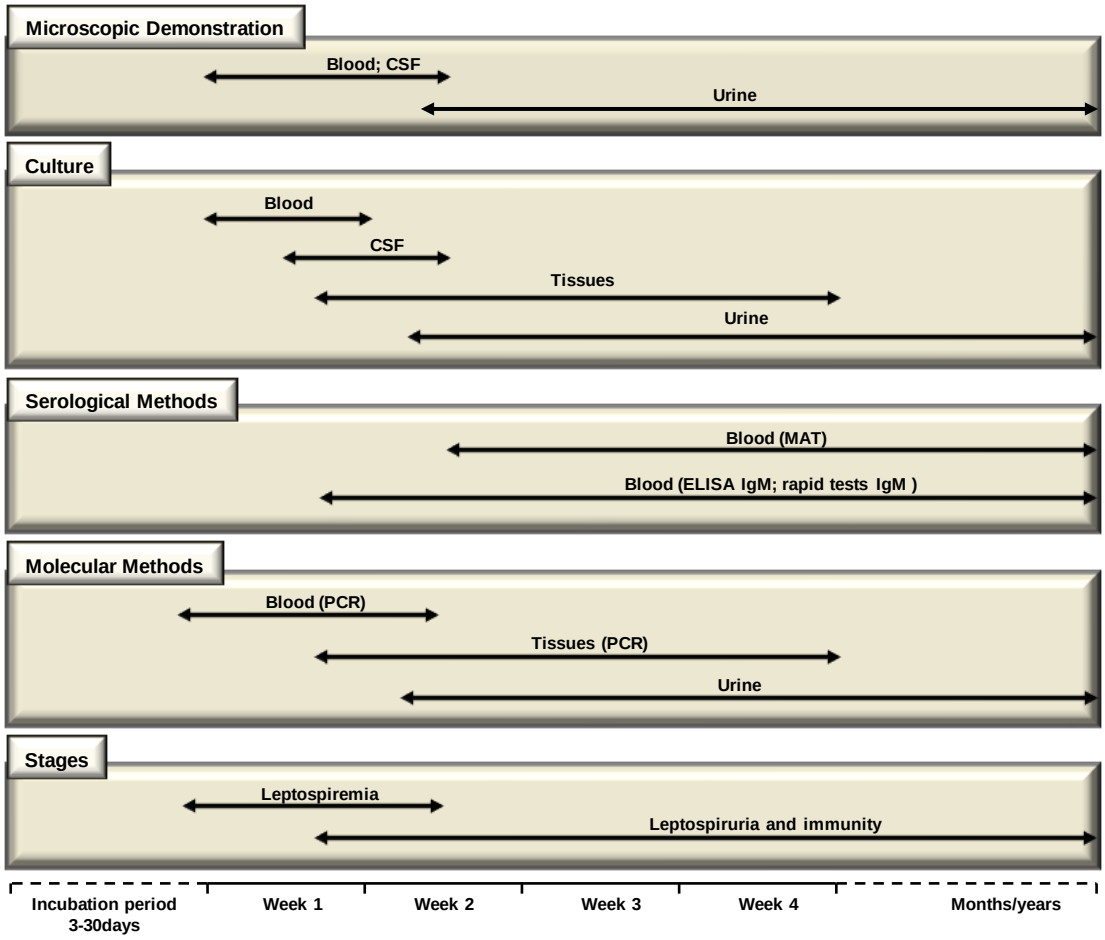

Figure 1.3. Selection of the suitable approach for the *Leptospira* detection given the specimens and the stage of infection (adapted from Ellis [8]).

The demonstration of *Leptospira* in live animals can be done by direct visualization of organisms in body fluids (such as blood or urine) using dark-ground microscopy, staining (silver and immunochemical), by culture and by DNA detection methods [8]. Dark-field microscopy (DFM) can, in theory, diagnose the leptospirosis by detecting the organism in body fluids, however the method requires highly trained staff, presenting limited specificity [6, 71] and leading to false positives due to artifacts that may be easily confused with leptospires [72].

Thus, DFM can provide only a presumptive diagnosis and should be confirmed by other methods [15]. Examination of tissue sections using direct immunofluorescence can confirm the presence of leptospires, however the sensitivity of this technique depends on high quality and serovar specific fluorescein-conjugated immunoglobulins and, as it also depends on the number of leptospires in the samples, it is less suitable for diagnosing the chronic carrier state [69]. For culture, anticoagulated blood is recommended, although the direct inoculation of blood into the culture medium is the ideal [13]. Conversely, several studies have found that plasma from EDTA anticoagulated whole blood gives the best results for detection of *Leptospira* DNA [73-75]. The nucleic acid-based diagnostic tests exhibit a higher sensitivity and ability to establish an early diagnosis, thus contributing to eliminate the need for a differential diagnosis, allowing for the start of appropriate treatment as soon as possible. There are several commercial kits available for the rapid nucleic acid purification from blood, tissues or urine. Nonetheless, for tissues a significant autolysis might impair the viability of the PCR assays due to the presence of inhibitors [76].

It is important to note that, in confirmed cases of leptospirosis, the absence of leptospiral DNA or antigen detection in blood may be due to a late specimen collection or a short leptospiremia or, otherwise, to the administration of antibiotics [71]. Moreover, in animals, the nonexistence of leptospires in urine should does not rule out carriers, it can only signify a lack of detectable numbers of the bacterium at the time of testing [8]. The disease is confirmed when accompanied by suggestive clinical signs or by the demonstration of generalized leptospirosis in organs taken at post mortem examination.

1.6.3. Isolation and storage of leptospires

Leptospires can be isolated from blood, urine (midstream), cerebrospinal fluid and *post mortem* tissues. Even though culture is the gold standard method for leptospirosis diagnosis, the success in isolating leptospires depends on the material, the stage of infection and other factors: (i) A successful in vitro leptospiral growth requires sufficient numbers of viable organisms in the inoculums [2]; (ii) The viability of leptospires is easily affected by multiple factors such as the advanced tissue autolysis, the late processing of the clinical sample after collection and yet, a range of temperature and contamination of samples with other bacteria (when preparing cultures from clinical specimens, serial dilutions should be made to minimize substances and contaminants that may interfere with leptospiral growth) [69]. To minimize this problem, selective media, supplemented with specific antibiotics or serum proteins are used to enhance efficiency in the isolation of fastidious leptospires [2, 6]. Being resistant to antimicrobial action of 5-fluorouracil (a pyrimidine analogue), this is the drug used in selective media to isolate leptospires from contaminated (clinical) samples. The most widely used medium in current practice is the semi-solid EMJH medium [77], supplemented with 100-200 µg/ml of 5-fluorouracil.

The major advantage of culture is that it can provide the subsequent identification of the isolate, which is useful in epidemiological studies and establishing of adequate treatment and control measures. Culture of leptospires constitutes the definitive diagnosis and the detection of the agent is necessary to identify animal carriers. However, this procedure is technically demanding and highly susceptible to failure besides being slow for routine use. The time needed for a positive culture, characterized by a visualization of a Dinger's ring beneath the surface of the medium [6], varies with the leptospiral serovar: in less fastidious serovars, such as Pomona group strains, growth may be observed in 7-10 days, however more fastidious serovars (such as Hardjo and Bratislava) can take up to 16 or even 26 weeks [8].

Leptospires can be regularly maintained by repeated subcultures and long-term storage can be done in semisolid agar (stock-cultures), kept at room temperature and away from light [2, 5]. It is preferable to subculture the strains at 3 months

intervals. Alternatively, Samir and Wasfy [78] indicated that cultures may be kept frozen in EMJH liquid medium, however liquid nitrogen is considered the preferred method of long-storage, especially for the maintenance of virulence [5].

1.6.4. Antibody detection

The microscopic agglutination test (MAT) is internationally considered the reference test for the serological diagnosis of leptospirosis and the serological classification of leptospires [69]. The original scheme of the MAT method dates back to the 1918 [79] and was further modified by several authors [80, 81]. The test consists in the reaction of suspected diluted sera with live antigen suspensions of leptospiral serovars representing different serogroups [15]. The inclusion of circulating serovars from the area where the test is performed should be done [69], because they may give higher titers than reference strains [15] and to avoid the possibility of false negative results [13]. These can happen especially if a reduced panel of antigens is used instead of the WHO recommended representative larger antigen battery [15, 69], suitable for covering the detection of all known or unknown serovars in all existing serogroups.

The MAT results are visualized under a dark-field microscope, after placing small drops of the serum/antigen reactions suspensions on a microscopic slide and the presence of agglutinations and their titers are determined, by comparing the serum reactions suspensions with negative (antigen with saline) and positive (antigen with known positive sera) controls. The endpoint is the highest serum dilution where 50% of antigen agglutination occurs, in comparison with the negative control [15].

However, interpretation of MAT results is difficult due to different factors: (i) cross-reactivity of antibodies; (ii) possible presence of antibodies induced by vaccination; and (iii) inconsistencies in the consensus of what antibody titers are indicative of infection. For example, for humans, an agglutinating titer of ≥ 1:200 is considered significant, when combined with existing symptoms, whereas a titer ≥ 1:100 is considered significant in animals as evidence of previous exposure [6]. In addition, different cut-off values of agglutination titers should be considered depending on the area, considering if the exposure to leptospirosis is common, such as in most

tropical countries, or not [13]. This subjective interpretation of results combined with other factors such as the inexperience of the test performers and the complex performance of the test, decreases the method accuracy for interlaboratory comparison.

MAT is insensitive in early acute-phase in human specimens [82, 83], prior to the antibody production of the immune phase. Besides, it cannot make an accurate distinction between current, recent, or past infections [13] when a single serum sample is tested, in spite that the observation of a rising titer in paired samples taken with a few days intervals is indicative of a current and recent infection.

In animals, leptospiral antibodies appear within a few days of onset of illness, persist for weeks or months and, in some cases, years [69]. It may be assumed that a low (a non-rising in repeated tests) titer corresponds to a previous infection, while titers ≥ 1:400 have been assumed as corresponding to recent infections [15]. Unfortunately, antibody titers may fall to undetectable levels while animals remain chronically infected. Thus, sensitive methods are needed to detect the organism in urine or the genital tract of chronic carriers [69].

To overcome some drawbacks of the MAT, many other screening immunological tests have been developed for determination of specific leptospiral IgM or IgG antibodies such us (i) the complement fixation test (CF) [84]; (ii) Microcapsule Agglutination test (MCAT) [85, 86]; (iii) Haemolysin test (HL) [87]; (iv) indirect immunofluorescent antibody test (IFA) [82]; (v) indirect haemagglutination test (IHA) [88, 89]; (vi) dipstick assay [90-92]; and (vii) enzyme-linked immunosorbent assay (ELISA) [93, 94]. Several IgM Elisa commercial kits are available on the market, based on the detection of antibodies against total extract of leptospires such as IgM ELISA, Dip-S-Tick (PanBio Inc.) and LeptoTek Dri-Dot (Biomerieux). Some IgM Elisa containing total cellular extracts of recombinant leptospiral proteins are also used [95, 96]. The specificity and the sensitivity of these ELISA are quite variable howsoever several authors have reported an earlier detection of the antibodies than with the MAT [83, 97-99]. Most of these tests, even if some are marketed, are rarely used and lack specificity or sensitivity [6, 12]. Less specific than MAT, they are mainly used as screening tests.

1.6.5. Evaluation of the serological tests for the infecting *Leptospira* identification

Serology, as already mentioned, claims the serovar as the basis taxon for the genus *Leptospira*. In general, the available serological tests are genus-specific or serogroup/serovar-specific.

The MAT, when used to test sera for the detection of anti-*Leptospira* antibodies, is a serogroup specific assay, with no ability to identify the reacting titers at serovar level [100]. Besides, as mentioned above, a broad range of serogroups should be represented in the panel of antigens used in the MAT for a presumptive serogroup identification [101]. Actually, the method is not the best option to provide a sensitive fingerprint (e.g., during an outbreak) of the infecting strains/serovars, nor to define phyletic relationships [101]. Repeated weekly subcultures are necessary for the test maintenance [6, 69]. It is also worth noting the risk that this represents for laboratory-acquired infections as panels of live leptospires are handled [6].

In what concerns the use of MAT for the identification of the serovar to which an isolated strain belongs, firstly the serogroup and related serovars to which the strain belongs is determined by testing it against 1) a set of hyperimmune rabbit sera representative of all serogroups, to determine the serogroup 2) afterwards, tests of the isolate are done against a set of hyperimmune rabbit sera representative of the serovars belonging to the assigned serogroup, to determine the possible serovar. Thereafter, the subsequent differentiation to the specific serovar level is done by the cross-agglutination absorption test, CAAT [52, 69]. CAAT allows for the identification of known serovars as well as it assigns new serovars [100]. Approximately 250 pathogenic serovars have been recognized by this method [43]. However, only a small part of the laboratories are able to perform this identification method [6, 100] because it is cumbersome and time-consuming with the laborious and specialized preparation of rabbit immune sera. A similar test, but no longer used, is the Factor sera method which was based on rabbit anti-*Leptospira* sera absorptions which led to a high degree of specificity [100].

Table 1.5. Summary of *non*-DNA-based tests used for *Leptospira* detection and characterization.

Detection/ Typing	Test	Advantages	Disadvantages	References
Isolation	Culture	- Definitive diagnosis - Provide the subsequently identification - Veterinary and human applicability	- Slow and difficult - Easy to contaminate - Cumbersome	[6, 69]
Direct detection of leptospires in specimens	DFM	- Simple; - Early diagnosis - Veterinary and human applicability	- Low sensitivity - requires highly trained staff - Lack sensitivity and specificity	[6, 69, 71]
	Staining	- Simple - Veterinary and human applicability	- Low sensitivity	[69]
Antibody detection	MAT	- Gold standard - Veterinary and human applicability - Serogroup specific - Veterinary and human applicability	- Requires a panel of live antigen - Laborious and difficult (expertise) - Problems in detecting carrier animals - Biohazard	[6, 69]
	CF	- technically complex procedure - Non biohazard	- Genus-specific	[84]
	MCAT	- Simple and easy to perform - Early detection	- Genus-specific	[85, 86]
	HL	- Non biohazard	- Genus-specific	[87]
	IFA	- Non biohazard	- Requires fluorescent microscope (expensive)	[82]
	IHA	- Simple and easy to perform - Non biohazard	- Genus-specific	[88, 89]
	Lateral flow test	- Simple, quick and easy to perform - Cost effective	- Genus-specific	[69]
	ELISA	- Early detection (IgM) - no need to maintain panel of cultures (commercially available) - Can combine with modern technology	- Genus-specific - Laborious	[69]
Typing	CAAT	- Viable pure isolates	- Cumbersome - Biohazard	[69]
	Monoclonal antibody	- Presumptive serovars identification	- Complicated - Expensive	[69]

Leptospira isolates can also be typed to serovar level, by performing a MAT with a panel of monoclonal antibodies (MAbs), based on the agglutination of characteristically serovars [102], although it requires some caution, as MAbs recognize a small number of epitopes on the LPS, which can be shared by different serovars [100]. Bourhy and collaborators [103] described distinct serovars with a similar agglutination pattern when testing with distinct panels of MAbs.

Other serological tests for detection of antibodies are also used (e.g., ELISA), however they're not suitable for the identification of the infecting serovar/serogroup. Recently, Doungchawee et al. [104] reported a whole-cell bacterial immunoblotting as an alternative method for differentiating between serogroups of *Leptospira.*

Overall, the use of serological techniques for the diagnosis and identification of the infecting serovars of *Leptospira*, either by detecting antibodies in sera or by identifying isolated strains is difficult because there are few laboratories equipped to perform either the MAT or the CAAT [9, 43]. Molecular methods, on the other hand, are reported as capable in identifying the serovars in easier tests, such as RFLP-based methods [105, 106] and PFGE [107].

1.6.6. DNA–based techniques for the rapid *Leptospira* detection

In the last decades, *Leptospira* detection based on molecular techniques has become easier following the development of alternative methods. DNA-based methods, which have been applied widely to the field of leptospires, improved the performance of diagnosis when compared to the conventional methods, by exhibiting higher sensitivity and subtyping accuracy, as discussed below, facilitating also the analysis by requiring less specific technical expertise.

Indeed, most molecular tests were developed as an alternative or supplementary approach to the currently existing serological and bacteriological methods. For an accurate and simplified diagnosis of leptospirosis it is required to have an assay that is able to detect the infection in an early stage, to detect a small number of leptospires in clinical specimens and that can be cheap, robust and simple enough to be used by minimally trained health technicians. Application of molecular techniques can render *Leptospira* laborious and cumbersome isolation

dispensable. Besides, the use of the above mentioned methods for *Leptospira* detection provides an improved biosafety, since they don't involve extended contact with live and possibly virulent *Leptospira* strains.

The polymerase chain reaction (PCR) is the most common methodology used for the molecular detection of leptospires; alternatives include Nucleic Acid Sequence-Based Amplification (NASBA) (Colebrander et al. 1994 *cited in* [108]), dot and in situ hybridization techniques [109-111] or isothermal amplification methods, such as loop-mediated isothermal amplification (LAMP) [112-114]. NASBA shows a high diagnostic sensitivity in amplifying multicopy RNA; DNA-hybridization methods are probably no more sensitive than serological methods [110], but the LAMP method, has advantages, including its simplicity with no need for thermocyclers, its specificity and a better sensitivity than standard PCR [114]. Nevertheless, PCR is more widely used and consequently better developed.

Conventional PCR assays are sensitive, rapid and require only small amounts of DNA. Consisting of an enzymatic DNA target amplification through a polymerization carried out by a thermocycle, the specificity of the assays is achieved by the development of short single-stranded pieces of DNA, or primers. PCR diagnosis for *Leptospira* was developed as early as 1989, and have been claimed to be more sensitive in detection than culture from urine [115]. Since then, a variety of conventional PCRs assays for detection and species identification have been described, targeting a variety of specific genes, namely 16S or 23S rRNA genes [116-121], *gyrB* [60] or *ompL1* [122]. Gravekamp et al.[123] developed two sets of primers (G1/G2 and B64-I/B64-II) that were able to detect and identify *Leptospira* species which were heavily used and validated in later studies for detection of DNA of leptospires in clinical samples [124-126]. The limit of detection of these assays varied from 1 cell/ml to 1,000 cells/ml, depending on the specimen and DNA extraction methods. More recently, several PCR-based assays were developed for genes restricted to pathogenic *Leptospira* such as *lig* [127], *hap1* [128], *lipL21* and *lipL41* [129] and *lipL32* [130]. A multiplex PCR format which combines more than one primer pair in a single reaction was also developed [131, 132]. Besides, a nested PCR format, based on conducting reactions using additional pairs of outer and inner primers, was also reported [133]. Such kind of methods have been described as having increased sensitivity and specificity to

detect pathogenic leptospires, however they have been more or less discarded due to the risk of contamination by PCR products.

Generally, the interest of all PCR methods mentioned lies in the ability to achieve a definitive diagnosis during the acute stage of the leptospirosis prior to the formation of detectable antibodies; however most methods have not been well evaluated, leaving its diagnostic value vague [117, 126]. Furthermore, the inability to differentiate between DNA from viable and dead bacterial cells also represents a limitation for DNA-based molecular diagnostics, since it impairs the estimation on the presence of living microorganisms and the accompanying potential pathogenic threats.

In contrast, the new technology and real-time PCR applications have been embraced by scientists and diagnostic techniques alike. Real-time PCR is faster than conventional PCR, sensitive, reproducible and with a considerably reduced risk of carry-over contamination [134]. The possibility that amplicon could be amplified as detected in “real” time makes this technique a useful tool towards a quick displacing of the traditional assays. This feature only has been made possible by the use of primers, oligoprobes or amplicon of molecules with the emission of a fluorescent signal, identified by sensitive detection platforms, after a direct or indirect interaction of the fluorescent dye and the target DNA [134]. The DNA quantification is also one of the advantages in the use of real-time PCR methodology since fluorescence is acquired each cycle [135]. Indeed, the amount of target DNA in the sample is directly correlated with the amplification cycle at which the level of the fluorescent signal exceeds the background fluorescence (threshold cycle or C_T value) [136].

The first use of real-time PCR technique was based on dsDNA-specific intercalating dye ethidium bromide [135] that exhibit little or no fluorescence while in free solution, but, when bounded to dsDNA and exposed to the optimum wavelength of light, produced a strong fluorescence signal [134]. Currently, there are many fluorogenic chemistries for the specific or non-specific detection of the target DNA. The most available formats are divided in two categories: (i) dyes that interact with any and all dsDNA depending on specific primer annealing for generating amplicon-specific fluorescence signals [e.g., SYBR Green 1 dye and Light Upon eXtension technology (LUX)] and (ii) sequence-specific fluorogenic

oligoprobes that combined with a pair of primers give an additional layer of specificity to the PCR (e.g., TaqMan probes, Molecular Beacons and Scorpions). Both approaches have similar sensitivity in detecting amplicon [134] and have been widely applied to *Leptospira* detection.

Although the use of other dyes has been described [137] SYBR Green and TaqMan technology are most used in the *Leptospira* field because of their simplicity. But although SYBR Green chemistry is most cost effective providing a sensitive detection it lacks on specific detection compared to the TaqMan probes [13]. Nevertheless, several studies were developed based on the SYBR Green melting curve analysis for the *Leptospira* detection targeting *lipL32* gene [138], *rrs* gene [139] or *secY* gene [73]. Most of them only differentiate between pathogenic and non-pathogenic leptospires, lacking the ability to distinguish between different species. Merien et al. [139] has developed a real-time PCR based on melting curves analysis with the use of SYBR Green I to distinguish between 7 pathogenic *Leptospira* species, however its detection sensitivity could be smaller when compared to a specific fluorescent TaqMan probe designed to a particular target sequence [134]. A TaqMan probe assay consists in a reaction of a set of primers and probe that binds to the amplified target sequence and is dually labeled with a 5'-end fluorescent reporter molecule and a 3'-end quencher molecule. When the probe is hydrolyzed by the DNA polymerase, a separation of the reporter dye and quencher molecule occurs and a fluorescent reporter signal is detected by the instrument (Figure 1.4.). There are multiple reporter dyes that can be used with a variety of quenchers that emitting fluorescence at different wavelengths enables the implementation of multiplex PCRs [136]. For a better sensitive detection amplicons are usually as short as possible. Besides, care should be taken when designing primers and interpreting PCR results since sequence data for their evaluation are mainly limited to data available in GenBank where several falsely annotated sequences exist.

TaqMan real time PCR assays were predominantly described for use with human samples such as whole-blood, serum or urine [70, 74, 75, 141-144]. However, only a few have been clinically validated [142-144]. This technique has also been used to identify a carrier state of animals from kidney and urine sample [145, 146].

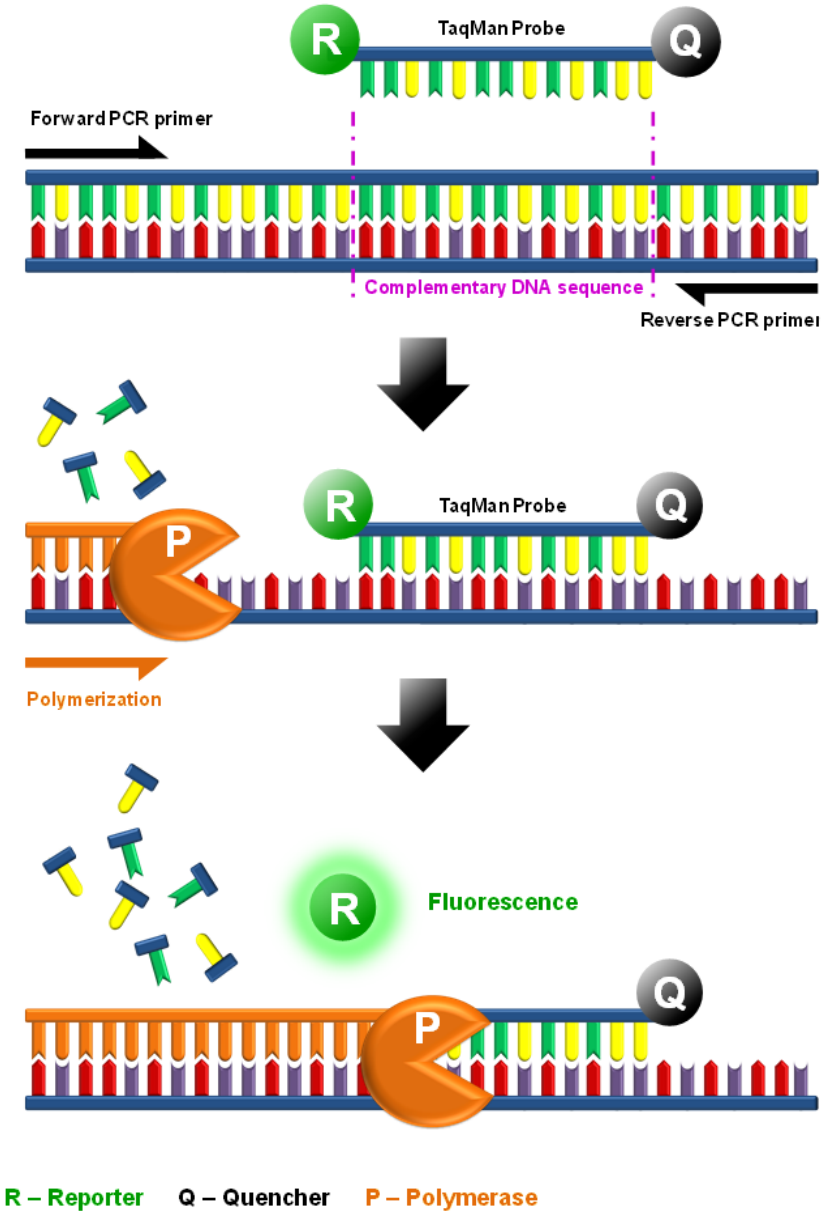

Figure 1.4. Graphic illustrating the principle underlying the TaqMan® real-time PCR assay (reproduced with permission from Costa [140]). Hydrolisis of the TaqMan probe by the DNA polymerase separates the 5'-end fluorescent reporter and the 3'-end quencher molecule allowing a fluorescence signal.

Although real-time PCR assays can theoretically detect a single copy of nucleic acid in a specimen, the presence of PCR inhibitors as well as a possible loss of nucleic acids during specimen processing can significantly compromise the efficacy of the assays. A positive amplification signal reveals the presence of pathogenic leptospires in the sample but in no case does it allow to directly identify the species. Recently, we reported an extended study based on TaqMan real-time PCR assays that are able to detect and identify four different pathogenic *Leptospira* species in tissues [147].

The detection of *Leptospira* from clinical material provided by the PCR techniques is essential for an earlier diagnosis. Nevertheless, the speciation on the subspecies is also an important requirement for determining the clinical significance and probable source of infection, to distinguish sporadic cases from possible outbreaks and to better access the epidemiology of the disease.

1.6.7. Characterization and genotyping of *Leptospira*

To overcome the cumbersome nature of classical serotyping and the high level of technical expertise it requires, there has been interest in developing alternative methods for the identification of species and specially serovars. Indeed, the correlation of *Leptospira* "molecular serotyping" systems turned out to be arduous since molecular and classical methods have intrinsic differences in their respective concepts (genes *vs* antigens). Although they will probably never match exactly and completely, genotyping is technically simpler becoming amenable to a highly successful level of use in future directions. Actually, from an epidemiological perspective, a molecular characterization of *Leptospira* would allow the extension of historical surveillance data analysis.

DNA-DNA hybridization is considered to be the gold-standard to assigned *Leptospira* spp. based on DNA homology and was first used for the genus *Leptospira* in 1969 [148]. Thereafter other studies were published based on DNA-DNA hybridization techniques for the *Leptospira* speciation [57, 58, 62, 149]. However, the major input information concerned the *Leptospira* molecular speciation was given by the group represent by Brenner et al. [57]. Therefore, its application is currently seldom used due to its complexity requiring considerable

amounts of isotope-labelled DNA of high quality. Alternatively, assays involving bacterial restriction-endonuclease DNA analysis (REA) [105, 150-155], Southern blot hybridization [156-159], ribotyping [106, 160], restriction fragment length polymorphism (RFLP) [161, 162], Pulsed-field gel electrophoresis (PFGE) [107, 163-165] and multilocus enzyme electrophoresis (MEE) [166] have also been used for characterization of leptospires. However, all these techniques require special expensive equipment and laborious procedures; further they show ambiguous interpretation, poor reproducibility, and need large quantities of high quality genomic DNA. PFGE, however, has been promoted as a standard test for genotyping since its patterns generally coincide with the serovar status.

Additionally, a large number of PCR-based typing methods have been studied extensively either accompanied with other molecular technique or coupled to subsequent sequencing of the amplicon [108]. The most commonly used as regards to this species include: Restriction Fragment Length Polymorphisms (RFLP) [167-171], Low Stringency Single Primer PCR (LSSP-PCR) [172-174], Single Strand Conformation Polymorphism analysis (SSCP) [175], IS-based PCR [176, 177] and hybridisation with specific labelled probes (e.g., MLPA) [154, 178]. These methods are part of a wide range of molecular PCR-based approaches that have been constantly improved aiming a standard speciation and serovar determination. However, most show poor reproducibility, require large quantities of good quality DNA, as previously mentioned, and/or need the availability of live leptospires. Another disadvantage resides in the fact that they do not directly produce digital data, although possible in some cases [100]. The use of both LSSP-PCR and SSCP, for example, produced reliable results on typing *Leptospira*, but those are complicated and laborious for an extensive application. On the other hand, the introduction of the sequence-based determination techniques that emerged over the last years has largely contributed for the knowledge of the molecular epidemiology and taxonomy of several bacteria. Phylogeny-based genotyping has been described for leptospires using sequences of several genes. Up to now, the *rrs* gene is the most commonly used target for sequence-based identification of *Leptospira* species [53, 63, 179-181]. Nonetheless, the *rrs* gene has shown to be not polymorphic enough because of its conserved nature. Furthermore, a phylogenetic analysis based on a single locus

may lead to erroneous results due to the plasticity of *Leptospira* genome reported [36, 39, 45]. Therefore, the use of multiple loci for the genotyping of *Leptospira* is imperative.

Variations of PCR formats can generate banding patterns that allow strains to be discriminated, but more recently, the molecular methods generating digital data or profiles are in demand. The sequence-based methods such as multi-locus variable number of tandem repeats analysis (MLVA) and multilocus sequencing typing (MLST) can yield significant information at a serovar level. MLVA technique relies on the detection of differences in copy numbers of tandem repeated DNA sequences (VNTRs) which allows information relating to the evolutionary *Leptospira* diversity. This approach is currently available for distinguish between serovars belonging to the *L. interrogans*. *L. kirschneri*, *L.borgpetersenii* [182-185] and, more recently, for *L. santarosai* [186]. The ability to detect VNTRs in such species has been greatly enhanced by the availability of their genome sequences. MLVA methods have highly discriminatory power being suitable for epidemiological studies (e.g., regional outbreaks) but the use of agarose gel electrophoresis to separate fragment sizes that is dependent on the subjective judgment by eye is a drawback [108]. MLST, therefore, is considered the most robust, phylogeny-based typing method for *Leptospira* providing an online availability and analysis of data (http://leptospira.mlst.net, http://pubmlst.org/leptospira/). In the MLST approach a number of housekeeping genes of an isolate are amplified and sequenced on both strands. The result is the assignment of a ST to that isolate that is underlain by the allelic profile for the loci studied. A first MLST scheme based on 6 loci (three housekeeping genes, two genes encoding outer membrane proteins and *rrs*) was developed in 2006 (6L scheme) [54]. This MLST scheme has the advantage that it can be applied on all pathogenic species of *Leptospira* [187]. Besides, a public database and website associated emerged less than a year ago and is now available in http://pubmlst.org/leptospira. Later, additional approaches were developed targeting either 4 loci (4L) [55, 188] or 7 loci (7L) (housekeeping genes) that were distributed across the genome [189-191]. A comparison of 7L, published by Thaipadungpanit et al. [189], and the 6L [54] MLST schemes showed that both approaches mostly yielded comparable results [192]. Victoria et al. [55], in turn,

demonstrated that one gene that encodes the SecY preprotein translocase, revealed a high discriminative power to the species level. More recently, a modified 7L MLST scheme was proposed by using a novel combination of target genes originally used in the 6L and extended 7L schemes [193]. These methods are reproducible and turned out to be efficient in discriminating serovars [191, 193] making it the most advanced molecular serotyping method currently available. MLST schemes used in characterization of *Leptospira* spp. are summarized in the Table 1.6.

Table 1.6. Summary of MLSTs schemes for leptospires characterization.

Schemes	Target species	Loci	References
4L scheme	*L. interrogans* *L. kirschneri*	*ligB; secY; rpoB; lipL41*	[188]
6L scheme	pathogenic and intermediate *Leptospira spp.*	*adk; icdA; lipL32; lipL41; rrs2; secY*	[54, 192]
7L scheme	*L. interrogans* *L. kirschneri*	*glmU; pntA; sucA; fadD; tpiA; pfkB; mreA*	[189]
7L scheme	*L. interrogans* *L. kirschneri*	*accA2; ccmF; czcA; gcvP; groEL; pola; recF*	[190]
7L scheme	*L. borgpetersenii* *L. noguchii* *L. santarosai* *L. weilii* *L. alexanderi*	*glmU; pntA; sucA; tpiA; pfkB; mreA; caiB*	[191]
7L scheme	*L. interrogans* *L. kirschneri* *L. noguchii* *L. weilii* *L. santarosai* *L.borgpetersenii*	*adk; glum; icdA; lipL32; lipL41; mreA; pntA*	[193]

The distinct advantage of this approach is that genetic relationships can be assigned on the basis of online data that are directly suitable for biocomputing and statistical analysis. However, highly skilled personnel and expensive equipment might limit its application widely. Besides, an extensive agreement towards the adoption of a unique consensus scheme for the *Leptospira* genotyping is still lacking.

In the near future, genotyping standardization is moving towards identification of serovars by whole genome sequencing (WGS) [108], which offers a powerful new approach for the knowledge of *Leptospira* characterization, promising rapid and unambiguous determination of significant evolutionary features. Surely the ongoing *Leptospira* Genomics and Human Health Project (http://gcid.jcvi.org/projects/gsc/leptospira/) results are being eagerly anticipated so that we can move on to another level of whole genome typing of the genus *Leptospira*.

1. 7. Thesis framework and Objectives

The diagnosis of leptospirosis has for a long time relied on long-established methods, mainly based on culture and serological techniques, which have been the gold standard for the diagnosis of leptospirosis in laboratories worldwide, and the use of these methods still persists, despite the huge potential of new molecular genetics studies. However, there are numerous limiting factors in those techniques, such as the fastidious growth of leptospires and the difficulties in their isolation, which can result in false negatives or a in a long time needed to obtain a *Leptospira* culture, the subjective interpretation of serological results as well as the cumbersome laboratory procedures and expertise required to perform those tests. Concomitantly, although the isolation of the bacterium is imperative, mainly to know and characterize local circulating strains, the elaborate nature of classical culture and serotyping of *Leptospira* strains also increases the interest in developing alternative diagnostic methods for the detection, identification and characterization of these agents. The application of molecular techniques can render *Leptospira* laborious isolation dispensable in a number of diagnostic needs.

At the time this project was originally envisioned, and throughout its course, it was clear that accurately identifying infecting leptospires is of utmost importance, both for animal and human health, to improve diagnosis and to make way for new insights on the epidemiology of the disease, ultimately leading to improved intervention strategies, particularly in the prevention of the disease.

Considering the above, a central aim of this work was to develop and implement improved methods for the laboratorial diagnosis of leptospirosis, including the detection and identification of the etiological agents directly in clinical specimens and the epidemiological characterization of the isolates. A crucial aspect was the need to develop effective tools allowing not only to detect pathogenic leptospires but also to discriminate between the most clinically-relevant species.

Nucleic acid-based tests usually exhibit higher specificity, sensitivity and ability to establish an early diagnosis, and allow an improved and reproducible discrimination for the typing of disease agents.

The objectives envisaged in this PhD project mainly relate to the development and application of DNA-based approaches for the efficient detection, identification and typing of *Leptospira* strains as a complement or alternative to conventional culture and serological approaches. These purposes have taken into account the use of bioinformatics applications as well as cost-efficient molecular techniques and easily electronically transferrable data.

Specifically, the following objectives were addressed:

Chapters 2 and 3:

a) To find DNA signatures or polymorphic regions in *Leptospira* genomes, allowing the discrimination between relevant pathogenic species;

b) To develop a novel and simple *TaqMan*-based multi-gene targeted real-time PCR assay for the detection and differentiation of the most relevant pathogenic species of *Leptospira* in clinical specimens, suitable for being introduced in the routine diagnostics of veterinary laboratories;

c) To evaluate and apply the developed real-time PCR assay to assess the infecting *Leptospira* species in animal tissue samples;

Chapter 4:

d) To evaluate the genetic diversity of pathogenic leptospires circulating in Portugal using a multilocus sequence typing (MLST) approach;

e) To feed relevant MLST international online databases with a new set of allele and sequence type information regarding European *Leptospira* isolates;

Chapter 5:

f) To contribute for a better knowledge on the genomic features of pathogenic leptospires, particularly of *L. kirschneri* serovar Mozdok types, by sequencing and announcing the draft whole-genome of a *L. kirschneri* serovar Mozdok type 2 strain, documented as being only isolated in Portugal.

1.8. References

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CHAPTER 2

Direct detection and differentiation of pathogenic *Leptospira* species using a multi-gene targeted real-time PCR approach

2. Direct detection and differentiation of pathogenic *Leptospira* species using a multi-gene targeted real-time PCR approach

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2.1. Summary

Leptospirosis is a growing public and veterinary health concern caused by pathogenic species of *Leptospira*. Rapid and reliable laboratory tests for the direct detection of leptospiral infections in animals are in high demand not only to improve diagnosis but also for understanding the epidemiology of the disease. In this work we describe a novel and simple *TaqMan*®-based multi-gene targeted real-time PCR approach able to detect and differentiate *Leptospira interrogans*, *L. kirschneri*, *L. borgpeteresenii* and *L. noguchii*, which constitute the veterinary most relevant pathogenic species of *Leptospira*. The method uses sets of species-specific probes, and respective flanking primers, designed from *ompL1* and s*ecY* gene sequences. To monitor the presence of inhibitors, a duplex amplification assay targeting both the mammal *β-actin* and the leptospiral *lipL32* genes was implemented. The analytical sensitivity of all primer and probe sets was estimated to be < 10 genome equivalents (GE) in the reaction mixture. Application of the amplification reactions on genomic DNA from a variety of pathogenic and non-pathogenic *Leptospira* strains and other non-related bacteria revealed a 100% analytical specificity. Additionally, pathogenic leptospires were successfully detected in five out of 29 tissue samples from animals (*Mus* spp., *Rattus* spp., *Dolichotis patagonum* and *Sus scrofa domesticus*). Two samples were infected with *L. borgpetersenii,* two with *L. interrogans* and one with *L. kirschneri*. The possibility to detect and identify these pathogenic agents to the species level in domestic and wildlife animals reinforces the diagnostic information and will enhance our understanding of the epidemiology of leptopirosis.

2.2. Introduction

Leptospirosis is a growing and underestimated public health and veterinary concern, caused by pathogenic spirochetes belonging to the family *Leptospiracea*, genus *Leptospira* [1, 2]. The disease is an important cause of abortion, stillbirths, infertility, poor milk production and death amongst livestock, harboring a significant economic impact [3-5]. Its transmission requires circulation of the agents among domestic and wild animal reservoirs, with rodents recognized as the most important sources that establish persistent renal carriage and urinary shedding of *Leptospira*. Humans are incidental hosts acquiring a systemic infection upon direct or indirect exposure to the urine, blood or tissue of an infected animal. Farmers, veterinarians, sewer workers, pet keepers, rodent catchers and those persons participating in aquatic leisure activities are more prone to acquire the disease.

Conventional classification of *Leptospira* is based on serological criteria, using the serovar as the basic taxon. To date over 250 pathogenic serovars separated into 25 serogroups are known [6]. The serological classification system is complemented by a genotypic one, in which 21 genetic species are currently recognized, including pathogenic, intermediate and non-pathogenic (or saprophytic) species [7-10]. Genetic species boundaries hardly correlate with the serological classification [8].

Serological approaches are used commonly for diagnosis of leptospirosis in animals. The reference method is the Microscopic Agglutination Test (MAT), which has the advantage of being specific for serogroups [3] but has several drawbacks of being laborious and requiring a panel of viable *Leptospira* cultures. Isolation of leptospires, from suspect clinical specimens, constitutes the definitive diagnosis but is also technically demanding, time consuming and subject to contamination and high rates of failure [4]. Isolates are traditionally classified to the serovar level by the Cross Agglutinin Absorption Test (CAAT) [8] which is cumbersome for routine use and is only performed in a few reference laboratories worldwide.

Rapid and reliable laboratory tests for the direct detection of leptospiral infections in animals are in high demand, particularly to support suitable control measures. Serology does not corroborate well with the presence of pathogenic

viable leptospires in the kidneys or urine and detection of the agents is necessary to identify healthy animal carriers. Molecular-based assays have been previously described for detecting leptospires in clinical samples. Most approaches are PCR-based and target specific genes or polymorphisms in the genome of pathogenic leptospires. Several real time PCR assays have been described predominantly for use with human samples such as whole-blood, serum or urine [11-16] but only few have been plentifully validated [17-20]. A few assays were evaluated or used for detecting *Leptospira* in kidney tissue, blood, urine and other clinical specimens from animals such as sheep [21], dogs [22, 23], pigs [5], deer [24], flying foxes [25] and rodents [26, 27]. Most assays rely on SYBR green detection chemistry and only differentiate between pathogenic and non-pathogenic leptospires, lacking the ability to distinguish between different species. Nevertheless, speciation of infecting *Leptospira* from clinical material may be important for determining the clinical significance, the probable source of infection, to distinguish sporadic cases from possible outbreaks and to better access the epidemiology of the disease.

In the present work we have developed a novel and simple *TaqMan*®-based multi-gene targeted real-time PCR approach yielding high sensitivity and specificity for the direct detection and differentiation of the most relevant pathogenic *Leptospira* species in animal samples, suitable for introduction into the routine diagnostics of veterinary laboratories.

2.3. Materials and Methods

2.3.1. Bacterial strains

Eighty five reference strains and clinical and environmental isolates of *Leptospira* spp. belonging to pathogenic, intermediate and non-pathogenic phylogenetic clades were used in this study (Table 2.1.). Strains were obtained from the collection maintained by the *Instituto Nacional de Investigação Agrária e Veterinária* (INIAV), Portugal, which is the Portuguese reference laboratory for animal diseases, from the Leptospirosis Laboratory at the *Instituto de Higiene e Medicina Tropical* (IHMT/UNL), Portugal, and from the WHO/FAO/OIE and National Leptospirosis Reference Centre in Amsterdam, The Netherlands.

Strains were grown in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium for up to 7 days.

Culturing *Leptospira* from tissue samples was performed as described by the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals [28]. Other bacterial strains were provided by INIAV for assessing the analytical specificity of the amplification reactions, representing the species: *Acinetobacter baumannii* (LNIV 1628/12), *Bacillus licheniformis* (VLA 1831), *Klebsiella pneumoniae* (VLA 1643), *Salmonella* Dublin (VLA 1272), *Streptococcus agalactiae* (VLA 33), *Proteus mirabilis* (LNIV 2269/II), *Yersinia enterocolitica* (VLA 1884), *Staphylococcus aureus* (VLA 1032), *Pseudomonas aeruginosa* (VLA 67), *Arcanobacterium pyogenes* (VLA 1321) and *Listeria monocytogenes* (VLA 1774).

2.3.2. Spiked tissue samples

A sample of kidney tissue from a bovine was used for testing as spiked sample. The kidney was acquired from a local official slaughterhouse (Raporal, Portugal), obtained from a bovine intended for normal human consumption, with no signs of leptospirosis. The bovine was not killed specifically for the purpose of this study. Approximately 200 mg portions of kidney tissue were excised with a sterile scalpel and homogenized with 5 ml of PBS buffer in a sterile plastic bag (Whirl-Pak bags) using a stomacher lab-blender. Kidney samples were individually spiked with the following strains, in order to determine the analytical detection sensitivity: *Leptospira interrogans* (serovar Autumnalis, strain Akiyami), *L. kirschneri* (serovar Mozdok, strain Portugal 1990) [29], *L. noguchii* (serovar Panama, strain CZ 214K) and *L. borgpetersenii* (serovar Tarassovi, strain Mitis Johnson). All the strains were grown at 29°C and the concentrations of leptospires were determined using a Petroff-Hausser counting chamber and adjusted to 10^8 cells/ml with PBS buffer. For each strain, tenfold serial dilutions from 10^7 to 10^0 cells/ml were prepared in PBS buffer and 0.1 ml aliquots were used to spike 0.9 ml of tissue homogenates. Tissue homogenate spiked with 0.1 ml PBS buffer was used as negative control. DNA extraction was performed as described in the paragraph “Genomic DNA extraction” below.

2.3.3. Tissue samples

INIAV IP is the Portuguese Reference Laboratory for animal diseases and provides diagnostic services to national veterinary authorities and private clients. Twenty seven dead wild rodents (25 *Mus* spp. and 2 *Rattus* spp.) were sent to the INIAV laboratory during the year 2011 for analysis and further used in this study (Table 2.2.). The rodents were captured in the Lisbon Zoo under routine operations for rodent population control, by the local veterinary authorities. No animals were sacrificed for the only purposes of research. Additionally, a Patagonian mara (*Dolichotis patagonum*), also from the zoo, and a swine (*Sus scrofa domesticus*) stillbirth fetus, from a private client, both suspect of dying with leptospirosis, were submitted for analysis to our reference laboratory and later included in this study (Table 2.2.). On arrival to the laboratory, animals were given a reference number and sent to the pathology where kidney, liver and/or lung tissue samples were collected. Specimens were then analyzed using culture-based methods according to the OIE standard procedures for leptospirosis [28]. Briefly, specimens were aseptically collected at necropsy, immediately emulsified in sterile buffered saline solution in a 10% tissue suspension, two to three drops were inoculated in a first tube of medium and two more tubes were similarly inoculated with increasing 10-fold dilutions of the tissue suspension. For the tissue culture, a semisolid *Leptospira* EMJH medium was used by adding 0.1% agar to commercial EMJH (Difco), to which rabbit serum (0.4%) and 5-Fluorouracil (100 µg/ml) were further added [28].
DNA was extracted directly from tissues homogenates as described below.

2.3.4. Genomic DNA extraction

Genomic DNA was extracted from both bacterial liquid cultures and tissue homogenates using the QIAamp DNA extraction kit according to the manufacturer's instructions (Qiagen, Hilden, Germany), with a final elution volume of 200 µl. The DNA concentration from the pure cultures was estimated spectrophotometrically using a Nanodrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and standardized to a concentration of 10^4 genome equivalents (GE)/µl for use in the reactions. The number of GE was

estimated using an average genome size of 4.6 Mb [30]. Genomic DNA suspensions were stored at - 20 ºC until further use.

2.3.5. Design of *TaqMan* probes and flanking primers

DNA sequences of representative strains and species of *Leptospira* were retrieved from NCBI-GenBank and aligned using the ClustalW algorithm implemented in the program MegAlign (vers. 5.03) (DNAStar, USA). Primers and dual labeled hydrolysis probes (*TaqMan*® probes) were designed to target selected species-specific genetic polymorphisms of the following pathogenic *Leptospira* spp.: *L. interrogans*, *L. borgpetersenii*, *L. kirschneri* and *L. noguchii* (Table 2.3.). Probes and primers specificities were assessed *in silico* using the BLAST tools from NCBI-GenBank. All probes and primers were synthesized by MWG Biotech (Germany).

2.3.6. Real-time PCR assays

We have implemented the following assay format for testing DNA templates extracted from biological samples: (i) a first duplex amplification step aiming the detection of pathogenic *Leptospira* spp. (by targeting the leptospiral *lipL32* gene; Table 2.3.) and including an internal control to monitor the presence of potential amplification inhibitors (by targeting the mammal *β-actin* gene; Table 2.3.); (ii) if pathogenic leptospires are detected in the first reaction, these may be further discriminated by testing each of the *L. interrogans*, *L. borgpetersenii*, *L. kirschneri* and *L. noguchii* targeted probes/primers (Table 2.3.). The CFX96 real-time PCR detection system (Bio-Rad, USA) was used for all assays. The amplification reactions were optimized individually for all the probes and associated primers using the SsoFast™ Probes Supermix (Bio-Rad, USA), according to the manufacturer's instructions. Each reaction was conducted in a total volume of 20 µl consisting of 1× SsoFast™ Probes Supermix, 400 nM of each primer, 150 nM of *TaqMan*® probe, DNase free water (GIBCO) and 5 µl of DNA template solution (extracted from pure cultures or tissues samples). Non-template negative controls (with PCR grade water) were included in each run to rule out the possibility of cross-contamination. The assay thermal conditions

were as follows: 95 °C for 2 min, followed by 45 cycles of 5 s at 95 °C and 15 s at the optimized annealing temperature for each probe (Table 2.3.). The thermal cycling conditions for the duplex amplification targeting *β-actin* and *lipL32* were 95° C for 2 min, followed by 45 cycles of 5 s at 95 °C and 35 s at 60 °C. Reproducibility of the assays was assessed by repeating the assays at least twice. Data analyses were performed by the detection system of the real-time PCR equipment, according to the manufacturer's instructions.

2.3.7. Analytical specificity and sensitivity

In order to determine if each set of probe and associated primers was specific for the respective *Leptospira* target species, the amplification assays were tested on DNA templates extracted from different strains belonging to pathogenic, intermediate and non-pathogenic *Leptospira* species (Table 2.1.), and from other non-related bacteria previously mentioned in "bacterial strains" section. The analytical sensitivity of the amplification assays (limits of detection – LODs) were determined using 10-fold serial dilutions of genomic DNA extracted from pure cultures of *L. interrogans* (serovar Autumnalis, strain Akiyami), *L. kirschneri* (serovar Mozdok, strain Portugal 1990), *L. noguchii* (serovar Panama, strain CZ 214K) and *L. borgpetersenii* (serovar Tarassovi, strain Mitis Johnson). LODs on tissue samples were assessed using DNA extracted from the serially diluted spiked macerates. Each template was tested in triplicate.

2.3.8. Sequencing

Leptospira isolates obtained from tissue samples were identified by comparative sequence analysis of a 245 bp region of the s*ecY* gene, as described by Victoria *et al.* [31]. Briefly, the region of interest was amplified using primers SecYII (5'-GAA TTT CTC TTT TGA TCT TCG-3') and SecYIV (5'-GAG TTA GAG CTC AAA TCT AAG-3'), which amplify *secY* sequences from all pathogenic strains of *Leptospira*. PCR amplifications were performed on a C1000 thermocycler (Bio-Rad) using the following program: an initial step of denaturation for 5 min at 95 ºC, followed by 34 cycles consisting of annealing,

45 s at 54 ºC, extension, 2 min at 72 ºC, and denaturation, 30 s at 94 ºC. Nucleotide sequences were determined, using the same primers, by commercially available sequencing services. Nucleotide sequence analysis and comparison with other relevant reference sequences were performed using the BLAST suite at NCBI-GenBank and aligned using Clustal X or MEGA software (version 5.0).

2.4. Results

2.4.1. Design of probes and primers

Species-specific sets of primers and probes targeting *L. interrogans*, *L. borgpetersenii*, *L. kirschneri* and *L. noguchii* are listed in Table 2.3. As shown in Figures 2.S1, 2.S2, 2.S3 and 2.S4 available under supporting information of this chapter, these sets of probes and primers contained sufficient polymorphisms to warrant *'in silico'* species specific amplification.

2.4.2. Analytical specificity and sensitivity

Execution of the PCRs on DNA extracted from various bacteria, revealed a highly specific amplification from any of the pathogenic strains belonging to the respective target *Leptospira* spp., i.e. *L. interrogans*, *L. kirschneri*, *L borgpetersenii* and *L. noguchii*. None of the other strains yielded a positive amplification reaction (Table 2.1.; Figure 2.1A.). The analytical sensitivity (LOD) of the amplification assays were found to be between 1 and 10 genome copies in the PCR mixture for each probe and primer set.

Table 2.1. *Leptospira* strains used in the present study and results of the real time PCR assays using the species-specific probes and flanking primers.

Species	Serogroup	Serovar	Strain	Source[1]	Set 1[2]	Set 2[3]	Set 3[4]	Set 4[5]	Set 5[6]
L. interrogans	Australis	Muenchen	München C 90	KIT	+	+	-	-	-
	Australis	Australis	Ballico	KIT	+	+	-	-	-
	Australis	Bratislava	Jez Bratislava	INIAV	+	+	-	-	-
	Autumnalis	Autumnalis	Akiyami A	INIAV	+	+	-	-	-
	Bataviae	Bataviae	Van Tienem	INIAV	+	+	-	-	-
	Canicola	Canicola	Hond Utrecht IV	INIAV	+	+	-	-	-
	Djasiman	Djasiman	Djasiman	KIT	+	+	-	-	-
	Hebdomadis	Hebdomadis	Hebdomadis	KIT	+	+	-	-	-
	Hebdomadis	Kremastos	Kremastos	KIT	+	+	-	-	-
	Icterohaemorrhagiae	Birkini	Birkin	KIT	+	+	-	-	-
	Icterohaemorrhagiae	Copenhageni	M20	INIAV	+	+	-	-	-
	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA	INIAV	+	+	-	-	-
	Icterohaemorrhagiae	Lai	Lai	KIT	+	+	-	-	-
	Pomona	Pomona	Pomona	INIAV	+	+	-	-	-
	Pyrogenes	Pyrogenes	Salinem	INIAV	+	+	-	-	-
	Sejroe	Hardjo type Prajitno	Hardjoprajitno	IHMT	+	+	-	-	-
L. borgpetersenii	Ballum	Ballum	Mus 127	INIAV	+	-	+	-	-
	Ballum	Castellonis	Castellon 3	KIT	+	-	+	-	-

Table 2.1. (cont.)

Species	Serogroup	Serovar	Strain	Source[1]	Set 1[2]	Set 2[3]	Set 3[4]	Set 4[5]	Set 5[6]
L. borgpetersenii	Hebdomadis	Jules	Jules	KIT	+	-	+	-	-
	Hebdomadis	Worsfoldi	Worsfold	KIT	+	-	+	-	-
	Javanica	Ceylonica	Piyasena	KIT	+	-	+	-	-
	Javanica	Poi	Poi	INIAV	+	-	+	-	-
	Javanica	Zhenkang	L 82	KIT	+	-	+	-	-
	Mini	Mini	Sari	IHMT	+	-	+	-	-
	Pyrogenes	Kwale	Julu	KIT	+	-	+	-	-
	Sejroe	Hardjo type bovis	Sponselee	KIT	+	-	+	-	-
	Sejroe	Hardjo type bovis	L550	KIT	+	-	+	-	-
	Sejroe	Hardjo type bovis	JB197	KIT	+	-	+	-	-
	Sejroe	Nyanza	Kibos	KIT	+	-	+	-	-
	Sejroe	Sejroe	M84	KIT	+	-	+	-	-
	Tarassovi	Kisuba	Kisuba	KIT	+	-	+	-	-
	Tarassovi	Tarassovi	Mitis Johnson	INIAV	+	-	+	-	-
L. kirschneri	Australis	Ramisi	Musa	KIT	+	-	-	+	-
	Autumnalis	Bulgarica	Nicolaevo	KIT	+	-	-	+	-
	Autumnalis	Butembo	Butembo	KIT	+	-	-	+	-
	Cynopteri	Cynopteri	3522C	IHMT	+	-	-	+	-

Table 2.1. (cont.)

Species	Serogroup	Serovar	Strain	Source[1]	Set 1[2]	Set 2[3]	Set 3[4]	Set 4[5]	Set 5[6]
L. kirschneri	Grippotyphosa	Grippotyphosa type Moskva	Moskva V	IHMT	+	-	-	+	-
	Grippotyphosa	Ratnapura	Wumalasena	KIT	+	-	-	+	-
	Grippotyphosa	Vanderhoedeni	Kipod 179	KIT	+	-	-	+	-
	Icterohaemorrhagiae	Bogvere	LT 60-69	KIT	+	-	-	+	-
	Pomona	Mozdok	5621	KIT	+	-	-	+	-
	Pomona	Mozdok	Portugal 1990	INIAV	+	-	-	+	-
	Pomona	Tsaratsovo	B 81/7	KIT	+	-	-	+	-
L. noguchii	Australis	Nicaragua	1011	KIT	+	-	-	-	+
	Autumnalis	Fortbragg	Fort Bragg	KIT	+	-	-	-	+
	Bataviae	Argentiniensis	Peludo	KIT	+	-	-	-	+
	Djasiman	Huallaga	M 7	KIT	+	-	-	-	+
	Louisiana	Louisiana	LSU 1945	KIT	+	-	-	-	+
	Panama	Panama	CZ 214	INIAV	+	-	-	-	+
	Pomona	Proechimys	1161 U	KIT	+	-	-	-	+
	Pyrogenes	Myocastoris	LSU 1551	KIT	+	-	-	-	+
	Shermani	Carimagua	9160	KIT	+	-	-	-	+
L. santarosai	Ballum	Peru	MW 10	KIT	+	-	-	-	-
	Bataviae	Balboa	735 U	KIT	+	-	-	-	-

Table 2.1. (cont.)

Species	Serogroup	Serovar	Strain	Source[1]	Set 1[2]	Set 2[3]	Set 3[4]	Set 4[5]	Set 5[6]
L. santarosai	Bataviae	Kobbe	CZ 320	KIT	+	-	-	-	-
	Grippotyphosa	Canalzonae	CZ 188	KIT	+	-	-	-	-
	Hebdomadis	Borincana	HS 622	KIT	+	-	-	-	-
	Hebdomadis	Maru	CZ 285	KIT	+	-	-	-	-
	Javanica	Fluminense	Aa 3	KIT	+	-	-	-	-
	Mini	Beye	1537 U	KIT	+	-	-	-	-
	Sarmin	Rio	Rr 5	KIT	+	-	-	-	-
	Sejroe	Guaricura	Bov.G.	KIT	+	-	-	-	-
	Shermani	Babudieri	CI 40	KIT	+	-	-	-	-
	Shermani	Shermani	1342 K	KIT	+	-	-	-	-
	Tarassovi	Atchafalaya	LSU 1013	KIT	+	-	-	-	-
L. weilii	Celledoni	Celledoni	Celledoni	INIAV	+	-	-	-	-
	Celledoni	Mengding	M 6906	KIT	+	-	-	-	-
	Javanica	Coxi	Cox	KIT	+	-	-	-	-
	Javanica	Mengma	S 590	KIT	+	-	-	-	-
	Javanica	Mengrun	A 102	KIT	+	-	-	-	-
	Mini	Hekou	H 27	KIT	+	-	-	-	-
	Pyrogenes	Menglian	S 621	KIT	+	-	-	-	-
	Sarmin	Sarmin	Sarmin	KIT	+	-	-	-	-

Table 2.1. (cont.)

Species	Serogroup	Serovar	Strain	Source[1]	Set 1[2]	Set 2[3]	Set 3[4]	Set 4[5]	Set 5[6]
L. weilii	Tarassovi	Topaz	94-79970/3	KIT	+	-	-	-	-
	Tarassovi	Vughia	LT 89-68	KIT	+	-	-	-	-
L. alexanderi	Hebdomadis	Manzhuang	A 23	KIT	nd	-	-	-	-
	Javanica	Mengla	A 85	KIT	nd	-	-	-	-
	Manhao	Manhao 3	L 60	KIT	nd	-	-	-	-
	Mini	Yunnan	A 10	KIT	nd	-	-	-	-
L. meyeri	Ranarum	Ranarum	ICF	KIT	nd	-	-	-	-
	Semaranga	Semaranga	Veldrat Semaranga 173	KIT	nd	-	-	-	-
L. inadai	Manhao	Lincang	L 14	KIT	nd	-	-	-	-
L.fainei	Hurstbridge	Hurstbridge	BUT 6T	KIT	nd	-	-	-	-
L.biflexa	Andaman	Andamana	CH 11	KIT	-	-	-	-	-
	Semaranga	Patoc	Patoc I	KIT	-	-	-	-	-

[1]INIAV - Instituto Nacional de Investigação Agrária e Veterinária, Lisbon, Portugal. IHMT - Instituto de Higiene e Medicina Tropical, Lisbon, Portugal. KIT - Royal Tropical Institute, Amsterdam, The Netherlands; [2]Set 1 targets the *lipL32* gene of pathogenic *Leptospira* spp.; [3]Set 2 targets the *secY* gene of *L. interrogans*; [4]Set 3 targets the *ompL1* gene of *L. borgpetersenii*; [5]Set 4 targets the *secY* gene of *L. kirschneri*; [6]Set 5 targets the *secY* gene of *L. noguchii*; nd - not done; Amplification (+) or no amplification (-).

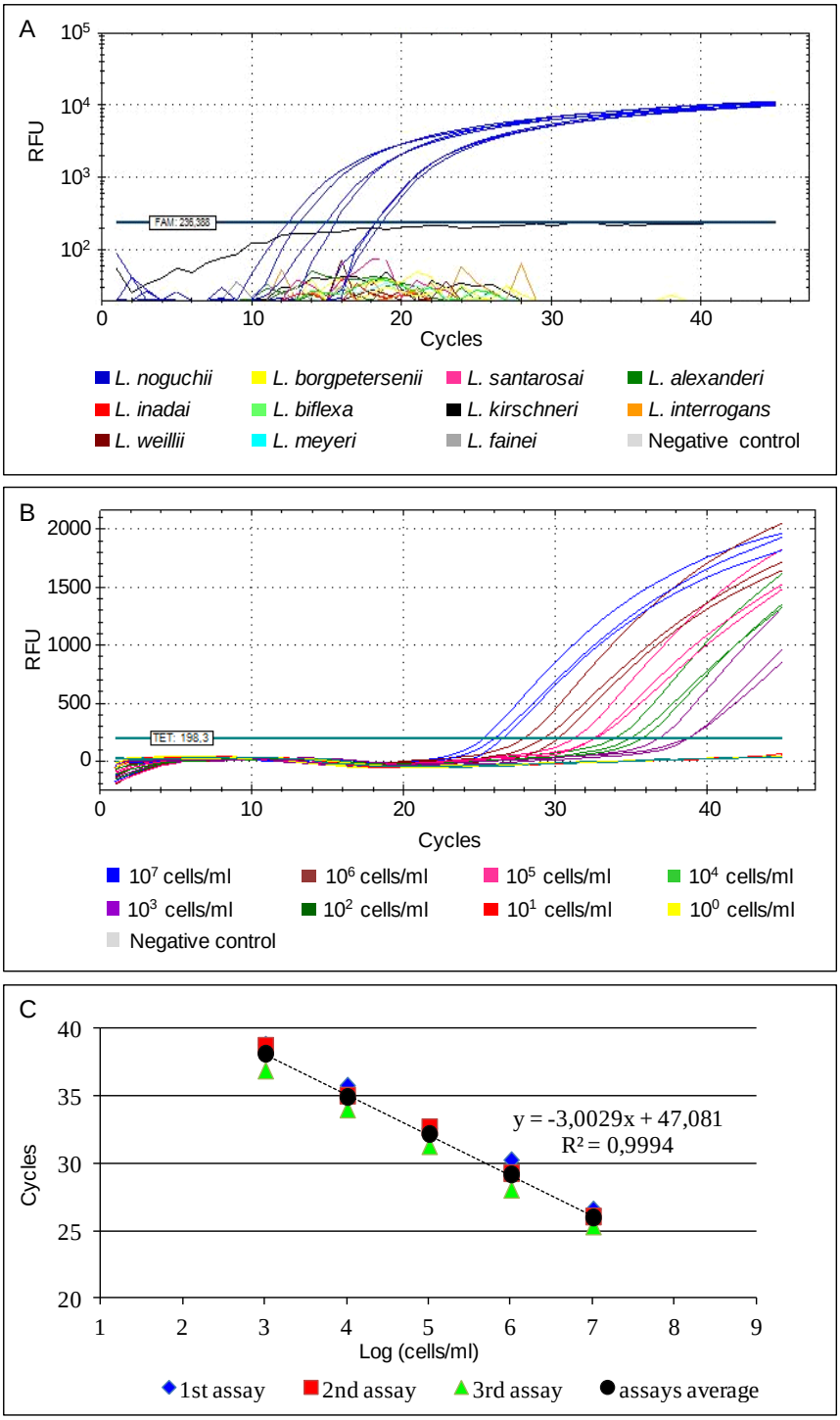

Figure 2.1. Illustration of the real-time PCR amplification curves obtained during the optimization of the assays. (A) Specificity tests of the *L. noguchii* targeted amplification assay using the TaqLnog probe combined with the flanking primers FLnog2 and RLnog2. Blue amplification curves represent *L. noguchii* strains. All other non-target strains yielded no amplification results. (B) Estimation of the limit of

detection of the amplification assay targeting *L. interrogans* (serovar Autumnalis, strain Akiyami) using DNA extracted directly from spiked bovine kidney samples as template as a typical example of all *Leptospira* probe and primer sets. The amplification curves obtained from different ten-fold serial dilutions of the target *Leptospira* are represented by different colours. Unspiked tissue homogenate (grey line) was used as negative control. (C) Standard curve obtained from the analysis of the amplification curves mentioned in the previous panel B. RFU - Relative Fluorescence Units.

2.4.3. Spiked tissue samples

The LOD of the PCRs on spiked tissue samples was similar for all probe/primers sets targeting the respective target species, and estimated to be 10^3 leptospires/ml of tissue homogenate (≈ per 200 mg of tissue) (Figure 2.1B.). Furthermore, the same LOD was estimated for the *lipL32*-targeted probe/primers when used in duplex amplification reactions with the mammal *β-actin* probe (not shown).

2.4.4. Clinical tissue samples

DNA extracted from 27 kidney samples of wild rodents were analysed with the *lipL32* and mammal *β-actin* targeted duplex assay (Table 2.2.; Figure 2.2A.). Leptospiral DNA was detected in three samples, as demonstrated by a positive amplification of the *lipL32* gene region (Table 2.2.; Fig. 2.2A.). Furthermore, the partial *β-actin* gene was amplified from all samples, showing that the PCR reactions were not significantly inhibited by potential contaminants.

When tested with each of the *L. interrogans*, *L. borgpetersenii*, *L. kirschneri* and *L. noguchii* targeted probes/primers, only these three samples showed amplification (Table 2.2.; Figure 2.2B.). Two of these DNA samples were identified as *L. borgpetersenii* and one sample as *L. interrogans*. Testing a pooled sample of kidney and liver tissues from a Patagonian mara, and a lung sample from an aborted swine fetus with the duplex PCR revealed a positive amplification for both samples (Table 2.2.). Subsequent testing with the species-specific sets of probes and primers showed that the Patagonian mara was infected with *L. interrogans* and the swine fetus with *L. kirschneri*.

Table 2.2. Results of the bacteriological culture and of the real time amplification assays for the tissue samples analyzed in the present study.

Sample	Origin	Set Actin[1]	Set 1[2]	Set 2[3]	Set 3[4]	Set 4[5]	Set 5[6]	Bacteriological analysis[7]
12-17433-Z1	*Mus* sp.	+	+	-	+	-	-	*L. borgpetersenii*
12-18078-Z6	*Mus* sp.	+	+	-	+	-	-	*L. borgpetersenii*
12-18458-Z13	*Mus* sp.	+	-	-	-	-	-	Negative
12-18458-Z14	*Mus* sp.	+	-	-	-	-	-	Negative
12-19472-Z15	*Mus* sp.	+	-	-	-	-	-	Negative
12-20553-Z16	*Mus* sp.	+	-	-	-	-	-	Negative
12-22955-Z17	*Mus* sp.	+	-	-	-	-	-	Negative
12-22955-Z18	*Mus* sp.	+	-	-	-	-	-	Negative
12-22955-Z19	*Mus* sp.	+	-	-	-	-	-	Negative
12-22955-Z20	*Mus* sp.	+	-	-	-	-	-	Negative
12-22955-Z22	*Mus* sp.	+	-	-	-	-	-	Negative
12-22955-Z23	*Mus* sp.	+	-	-	-	-	-	Negative
12-22955-Z24	*Mus* sp.	+	-	-	-	-	-	Negative
12-22955-Z25	*Mus* sp.	+	-	-	-	-	-	Negative
12-22955-Z26	*Mus* sp.	+	-	-	-	-	-	Negative
12-22955-Z27	*Mus* sp.	+	-	-	-	-	-	Negative
12-22955-Z28	*Mus* sp.	+	-	-	-	-	-	Negative
12-22955-Z29	*Mus* sp.	+	-	-	-	-	-	Negative
12-22955-Z30	*Mus* sp.	+	-	-	-	-	-	Negative

Table 2.2. (cont.)

Sample	Origin	Set Actin[1]	Set 1[2]	Set 2[3]	Set 3[4]	Set 4[5]	Set 5[6]	Bacteriological analysis[7]
12-22955-Z31	*Mus* sp.	+	-	-	-	-	-	Negative
12-22955-Z32	*Mus* sp.	+	-	-	-	-	-	Negative
12-22955-Z33	*Mus* sp.	+	-	-	-	-	-	Negative
12-22955-Z34	*Mus* sp.	+	-	-	-	-	-	Negative
12-22955-Z36	*Mus* sp.	+	-	-	-	-	-	Negative
12-22955-Z37	*Rattus* sp.	+	+	+	-	-	-	*L. interrogans*
12-22955-Z38	*Mus* sp.	+	-	-	-	-	-	Negative
12-22955-Z39	*Rattus* sp.	+	-	-	-	-	-	Negative
11-36840	*Dolichotis patagonum*	+	+	+	-	-	-	*L. interrogans*
12-494	*Sus scrofa domesticus* (fetus)	+	+	-	-	+	-	*L. kirschneri*

[1] Set Actin targets the *β-actin* gene of mammals, [2]Set 1 targets the *lipL32* gene of pathogenic *Leptospira*; [3]Set 2 targets the *secY* gene of *L. interrogans*; [4]Set 3 targets the *ompL1* gene of *L. borgpetersenii*; [5]Set 4 targets the *secY* gene of *L. kirschneri*; [6]Set 5 targets the *secY* gene of *L. noguchii*; [7]The analysis of the partial sequences of the *secY* gene of each isolate allowed to identify the *Leptospira* species; Amplification (+) or no amplification (-)

Leptospira isolates were only cultured from the samples that also yielded PCR-positive results, thus confirming the presence of viable leptospires (Table 2.2.).

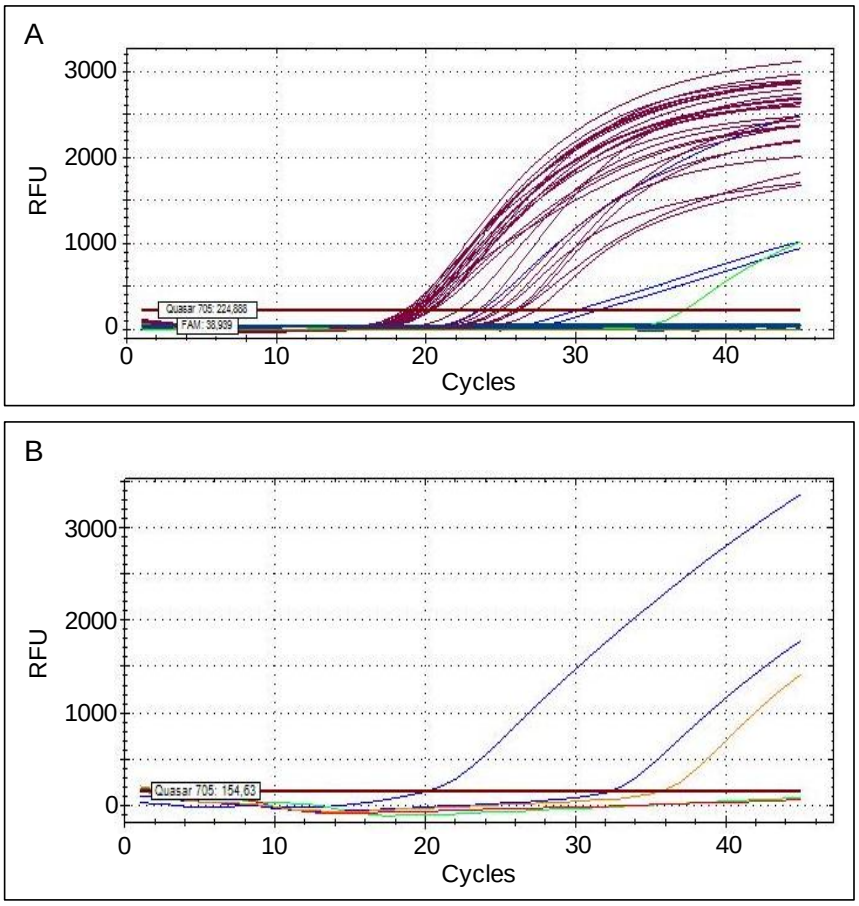

Figure 2.2. Illustration of the real-time PCR amplification curves obtained during the testing of naturally-infected tissue samples. (A) Results of the *β-actin* and *lipL32* targeted duplex amplification assay when testing representative samples from the wild rodents. The partial *β-actin* gene was amplified from all tissue samples (dark pink lines). Leptospiral DNA was detected in three samples by a positive amplification of the *lipL32* gene (blue lines). A spiked positive control with *L. interrogans* (serovar Autumnalis, strain Akiyami) is shown (green line). (B) From the previous leptospiral positive amplification results, two samples were assessed as infected with *L. borgpetersenii* using the respective targeted amplification assay with probe TqM_bpn and flanking primers F_bpn and R_bpn1 (blue lines). The positive and negative controls are illustrated by the orange and red lines, respectively.

Molecular speciation through analysis of the partial sequences of the *secY* gene was in concordance with the results obtained by the species-specific PCRs. Two isolates were identified as *L. borgpetersenii* (from wild rodents; GenBank accession numbers KM066006 and KM066007), one as *L. kirschneri* (from the swine fetus; accession number KM066009) and two as *L. interrogans* (from a wild rodent and the Patagonian mara; accession numbers KM066008 and KM066010, respectively).

Table 2.3. Primers and probes used in this study targeting selected genes of pathogenic species of *Leptospira*

Set	Primer/Probe	Sequence (5'- 3')	Annealing temperature	Complementary target species
Set Actin[1]	F_Actin	GGC TCY ATY CTG GCC TC	60 ºC	*β-actin* gene of mammals
	R_Actin	GCA YTT GCG GTG SAC RAT G		
	P_Actin	Cy5.5 (Quasar 705) -TAC TCC TGC TTG CTG ATC CAC ATC-BHQ2		
Set 1[2]	45F	AAG CAT TAC CGC TTG TGG TG	60 ºC	*lipL32* gene of pathogenic *Leptospira* spp.
	286R	GAA CTC CCA TTT CAG CGA TT		
	taq-189P	FAM-AAA GCC AGG ACA AGC GCC G-BHQ1		
Set 2	PFLint2	CTT GAG CCT GCG CGT TAY C	63 ºC	*secY* gene of *L. interrogans*
	PRLint2	CCG ATA ATT CCA GCG AAG ATC		
	TaqLint2	TET-CTC ATT TGG TTA GGA GAA CAG ATC A-BHQ1		

Table 2.3. (cont.)

Set	Primer/Probe	Sequence (5'- 3')	Annealing temperature	Complementary target species
Set 3	F_bpn	GAT TCG GGT TAC AAT TAG ACC	65 ºC	*ompL1* gene of *L. borgpetersenii*
	R_bpn1	TTG ATC TAA CCG GAC CAT AGT		
	TqM_bpn	Cy5.5 (Quasar 705) -TAC TAA GGA TGG TTT GGA CGC TGC-BHQ2		
Set 4	F_nery	CTG GCT TAA TCA ATG CTT CTG	60 ºC	*secY* gene of *L. kirschneri*
	R_nery	CTC TTT CGG TGA TCT GTT CC		
	TqM_nery	Texas Red-CAG TTC CAG TTG TAA TAG ATA AGA TTC-BHQ2		
Set 5	FLnog2	TCA GGG TGT AAG AAA GGT TC	63 ºC	*secY* gene of *L. noguchii*
	RLnog2	CAA AAT TAA AGA AGA AGC AAA GAT		
	TaqLnog	FAM-CGA TTG GCT TTT TGC TTG AAC CATC-BHQ1		

[1]Retrieved from Costa et al. [32]; [2]Retrieved from Stoddard et al. [16].

2.5. Discussion

In this work we present a two step real-time PCR strategy to infer the presence of pathogenic leptospires in clinical and veterinary samples. In the first step, we assess if an animal tissue sample is infected with a pathogenic leptospire by targeting its *lipL32* gene. The *lipL32* gene encodes an outer membrane lipoprotein that is confined to pathogenic *Leptospira* species [16]. The second step identifies the four most common and veterinary relevant pathogenic

Leptospira species, *L. interrogans*, *L. borgpetersenii*, *L. kirschneri* and *L. noguchii* using dedicated sets of probes and primers.

Probes and flanking primers were developed by *in silico* analysis and further tested for their practical utility on DNA extracted from cultured bacteria, spiked tissues and clinical specimens. The amplification assays have proved to be specific to the respective targeted species, with no cross-reactions when non-pathogenic leptospires or other pathogens were tested. The amplification of the *β-actin* gene was included in the initial *lipL32*-based PCR to assess the presence of amplification inhibitors in tissue samples [32]. However, the abundant presence of *β-actin* gene copies in DNA samples extracted from tissues may ensure some amplification even when low levels of potential inhibitors are present (but amplification curves are usually weaker and anomalous). The analytical sensitivity deduced for the amplification assays, i.e. 1 to 10 GE on DNA extracted from cultured leptospires and 10^3 leptospires/ml tissue homogenate, were similar to the ones of other previous studies concerning the molecular detection of leptospires [15, 16, 18, 19, 23].

The panel of species-specific probes and flanking primers may be extended with the design of novel oligonucleotides, e.g. for use in regions where the occurrence of additional species of pathogenic leptospires is common. As far as we know, this is the first report describing a strategy capable of clearly identifies four most frequently found pathogenic *Leptospira* species based on the use of *TaqMan*® probes.

From 27 kidney samples of wild rodents, and samples from a Patagonian mara and a porcine fetus suspected of leptospirosis, three rodent samples and the samples from the Patagonian mara and fetus all yielded a positive PCR test for the presence of pathogenic leptospires. In concordance, these samples were also positive by culture. Culture provides proof of infection and thus is an ideal reference standard. Consequently, these results are consistent with a 100% clinical sensitivity and specificity of the PCR. Subsequent prospective analysis of a larger sample set would allow substantiating this conclusion.

Phylogenetic identification of the cultures also allowed supporting the findings obtained with the species-specific PCRs. Indeed, speciation by phylogeny was in all cases in concordance with the results obtained via the PCR method.

Initially, we anticipated that more samples would be positive by the real time PCR assay than by culture [5, 33-35]. Recently, Fornazari *et al.* [21] reported that quantitative PCR presented the highest sensitivity among several techniques to detect leptospires in tissues samples, the bacteriological culture being the least sensitive. Apparently, our procedure of culturing, using macerated fresh tissue has been highly effective. Alternatively, it cannot be excluded that the bacterial load of the tissues might have been very high. Nevertheless, the low rate of positive animals (11%) is not too discrepant from the prevalence values found in other studies where leptospiral DNA was detected in rodents tissues by PCR-based assays, which ranged from 13% to 20% [26, 36, 37]. Furthermore, as far as we know, the region of Lisbon, where the rodents were captured, is not usually regarded as having major leptospirosis problems [2], which may also reflect a lower prevalence of the agent in reservoirs such as wild rodents. We anticipate that our assays may be useful in studies inferring the prevalence of pathogenic leptospires in wild rodents and other animals, with the advantage of differentiating the infecting *Leptospira* species.

The amplification assays described were able to detect pathogenic leptospires in samples of animal tissues, such as kidney or lung. Although the analysis of this kind of samples is not essential for an early diagnosis of leptospirosis, it has a great value in situations such as epidemiological and post-mortem investigations. The last situation is very well illustrated in this work with the detection of pathogenic leptospires in tissues of a Patagonian mara and a swine fetus. Both animals were suspect of having leptospirosis, which was confirmed by this study. The porcine fetus was infected with a strain belonging to *L. kirschneri*. Pigs may be infected by several *Leptospira* species (and serovars) that may cause infertility, fetal death and abortion. *Leptospira kirschneri* has been reported but seems to be less frequently found in pigs in Portugal than other species [38]. The Patagonian mara, a relatively large rodent that lived in the local zoo, was found to be infected with *L. interrogans*. To our knowledge, this is the first report describing the molecular detection or the isolation of a pathogenic leptospire from that rodent, which proved to have died of leptospirosis. Zoos are often infested with rats that are notorious reservoirs of *L. interrogans*. We hypothesise that this Patagonian mara has been infected by

rats as the primary infection reservoir, which would support the potential hazard of rodents in zoos for both (exotics) animals and public.

The amplification assay described in this work is able to indentify the four most relevant pathogenic species of *Leptospira* infecting farm and wild animals. While the approach can be extended to other *Leptospira* species, it is important to continually evaluate the specificity of previously designed probes and primers and, if necessary, modify and improve the sequences, in order to ensure an effective and specific detection and identification of the circulating *Leptospira* species.

2.6. Conclusions

The molecular assays presented in this work allow the detection and identification of four relevant pathogenic species of *Leptospira*, directly from animal tissues. The assays proved to be specific and sensitive, and much faster than the bacteriological culture, reducing the time for confirmatory leptospirosis diagnosis. The assays are amenable to future automation possibilities and will reinforce the diagnostic information and enhance our knowledge about the epidemiology of leptopirosis.

2.7. Acknowledgments

Madalena Monteiro (INIAV, I.P.) is acknowledged for excellent technical assistance in performing pathological analysis. Lurdes Clemente and Ana Botelho are acknowledged for providing non-*Leptospira* bacterial strains. Narciso Lapão, Nuno Gaspar and António Crespo are acknowledged for their help in providing samples and respective information.

2.8. Supporting Information

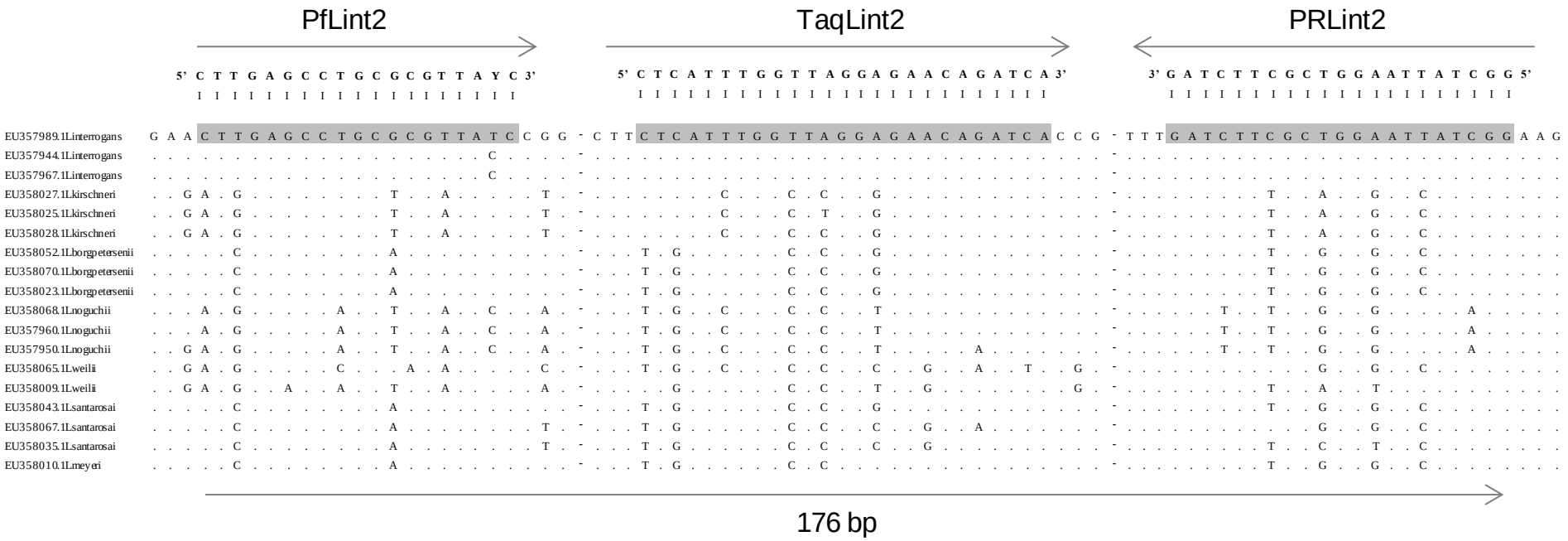

Figure 2.S1. Complementary targets of the species-specific *Leptospira interrogans* probe and respective flanking primers. Partial alignment of *secY* gene sequences of representative *Leptospira* species showing the complementary targets of the probes TaqLint2 (and respective flanking primers PFLint2 and PRLint2). Mismatches in sequences are highlighted. The GenBank access numbers from which the partial sequences were retrieved are indicated for each species.

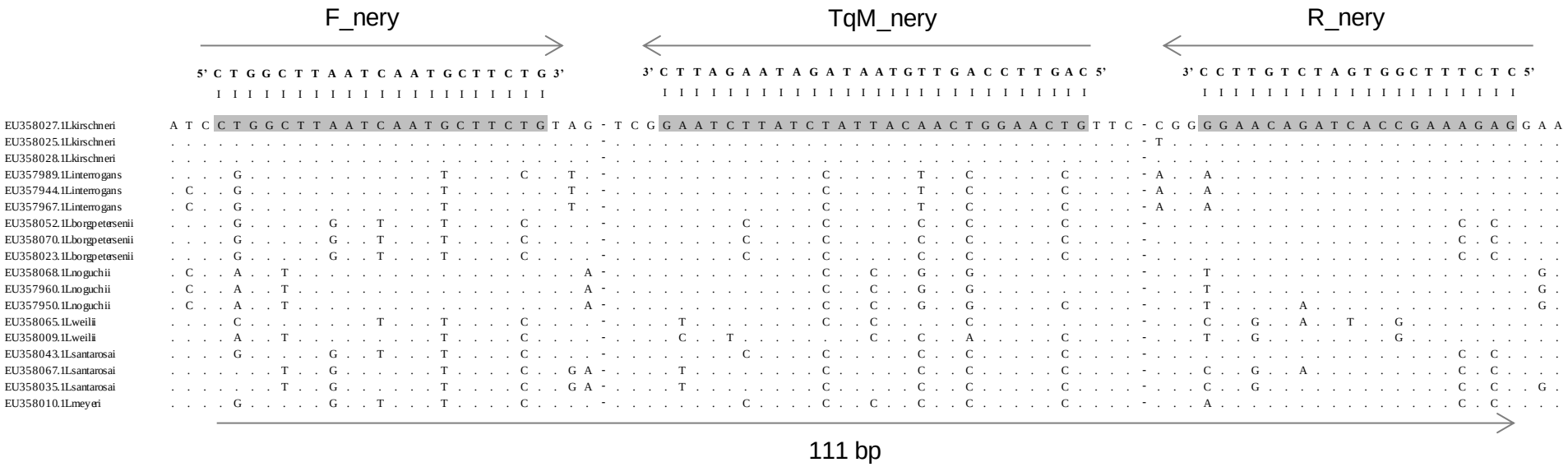

Figure 2.S2. Complementary targets of the species-specific *Leptospira kirschneri* probe and respective flanking primers. Partial alignment of *secY* gene sequences of representative *Leptospira* species showing the complementary targets of the probe TqM_nery (and respective flanking primers F_nery and R_nery). Mismatches in sequences are highlighted. The GenBank access numbers from which the partial sequences were retrieved are indicated for each species.

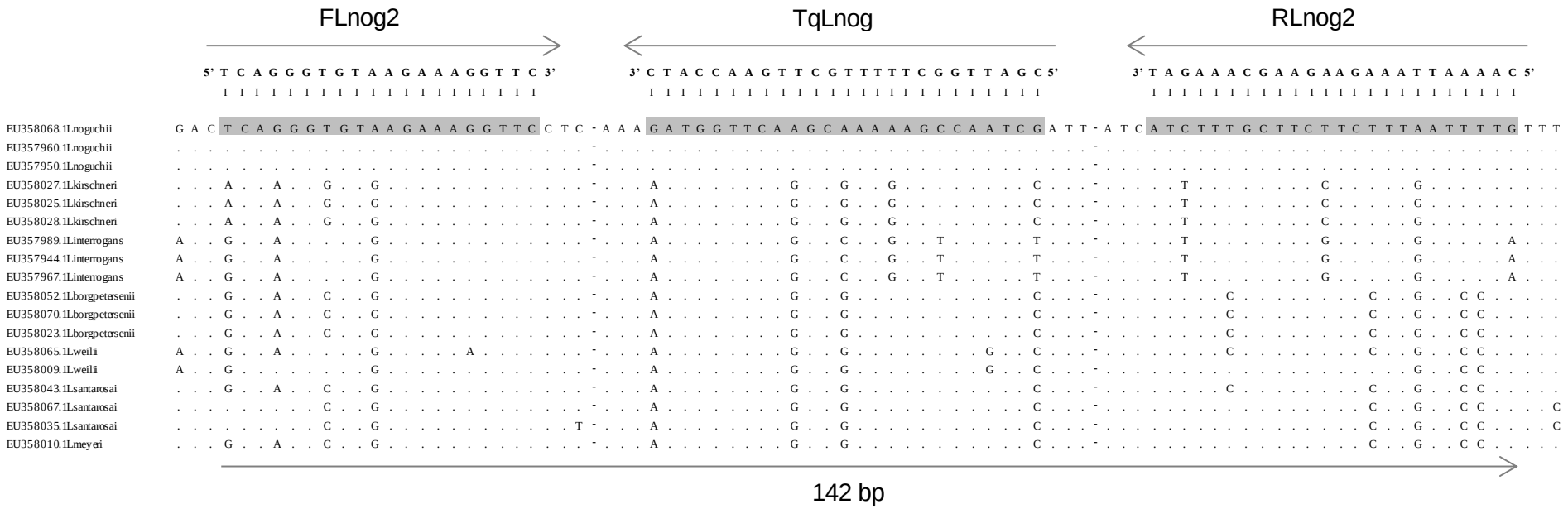

Figure 2.S3. Complementary targets of the species-specific *Leptospira noguchii* probe and respective flanking primers. Partial alignment of *secY* gene sequences of representative *Leptospira* species showing the complementary targets of the probe TaqLnog (and respective flanking primers FLnog2 and RLnog2). Mismatches in sequences are highlighted. The GenBank access numbers from which the partial sequences were retrieved are indicated for each species.

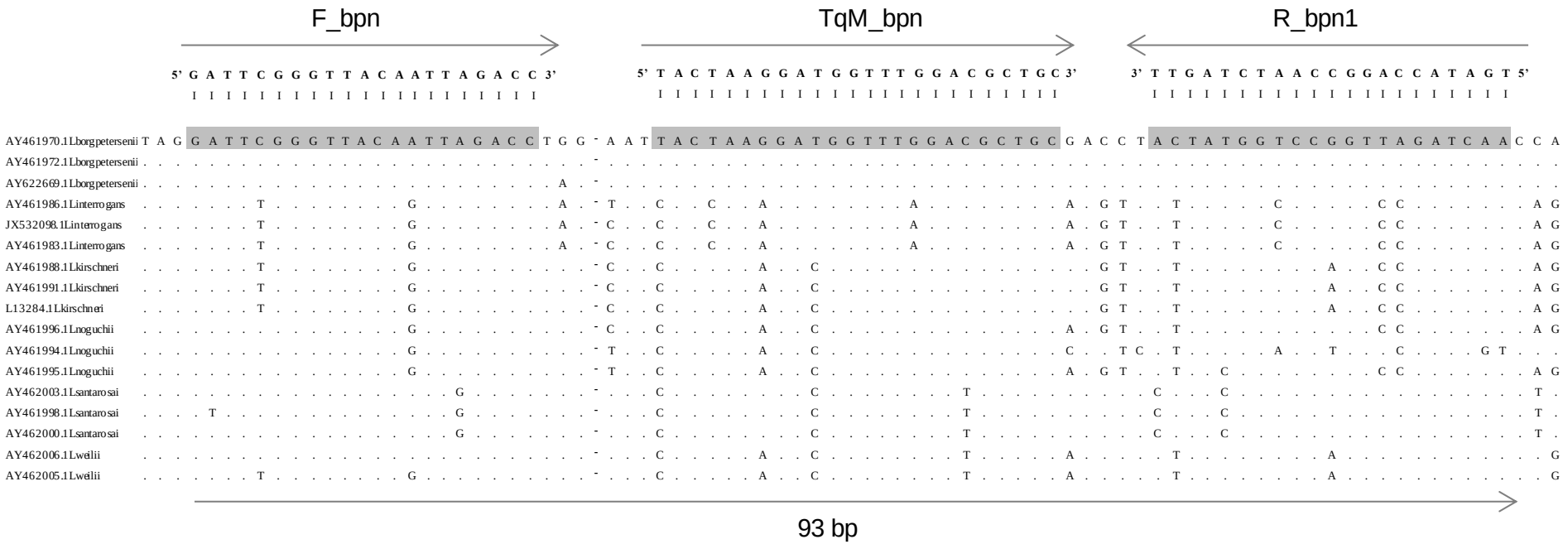

Figure 2.S4. Complementary targets of the species-specific *Leptospira borgpetersenii* probe and respective flanking primers. Partial alignment of ompL1 gene sequences of representative *Leptospira* species showing the complementary targets of the probe TqM_bpn (and respective flanking primers F_bpn and R_bpn1). Mismatches in sequences are highlighted. The GenBank access numbers from which the partial sequences were retrieved are indicated for each species.

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CHAPTER 3

Extended panel of species-specific probes for the real time PCR detection and identification of pathogenic leptospires in tissues

3. Extended panel of species-specific probes for the real time PCR detection and identification of pathogenic leptospires in tissues

Manuscript in preparation

3.1. Summary

Leptospirosis caused by pathogenic spirochetes of the genus *Leptospira* occurs in humans and animals worldwide and causes important economic losses in the cattle and sheep industries. Recently we developed a real time PCR approach for the detection and differentiation of pathogenic *Leptospira* species, particularly of *L. interrogans*, *L. kirschneri*, *L. borgpetersenii* and *L. noguchii*, which constitute the veterinary most relevant pathogenic species of *Leptospira*. In this work we extended the panel of species-specific probes in order to additionally detect the important *L. santarosai* and *L. weilii*, and assessed the efficiency of the extended assay for the detection of leptospiral infection in a large set of 154 kidney tissues originally collected from rodents with origin in the north of Portugal. The *lipL32* gene was detected in 11% of these samples. Considering culture as a gold standard diagnostic method, the *lipL32*-targeted PCR assay showed a diagnostic specificity and sensitivity of respectively 97.1% and 81.3% for the detection of pathogenic leptospires in tissue samples. An observed kappa coefficient was estimated in 0.762 for the overall "substantial" agreement between the real time PCR assay and the bacteriological culture. All samples yielding *lipL32* positive amplification were further tested with the *Leptospira* species-specific duplex real time PCR assays. Two rodent samples were found to be infected with *L. kirschneri*, and ten samples were found to be infected with *L. borgpetersenii*. One sample gave positive amplification signals for both *L. kirschneri* and *L. borgpetersenii* probes, potentially representing mixed leptospiral infection. Four samples with a positive *lipL32* amplification did not show any amplification signals with species-specific probes. Our two-step approach allows a simple, rapid and robust identification of the most commonly infecting *Leptospira* species, becoming an enhanced tool for the study of the epidemiology of leptospirosis.

3.2. Introduction

Leptospirosis caused by pathogenic spirochetes of the genus *Leptospira* occurs in humans and animals worldwide and causes important economic losses in the cattle and sheep industries primarily due to reproductive wastage and to decrease milk production [1-3]. Chronic infection and urinary shedding of *Leptospira* into the environment play a crucial role in transmission and spreading of infection which might lead to acute clinical disease or incidental infections both in humans and animals [2, 3]. Mammalian species, especially rodent species, are considered to be main reservoirs of pathogenic leptospires. However, detailed information about the epidemiology of the disease is still scarce and disperse, also related with the increased difficulty of the laboratorial detection and characterization of the agents. Serological assays as well as evidence of the occurrence of leptospires in tissues are commonly used for assessing leptospirosis in animals but usually lack sensitivity and specificity, which contribute to high rates of diagnostic failure [4]. Serological tools are also not reliable for detecting the presence of bacteria in the kidneys or urine and so the direct detection of the agent is necessary to identify chronically infected or carrier animals [2, 3]. Although the isolation of the infecting bacterium is imperative to better know the local strains circulating in a geographical region, polymerase chain reaction (PCR) assays can be helpful for an easier and rapid detection of leptospiral DNA in a wide variety of clinical samples such as serum, aborted foetuses, cerebrospinal fluid and urine, and environmental samples [5-17]. However, most PCR assays only differentiate between pathogenic and non-pathogenic leptospires and the identification of the infecting species is not usually directly achieved [18].

Recently we developed a multi-gene targeted real time PCR approach for the detection and differentiation of pathogenic *Leptospira* [19]. The assay was able to detect and differentiate *Leptospira interrogans*, *L. kirschneri*, *L. borgpetersenii* and *L. noguchii*, which constitute the veterinary most relevant pathogenic species of *Leptospira*. In this work we extended the panel of species-specific probes in order to additionally detect the important *L.*

santarosai and *L. weilii*, and assessed the efficiency of the extended assay for the detection of leptospiral infection in a large set of rodent kidney tissues.

3.3. Materials and Methods

3.3.1. Bacterial strains

Sixty seven reference reference strains and clinical and environmental isolates of *Leptospira* spp. belonging to pathogenic, intermediate and non-pathogenic phylogenetic clades were used in this study to validate the novel real time PCR assays targeting *L. santarosai* and *L. weilii* as shown in Table 3.S1 available under supporting information of this chapter. These strains were also previously used for validating the *L. interrogans*, *L. kirschneri*, *L. borgpetersenii* and *L. noguchii* probes [19]. Strains were obtained from the collections maintained by the *Instituto Nacional de Investigação Agrária e Veterinária* (INIAV), Portugal, which is the Portuguese reference laboratory for animal diseases, from the Leptospirosis Laboratory at the *Instituto de Higiene e Medicina Tropical* (IHMT/UNL), Portugal, and from the WHO/FAO/OIE and National Leptospirosis Reference Centre in Amsterdam, The Netherlands. Details about the culture and genomic DNA extraction from these strains can be found in Ferreira et al. [19].

3.3.2. Tissue samples

One hundred and fifty-four kidney samples from *Apodemus sylvaticus, Crocidura russula*, *Mus musculus, Mus spretus, Rattus norvegicus* and*, Rattus rattus* were used in this study for the detection of leptospires using real time PCR assays. Animals were captured in a previous study from several cattle farms in the North of Portugal [20], from July 2002 to August 2004, and the tissue samples were kindly provided by the Department of Veterinary Sciences of *Universidade de Trás-os-Montes e Alto Douro*, Portugal. *M. musculus* was the most abundant species captured (~ 83%), followed by *M. spretus* (~ 8%), *R. norvegicus* (~ 6%), *A. sylvaticus* (~ 1%), *R. rattus* (~ 1%) and *C. russula* (~ 1%), and details about the capture of the rodents and collection of tissue samples are

described by Paiva-Cardoso [20]. Frozen kidneys samples were thawed and, approximately, 200 mg of kidney tissue were suspended in 0.6 ml of cell lysis buffer (Citogene® genomic DNA purification kit, Citomed, Portugal) and used for DNA extraction according to the manufacturer's instructions, with a final elution volume of 50 µl. Genomic DNA suspensions were stored at -20ºC until further use. Additional samples of kidney were kindly provided by the Thymus Development and Function Laboratory of the Institute for Molecular and Cell Biology, Porto, Portugal, and used as negative controls.

3.3.3. Probes and primers

Species-specific probes and respective flanking primers targeting *L. interrogans*, *L. kirschneri*, *L. borgpetersenii* and *L. noguchii* were previously described [19]. Novel probes and primers were developed in this study targeting *L. santarosai* and *L. weilii*. Nucleotide sequences of relevant *Leptospira* species were retrieved from NCBI-GenBank database and aligned using MEGA software version 5.1 [21]. A comparative analysis was performed and specific primers and probes (*TaqMan* probes) were designed targeting the *rrs* and *secY* genes of respectively *L. santarosai* and *L. weilii*. The specificity of primers and probes were assessed *in silico* by comparing their sequences with sequences in GenBank using BLAST tools (http://www.ncbi.nlm.nih.gov/). All probes and primers used in this study are described in Table 3.1. and were synthesized by MWG Biotech (Germany).

3.3.4. Real time PCR assays

Amplification assays were mostly performed as previously described [19], with some modifications. For the analysis of tissues samples, the first step was the detection of pathogenic leptospires with the duplex reaction *β-actin/lipL32*. In our previous study [19], the *lipL32*-positive samples were subsequently tested with the individual species-specific assays (for a total of four probe/primers sets targeting respectively *L. interrogans*, *L. noguchii*, *L. borgpetersenii* and *L. kirschneri*). In order to reduce the number of real time PCR reactions to

perform, with the addition of two novel probes targeting *L. santarosai* and *L. weilii*, we optimized the duplex use of combined probe/primer sets (Table 3.1.). Probes were combined mainly according to their optimal annealing temperatures. The CFX96 real-time PCR detection system (Bio-Rad, USA) was used for all assays, also using the SsoFast™ Probes Supermix (Bio-Rad, USA), according to the manufacturer's instructions. Each reaction was conducted in a total volume of 20 µl consisting of 1× SsoFast™ Probes Supermix, 400 nM of each primer, 150 nM of each TaqMan® probe, DNase free water (GIBCO) and 5 µl of DNA template solution. Amplification conditions were: 95ºC for 2 min, followed by 45 cycles of 5 s at 95ºC and 35 s at the optimized annealing temperature for each set of duplex reactions (Table 3.1.). Positive controls (with tissue infected with *Leptospira* DNA as template) and negative controls (PCR grade water as template) were included in each PCR run. Amplification assays were performed in triplicate and considered to be positive when at least one reaction showed a positive cycle-to-threshold (Ct) value (≤ 39 cycles).

3.3.5. Sequencing

DNA extracts from selected kidney samples were used for the amplification and sequencing of a 245 bp segment of *secY* gene, as described by Victoria et al. [22], using primers SecYII (5'-GAA TTT CTC TTT TGA TCT TCG-3') and SecYIV (5'-GAG TTA GAG CTC AAA TCT AAG-39). Sequencing was performed by STAB VIDA Genomics LAB, (Caparica, Portugal) and nucleotide sequences were compared to sequences of reference strains retrieved from NCBI-GenBank using MEGA software version 5.0. [21].

3.3.6. Statistical analysis

Diagnostic sensitivity, specificity, and kappa coefficient [for measuring the agreement between the method of bacteriological culture [20] and the real-time PCR assays] were computed using the clinical research calculators of the online VassarStats software (http://vassarstats.net).

Table 3.1. Real time PCR assays and respective primers and probes used in this study targeting pathogenic species of *Leptospira*.

Duplex assay	Annealing temperature	Primer/Probe	Sequence (5'- 3')	Complementary target species
β-actin/lipL32	60 ºC	F_Actin[1]	GGC TCY ATY CTG GCC TC	*β-actin* gene of mammals
		R_Actin[1]	GCA YTT GCG GTG SAC RAT G	
		P_Actin[1]	Cy5.5 (Quasar 705) -TAC TCC TGC TTG CTG ATC CAC ATC-BHQ2	
		45F[2]	AAG CAT TAC CGC TTG TGG TG	*lipL32* gene of pathogenic *Leptospira*
		286R[2]	GAA CTC CCA TTT CAG CGA TT	
		taq-189P[2]	FAM-AAA GCC AGG ACA AGC GCC G-BHQ1	
Lint/Lnog	63 ºC	PFLint2[3]	CTT GAG CCT GCG CGT TAY C	*secY* gene of *L. interrogans*
		PRLint2[3]	CCG ATA ATT CCA GCG AAG ATC	
		TaqLint2[3]	TET-CTC ATT TGG TTA GGA GAA CAG ATC A-BHQ1	
		FLnog2[3]	TCA GGG TGT AAG AAA GGT TC	*secY* gene of *L. noguchii*
		RLnog2[3]	CAA AAT TAA AGA AGA AGC AAA GAT	
		TaqLnog[3]	FAM-CGA TTG GCT TTT TGC TTG AAC CATC-BHQ1	
Lborg/Lsant	65 ºC	F_bpn$_3$	GAT TCG GGT TAC AAT TAG ACC	*ompL1* gene of *L. borgpetersenii*
		R_bpn1[3]	TTG ATC TAA CCG GAC CAT AGT	
		TqM_bpn[3]	Cy5.5 (Quasar 705) -TAC TAA GGA TGG TTT GGA CGC TGC-BHQ2	
		PFLsanta	GGG AGC TAA TAC TGG ATA GTC C	*rrs* gene of *L. santarosai*
		PRLsanta	TTA CCT CAC CAA CTA GCT AAT CG	
		TqMsanta	Cy5- CAA TGA ATC TTT ACC CGA TAC ATC C-BHQ2	
Lkir/Lwei	60 ºC	F_nery[3]	CTG GCT TAA TCA ATG CTT CTG	*secY* gene of *L. kirschneri*
		R_nery[3]	CTC TTT CGG TGA TCT GTT CC	
		TqM_nery[3]	Texas Red-CAG TTC CAG TTG TAA TAG ATA AGA TTC-BHQ2	
		Fweil	TGG ATT GGC ATT AGC ACC TTA T	*secY* gene of *L. weilii*
		Rweil	CGT GGA AAT YTG AGG GAT AG	
		Lweil	HEX-CAG GCA AAR ATT CTT TGC ACA AAG C-BHQ1	

[1]Costa et al. [28]; [2]Stoddard et al. [14]; [3]Ferreira et al. [19]

3.4. Results

The analytical specificity and sensitivity of the novel real time PCR probes and primers targeting *L. santarosai* and *L. weilii* were evaluated with 67 *Leptospira* strains belonging to eleven different species (pathogenic, intermediate and non-pathogenic). Strong fluorescence signals were only obtained with reactions containing *L. santarosai* and *L. weilii* DNA templates tested with the corresponding species-specific targeted probe and primers (Table 3.S1.).

The results of the *lipL32*-targeted real time PCR assays for the 154 kidneys samples analyzed in this study are summarized in Table 3.2. The *lipL32* gene was detected in 17 (11%) samples. Results for the culture-based detection of leptospires from these samples and serological characterization of the isolates were also available from Paiva-Cardoso [20] (Table 3.2.). Thirteen samples were positive by both culture and real time PCR detection methods and seven samples showed discrepant results (Table 3.2.). All remaining 134 (87%) samples were negative for the presence of pathogenic leptospires by both methods (Table 3.2) and others presented in the study of Paiva-Cardoso [20], such as LipL21, LipL32 and G1/G2-based conventional PCR assays. The control *β-actin* mammal gene was amplified from all samples, which means that reactions were not significantly inhibited by the occurrence of potential contaminants.

Table 3.2. Results of culture and *lipL32*-targeted real time PCR assay for the detection of *Leptospira* in rodents kidney samples.

Number of samples	**Culture**[1]	***lipL32***[2]
13	+	+
3	+	-
4	-	+
134	-	-

[1]Data retrieved from Paiva-Cardoso [20]; [2]Amplification signal detected with the *lipL32*-targeted real time PCR assay

Considering culture as a gold standard diagnostic method, the *lipL32*-targeted PCR assay showed a diagnostic specificity and sensitivity of respectively 97.1% and 81.3% for the detection of pathogenic leptospires in tissue samples. An observed kappa coefficient was estimated in 0.762 ($CI_{P95\%}$ 0.605 - 0.920) for the overall agreement between the real time PCR assay and the bacteriological culture.

Table 3.3. shows details about all kidneys samples that yielded a positive result for the detection of pathogenic leptospires, either by bacteriological analysis and/or by *lipL32*-targeted real time PCR. All samples yielding *lipL32* positive detection were further tested with the *Leptospira* species-specific duplex real time PCR assays (Table 3.3.; Figure 3.S1.). Two *M. musculus* samples were found to be infected with *L. kirschneri*, and eight *M. musculus* and two *M. spretus* samples were found to be infected with *L. borgpetersenii* (Table 3.3.). One sample (Rim125) gave positive amplification signals for both *L. kirschneri* and *L. borgpetersenii* probes, potentially representing a mixed leptospiral infection (Table 3.3.). Additionally, four samples with a positive *lipL32* amplification, but culture negative, did not show any amplification signals with species-specific probes (Table 3.3.). No isolates or positive PCR results were obtained from *A. sylvaticus*, *R. rattus* and *C. russula* samples.

Partial *secY* sequences were obtained from samples Rim13, Rim 125 and Rim139. The nucleotide sequences from samples Rim13 and Rim139 were fully identical to other sequences retrieved from NCBI-Genbank representing, respectively, *L. borgpetersenii* and *L. kirschneri*. The sample Rim125 was suspect of harboring mixed infection with *L. kirschneri* and *L. borgpetersenii*, after the real time PCR assays with species-specific probes (Table 3.3.). The *secY* sequence obtained from sample Rim125 did not show a good quality, with several overlapping peaks, which may corroborate the hypothesis of a mixed infection.

Table 3.3. Origin of samples showing culture and/or *lipL32* amplification positive results and respective results after being tested with *Leptospira* species-specific real time PCR.

Sample	Host[1]	Place of colection[1]	Culture[1]	Serogroup[1]	Serovar[1]	*lipL32*	*L. interrogans*	*L. borgpetersenii*	*L. kirschneri*	*L. noguchii*	*L. santarosai*	*L. weilii*
Rim109	*M. musculus*	Vreia de Jales, Vila Pouca de Aguiar	-	nd	nd	+	-	-	-	-	-	-
Rim190	*M. musculus*	Adoufe, Vila Real	-	nd	nd	+	-	-	-	-	-	-
Rim193	*M. musculus*	Adoufe, Vila Real	-	nd	nd	+	-	-	-	-	-	-
Rim196	*M. spretus*	Borbela, Vila Real	-	nd	nd	+	-	-	-	-	-	-
Rim139	*M. musculus*	Agarez, Vila Real	+	Pomona	Altodouro[2]	+	-	-	+	-	-	-
Rim201	*M. musculus*	Testeira, Vila Real	+	Pomona	Altodouro[2]	+	-	-	+	-	-	-
Rim130	*M. musculus*	Lixa do Alvão, Vila Pouca de Aguiar	+	Unknown	Unknown	+	-	+	-	-	-	-

Table 3.3. (cont.)

Sample	Host[1]	Place of colection[1]	Culture[1]	Serogroup[1]	Serovar[1]	*lipL32*	*L. interrogans*	*L. borgpetersenii*	*L. kirschneri*	*L. noguchii*	*L. santarosai*	*L. weilii*
Rim156	*M. musculus*	Pena, Vila Real	+	Sejroe	Saxkoebing	+	-	+	-	-	-	-
Rim13	*M. musculus*	Pereira, Vila Real	+	Ballum	Ballum	+	-	+	-	-	-	-
Rim33	*M. musculus*	Pereira, Vila Real	+	Ballum	Ballum	+	-	+	-	-	-	-
Rim69	*M. musculus*	Aveção do Cabo, Vila Real	+	Ballum	Ballum	+	-	+	-	-	-	-
Rim165	*M. musculus*	Agarez, Vila Real	+	Ballum	Ballum	+	-	+	-	-	-	-
Rim203	*M. musculus*	São Mamede, Vila Real	+	Ballum	Ballum	+	-	+	-	-	-	-
Rim283	*M. musculus*	Bragadas, Ribeira de Pena	+	Ballum	Ballum	+	-	+	-	-	-	-
Rim90	*M. spretus*	Vila Seca, Vila Real	+	Ballum	Ballum	+	-	+	-	-	-	-

Table 3.3. (cont.)

Sample	Host[1]	Place of colection[1]	Culture[1]	Serogroup[1]	Serovar[1]	*lipL32*	*L. interrogans*	*L. borgpetersenii*	*L. kirschneri*	*L. noguchii*	*L. santarosai*	*L. weilii*
Rim102	*M. spretus*	Sanguinhedo, Vila Real	+	Ballum	Ballum	+	-	+	-	-	-	-
Rim125	*M. spretus*	Sanguinhedo, Vila Real	+	Unknown	Unknown	+	-	+	+	-	-	-
Rim70	*M. musculus*	Aveção do Cabo, Vila Real	+	Ballum	Ballum	-	*	*	*	*	*	*
Rim143	*M. musculus*	Pena, Vila Real	+	Unknown	Unknown	-	*	*	*	*	*	*
Rim144	*R. norvegicus*	Agarez, Vila Real	+	Ballum	Ballum	-	*	*	*	*	*	*

[1]Data retrieved from Paiva-Cardoso [20]; [2]Paiva-Cardoso et al. [28]; nd - not done; *Species-specific real time PCR assays not performed

3.5. Discussion

The current study sought to expand and evaluate the performance of a probe-based real time PCR assay to directly detect and differentiate pathogenic *Leptospira* in animal tissue samples. A large number of kidney tissues was used in this study. These samples were originally collected from rodents with origin in the north of Portugal [20]. The bacteriological culture tests originally performed by Paiva-Cardoso [20] were used as a gold standard test to assess the diagnostic sensitivity and specificity of the PCR assay. The first step of our assay, the *lipL32*-targeted PCR, showed a high sensitivity and specificity for the detection of pathogenic leptospires when compared with the gold standard method. We found the *lipL32* gene in 17 out of 154 (11%) tissues samples, which were considered to be infected with pathogenic leptospires. An observed kappa coefficient was estimated in 0.762 for the overall agreement between the real time PCR assay and the bacteriological culture. Although the criteria for judging kappa statistic are not completely objective nor universally accepted, this value may allow us to infer a "substantial" agreement between the two methods [23]. Noteworthy, a few discrepancies were found between the bacteriological culture and the real time PCR assay (Table 3.1.). Three samples were culture positive but PCR negative. False-negative PCR results may happen when inhibitors are present in the samples or the template DNA is degraded, e.g. due to prolonged or inadequate storage of samples (although in our case a normal *β-actin* mammal control gene amplification was reported for all samples). For example, Subharat et al. [24] reported a higher sensitivity when detecting leptospiral DNA using fresh samples, when compared with the use of DNA templates extracted from previously frozen samples. Four samples were culture negative but PCR positive. Previous studies reported a high sensitivity compared with culture when PCR was applied to biological samples [4, 5, 25, 26]. However, it should be noted the full agreement between our results and the PCR-based results obtained by Paiva-Cardoso [20] concerning the molecular detection of leptospires.

In the second step of our approach, a total of six most important pathogenic *Leptospira* species may be detected and differentiated: *L. interrogans*, *L.*

borgpetersenii, *L. kirschneri*, *L. noguchii*, *L. santarosai* and *L. weilii*. This study extended the panel of species-specific probes and primers for detecting *L. weilii* and *L. santarosai*, which constitutes an upgrade to our previous study [19]. Developed probe and primers sets proved to be specific when tested with strains of the targeted species (*L. weilii* or *L. santarosai*), showing no cross-reactions with other pathogenic or non-pathogenic leptospires (Table 3.S1.). The real time PCR assays were able to detect pathogenic leptospires in kidney samples and the species-specific assays showed the presence of two distinct species: *L. borgpetersenii*, and *L. kirschneri*, corroborating the previous culture-based studies from Paiva-Cardoso [20] who used the same tissue samples. Previously, [27, 28] also reported *L. borgpetersenii* and *L. kirschneri* among the most prevalent *Leptospira* pathogenic species infecting rodents in Portugal. One sample (Rim125) showed positive amplification results for both *L. borgpetersenii* and *L. kirschneri* real time PCR assays (Table 3.3.). These results suggest that this sample potentially harbored a mixed leptospiral infection. We tried to sequence a segment of the *secY* gene using DNA extracted from this sample as template. If there was a mixed infection, we should expect to obtain a bad quality sequence due to the presence of overlapping base peaks, as a result of the presence of at least two different gene fragments originating from different *Leptospira* species. This was the case for the Rim125 sample. Also corroborating the hypothesis of a mixed infection, Paiva-Cardoso [20] was not able to serotype the isolate collected from Rim125 sample (Table 3.3.). The incongruence of serotyping results may also potentially arise from the occurrence of a mixed culture. Although the occurrence of mixed infections with different species of *Leptospira* should be feasible, since animals are hosts for distinct leptospiral species, we know of no published report describing such situation.

There are several published reports using hydrolysis probes to detect pathogenic leptospires but most are only directed towards the discrimination between pathogenic and non-pathogenic *Leptospira* species [13-16]. Our two-step approach allows a simple, rapid and robust identification of the most commonly infecting *Leptospira* species, becoming an enhanced tool for the study of the epidemiology of leptospirosis.

3.6. Acknowledgments

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3.7. Supporting Information

Table 3.S1. *Leptospira* strains used in the present study and results of the real time PCR assays using the *L. santarosai* and *L. weilii* species-specific probes and flanking primers.

Species	Serogroup	Serovar	Strain	Source[1]	*L. santarosai* [2]	*L. weilii* [3]
L. interrogans	Australis	Muenchen	München C 90	KIT	-	-
	Australis	Australis	Ballico	KIT	-	-
	Australis	Bratislava	Jez Bratislava	INIAV	-	-
	Canicola	Canicola	Hond Utrecht IV	INIAV	-	-
	Hebdomadis	Hebdomadis	Hebdomadis	KIT	-	-
	Icterohaemorrhagiae	Birkini	Birkin	KIT	-	-
	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA	INIAV	-	-
	Icterohaemorrhagiae	Lai	Lai	KIT	-	-
	Pomona	Pomona	Pomona	INIAV	-	-
	Pyrogenes	Pyrogenes	Salinem	INIAV	-	-
	Sejroe	Hardjo type Prajitno	Hardjoprajitno	IHMT	-	-
L. borgpetersenii	Ballum	Ballum	Mus 127	INIAV	-	-
	Hebdomadis	Jules	Jules	KIT	-	-
	Javanica	Ceylonica	Piyasena	KIT	-	-

Table 3.S1. (cont.)

L. borgpetersenii	Javanica	Poi	Poi	INIAV	-	-
	Javanica	Zhenkang	L 82	KIT	-	-
	Pyrogenes	Kwale	Julu	KIT	-	-
	Sejroe	Hardjo type bovis	Sponselee	KIT	-	-
	Sejroe	Hardjo type bovis	L550	KIT	-	-
	Sejroe	Hardjo type bovis	JB197	KIT	-	-
	Sejroe	Sejroe	M84	KIT	-	-
	Tarassovi	Kisuba	Kisuba	KIT	-	-
L. kirschneri	Australis	Ramisi	Musa	KIT	-	-
	Autumnalis	Bulgarica	Nicolaevo	KIT	-	-
	Autumnalis	Butembo	Butembo	KIT	-	-
	Cynopteri	Cynopteri	3522C	IHMT	-	-
	Grippotyphosa	Grippotyphosa type Moskva	Moskva V	IHMT	-	-
	Grippotyphosa	Ratnapura	Wumalasena	KIT	-	-
	Grippotyphosa	Vanderhoedeni	Kipod 179	KIT	-	-
	Icterohaemorrhagiae	Bogvere	LT 60-69	KIT	-	-
	Pomona	Mozdok	5621	KIT	-	-
	Pomona	Tsaratsovo	B 81/7	KIT	-	-

Table 3.S1. (cont.)

L. noguchii	Australis	Nicaragua	1011	KIT	-	-
	Autumnalis	Fortbragg	Fort Bragg	KIT	-	-
	Bataviae	Argentiniensis	Peludo	KIT	-	-
	Djasiman	Huallaga	M 7	KIT	-	-
	Louisiana	Louisiana	LSU 1945	KIT	-	-
	Panama	Panama	CZ 214	INIAV	-	-
	Pomona	Proechimys	1161 U	KIT	-	-
	Pyrogenes	Myocastoris	LSU 1551	KIT	-	-
	Shermani	Carimagua	9160	KIT	-	-
L. santarosai	Ballum	Peru	MW 10	KIT	+	-
	Bataviae	Balboa	735 U	KIT	+	-
	Bataviae	Kobbe	CZ 320	KIT	+	-
	Grippotyphosa	Canalzonae	CZ 188	KIT	+	-
	Hebdomadis	Maru	CZ 285	KIT	+	-
	Javanica	Fluminense	Aa 3	KIT	+	-
	Mini	Beye	1537 U	KIT	+	-
	Sarmin	Rio	Rr 5	KIT	+	-
	Sejroe	Guaricura	Bov.G.	KIT	+	-
	Shermani	Shermani	1342 K	KIT	+	-

Table 3.S1. (cont.)

L. weilii	Celledoni	Celledoni	Celledoni	INIAV	-	+
	Celledoni	Mengding	M 6906	KIT	-	+
	Javanica	Coxi	Cox	KIT	-	+
	Javanica	Mengma	S 590	KIT	-	+
	Javanica	Mengrun	A 102	KIT	-	+
	Mini	Hekou	H 27	KIT	-	+
	Sarmin	Sarmin	Sarmin	KIT	-	+
L. alexanderi	Hebdomadis	Manzhuang	A 23	KIT	-	-
	Javanica	Mengla	A 85	KIT	-	-
	Manhao	Manhao 3	L 60	KIT	-	-
L. meyeri	Ranarum	Ranarum	ICF	KIT	-	-
	Semaranga	Semaranga	Veldrat Semaranga 173	KIT	-	-
L. inadai	Manhao	Lincang	L 14	KIT	-	-
L.fainei	Hurstbridge	Hurstbridge	BUT 6T	KIT	-	-
L.biflexa	Andaman	Andamana	CH 11	KIT	-	-
	Semaranga	Patoc	Patoc I	KIT	-	-

[1]INIAV - Instituto Nacional de Investigação Agrária e Veterinária, Lisbon, Portugal. IHMT - Instituto de Higiene e Medicina Tropical, Lisbon, Portugal. KIT - Royal Tropical Institute, Amsterdam, The Netherlands; [2]Assay targets the *rrs* gene of *L. santarosai*; [3]Assay targets the *secY* gene of *L. weilii*; Amplification (+) or no amplification (-).

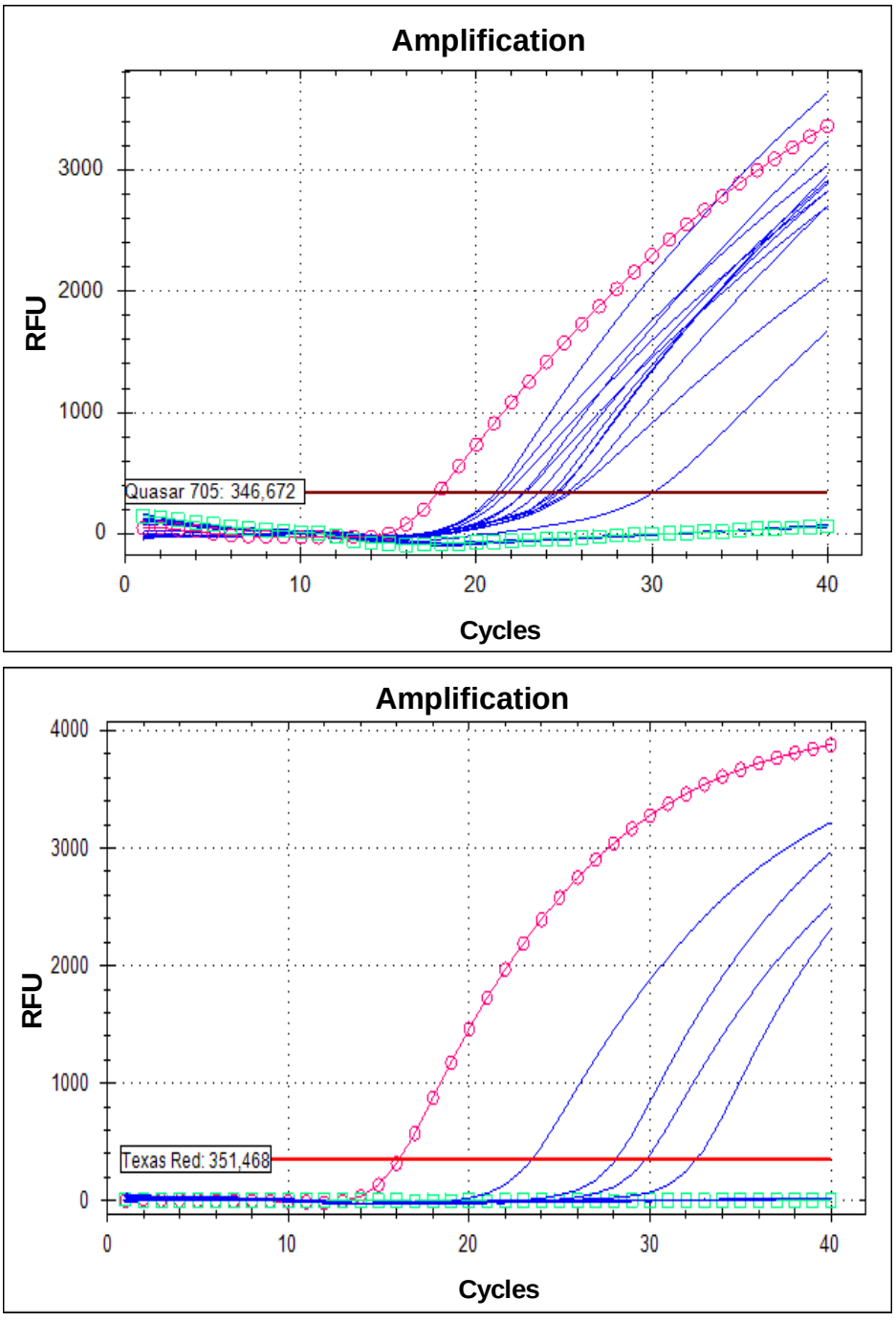

Figure 3.S1. Illustration of the species-specific real-time PCR amplification curves obtained after testing infected kidney samples positive for the *lipL32* gene. (A) Amplification results obtained with the *L. borgpetersenii* targeted real time PCR assay. **(B)** Amplification results obtained with the *L. kirschneri* targeted real time PCR assay. Blue lines - kidney samples; Pink line - positive control; Green line - negative control.

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CHAPTER 4

Genetic diversity of pathogenic leptospires from wild, domestic and captive host species in Portugal

4. Genetic diversity of pathogenic leptospires from wild, domestic and captive host species in Portugal

Manuscript in preparation

4.1. Summary

Leptospirosis is an important yet underestimated and neglected zoonotic disease caused by pathogenic *Leptospira* species, with a worldwide distribution and posing significant challenges to the veterinary and public health. The availability of effective tools to accurately identify and type infecting leptospires is of utmost importance for the diagnosis of the disease and for assessing its epidemiology. Several Multi-Locus Sequence Typing (MLST) approaches were described for the typing of global isolates of *Leptospira* and an extensive agreement towards the adoption of a unique consensus scheme for this agent is still lacking. Additionally, the public databases supporting these MLST schemes still suffers from a small number of *Leptospira* strains typed, and there is a large disequilibrium in the number of representative isolates from different regions. Most strains currently typed have their origins in a few Asian and South American countries, with only a minority of the isolates from Europe (being most countries represented only by one or a few isolates). In this study we revisited several isolates of pathogenic *Leptospira* collected from domesticated, wild and captive animals in Portugal, spanning the period 1990 to 2012, and a selection of these isolates was genotyped using three previously published MLST schemes. The most useful MLST approach for typing the Portuguese isolates was the $7L_{Boonsilp}$ scheme supported by the online database available at http://leptospira.mlst.net, denoted as *Leptospira* database throughout this chapter. A total of seven distinct sequence types (STs) was detected among the Portuguese isolates, with two STs representing *L. borgpetersenii* (ST149 and ST152), two STs representing *L. kirschneri* (ST117 and a new ST) and three STs representing *L. interrogans* (ST17, ST24 and ST140), and most distinct serovars were assigned a distinct sequence type. Global widespread (and maybe more virulent) *Leptospira* genotypes seem to circulate in Portugal, particularly the *L. interrogans* ST17 isolates which are associated with several outbreaks of leptospirosis among humans and animals in different regions of the world. This study makes a contribution for enriching the global MLST databases with a new set of allele and sequence type information regarding Portuguese (European) pathogenic *Leptospira* isolates.

4.2. Introduction

Leptospirosis is an important yet underestimated and neglected zoonotic disease with a worldwide distribution posing significant challenges to the veterinary and public health [1, 2]. The agents of this disease are helically coiled gram negative bacteria of the genus *Leptospira*. The epidemiological cycle of leptospirosis depends upon the circulation of the agents among animal reservoirs which establish persistent renal carriage and urinary shedding of pathogenic *Leptospira* cells [2]. Many species can act as reservoirs but small mammals and wild rodents are usually considered most important. Domestic animals such as bovines [3] and pigs [4] can also become chronic renal carriers after infection [5, 6]. In livestock, leptospirosis is an important cause of abortion, stillbirths, infertility, poor milk production and death, all of which cause important economic losses [7]. Both wild and domesticated animals can be a source of infection for humans, which are incidental hosts, acquiring a systemic infection after direct or indirect exposure to the urine of infected animals, particularly rodents. Pathogenic *Leptospira* penetrate skin and mucous membranes, causing a febrile illness. A subset of the patients develops severe manifestations known as Weil's disease [1, 2].

Traditionally, free-living environmental saprophytic and host-requiring pathogenic leptospires have been assigned respectively to *Leptospira biflexa* and *Leptospira interrogans* presently referred to as *sensu lato* (s.l.), due to the existence of genomospecies with the same names, that are referred to as *sensu stricto*. More than 250 serovars were recognised within the traditional circumscription of *L. interrogans* [8]. Serovars have been considered the basic taxon of leptospires which, can be determined by cross agglutinin absorption test (CAAT), on the basis of the structural heterogeneity of the leptospiral lipopolysaccharide [9]. These leptospiral serological entities may demonstrate specific, although not entirely exclusive, host preferences (e.g. cattle and swine are reservoirs for Hardjo and Pomona serovars, respectively) [2, 10], and are grouped into serogroups presenting antigenically related characteristics [2, 8]. Serogroups are determined by the Microscopic Agglutination Test (MAT) [10].

Leptospirosis has been confirmed as a relevant public health concern in Portugal, particularly in the central mainland and in the semi-tropical São Miguel and Terceira islands (in Azores) [1]. Serovars Copenhageni (serogroup Icterohaemorrhagiae) and Arborea (serogroup Ballum) are known to circulate in the endemic areas, by kidney isolation from rodents (*Mus* spp. and *Rattus* spp.) and European hedgehog (*Erinaceus europaeus*) [11], and also from humans [12]. Isolates of serogroup Pomona also occur in mainland Portugal, being detected both in domestic animals, such as pigs and horses, and wild animals, particularly rodents [4, 11, 13-15]. Strains belonging to serogroups Icterohaemorragiae, Sejroe (serovar Hardjo), Australis (serovar Bratislava) and Canicola (serovar Canicola) have also been recovered from domestic animals in Portugal such as cattle, pigs, horses and dogs [1, 3, 4, 13, 16]. A recent study highlighted the presence of antibodies against nine pathogenic serovars of *Leptospira* (Tarassovi, Altodouro, Autumnalis, Bratislava, Copenhageni, Mozdok, Arboreae, Ballum, Icterohaemorrhagiae) in the wild boar (*Sus scrofa*) population from northern Portugal [17]. Previous serological and bacteriological evidence also pointed to the widespread occurrence of leptospirosis in Portuguese farm animals, particularly in cattle and pigs [15].

Molecular approaches have been increasingly used to study the taxonomy of leptospires and, currently, 22 distinct *Leptospira* species were formally described consisting of three clades comprising ten pathogenic, five intermediate and seven saprophytic species [18-20]. Noteworthy, the molecular and serological classification of leptospires show little correlation. For example, serovars of the same serogroup can be found in a single or more different species, suggesting that the genes determining serotype may be transferred horizontally among these bacteria [8, 9]. *Leptospira interrogans*, *L. kirschneri* and *L. borgpetersenii* are associated to most leptospirosis cases worldwide [21, 22], although the clinical manifestations caused by different species may be identical.

The availability of effective tools to accurately identify and type infecting leptospires is of utmost importance for the diagnosis of the disease and for assessing its epidemiology. Phylogeny-based genotyping has been described for leptospires using sequences of several genes such as *rrs* (16S ribosomal

RNA), *secY* (translocase preprotein secY), *gyrB* (DNA gyrase subunit B), *flaB* (flagellar protein B), *ligB* (leptospiral immunoglobulin-like gene) and *rpoB* (RNA polymerase beta-subunit), some of these genes presenting a high discriminative power [18, 23-26]. However, as mentioned above, horizontal gene transfer has been described for *Leptospira* [27] and thus the use of typing methods targeting multiple genomic targets is required in order to limit the risk of misclassification. Multi-locus sequence typing (MLST) is one of such methods, and currently the most robust one. The first *Leptospira* MLST scheme has been developed in the early 2000s and uses six loci (6L scheme) [28]. This 6L scheme does not conform to the original concept of MLST as it includes a non-housekeeping gene (*rrs*), and genes that encode cell surface proteins (*LipL32* and *LipL41*), but has the advantage that it can be applied on all pathogenic species of *Leptospira* [29]. Later, more conventional alternative MLST approaches were developed, targeting seven housekeeping genes (7L) that were distributed across the genome and were not under positive selection [30, 31]. These 7L MLST schemes were originally described for application with *L. interrogans* and, to some extent, the closely related species *L. kirschneri*. The 7L scheme originally described by Thaipadungpanit et al. [30] was further expanded for the genotyping of additional pathogenic species, namely *L. borgpetersenii*, *L. noguchii*, *L. santarosai*, *L. weilii* and *L. alexanderi* ($7L_{Boonsilp}$ scheme) [29, 32]. A comparison of the 7L [30] and the 6L [28] MLST schemes showed that both approaches mostly yielded comparable results [33]. More recently, a modified 7L MLST scheme was proposed by using a novel combination of target genes originally used in the 6L and $7L_{Boonsilp}$ schemes [34].

Clearly, a consensus MLST scheme for the typing of global isolates of *Leptospira* must be agreed by the scientific and medical community devoted to the study of this agent. Additionally, the main studies setting and using these MLST schemes, and the respective online databases, still suffers from a small number of *Leptospira* strains typed, and there is a large disequilibrium in the number of representative isolates from different regions and countries. For example, for the 6L MLST scheme [28, 35], most of the *Leptospira* isolates typed (> 70%) were originally collected from Asian and South American countries, particularly from India and Brazil, with only around 13% of European

isolates. Similarly, more than 77% of the isolates typed with the $7L_{Boonsilp}$ MLST scheme [30, 32] were originally collected from Asian countries, with a major proportion of isolates from Thailand and China, and only around 8% of the isolates were collected from European countries (being most countries only represented by one or two isolates).

In this study we revisit several isolates of pathogenic *Leptospira* collected from domesticated (*Bos taurus*, *Sus scrofa domesticus* and *Equus caballus*), wild (*Mus musculus* and *Rattus norvegicus*) and captive (*Callithrix jacchus*, *Suricata suricatta*, *Lemur catta* and *Dolichotis patagonum*) animals in Portugal, spanning the period 1990 to 2012. A selection of these isolates was genotyped with MLST schemes based in the above mentioned 6L [28, 33, 35], $7L_{Boonsilp}$ [32] and modified 7L ($7L_{Varni}$) [34] schemes. We aimed to assess the feasibility of using these three MLST schemes, and of the respective available online databases, for genotyping Portuguese *Leptospira* isolates, to evaluate the genetic diversity of pathogenic leptospires circulating in Portugal, and ultimately to feed the relevant MLST online databases with a new set of allele and sequence type information regarding European isolates.

4.3. Materials and Methods

4.3.1. Pathogenic *Leptospira* isolates

Twenty seven *Leptospira* isolates were analyzed in this work which are maintained in the collections of *Instituto de Higiene e Medicina Tropical* (IHMT) and *Instituto Nacional de Investigação Agrária e Veterinária, IP* (INIAV, IP), in Lisbon, and Universidade de Trás-os-Montes e Alto Douro (UTAD), in Vila Real, Portugal (Table 4.1.). Some of these strains were previously mentioned in international publications (Table 4.1.). Isolates were cultured in EMJH medium. Serogroup and serovar information for strains IHMT A02, IHMT 8A, 16A, 54A, 62B, 71A, 102A, 105A, 214A, 216A, 227B and UTAD Rim283, Rim156, Rim 139 was obtained from these institutes (Table 4.1.). The serogroup and serovar for all remaining isolates were tested (or retested) at the WHO/FAO/OIE and National Collaborating Centre for Reference and Research on Leptospirosis,

Royal Tropical Institute (KIT), Amsterdam, The Netherlands, according to the standard serological methods used in this reference laboratory (Table 4.1.).

4.3.2. DNA extraction

Leptospira isolates were cultivated in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium according to the standard protocol, genomic DNAs were extracted from pure cultures by using QIAamp DNA extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, with a final elution volume of 200 µl.

4.3.3. Confirmation of *Leptospira* species

The species assignment of the isolates was confirmed by a multi-gene targeted real-time PCR assay using species-specific *TaqMan*® probes as described by Ferreira et al. [36]. This assay allows the differentiation between the pathogenic *L. interrogans*, *L. borgpetersenii*, *L. kirschneri* and *L. noguchii*.
Partial amplification and sequencing of the *secY* gene was achieved for all strains, according to Victoria et al. [26], using primers SecYII (5'- GAA TTT CTC TTT TGA TCT TCG -3') and SecYIV (5'- GAG TTA GAG CTC AAA TCT AAG -3'). Sequencing of the PCR products was performed at Macrogen sequencing facility (Macrogen Inc., Amsterdam, Netherlands).

4.3.4. Multi-locus sequence typing

A subset of 12 isolates was selected for MLST analysis (Table 4.2.). The partial amplification and sequencing of the genes selected for the 6L scheme (*adk*, *icdA*, *lipL41*, *rrs*, *secY* and *lipL32*), originally developed by Ahmed et al. [28] and later slightly improved by Ahmed et al. [33], and for the $7L_{Boonsilp}$ scheme (*glmU*, *pntA*, *sucA*, *tpiA*, *pfkB*, *mreA* and *caiB*) [32] was achieved as detailed by Ahmed et al. [29]. Sequencing of the PCR products was performed at Macrogen sequencing facility (Macrogen Inc., Amsterdam, Netherlands).

4.3.5. Sequence analysis

The *secY* gene sequences were trimmed and aligned using the CLUSTAL X v2.0 software [37]. Additional sequences used in this alignment were retrieved from GenBank (accession numbers are indicated in the phylogenetic tree). A phylogenetic tree was computed using PAUP software (Sinauer Associates, Inc., Sunderland, MA) using the neighbor-joining method and the Kimura two-parameter model for calculating distances. Trees were visualized and edited with Treeview (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

MLST 6L scheme analysis was performed as described by Ahmed et al. [29] with some modifications. Nucleotide sequences for each of the six genes were trimmed to the reference sizes, concatenated according to the order *adk-icdA-lipL32-lipL41-rrs2-secY* and aligned using Clustal X. Additional sequences included in the alignment were retrieved from Nalam et al. [35]. A phylogenetic tree was then computed as described above. At the time of writing the book chapter by Ahmed et al. [29], there was no public database and website associated to the 6L MLST scheme. However, an online database is now available at http://pubmlst.org/leptospira (under scheme#3), denoted in this chapter as *Pubmlst* database. By assessing *Pubmlst* database it is now possible to assign a sequence type (ST) to the isolates, according to their allelic profiles. Sequences used to define the locus for genes *lipL32*, *lipL41* and *secY* were trimmed by approximately 20 bp at either end of the amplified products, as described by Ahmed et al. [29, 33]. These sequences are thus shorter than the sequences used in the above mentioned *Pubmlst* database, which correspond to the original MLST approach [28, 35].

MLST $7L_{Boonsilp}$ scheme analysis was performed as described by Ahmed et al. [29]. Nucleotide sequences for each of the seven genes were trimmed to the reference sizes, concatenated according to the order *glmU-pntA-sucA-tpiA-pfkB-mreA-caiB* and aligned using Clustal X. Additional sequences included in the alignment were retrieved from the public *Leptospira* database http://leptospira.mlst.net and a phylogenetic tree was computed as described above. Sequence types were assigned to the isolates according to their allelic profiles.

Finally, the sequence information obtained for the genes *adk*, *glmU*, *icdA*, *lipL32*, *lipL41*, *mreA* and *pntA* was also used to assign sequence types to the isolates according the modified 7L ($7L_{Varni}$) MLST scheme as proposed by Varni et al. [34], using the online *Pubmlst* database available at http://pubmlst.org/leptospira (under scheme#2).

In this paper, the $7L_{Boonsilp}$, 6L and modified 7L MLST schemes are referred to as $7L_{Boonsilp}$, 6L and $7L_{Varni}$ scheme and associated ST's are coded accordingly , i.e., $ST_{(7LBoonsilp)}$, $ST_{(6L)}$, and $ST_{(7LVarni)}$. Please note that each strain included in this study thus has three STs according to each of the three MLST schemes.

4.4. Results

The results obtained for the assignment of *Leptospira* species using species-specific *TaqMan*® probes are disclosed in Table 4.1. for all the 27 strains studied in this work, as well as the results obtained for the serogroup/serovar determination. The phylogenetic analysis of the *secY* gene corroborated the identification obtained by the real time PCR approach (Table 4.1.; Figure 4.1.). The 14 isolates identified as *L. borgpetersenii* were distributed by two closely related clusters in the *secY* phylogenetic tree (Figure 4.1.): cluster A (containing 10 isolates from cattle, from two Azorean islands); and cluster B (containing 4 isolates from *M. musculus*). Five isolates were identified as *L. kirschneri*, also distributed by two clusters in the phylogenetic tree (Figure 4.1.): cluster C (with 3 isolates from pigs and one isolate from a horse); and cluster D (with one isolate from *M. musculus*). Finally, 8 isolates were identified as *L. interrogans*, distributed by 3 clusters (Figure 4.1.): cluster E (with one isolate from cattle); cluster F (with one isolate from a horse); and cluster G (with 6 isolates from rats and wild captive animals). Noteworthy, with the exception of one isolate from a rat (IHMT A02), all the remaining isolates in cluster G were obtained from animals of the Lisbon zoo (including an infesting rat) (Table 4.1.).

Table 4.1. *Leptospira* strains used in this study and respective species, origin, serogroup/serovar/type and *secY* cluster.

Strain	Species	Origin	Serogroup	Serovar	*secY* cluster[9]
IHMT 8A	*L. borgpetersenii*	*Bos taurus* (São Miguel, Azores, Portugal; 2008)	Sejroe	Hardjo, Hardjo-bovis	A
IHMT 16A	*L. borgpetersenii*	*Bos taurus* (São Miguel, Azores, Portugal; 2008)	Sejroe	Hardjo, Hardjo-bovis	A
IHMT 102A	*L. borgpetersenii*	*Bos taurus* (São Miguel, Azores, Portugal; 2008)	Sejroe	Hardjo, Hardjo-bovis	A
IHMT 54A	*L. borgpetersenii*	*Bos taurus* (Terceira, Azores, Portugal; 2008)	Sejroe	Hardjo, Hardjo-bovis	A
IHMT 62B	*L. borgpetersenii*	*Bos taurus* (Terceira, Azores, Portugal; 2008)	Sejroe	Hardjo, Hardjo-bovis	A
IHMT 71A	*L. borgpetersenii*	*Bos taurus* (Terceira, Azores, Portugal; 2008)	Sejroe	Hardjo, Hardjo-bovis	A
IHMT 105A	*L. borgpetersenii*	*Bos taurus* (Terceira, Azores, Portugal; 2008)	Sejroe	Hardjo, Hardjo-bovis	A
IHMT 214A	*L. borgpetersenii*	*Bos taurus* (Terceira, Azores, Portugal; 2008)	Sejroe	Hardjo, Hardjo-bovis	A

Table 4.1. (cont.)

Strain	Species	Origin	Serogroup	Serovar	*secY* cluster[9]
IHMT 216A	*L. borgpetersenii*	*Bos taurus* (Terceira, Azores, Portugal; 2008)	Sejroe	Hardjo, Hardjo-bovis	A
IHMT 227B	*L. borgpetersenii*	*Bos taurus* (Terceira, Azores, Portugal; 2008)	Sejroe	Hardjo, Hardjo-bovis	A
INIAV 17433 Z1[1]	*L. borgpetersenii*	*Mus musculus* (Lisbon zoo, Portugal; 2012)	Ballum	Castellonis	B
INIAV 18078 Z6[1]	*L. borgpetersenii*	*Mus musculus* (Lisbon zoo, Portugal; 2012)	Ballum	Castellonis	B
UTAD Rim156[6]	*L. borgpetersenii*	*Mus musculus* (Pena, Portugal; 2009)	Sejroe	Saxkoebing[6]	B
UTAD Rim283[6]	*L. borgpetersenii*	*Mus musculus* (Bragadas, Portugal; 2009)	Ballum	Ballum[6]	B
INIAV 25318	*L. kirschneri*	*Sus scrofa domesticus* (Lisbon, Portugal; 2011)	Pomona	Tsaratsovo	C
INIAV 494[1]	*L. kirschneri*	*Sus scrofa domesticus* (Lisbon, Portugal; 2012)	Pomona	Tsaratsovo	C

Table 4.1. (cont.)

Strain	Species	Origin	Serogroup	Serovar	*secY* cluster[9]
INIAV Mozdok PT[4]	*L. kirschneri*	*Sus scrofa domesticus* (Lourinha, Portugal; 1990)	Pomona	Mozdok type 1	C
INIAV Horse 112[3]	*L. kirschneri*	*Equus caballus* (Lisbon, Portugal; 2004)	Pomona	Mozdok type 2	C
UTAD Rim139[2]	*L. kirschneri*	*Mus musculus* (Vila Real, Portugal; 2009)	Pomona[2]	Altodouro[2]	D
INIAV 13843	*L. interrogans*	*Bos taurus* (Lisbon, Portugal; 2006)	Pomona	Pomona	E
INIAV Horse 133[3]	*L. interrogans*	*Equus caballus* (Lisbon, Portugal; 2004)	Australis[3]	Bratislava[3]	F
IHMT A02[5]	*L. interrogans*	*Rattus norvegicus* (Terceira, Azores, Portugal; 1993)	Icterohaemorrhagiae	Icterohaemorrhagiae	G
INIAV 22955 Z37[1]	*L. interrogans*	*Rattus norvegicus* (Lisbon zoo, Portugal; 2012)	Icterohaemorrhagiae	Copenhageni	G
INIAV 17191[7]	*L. interrogans*	*Callithrix jacchus* (Lisbon zoo, Portugal; 2003)	Icterohaemorrhagiae	Copenhageni	G

Table 4.1. (cont.)

Strain	Species	Origin	Serogroup	Serovar	*secY* cluster[9]
INIAV 37276[8]	*L. interrogans*	*Suricata suricatta* (Lisbon zoo, Portugal; 2006)	Icterohaemorrhagiae	Copenhageni	G
INIAV 3847[8]	*L. interrogans*	*Lemur catta* (Lisbon zoo, Portugal; 2006)	Icterohaemorrhagiae	Copenhageni	G
INIAV 36840[1]	*L. interrogans*	*Dolichotis patagonum* (Lisbon zoo, Portugal; 2011)	Icterohaemorrhagiae	Copenhageni	G

[1]Ferreira et al. [36]; [2] Paiva-Cardoso et al. [14]; [3] Rocha et al. [13]; [4] Rocha [4];[5]Collares-Pereira et al. [11, 50]; [6]Paiva-Cardoso [51]; [7]Rocha et al. [42]; [8]Rocha, 2006 (Unpublished data); [9]According to Figure 4.1.; IHMT – Instituto de Higiene e Medicina Tropical, INIAV – Instituto Nacional de Investigação Agrária e Veterinária; UTAD – Universidade de Trás-os-Montes e Alto Douro

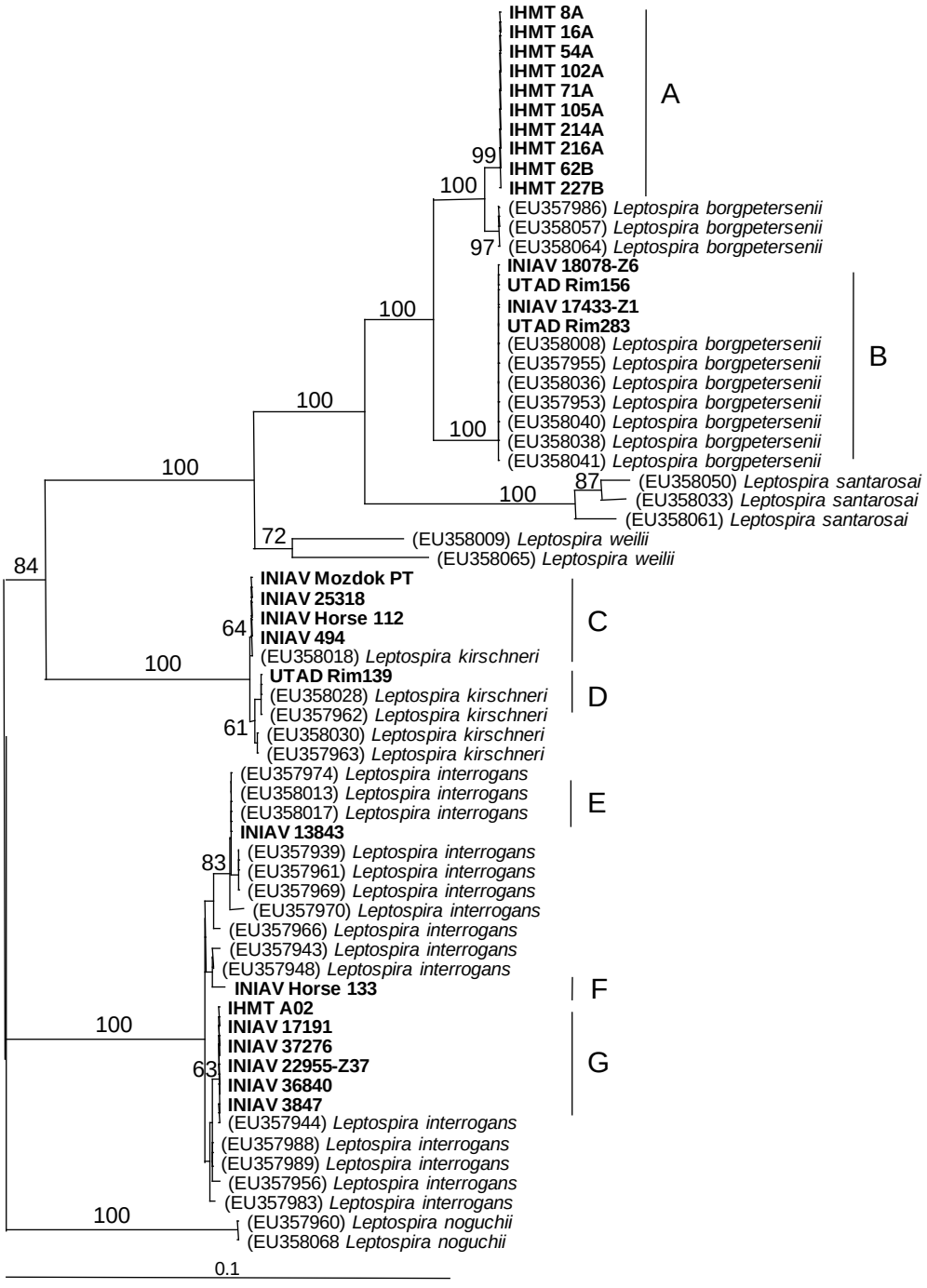

Figure 4.1. Unrooted phylogenetic tree of *Leptospira* isolates obtained by neighbour-joining analysis of partial *secY* gene sequences using PAUP software. The numbers given on the branches are the frequencies (> 50%) with which a given branch appeared in 1000 bootstrap replications. Portuguese *Leptospira* isolates are highlighted in bold and the GenBank accession numbers are indicated for the remaining sequences. Letters A - G identify the clades containing Portuguese isolates.

A summary of the results of the MLST analysis for a subset of 12 strains is presented in Table 4.2., regarding the sequence types obtained by using each of the three schemes. Tables 4.S1., 4.S2. and 4.S3., included in the section of supporting information of this chapter, present the allelic profiles found for each isolate according to, respectively, the $7L_{Boonsilp}$, the 6L and $7L_{Varni}$ MLST schemes. The phylogenetic relation between sequence types assigned by the $7L_{Boonsilp}$ and 6L schemes can be assessed, respectively, in the phylogenetic trees shown in Figures 4.S1. and 4.S2. Figures 4.S3-4.S9 (see Supporting information) represent the phylogenetic trees obtained by analyzing the individual genes of the $7L_{Boonsilp}$ scheme (respectively, *glmU*, *pntA*, *sucA*, *tpiA*, *pfkB*, *mreA* and *caiB*).

The $7L_{Boonsilp}$ scheme assigned the same ST to all isolates clustering together on the *secY* phylogenetic tree (Table 4.2.). A total of seven distinct STs were therefore detected among the Portuguese isolates, with two STs representing *L. borgpetersenii* ($ST_{(7LBoonsilp)}149$ and $ST_{(7LBoonsilp)}152$), two STs representing *L. kirschneri* ($ST_{(7LBoonsilp)}117$ and a new ST not yet recognized in the *Leptospira* database) and three STs representing *L. interrogans* ($ST_{(7LBoonsilp)}17$, $ST_{(7LBoonsilp)}24$ and $ST_{(7LBoonsilp)}140$). The relation between each of these STs and other global *Leptospira* STs is shown in a phylogenetic tree based in the concatenated gene sequences of the $7L_{Boonsilp}$ scheme (Figure 4.S1.). The clusters formed in this tree for the Portuguese isolates are similar to the clusters formed when phylogenetic trees are inferred from the individual gene sequences of the scheme (Figures 4.S3 – 4.S9). Sequence types and gene alleles cluster together according to the *Leptospira* species and are mostly concordant with the *secY* phylogeny (Figure 4.1.). The allelic profiles found under the $7L_{Boonsilp}$ scheme are shown in Table 4.S1. Strain UTAD Rim139 represents a new ST due to the occurrence of novel alleles for genes *glmU*, *pfkB* and *mreA* (Table 4.S1.).

Table 4.2. MLST results for selected strains used in this study.

Strain	*secY* cluster[1]	$ST_{(7LBoonsilp)}$[2]	$ST_{(6L)}$[3]	$ST_{(7LVarni)}$[4]
IHMT 102A	A	152	128	new ST I
IHMT 105A	A	152	128	new ST I
INIAV 18078 Z6	B	149	new ST I	new ST II
UTAD Rim283	B	149	new ST I	new ST II
INIAV 25318	C	117	new ST II	new ST III
INIAV Mozdok PT	C	117	new ST II	new ST III
INIAV Horse 112	C	117	98	new ST IV
UTAD Rim139	D	New ST	new ST III	new ST V
INIAV 13843	E	140[5]	58	52[5]
INIAV Horse 133	F	24	17	new ST VI
INIAV 22955 Z37	G	17	2	new ST VII
INIAV 36840	G	17	2	new ST VII

[1]According to Figure 1; [2]Sequence type assigned by the $7L_{Boonsilp}$ scheme [29, 32]; [3]Sequence type assigned by the 6L scheme [28, 29, 33]; [4]Sequence type assigned by the $7L_{Varni}$ scheme [34]; [5]ST assigned with the absence of allelic information for locus *pntA*; IHMT – Instituto de Higiene e Medicina Tropical, INIAV – Instituto Nacional de Investigação Agrária e Veterinária; UTAD – Universidade de Trás-os-Montes e Alto Douro

The 6L scheme was slightly more discriminative than the $7L_{Boonsilp}$ scheme, with the division of the Portuguese isolates into eight distinct STs (Table 4.2.). The results of both MLST schemes were mostly concordant but the isolates with $ST_{(7LBoonsilp)}$117 were further divided into two STs by the 6L scheme (Table 4.2.). These two STs are closely related (one being $ST_{(6L)}$98), comprising strains

INIAV MozdokPT [4], INIAV 25318 and INIAV Horse 112 [13], clustering together in the phylogenetic tree based in the respective concatenated 6L gene sequences (Figure 4.S2.).

The number of new STs not recognized by the *Pubmlst* database was higher for the 6L scheme (Table 4.2.), with three new STs, due to new alleles found for genes *adk*, *icdA* and *secY* (Table 4.S2.).

The $7L_{Varni}$ MLST scheme also divided the Portuguese isolates into eight STs, fully concordant with the 6L scheme (Table 4.2.). However, the online *Pubmlst* database supporting this $7L_{Varni}$ scheme revealed to be the most incomplete, with all the Portuguese isolates being assigned to new STs, with the exception of isolate INIAV 13843 (Table 4.2.). New alleles were found for the genes *adk*, *glmU*, *icdA*, *lipL41*, *mreA* and *pntA*, which are still missing from the *Pubmlst* database (Table 4.S3.).

4.5. Discussion

In this work we assessed the feasibility of using three previously published MLST schemes to type a range of host and geographically diverse Portuguese isolates of pathogenic *Leptospira*. We did not find any particular difficulty by directly using the conditions and primers summarized by Ahmed et al. [29] to successfully amplify the respective gene fragments from the Portuguese isolates, although difficulties in analyzing some of these genes were previously noticed by other authors [38]. We did not detect any non-standard length alleles among the Portuguese isolates, although it was previously described that some *Leptospira* strains might become nontypeable due to deletions in some loci, such as in the *caiB* [32] and *lipL32* [33] genes.

Ten isolates analyzed in this work were originally collected from cattle in 2008, from two islands of Azores (São Miguel and Terceira, separated by 170 km in the Atlantic Ocean). All these isolates were identified as *L. borgpetersenii* and clustered together in the *secY* phylogenetic tree under cluster A (Table 4.1., Figure 4.1.). These isolates were serovar Hardjo type Hardjo-bovis (serogroup Sejroe), a serovar found in most parts of the world and with cattle known to be the main carriers [7], both dairy and meat producing. Two isolates (IHMT 102A

and IHMT 105A), one from each island, were typed by MLST and showed to belong to a same sequence type, regardless of the MLST scheme used (Table 4.2.): $ST_{(7LBoonsilp)}$152, $ST_{(6L)}$128 and a new $ST_{(7LVarni)}$. *Leptospira* isolates from cattle in Azores seems to be therefore genetically homogeneous. However, cattle isolates circulating on these somewhat isolated islands do not seem to be geographically restricted, being also present in other diverse regions of the world. *Leptospira* $ST_{(7LBoonsilp)}$152 isolates were found associated with cattle in Netherlands and USA, and with a human host in Australia [32]. Also, $ST_{(6L)}$128 isolates were previously found in China but there is not much information about this ST in the *Pubmlst* database.

Four additional isolates were also identified as *L. borgpetersenii* and clustered together in the *secY* phylogenetic tree under cluster B (Table 4.1., Figure 4.1.). Two of these isolates (INIAV 17433 Z1 and INIAV 18078 Z6) were obtained from *M. musculus* captured in Lisbon zoo, in 2012 [36], and a third isolate (UTAD Rim283) was also obtained from *M. musculus*, but from Vila Real, north of Portugal, in 2009. The three isolates were serovar Castellonis (INIAV 17433 Z1 and INIAV 18078 Z6) and Ballum (UTAD Rim283) belonging to serogroup Ballum. Isolates INIAV 18078 Z6, from Lisbon, and UTAD Rim283, from Vila Real, showed to belong to a same ST (Table 4.2.): $ST_{(7LBoonsilp)}$149 and new STs by both the 6L and the $7L_{Varni}$ MLST schemes. *Leptospira* $ST_{(7LBoonsilp)}$149 isolates were found associated to mouse hosts in Japan, Denmark and Spain [32]. The fourth isolate UTAD Rim156 was also obtained from *M. musculus*, in the north of Portugal, but was serovar Saxkoebing (serogroup Sejroe) instead, a serovar also known to be associated with mice. We did not type this isolate in the present work but a sequence type is available under the 6L scheme in the *Pubmlst* database. According to *Pubmlst* database (under scheme#2), isolate UTAD Rim156 represents $ST_{(6L)}$132. This $ST_{(6L)}$132 (and thus the isolate UTAD Rim156) does not belong to the same cluster as isolates INIAV 18078 Z6 and UTAD Rim283 (Figure 4.S2.). These results suggest that *L. borgpetersenii* isolates associated with *Mus musculus* hosts in Portugal are genetically diverse, with the finding of two distinct genotypes occurring in three strains typed by MLST.

Isolate UTAD Rim139, originally also collected from *M. musculus* in the north of Portugal, was confirmed to represent *L. kirschneri* serovar Altodouro (serogroup Pomona) (Table 4.1.). This isolate was assigned a new ST for all the MLST schemes used (Table 4.2.). Noteworthy, this isolate was typed by Ahmed et al. [28] and is included in the *Pubmlst* database available (under scheme#2), and was assigned an $ST_{(6L)}$131 (representing *L. borgpetersenii*, also typed as serovar Kunming, serogroup Pomona). This initial ST assignment of serovar Altodouro presents an error since this isolate was described and extensively characterized in a recent study as *L. kirschneri* serovar Altodouro (Paiva-Cardoso et al., 2013), as in our study. Additional four isolates were also confirmed to represent *L. kirschneri*: three isolates from pigs (with an isolation interval of more than 20 years) and one isolate from a horse (Table 4.1.). These isolates clustered together in the *secY* phylogenetic tree under cluster C (Figure 4.1.) but represented distinct serovars: Tsaratsovo (INIAV 25318 and INIAV 494), and Mozdok (INIAV Mozdok PT and INIAV Horse 112), both of serogroup Pomona. The INIAV horse112 isolate has been previously considered to belong to serovar Tsaratsovo, when identified by REA [13], however, when typed by MAbs in the course of this study, it was identified as Mozdok type 2. The classification of Pomona serogroup serovars has always been difficult, the antigenic differences between serovars are small and the work of different authors, using several methods of identification, has given rise to controversial and sometimes contradictory observations [16, 39]. Several studies have reported that serovars Mozdok and Tsaratsovo are identical [39, 40], and it has been suggested that the designation of Tsaratsovo as a separate serovar should be abandoned. Terpstra et al. [41] found that Tsaratsovo, Dania and Mozdok were identical, by REA, although Tsaratsovo could easily be distinguished by MAbs analysis. However, In the REA study of Portuguese strains INIAV Horse 112 and INIAV Mozdok PT performed by Rocha et al. [13], slight differences were observed between Tsaratsovo (INIAV Horse 112 and Tsaratsovo type strain B 81/7 reference strain) and Mozdok (INIAV Mozdok PT and Mozdok 5621 reference strain) serovars, contrarily to the previous REA observations of Terpstra et al. [41] on the same serovars. One serovar Tsaratsovo (INIAV 25318) and the two serovar Mozdok isolates were typed with

MLST. The $7L_{Boonsilp}$ scheme assigned the same $ST_{(7LBoonsilp)}117$ to all three isolates (Table 4.2). There is not much information about this ST in public databases, but an $ST_{(7LBoonsilp)}117$ isolate serovar Mozdok was found in a field vole in Russia [32]. Noteworthy, the 6L scheme assigned different STs to the horse ($ST_{(6L)}98$) and pigs (new ST) isolates (Table 4.2.), although both STs are closely related (Figure 4.S2.). The $ST_{(6L)}98$ comprises isolates typed both as $ST_{(7LBoonsilp)}117$ and $ST_{(7LBoonsilp)}115$ in the *Pubmlst* database, and these are closely related sequence types (Figure 4.S1.). Ahmed et al. [33] also previously noticed that the 6L scheme had a tendency to split $7L_{Boonsilp}$ single STs into closely related clusters. The $7L_{Varni}$ scheme agreed with the 6L scheme but no STs were assigned to the isolates (Table 4.2.).

Five isolates from distinct captive animals and an infesting rat from Lisbon zoo (INIAV 22955 Z37, INIAV 17191, INIAV 37276, INIAV 3847 and INIAV 36840), collected between 2003 and 2012, were identified as *L. interrogans* and clustered together in the *secY* phylogenetic tree under cluster G (Table 4.1., Figure 4.1.). As typed by MAbs all these isolates were serovar Copenhageni (serogroup Icterohaemorrhagiae), with black and brown rats as common hosts. Two of these isolates (INIAV 22955 Z37, from a rat, and INIAV 36840, from a Patagonian Mara) were found to belong to a same sequence type: $ST_{(7LBoonsilp)}17$, $ST_{(6L)}2$ and a new ST for $7L_{Varni}$ scheme, respectively (Table 4.2.). The Patagonian Mara died of leptospirosis [36], as well as other captive animals, and our typing results suggest that the strain responsible for these deaths is also circulating (and seems to be perpetuated) among infesting rats in the same zoo. Previously, Icterohaemorragiae serogroup strains had been isolated from fatal leptospirosis cases of other captive animals (*Saguinus Midas* and *Pithecia pithecia*) in the Lisbon Zoo [42]. Noteworthy, $ST_{(7LBoonsilp)}17$ isolates seems to be widespread and were found to be also associated with human infections in Japan, Brazil, Belgium and Denmark [32]. This sequence type seems to be responsible for up to 90% of human leptospirosis cases in São Paulo, Brazil [43], and was also identified in a rat, pig and human hosts in Argentina [44]. It seems to be also frequent in Russia [45] and China [46]. Most interesting, $ST_{(7LBoonsilp)}17$ isolates were recently responsible for an outbreak of

severe leptospirosis among capuchin monkeys in a Colombian wildlife rehabilitation center [47].

Finally, two isolates were confirmed to represent *L. interrogans*, one from cattle (INIAV 13843; serovar Pomona, serogroup Pomona) and one from a horse (INIAV Horse 133 [13]; serovar Bratislava, serogroup Australis) (Table 4.1.), also clustering differently in the *secY* phylogenetic tree under clusters E and F, respectively (Figure 4.1.). Distinct sequence types were assigned to each of these isolates by the MLST schemes (Table 4.2.). Isolate INIAV 13843, from cattle, was assigned $ST_{(7LBoonsilp)}140$, $ST_{(6L)}58$ and $ST_{(7LVarni)}52$, respectively (Table 4.2.). *Leptospira* $ST_{(7LBoonsilp)}140$ isolates were found associated to humans (Australia, Sri Lanka) and opossum (Brazil) [32]. The $ST_{(6L)}58$ also comprises isolates typed as $ST_{(7LBoonsilp)}140$, according to the *Pubmlst* database. The only sequence type number assigned by the $7L_{Varni}$ scheme in this work was $ST_{(7LVarni)}52$, for isolate INIAV 13843, and this ST was found to be frequently associated to cattle and pigs in Argentina [34]. Isolate INIAV Horse 133 was assigned $ST_{(7LBoonsilp)}24$, $ST_{(6L)}17$ and a new $ST_{(7LVarni)}$. $ST_{(7LBoonsilp)}24$ isolates were found associated to hedgehog and mouse (in Czech Republic) and human (in Germany) hosts [32]. These $ST_{(7LBoonsilp)}24$ isolates were also recently suspect of causing the dead of beavers in south-west Germany [48], and were among the most common isolates collected from small mammals in Eastern Croatia [49]. Isolates with $ST_{(6L)}17$ were also found associated to humans (Italy, Germany) and rodents (Tanzania, Panama) hosts.

All the three MLST schemes revealed a similar discriminatory power for typing the Portuguese isolates, allowing the correct assignment of *Leptospira* species and, within each species, with some propensity to differentiate isolates according to their hosts. Previous studies noticed that MLST approaches may contribute to unravel potential associations between specific *Leptospira* STs and their hosts and geographic regions of origin [32, 38]. For example, recently, Dietrich et al. [38] found a distinct clustering of *Leptospira* isolates according to their different small mammal host species in Madagascar. Most distinct *Leptospira* serovars were assigned a distinct ST in our study. The exception was $ST_{(7LBoonsilp)}117$, which was assigned to isolates with both serovars Tsaratsovo or Mozdok. Other authors also noticed that serovar might be a

limited indicator of genetic relatedness, which may be due to horizontal gene transfer of genes encoding the surface determinants that confer serovar designation [29, 32].

The most useful MLST approach in our study seems to be the $7L_{Boonsilp}$ scheme [29, 32], although the 6L and the $7L_{Varni}$ schemes showed to be slightly more discriminant due to the differentiation of $ST_{(7LBoonsilp)}117$ into two closely related sequence types. The major advantage of the $7L_{Boonsilp}$ scheme was its discriminative power in many cases. However, a current limitation of this *Leptospira* database is the lack of information available for most of the sequence types assigned, with only some anecdotally data about e.g. the host and geographic origins of a few representative isolates. Anyway, it seems that globally widespread (and maybe more virulent) *Leptospira* genotypes are circulating in Portugal, particularly the *L. interrogans* $ST_{(7LBoonsilp)}17$ isolates. Other genotypes seem to occur less frequently in other geographic regions, namely in other European countries, such as the new STs assigned for all the MLST schemes represented by *L. kirschneri* UTAD Rim139. However, this situation may be the result of the very limited number of currently published MLST-based surveys addressing the epidemiology of leptospirosis.

Leptospira MLST approaches were only recently described, these are not particularly affordable techniques, and other important constrain is that several distinct schemes are currently used by different authors, which difficult the comparative analysis between different studies. An integrated consensual MLST scheme should therefore be agreed by the scientific community studying the epidemiology of these important agents. Additionally, most isolates typed so far have their origin in a few Asian and South American countries, which still significantly limits our understanding about the global epidemiology of leptospirosis based in genotyping data. Our study makes a contribution for enriching the global MLST databases with a new set of allele and sequence type information regarding Portuguese, and European, pathogenic *Leptospira* isolates.

4.6. Acknowledgments

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4.7. Supporting information

Table 4.S1. Typing results for the $7L_{Boonsilp}$ MLST scheme.

Strain	Species	Origin	$ST_{(7LBoonsilp)}$[1]	*glmU*	*pntA*	*sucA*	*tpiA*	*pfkB*	*mreA*	*caiB*
IHMT 102A	*L. borgpetersenii*	*Bos taurus* (São Miguel, Azores, Portugal; 2008)	152	26	30	28	35	39	29	29
IHMT 105A	*L. borgpetersenii*	*Bos taurus* (Terceira, Azores, Portugal; 2008)	152	26	30	28	35	39	29	29
INIAV 18078 Z6	*L. borgpetersenii*	*Mus musculus* (Lisbon zoo, Portugal; 2012)	149	24	32	30	36	67	26	12
UTAD Rim283	*L. borgpetersenii*	*Mus musculus* (Vila Real, Portugal; 2009)	149	24	32	30	36	67	26	12
INIAV 25318	*L. kirschneri*	*Sus scrofa domesticus* (Lisbon, Portugal; 2011)	117	13	25	15	22	33	18	23
INIAV Mozdok PT	*L. kirschneri*	*Sus scrofa domesticus* (Lourinha, Portugal; 1990)	117	13	25	15	22	33	18	23

Table 4.S1. (cont.)

Strain	Species	Origin	$ST_{(7LBoonsilp)}$[1]	*glmU*	*pntA*	*sucA*	*tpiA*	*pfkB*	*mreA*	*caiB*
INIAV Horse 112	*L. kirschneri*	*Equus caballus* (Lisbon, Portugal; 2004)	117	13	25	15	22	33	18	23
UTAD Rim139	*L. kirschneri*	*Mus musculus* (Vila Real, Portugal; 2009)	New ST	new	20	15	22	new	new	23
INIAV 13843	*L. interrogans*	*Bos taurus* (Lisbon, Portugal; 2006)	140[2]	3	-	3	3	4	5	16
INIAV Horse 133	*L. interrogans*	*Equus caballus* (Lisbon, Portugal; 2004)	24	1	4	2	1	5	3	4
INIAV 22955 Z37	*L. interrogans*	*Rattus norvegicus* (Lisbon, Portugal; 2012)	17	1	1	2	2	10	4	8
INIAV 36840	*L. interrogans*	*Dolichotis patagonum* (Lisbon zoo, Portugal; 2011)	17	1	1	2	2	10	4	8

[1]Sequence type assigned by the $7L_{Boonsilp}$ scheme [29, 32]; [2]ST assigned with the absence of allelic information for locus *pntA*; IHMT – Instituto de Higiene e Medicina Tropical, INIAV – Instituto Nacional de Investigação Agrária e Veterinária; UTAD – Universidade de Trás-os-Montes e Alto Douro

Table 4.S2. Typing results for the 6L MLST scheme.

Strain	Species	Origin	$ST_{(6L)}$[1]	*adk*	*icdA*	*lipL32*[2]	*lipL41*[3]	*rrs2*	*secY*[4]
IHMT 102A	*L. borgpetersenii*	*Bos taurus* (São Miguel, Azores, Portugal; 2008)	128	57	54	30	39	20	47
IHMT 105A	*L. borgpetersenii*	*Bos taurus* (Terceira, Azores, Portugal; 2008)	128	57	54	30	39	20	47
INIAV 18078 Z6	*L. borgpetersenii*	*Mus musculus* (Lisbon zoo, Portugal; 2012)	new ST I	new I	61	10	15	20	49[5]
UTAD Rim283	*L. borgpetersenii*	*Mus musculus* (Vila Real, Portugal; 2009)	new ST I	new I	61	10	15	20	49
INIAV 25318	*L. kirschneri*	*Sus scrofa domesticus* (Lisbon, Portugal; 2011)	new ST II	15	new I	11	16	12	21
INIAV Mozdok PT	*L. kirschneri*	*Sus scrofa domesticus* (Lourinha, Portugal; 1990)	new ST II	15	new I	11	16	12	21
INIAV Horse 112	*L. kirschneri*	*Equus caballus* (Lisbon, Portugal; 2004)	98	15	25	11	16	12	21
UTAD Rim139	*L. kirschneri*	*Mus musculus* (Vila Real, Portugal; 2009)	new ST III	new II	28	11	21	12	new I
INIAV 13843	*L. interrogans*	*Bos taurus* (Lisbon, Portugal; 2006)	58	2	2	3	4	2	6

Table 4.S2. (cont.)

Strain	Species	Origin	$ST_{(6L)}$[1]	*adk*	*icdA*	*lipL32*[2]	*lipL41*[3]	*rrs2*	*secY*[4]
INIAV Horse 133	*L. interrogans*	*Equus caballus* (Lisbon, Portugal; 2004)	17	1	2	1	2	1	4
INIAV 22955 Z37	*L. interrogans*	*Rattus norvegicus* (Lisbon, Portugal; 2012)	2	1	1	2	2	1	1
INIAV 36840	*L. interrogans*	*Dolichotis patagonum* (Lisbon zoo, Portugal; 2011)	2	1	1	2	2	1	1

[1]Sequence type assigned by the 6L scheme [28, 29, 33]; [2]First 30 and last 8 bases are missing from sequences when compared with sequences at the database; [3]First 42 and last 13 bases are missing from sequences when compared with sequences at the database; [4]First 22 bases are missing from sequences when compared with sequences at the database; [5]Part of the 3' end sequence is missing; IHMT – Instituto de Higiene e Medicina Tropical, INIAV – Instituto Nacional de Investigação Agrária e Veterinária; UTAD – Universidade de Trás-os-Montes e Alto Douro

Table 4.S3. Typing results for the $7L_{Varni}$ MLST scheme.

Strain	Species	Origin	$ST_{(m7L)}$[1]	*adk*[3]	*glmU*	*icdA*[4]	*lipL32*[5]	*lipL41*[6]	*mreA*	*pntA*
IHMT 102A	*L. borgpetersenii*	*Bos taurus* (São Miguel, Azores, Portugal; 2008)	new ST I	29	new I	new I	32	new I	31	new I
IHMT 105A	*L. borgpetersenii*	*Bos taurus* (Terceira, Azores, Portugal; 2008)	new ST I	29	new I	new I	32	new I	31	new I
INIAV 18078 Z6	*L. borgpetersenii*	*Mus musculus* (Lisbon zoo, Portugal; 2012)	new ST II	new I	26	new II	25	29	36	new II
UTAD Rim283	*L. borgpetersenii*	*Mus musculus* (Vila Real, Portugal; 2009)	new ST II	new I	26	new II	25	29	36	new II
INIAV 25318	*L. kirschneri*	*Sus scrofa domesticus* (Lisbon, Portugal; 2011)	new ST III	7	5	new III	23	7	7	5
INIAV Mozdok PT	*L. kirschneri*	*Sus scrofa domesticus* (Lourinha, Portugal; 1990)	new ST III	7	5	new III	23	7	7	5
INIAV Horse 112	*L. kirschneri*	*Equus caballus* (Lisbon, Portugal; 2004)	new ST IV	7	5	22	23	7	7	5
UTAD Rim139	*L. kirschneri*	*Mus musculus* (Vila Real, Portugal; 2009)	new ST V	new II	new I	new IV	23	new II	new I	11

Table 4.S3. (cont.)

Strain	Species	Origin	$ST_{(m7L)}$[1]	*adk*[3]	*glmU*	*icdA*[4]	*lipL32*[5]	*lipL41*[6]	*mreA*	*pntA*
INIAV 13843	*L. interrogans*	*Bos taurus* (Lisbon, Portugal; 2006)	52[2]	3	2	2	10	4	1	-
INIAV Horse 133	*L. interrogans*	*Equus caballus* (Lisbon, Portugal; 2004)	new ST VI	5	1	2	3	17	11	9
INIAV 22955 Z37	*L. interrogans*	*Rattus norvegicus* (Lisbon, Portugal; 2012)	new ST VII	5	1	3	28	17	4	2
INIAV 36840	*L. interrogans*	*Dolichotis patagonum* (Lisbon zoo, Portugal; 2011)	new ST VII	5	1	3	28	17	4	2

[1]Sequence type assigned by the $7L_{Varni}$ scheme [34]; [2]ST assigned with the absence of allelic information for locus *pntA*; [3]Last base is missing from sequences when compared with sequences at the database; [4]Last two bases are missing from sequences when compared with sequences at the database; [5]First 30 and last 8 bases are missing from sequences when compared with sequences at the database; [6]First 42 and last 14 bases are missing from sequences when compared with sequences at the database; IHMT – Instituto de Higiene e Medicina Tropical, INIAV – Instituto Nacional de Investigação Agrária e Veterinária; UTAD – Universidade de Trás-os-Montes e Alto Douro

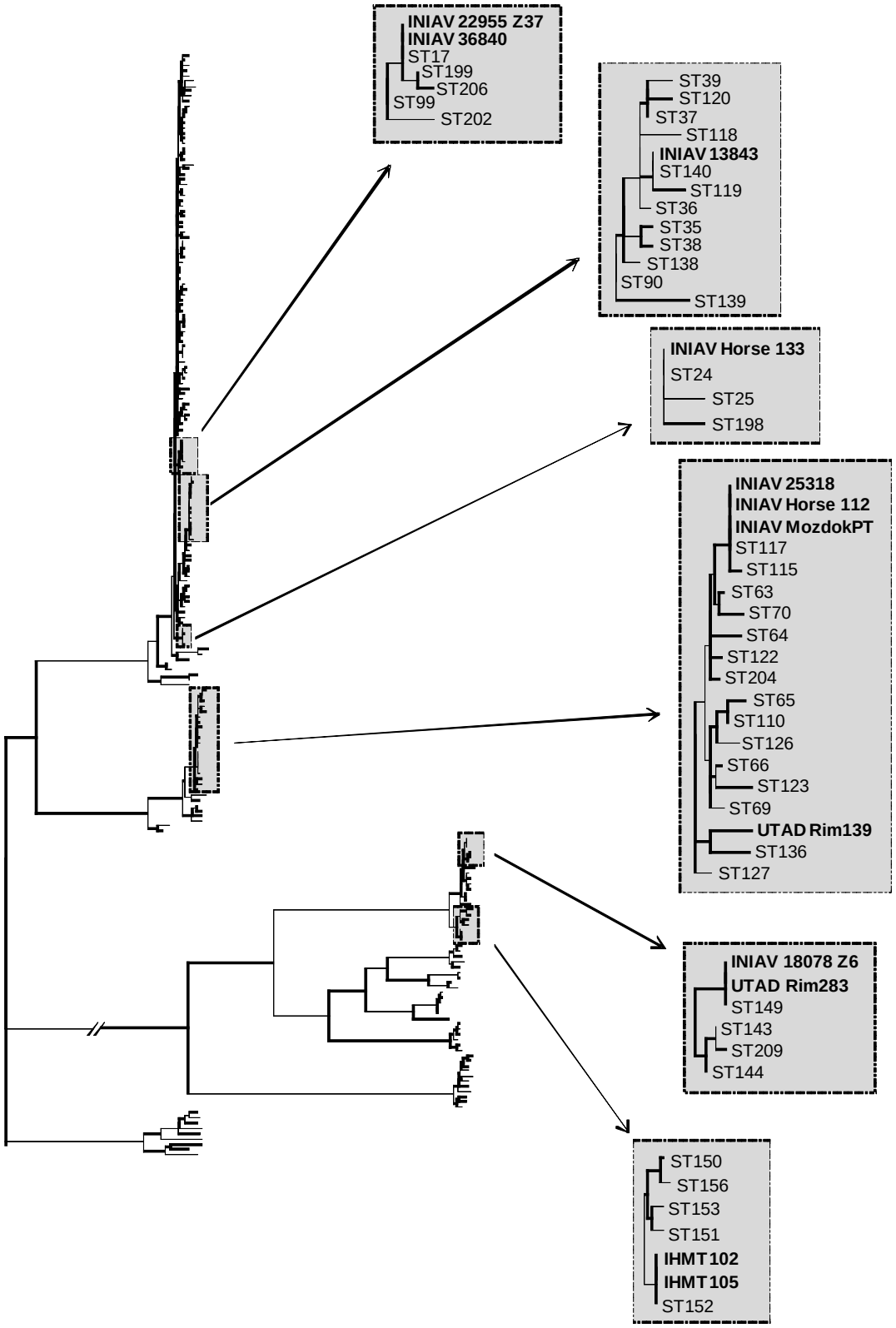

Figure 4.S1. Unrooted phylogenetic tree obtained by neighbour-joining analysis of concatenated sequences of the genes used in the $7L_{Boonsilp}$ MLST scheme (order *glmU-pntA-sucA-tpiA-pfkB-mreA-caiB*) using PAUP software. Grey boxes highlight the parts of the tree containing the Portuguese isolates, which are in bold. ST - Sequence type.

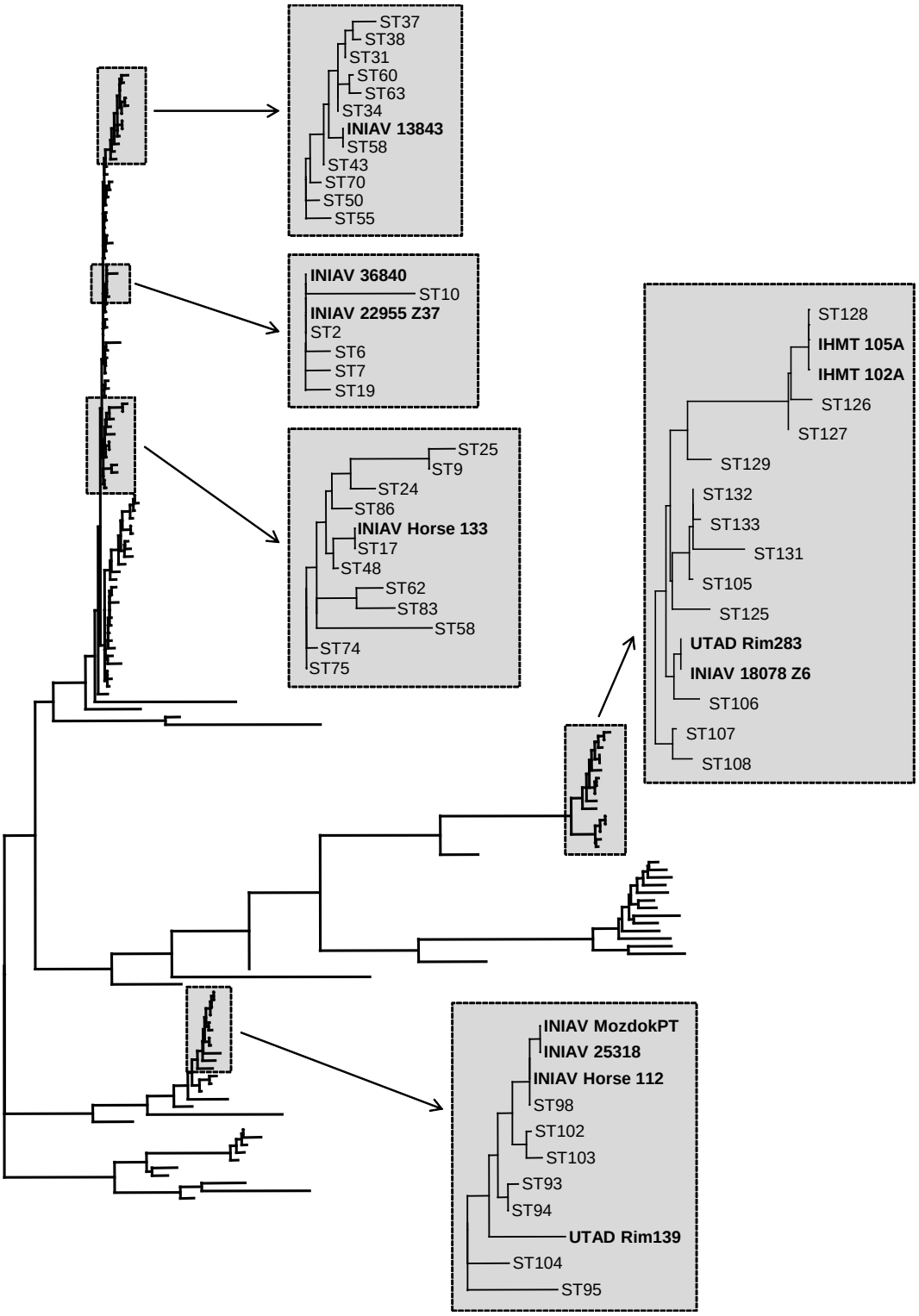

Figure 4.S2. Unrooted phylogenetic tree obtained by neighbour-joining analysis of concatenated sequences of the genes used in the 6L MLST scheme (order *adk-icdA-lipL32-lipL41-rrs2-secY*) using PAUP software. Grey boxes highlight the parts of the tree containing the Portuguese isolates, which are in bold. ST - Sequence type.

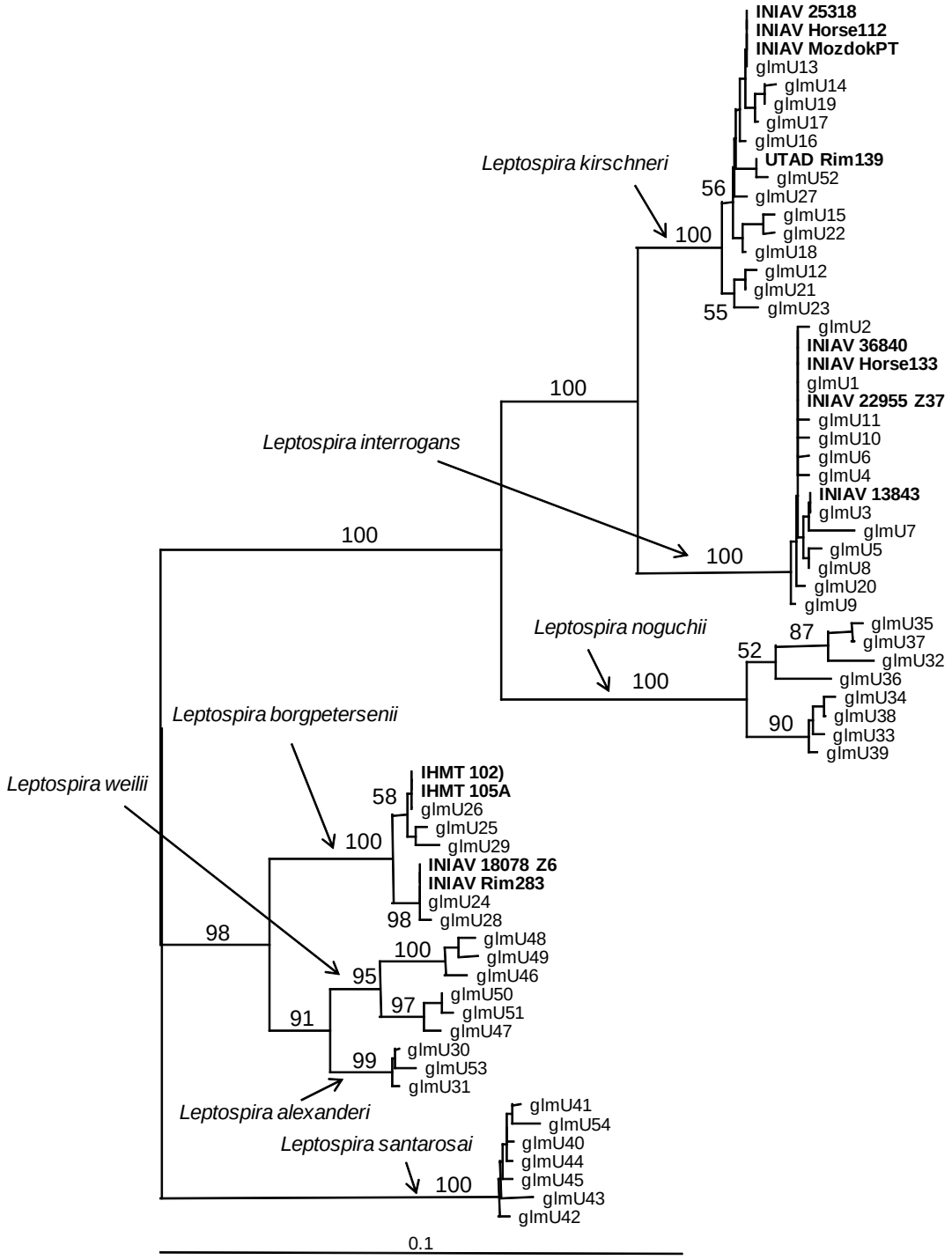

Figure 4.S3. Unrooted phylogenetic tree obtained by neighbour-joining analysis of sequences of the gene *glmU* using PAUP software. The numbers given on the branches are frequencies with which a given branch appeared in 1000 bootstrap replications. Portuguese *Leptospira* isolates are highlighted in bold. Other sequences represent distinct alleles of the *glmU* gene.

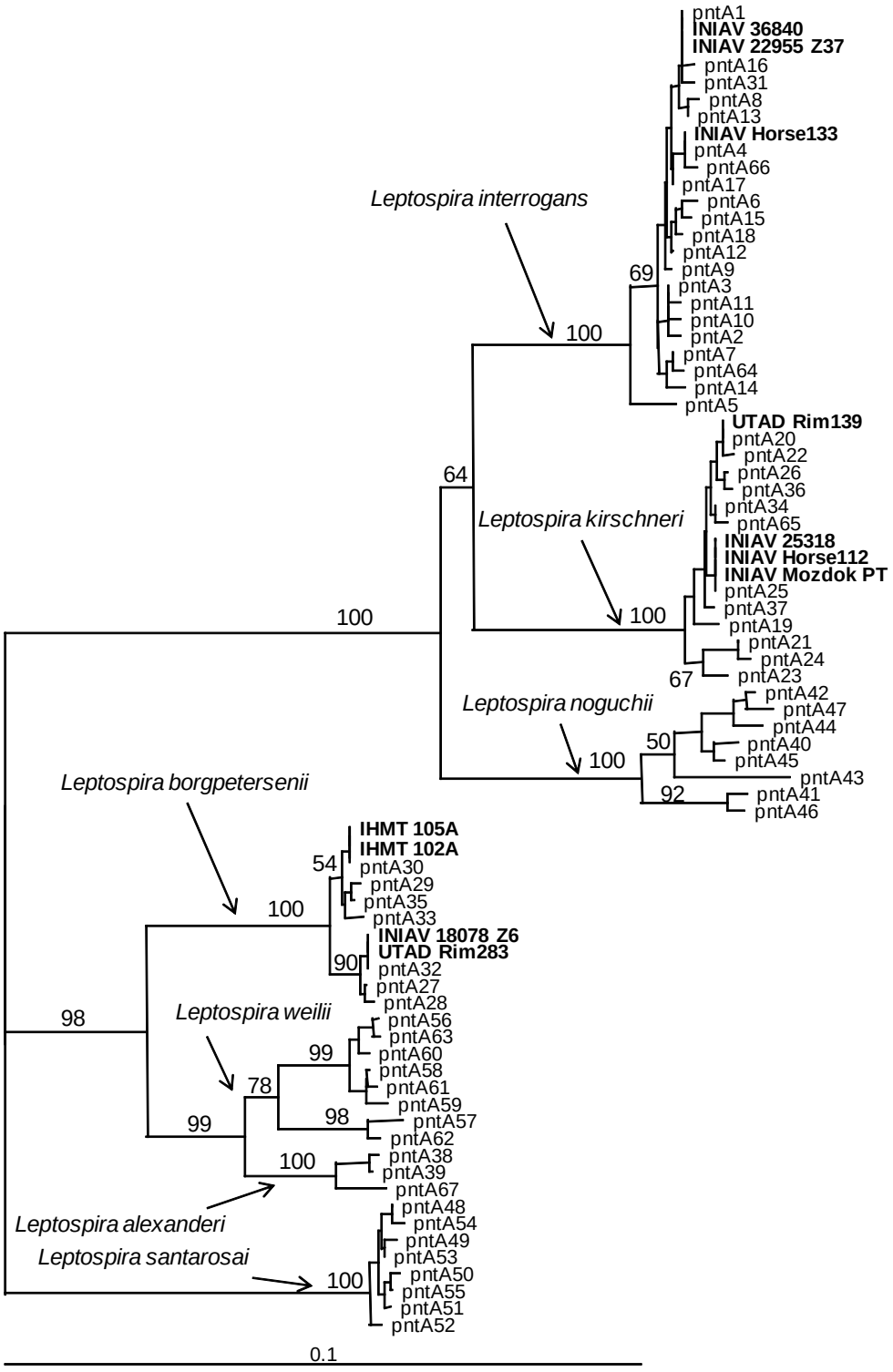

Figure 4.S4. Unrooted phylogenetic tree obtained by neighbour-joining analysis of sequences of the gene *pntA* using PAUP software. The numbers given on the branches are frequencies with which a given branch appeared in 1000 bootstrap replications. Portuguese *Leptospira* isolates are highlighted in bold. Other sequences represent distinct alleles of the *pntA* gene. Note: strain INIAV 13843 is not included in this analysis.

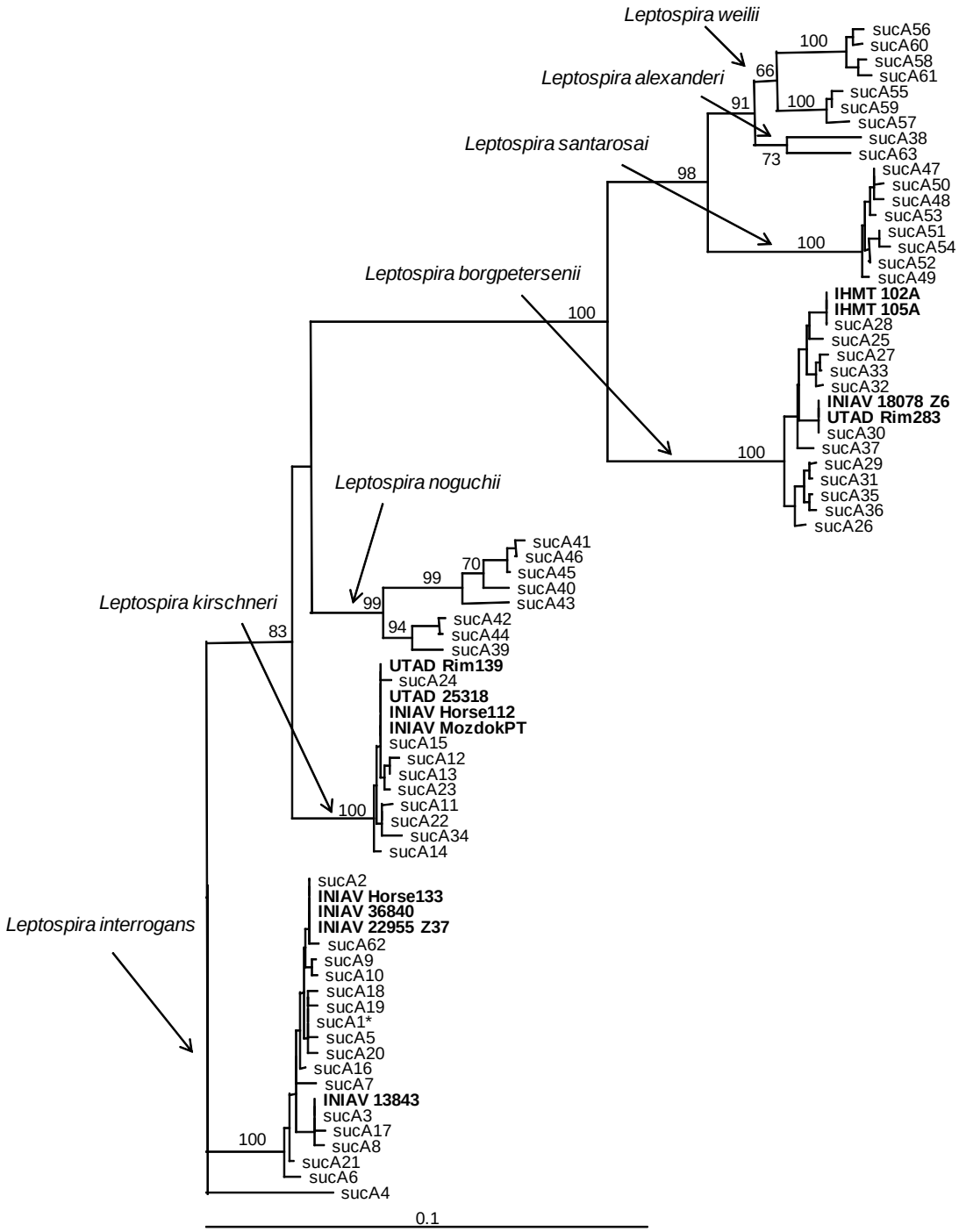

Figure 4.S5. Unrooted phylogenetic tree obtained by neighbour-joining analysis of sequences of the gene *sucA* using PAUP software. The numbers given on the branches are frequencies with which a given branch appeared in 1000 bootstrap replications. Portuguese *Leptospira* isolates are highlighted in bold. Other sequences represent distinct alleles of the *sucA* gene. *Of the 46 strains with the *sucA* alele 1 included in the *Leptospira* MLST database, apparently two strains are assigned to *L. kirschneri*.

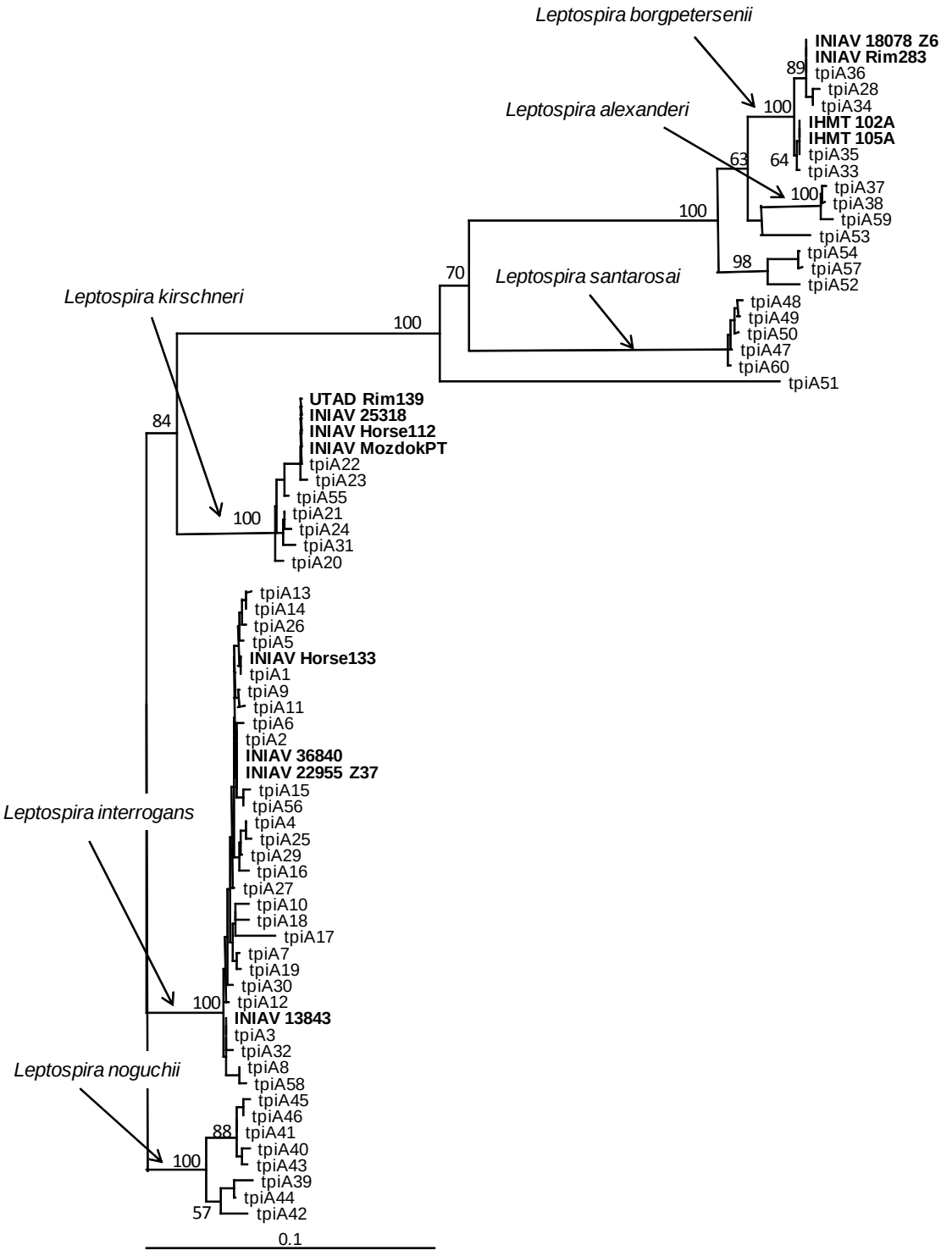

Figure 4.S6. Unrooted phylogenetic tree obtained by neighbour-joining analysis of sequences of the gene *tpiA* using PAUP software. The numbers given on the branches are frequencies with which a given branch appeared in 1000 bootstrap replications. Portuguese *Leptospira* isolates are highlighted in bold. Other sequences represent distinct alleles of the *tpiA* gene.

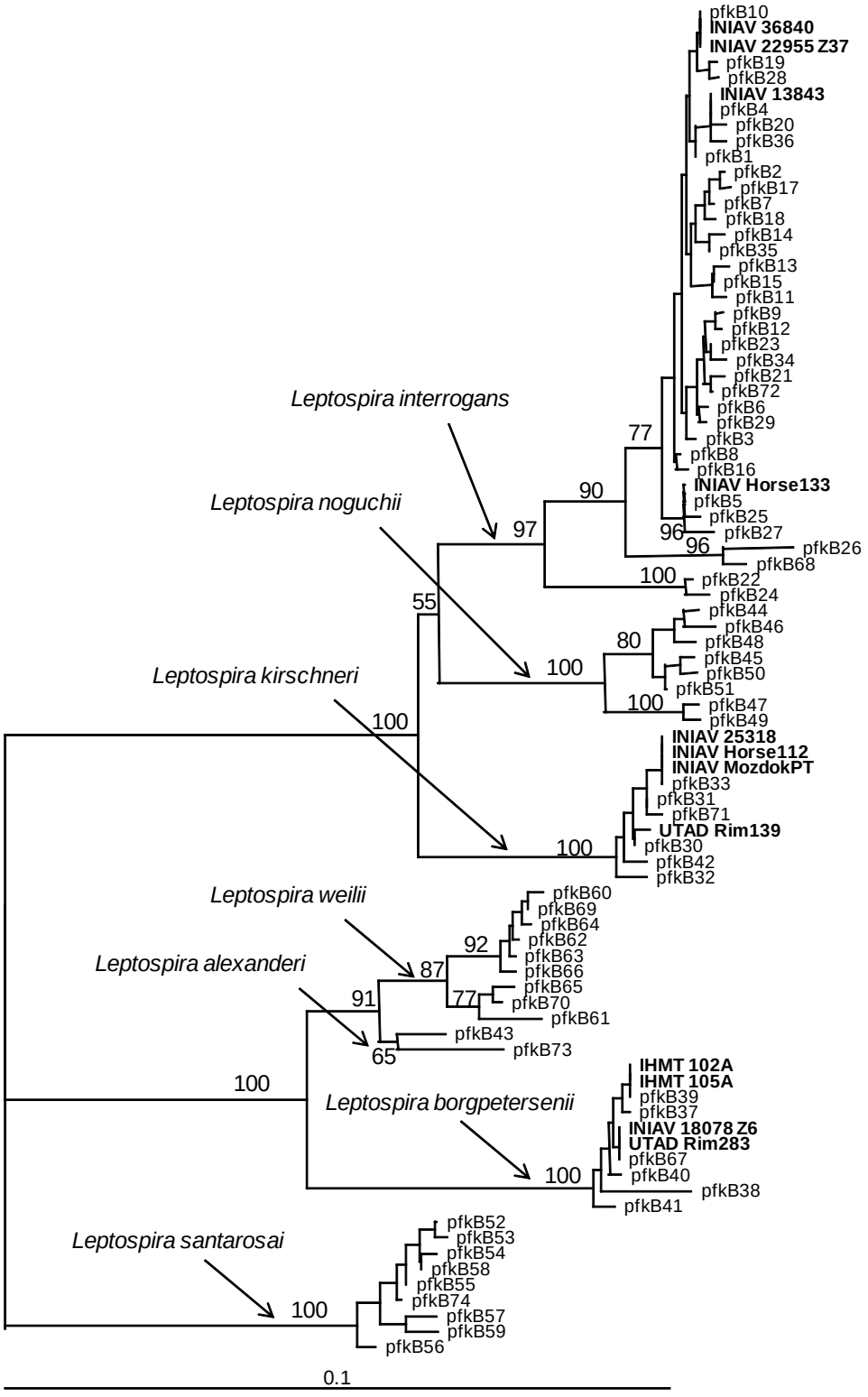

Figure 4.S7. Unrooted phylogenetic tree obtained by neighbour-joining analysis of sequences of the gene *pfkB* using PAUP software. The numbers given on the branches are frequencies with which a given branch appeared in 1000 bootstrap replications. Portuguese *Leptospira* isolates are highlighted in bold. Other sequences represent distinct alleles of the *pfkB* gene.

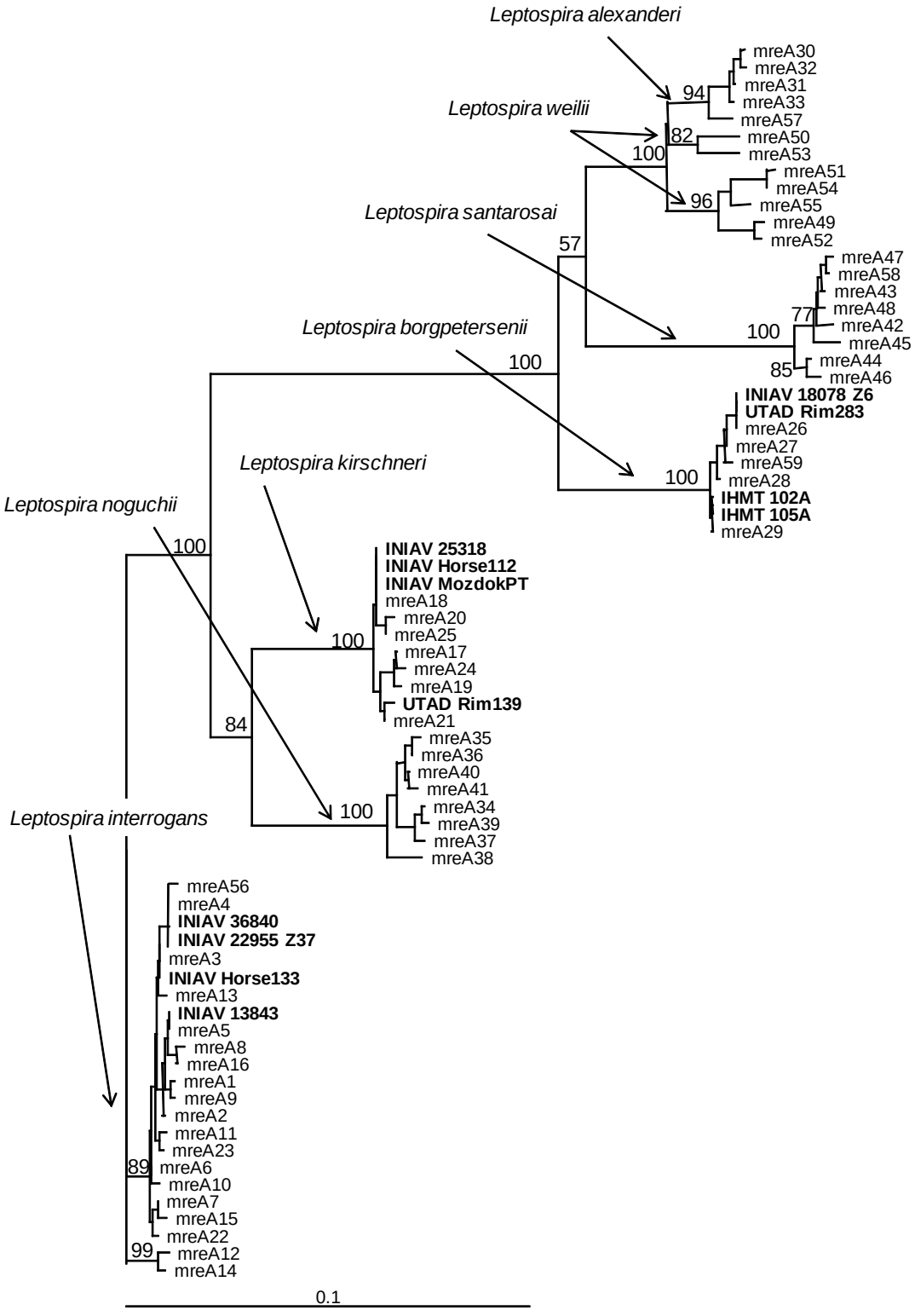

Figure 4.S8. Unrooted phylogenetic tree obtained by neighbour-joining analysis of sequences of the gene *mreA* using PAUP software. The numbers given on the branches are frequencies with which a given branch appeared in 1000 bootstrap replications. Portuguese *Leptospira* isolates are highlighted in bold. Other sequences represent distinct alleles of the *mreA* gene.

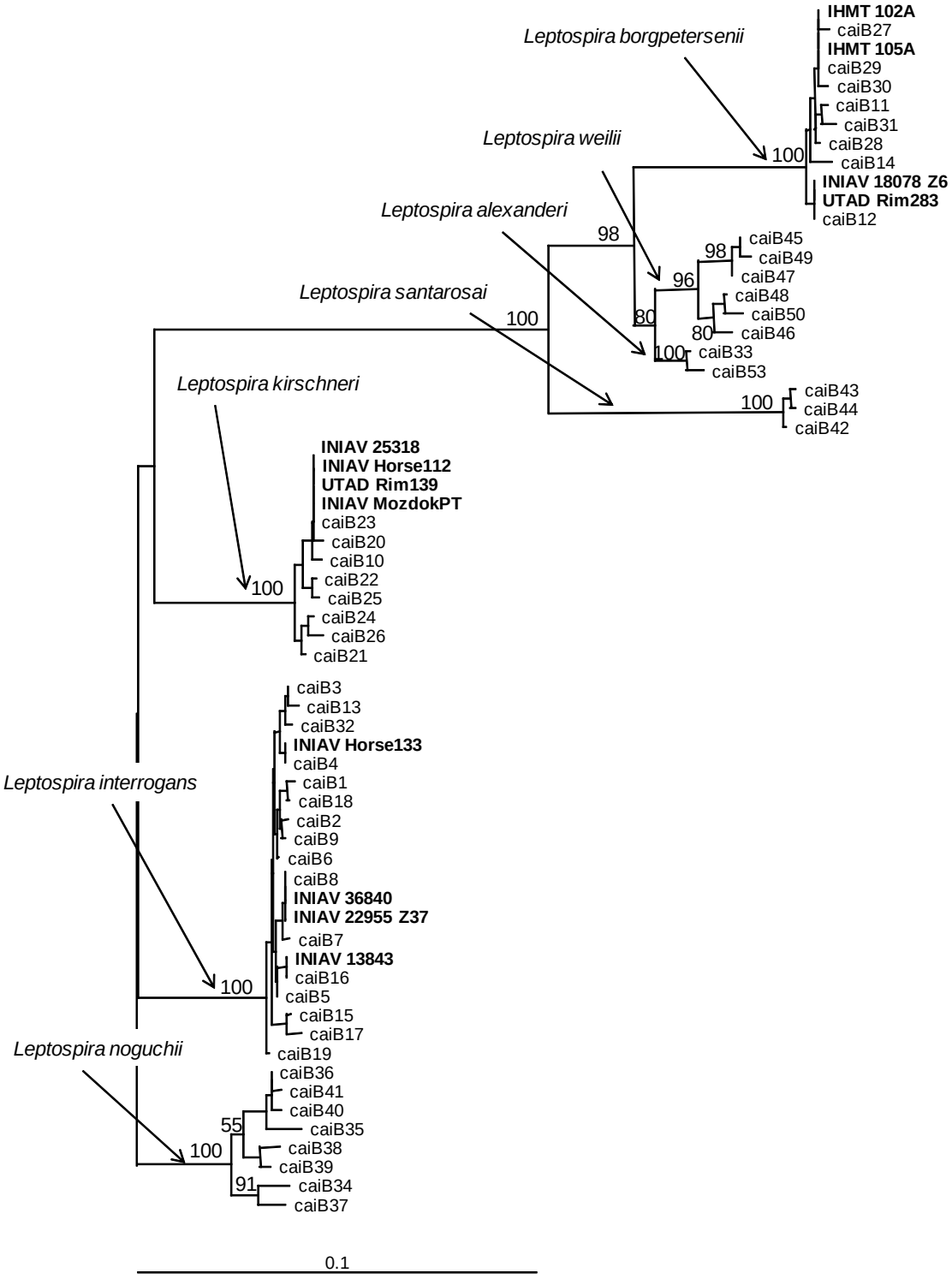

Figure 4.S9. Unrooted phylogenetic tree obtained by neighbour-joining analysis of sequences of the gene *caiB* using PAUP software. The numbers given on the branches are frequencies with which a given branch appeared in 1000 bootstrap replications. Portuguese *Leptospira* isolates are highlighted in bold. Other sequences represent distinct alleles of the *caiB* gene.

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CHAPTER 5

General features of the *Leptospira kirschneri* serovar Mozdok type 2 genome organization

5. General features of the *Leptospira kirschneri* serovar Mozdok type 2 genome organization

Manuscript in preparation

5.1. Summary

Leptospirosis is a worldwide underestimated zoonotic disease caused by pathogenic species of *Leptospira*, a genus belonging to the family *Leptospiraceae* and phylogenetically related to other spirochetes. Currently, six complete genome sequences are published such as *L. interrogans* serovars Lai and Copenhageni, whose strains belonged to the serogroup Icterohaemorrhagiae, the saprophyte *L. biflexa*, the intermediate *L. licerasiae* and other pathogenic species, *L. borgpeterseni* and *L. santarosai*. The whole genome of *L. kirschneri* was not studied to date. *L. kirschneri* is a pathogenic species containing pathogenic serovars such as those of the Pomona serogroup that are associated as the cause of occasional small outbreaks of leptospirosis in animals. *L. kirschneri* serovar Mozdok is one of those serovars belonging to the species and serogroup mentioned comprising three types of Mozdok strains, i.e., type 1, type 2 and type 3, based on the different patterns obtained from the agglutination of a panel of monoclonal antibodies (MAbs). There is very limited information about the genetic differentiation of these three types of serovar Mozdok. As far as we know serovar Mozdok type 2 was only documented as isolated in Pancas, Portugal. In this study we announce the first draft genome sequence of *L. kirschneri* serovar Mozdok type 2 strain Horse 112 that was isolated from a 5–10 years old horse in 2004. The draft genome sequenced *de novo* had a total of 4,302,078 bases and a total of 3766 genes coding for proteins getting the best result in most of cases belonging to *Leptospira* family. A total number of 91 SNPs were assessed in strain Horse 112 genome against the other four serovar Mozdok strains, namely Brem 166, B 81/7, Vehlefans 3 and Vehlevans 2. Remarkably, 17 SNPs are shared between strains Horse 112 and two genomes analyzed, including strains Brem 166 and B 81/7 (Mozdok type 3), sugesting the accuracy of these SNPs and the possibility of the two isolates being likely identical to each other. By generating the first draft genome of a serovar Mozdok type 2 strain we are able to provide insights for a more detailed and comparative analysis to correlate serovar Mozdok's types characteristics and genomic sequences contributing to a deeper understanding of these serovars evolution.

5.2. Introduction

Leptospirosis is a worldwide underestimated zoonotic disease [1]. This infection occurs in humans and animals presenting a severity ranging from very mild febrile illness to severe manifestations, such as organ dysfunction, which may be fatal. Leptospirosis is caused by pathogenic species of *Leptospira*, a genus belonging to the family *Leptospiraceae* and phylogenetically related to other spirochetes. Currently, genus *Leptospira* comprises nine pathogenic, five intermediate and seven non-pathogenic species classified according to their 16S rRNA gene sequences and pathogenicity [2]. Traditionally, leptospires are subdivided into serovars, i.e. the basic taxon, that are grouped into serogroups based on their antigenic relatedness [3]. Approximately 250 pathogenic serovars are recognized by the cross agglutinin absorption test (CAAT) [4]. In general, the molecular and serological classifications of leptospires show little correlation; and to date, is commonly accepted that the identification of leptospires should be done based on both species and the serovar [2]. Significant efforts aimed a standard speciation and serovar determination. Available serological tests are genus-specific or serogroup/serovar-specific. Nevertheless, molecular subtyping methods are also reported as capable of identifying the serovars such as RFLP- based methods [5, 6] and PFGE [7]. The sequence-based methods such as multi-locus variable number of tandem repeats analysis (MLVA) [8-12] and multilocus sequencing typing (MLST) [13, 14] can also yield significant information at serovar level. Nonetheless, these methods still give us insufficient information. Indeed, the correlation of *Leptospira* "molecular serotyping" systems turned out to be arduous since molecular and classical methods have intrinsic differences in their respective concepts (genes *vs* antigens). Genotyping standardization is moving towards identification of serovars by whole genome sequencing (WGS) [15].

In 2003 and 2004, two *Leptospira* genomes were the first to be sequenced, namely *L. interrogans* serovars Lai [16] and Copenhageni [17], whose strains belonged to the serogroup Icterohaemorrhagiae. Other complete genome sequences are currently available, such as the saprophyte *L. biflexa* [18], the intermediate *L. licerasiae* [19] and other pathogenic species, *L. borgpeterseni* [20] and *L. santarosai* [21]. All *Leptospira* species have at least two circular

replicons, with exception of *L. biflexa* that possesses a third circular replicon of 74 kb, designated p74, which is not present in the pathogenic species [22]. Naturally occurring plasmids have not been reported in *Leptospira* and the mechanisms of gene transfer are largely undiscovered. Comparative genomics might allow the recognition of pathogen-specific genes and as far as we know, 893 pathogen-specific genes were identified wherein 1,547 proteins are common for *Leptospira* genus [19, 22]. The ongoing *Leptospira* Genomics and Human Health Project (http://gcid.jcvi.org/projects/gsc/leptospira/) will provide detailed studies on *Leptospira* evolution and distribution, identifying features that are unique to pathogenic species of *Leptospira*.

The whole genome of *L. kirschneri* was not studied to date. *L. kirschneri* is a pathogenic species containing pathogenic serovars such as those of the Pomona serogroup that are associated as the cause of occasional small outbreaks of leptospirosis in animals [23-25]. *L. kirschneri* serovar Mozdok is one of those serovars belonging to the species and serogroup mentioned that is highlighted in this report. Collares-Pereira et al. [26] reported three types of Mozdok strains, i.e., type 1, type 2 and type 3, based on the different patterns obtained from the agglutination of a panel of monoclonal antibodies (MAbs). There is very limited information about the genetic differentiation of these three types of serovar Mozdok. As far as we know serovar Mozdok type 2 was only documented as isolated in Pancas, Portugal [26]. A reassessment of the serological characterization based on the agglutination of MAbs for some serovars from another study [27], allowed to reassign isolate No. Horse 122, belonging to the Pomona serogroup, as Mozdok type 2 (see Chapter 4). This discovery once again in Portugal, following the lack of information about this strain worldwide, ensures the need to analyze its genome so as to identify possible important mechanisms in clinical outcome. Recently, draft genomes sequences have been available on the NCBI database (http://www.ncbi.nlm.nih.gov/) for two type's serovars of *L. kirschneri* serovar Mozdok, namely the type 1 and type 3 and there is no genome information available for the type 2. Here, we announce the draft genome sequence of *L. kirschneri* serovar Mozdok type 2 strain Horse 112 mentioned above was isolated from a 5–10 years old horse in 2004. The sequenced genome will

contribute for a future genome comparative analysis providing the identification of important molecular markers of *L. kirschneri* serovar Mozdok types and perhaps even an income to explore a genetic basis for disease severity.

5.3. Materials and Methods

5.3.1. Bacterial culture and genomic DNA extraction

The *L. kirschneri* serovar Mozdok type 2 str. Horse 112 strain was grown at 28 °C under aerobic conditions in liquid Ellinghausen–McCullough–Johnson–Harris (EMJH) medium. High-quality genomic DNA was extracted from 25 ml EMJH liquid cultures using the QIAamp DNA extraction kit (Qiagen, Hilden, Germany), following the manufacturer's instructions with modifications. The bacterial culture was centrifuged for 45 min at full speed. Given the high amount of culture the volumes were adjusted to 3 times more the volume of the ATL buffer (supplied in the QIAamp DNA extraction kit) and proteinase K added as well as 2 times more the RNAase.

5.3.2. Sequencing

Genome was sequenced using Ion Torrent's PGM, according to the manufacturer's instructions. Adapter-trimmed high-quality reads were assembled using CLC Genomic Workbench version 7.0.3 *de novo* assembler.

5.3.3. Gene finding and annotation

The chosen tool for gene detection was Prodigal 2.6.2 [28] that has shown to be specific for prokariotic gene prediction. Next step was to identify the function for all these genes in comparison with the sequences from the database belonging to the taxonomic group Bacteria. The search was carried out using Blastp using the translated protein from every gene identified with a minimal p-value of 10^{-3}.

5.3.4. SNP calling and intraspecific variation

Using the resultant contigs from the assembly using CLC as reference, a SNP calling was made by mapping reads coming from sequencing in *L. kirschneri* serovar Mozdok type 2 str. Horse 112, as well as reads coming from a set of different strains from NCBI. The SRA codes for these new strains to compare were: SRR353570, SRR353572, SRR712415, SRR712414 belonging to *L. kirschneri* serovar Mozdok str. Brem 166, serovar Mozdok str. B 81/7, serovar Mozdok 1 str. Vehlefans 3 and serovar Mozdok 1 str. Vehlevans 2, respectively. To detect the SNPs a first step of mapping reads was performed using BWA. After this, SAM files were processed using SAMtools to get the final VCF file containing the SNPs across the five strains of *Leptospira*. SNPs belonging exclusively to *L. kirschneri* serovar Mozdok type 2 str. Horse 112 were quantified. SNPs shared between strain Horse 112 and the other strains were also quantified.

5.3.5.Search for rRNA

The search for rRNAs was divided in two since the used databases were divided in small subunit (SSU) and large subunit (LSU), both of them retrieved from SILVA database (www.arb-silva.de).

5.4. Results

L. kirschneri serovar Mozdok type 2 str. Horse 112 genome was sequenced using Ion Torrent's PGM sequencing platform, and the data set was made up of 2,675,719 paired-end reads with a 56,512 bp average length and a raw coverage depth of 183×. The draft genome sequenced *de novo* had a total of 4,302,078 bases with the primary assembly consisted of 485 contigs ($N_{50\ [bp]}$=15,231). With the set of contigs free of redundancy, a gene detection was performed in order to identify the genic structures along the sequences. The results of gene prediction by Blastp revealed a total of 3766 genes coding for proteins getting the best result in most of cases belonging to *Leptospira* family

what indicates a good prediction in the genes. The majority of the start codons were ATG with a total of 2979.

Approximately half of the genes (i.e., 1750 genes) were annotated to encode hypothetical proteins. Besides, there are 120 predicted coding sequences annotated as uncharacterized protein, i.e., failed to exhibit similarity to any known genes. The rRNAs search for strain Horse 112 sequenced genome showed that it contains 24 LSU and 14 SSU genes. General genome features are summarized in Table 5.1.

Table 5.1. Summary of general genome features of *L. kirschneri* serovar Mozdok type 2 str. Horse 112 compared to strains Brem 166, B 81/7, Vehlefans 3 and Vehlevans 2.

Serovar/strain	**Feature**			
	Size (Mp)	**GC%**	**Gene**	**Protein**
Mozdok 2 str. Horse 112[1]	4.30	35.90	3766	3762
Mozdok str. Brem 166[2]	4.35	35.90	3665	3594
Mozdok 3 str. B 81/7[2]	4.25	35.90	3611	3445
Mozdok 1 str. Vehlefans 3[2]	4.40	35.90	3702	3610
Mozdok 1 str. Vehlevans 2[2]	4.41	35.90	3707	3618

[1]Rocha et al. [27]; [2]data obtained from the database Genbank (http://www.ncbi.nlm.nih.gov/genome).

We assessed the presence of SNP markers across the genome between strain Horse 112 and the panel of *L. kirschneri* serovar Mozdok genomes surveyed in this work. Among 91 SNPs assessed in strain Horse 112, 19 were common to the other serovar Mozdok genomes (Figure 5.1.). Wherein 17 are shared between strains Horse 112 and Brem 166 and B 81/7 (Figure 5.1.).

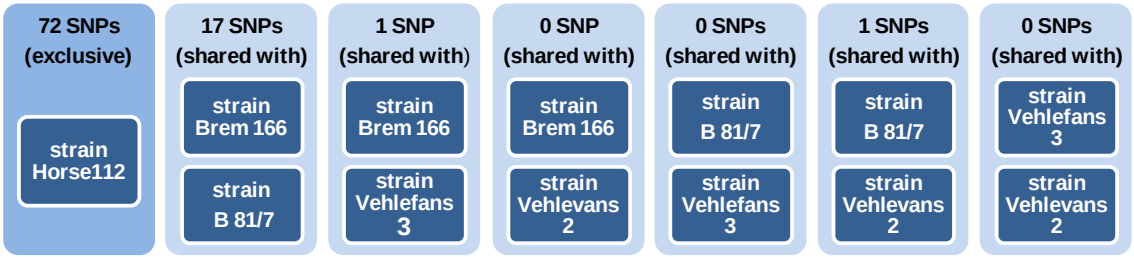

Figure 5.1. Total number of SNPs assessed for *L. kirschneri* serovar Mozdok type 2 str. Horse 112 against strains Brem 166, B 81/7, Vehlefans 3 and Vehlevans 2.

5.5. Discussion

The work presented here is an announcement of the whole-genome sequence of *L. kirschneri* serovar Mozdok type 2 str. Horse 112. This strain represents a serovar Mozdok type 2 of Pomona serogroup referred to as the cause of occasional small outbreaks of leptospirosis in animals [23-25]. The serovar Mozdok type 2 was assigned to Horse 112 strain by the use of an MAbs panel at the WHO/FAO/OIE and National Collaborating Centre for Reference and Research on Leptospirosis, Royal Tropical Institute (KIT), Amsterdam (Chapter 4). This strain was originally isolated from an apparently healthy horse and was previously typed as *L. kirschneri* serovar Tsaratsovo using a restriction endonuclease analysis (REA) approach [27]. Discrepancies between MAbs panel serotyping and genome-wide molecular fingerprints at the serovar level were previously noticed for *Leptospira* serovar Pomona strains [26, 29, 30]. Noteworthy, several Portuguese field and other reference *Leptospira* strains assigned to serovar Tsaratsovo by genetic methods were found to represent Mozdok types 1 and 3 when typed by the use of MAbs panels [26]. In the REA study of Portuguese strain Horse 112 performed by Rocha et al. [27], slight differences were observed between Tsaratsovo (Horse 112 and Tsaratsovo type strain B 81/7 reference strain) serovars, contrarily to the previous REA observations of Terpstra et al. [29] on the same serovars.

To date, no genome sequences of serovar Mozdok type 2 strains are publicly available. Sequencing of several genomic regions represents a powerful approach to identify variations in the DNA sequence. Data presented in this work provide general features on the draft genome generated from *L. kirschneri* serovar Mozdok type 2 str. Horse 112, and report SNP variation between the strain studied and phylogenetic related strains deposited as a public resource on GenBank database, namely *L. kirschneri* serovar Mozdok str. Brem 166, *L. kirschneri* serovar Mozdok str. B 81/7, *L. kirschneri* serovar Mozdok 1 str. Vehlefans 3 and *L. kirschneri* serovar Mozdok 1 str. Vehlevans 2. These draft genomes available are included in the ongoing "*Leptospira* Genomics and Human Health" project which does not include a serovar Mozdok type 2 (www.jcvi.com).

Strain Horse 112 has a genome size equivalent when compared to other *Leptospira* genomes [22]. High quality sequencing reads of this strain were mapped to the reference sequence (RefSeq) *L. interrogans* serovar Lai str. 56601 and assuming 100% identity between sequencing reads only 6 % of the reference was found to be covered by sequencing reads (data not shown). Thus it was performed less stringent analysis and assumed mapping condition that at least 80% of sequencing read must be at least 80% identical to the reference and so 82% of the reference was found to be covered. Although genetically similar, *L. kirschneri* serovar Mozdok type 2 str. Horse 112 and *L. interrogans* serovar Lai str. 56601 belong to different species so it would be expectable the genome variation observed. Our preliminary findings are based on a general comparison between strains of the same species and, especially, at the same serovar, i.e. *L. kirschneri* serovar Mozdok. Accurate detection and genotyping of SNPs is crucial to detect rare, as well as common, variants between strains. It determines the genotype for each individual at each site contributing to better elucidate and determine the origin and dissemination of the strains. Our data show a total number of 91 SNPs assessed in *L. kirschneri* serovar Mozdok type 2 str. Horse 112 genome against the other four *L. kirschneri* serovar Mozdok strains that were analyzed in groups of three combinations of strains of time (Figure 5.1.). Although substantial, this number represents only a reference since our analysis cannot filter out false SNPs that may be attributable to

sequencing errors. Recognizing rare SNPs instead of sequencing errors remains a challenge [31, 32]. Bansal and collaborators [31] mentioned that analysis of population sequencing data can potentially allow an accurate detection of SNPs, distinguishing between false SNPs (i.e., sequencing errors) and real SNPs. Efforts are needed in order to increase the number of available complete (nondraft) genome sequences from the different types of serovars Mozdok that can contribute to filter out SNPs that represent artifacts of systematic sequencing errors. Additionally, obtaining well-calibrated quality scores is important to determine in which positions there are polymorphisms or in which positions at least one of the bases differs from a reference sequence [32]. Remarkably, 17 SNPs are shared between strains Horse 112 and two genomes analyzed, including strains Brem 166 and B 81/7 (Mozdok type 3), sugesting the accuracy of these SNPs and the possibility of the two isolates being likely identical to each other. This does not exclude the fact that the majority of 72 SNPs assessed as exclusive for serovar Mozdok type 2 str. Horse 112 studied are false, which may have biological significance. Moreover it is noted that those strains used for comparison were manipulated for sequencing in different laboratories, after serial passage, which can influence the results. Further sequencing analysis should be done at those SNP positions to clarify our results.

The automatic annotation of strain Horse 112 genome sequence revealed 3766 genes which is similar with the other strains analyzed. Most of these genes are referred to as belonging to *Leptospira* family indicating a good prediction in the genes. Interspecies comparison of gene layout reveals extensive reorganization of genes. However, a genome comparison using the total number of genes features is misleading because of the multitude of insertion sequence (IS) elements present in the *Leptospira* genomes [33]. Comparative genomics of the pathogenic strains of *L. borgpetersenni* and *L. interrogans* and the non-pathogenic *L. biflexa* has assessed the identification of 893 pathogen-specific genes wherein genes encoding proteins of unknown functions are overrepresented [22]. This is a key point in the post-genomic analysis since it suggests the presence of pathogenic mechanisms unique to *Leptospira* [34]. However, to date all studies done are insufficient to bridge this gap. Bulach et

al. [33] mentioned that an accurate estimation of the start of the coding regions is important for a prediction of the subcellular location of the encoded protein. The majority of the start of the coding regions obtained in this study was ATG with a total number of 2979. This value is in agreement with those presented by Bulach et al. [33] wherein documented 2733, 2807 and 2572 for the serovars Copenhageni, Lai and Hardjobovis, respectively. Conversely, CTG and ATT did not occur in our study.

The analysis of whole genome sequences allowed findings of significant structural differences, such as the large chromosomal inversion and the distribution of several insertion sequences [17, 35, 36]. Furthermore, lateral DNA transfer has been reported, corroborating the concept of genome plasticity as suggested by several studies [17, 20, 37]. The availability of whole-genome sequences of relevant *Leptospira* strains provides a genetic basis for such studies enhancing the possibility of someday being used in order to accurately predict magnitude of virulence. By generating the first draft genome of a serovar Mozdok type 2 strain we are able to provide insights for a more detailed and comparative analysis to correlate serovar Mozdok's types characteristics and genomic sequences contributing to a growing awareness and deeper understanding of this serovar.

5.6. References

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CHAPTER 6

General Discussion and Concluding Remarks

6.1. General Discussion

The main objective of this work was essentially to develop and apply improved methods for the laboratory diagnosis of leptospirosis aiming the detection and identification of *Leptospira* strains in clinical specimens and the typing of these agents. An effective surveillance and control of leptospirosis in the veterinary field requires a rapid and accurate detection of the etiological agent, i.e. *Leptospira*, by the use of effective diagnostic tests. Detection of the agent is also necessary to identify animal carriers. Moreover, the speciation of infecting *Leptospira* from clinical material may be important for determining the clinical significance, the probable source of infection, to distinguish sporadic cases from possible outbreaks and to better access the epidemiology of the disease.

DNA-based methods were selected as the main means to fulfill the central objectives of this project. The most used DNA amplification technology in veterinary microbiology laboratories is, unquestionably, the polymerase chain reaction (PCR), introduced in the mid-1980s [1]. The increasing affordability of thermal cyclers and automated DNA extraction platforms makes PCR attractive for the diagnostic microbiology laboratories [2]. It is amazing how rapidly the technological advances in the molecular diagnostic field have developed, in the latest years. Currently, several PCR-based assays are available to shorten the analysis time and the specific detection of leptospiral DNA without culture procedures [3-9] but only a few of these assays have been clinically validated [10-13]. Despite, these methods still show limitations to detect *Leptospira* in clinical samples they are more advantageous compared either to conventional microscopy-based techniques or serological methods, which require trained staff and complex laboratorial procedures. From a diagnostic perspective, PCR is also the only sensitive and specific test for *Leptospira* detection within the first days of the disease after the onset of symptoms and prior to the production of detectable antibodies, which is particularly useful in human leptospirosis, where early diagnosis is crucial for establishing a prompt initiation of effective antibiotic therapy in individuals, to avoid a possible fatal evolution of acute disease [14]. In animals, bacteraemia is transient and, although some acute infections can cause expressive symptoms like icterus and haemoglobinuria, especially in

young animals, in most cases the acute phase of the disease is not accompanied by clinical signs and emphasis goes to the diagnosis on a herd basis, often related to chronic infections, which can cause reproductive problems like abortion, stillbirth, birth of weak offspring and infertility, resulting in serious economic losses [15]. Despite that differences in its application exist related to the cycle of infection in animals and purpose of diagnosis, the PCR approach for the demonstration of leptospires or their genetic material in animal tissues or body fluids can be very useful to establish a more rapid diagnosis and overcome the limitations of isolation in the detection of those chronical infections and of carriers animals.

PCR methods have shown to be particularly useful for the study of fastidious organisms that are difficult or impossible to cultivate in artificial media [16]. This powerful technology also allowed achieving important milestones in the field of leptospirosis, including the ongoing *Leptospira* Genomics and Human Health Project (http://gcid.jcvi.org/projects/gsc/*Leptospira*/).

The objectives originally proposed for this project (Chapter 1), were considered to be accomplished. A detailed discussion of the results obtained during this study was made in the previous chapters, so the current chapter overviews the main findings and their contribution for the *Leptospira* study. Perspectives that may promote the advancement of *Leptospira* detection and typing are also discussed. The main goals resulting from this work may be shared between two important topics: i) Simultaneous detection and identification of leptospiral DNA from pure cultures and biological samples (Chapters 2 and 3); and ii) Typing of *Leptospira* strains (Chapters 4 and 5).

6.1.1. Simultaneous detection and identification of leptospiral DNA

To meet the objectives outlined for this task, it was essential to carefully select the most suitable PCR-based technique, balancing budget constraints and requirements for special equipment and technical expertise. Choosing a real-time *TaqMan*-based PCR approach (Chapters 2 and 3) focused on its ability to detect and identify bacterial species simultaneously within a single reaction, thus further reducing cost and hands-on time. Prior to the writing of this project,

there were no studies describing a strategy capable of clearly detecting and identifying simultaneously the most frequently found pathogenic *Leptospira* species based on the real-time PCR approach. Most of the described assays have relied on SYBR green detection chemistry and were only able to differentiate between pathogenic and non-pathogenic leptospires, lacking the ability to distinguish between different species. Merien *et al.* [17] has developed a real-time PCR based on melting curves analysis with the use of SYBR Green I to distinguish between seven pathogenic *Leptospira* species. However the detection sensitivity of this assay was limited when compared to a specific fluorescent *TaqMan* probe designed towards a particular target sequence [18]. A major challenge was the selection of high quality DNA markers (i.e., DNA regions) that were specific for the target *Leptospira* species, in order to avoid false positives, and simultaneously enabling the differentiation amongst the other species. At the development stage of this study, the issues concerning the "*in silico*" comparative sequence analysis was further complicated by the limited data available in the Genebank where the occurrence of several mistakenly annotated sequences were also found. With an extensive screening on several potential target sequences it was possible to design a novel and simple *TaqMan*®-based multi-gene targeted real-time PCR approach. This PCR approach used molecular targets comprising a sufficient number of polymorphisms allowing the species specific amplification of *L. interrogans*, *L. kirschneri*, *L. borgpeteresenii*, *L. noguchii* (Chapter 2), *L. santarosai* and *L. weilii* (Chapter 3). These six species constitute the veterinary most relevant pathogenic species of *Leptospira*. The method uses sets of species-specific probes, and respective flanking primers, designed with complementary targets on *rrs, ompL1* and s*ecY* gene sequences. These genes are present and moderately well conserved across a broad range of pathogenic *Leptospira* species. However, these genes also present polymorphisms that make their use highly valuable for the differentiation amongst *Leptospira* species. For example, Victoria et al. [19], in turn, demonstrated that *secY* gene, encoding the SecY preprotein translocase, has a high discriminative power to the species level.

Our PCR assays, revealed a high specificity, yielding amplification signals only from respective target species. The specificity was validated by an inter-

laboratory study in cooperation with the National Collaborating Centre for Reference and Research on Leptospirosis, KIT Biomedical Research (Amsterdam, The Netherlands) by testing several reference strains *Leptospira*, representative of the genomic and geographic diversity of the genus. This validation step is essential to incorporate such a test in diagnostic routine methods. Apart from continuous technical improvements, an accurate standardization of this method is necessary to ensure laboratory-to-laboratory analysis applicability and reproducibility under more different circumstances. Consequently, it is important to continually evaluate the specificity of the designed probes and primers and, if necessary, modify and improve the sequences, in order to ensure an effective and specific detection and identification of the circulating *Leptospira* species.

Culture of leptospires constitutes the definitive diagnosis of leptospirosis. The major advantage of culture is that it can provide the subsequent identification of the isolate, which is useful in epidemiological studies and to establish adequate treatment and control measures. However the success in isolating leptospires depends on the clinical specimen's material, the stage of infection and other factors (Chapter 1), being highly susceptible to failure. Culture approaches are also slow for routine use. In this context, this work emphasizes the utility of a real-time *TaqMan* PCR approach to circumvent these limitations. Application of this method on animal samples and its results in comparison with culture, as the gold-standard diagnostic method, were discussed in Chapter 3. The developed DNA-based detection assay exhibits a higher sensitivity in the identification of leptospires from biological samples, allowing the direct detection and identification of infecting leptospires in those samples. In the *TaqMan*®-based multi-gene targeted real-time PCRs developed, pathogenic leptospires were successfully detected in tissue samples from animals (*Mus* spp., *Rattus* spp., *Apodemus sylvaticus, Crocidura* russula, *Dolichotis patagonum* and *Sus scrofa domesticus*). The inclusion of an internal control PCR (targeting the mammal *β-actin* gene) in the initial duplex *β-actin/lipL32* PCR, when testing tissue samples, allowed to assess the presence of amplification inhibitors [20] or failure of DNA extraction. Despite the great advantages presented by real-time PCR approaches, in terms of specificity and sensitivity in detection and identification

of *Leptospira* in clinical specimens (Chapter 3), these approaches still face several challenges, especially in the interpretation of the clinical relevance of the results. Important in this context is the availability of reference materials and an accurate gold standard diagnostic method to validate and assess the performance of this and other molecular methods for use in veterinary microbiology laboratories. Efforts are been made by the OIE, making available relevant guidelines and procedures towards that aim [16]. On the other hand, it is remarkable the fundamental role played by the veterinary microbiology laboratories in this context, since their main purposes include the detection, identification, and characterization of any pathogenic organisms present in a broad range of biological samples, such as tissues, blood, urine, and other fluids collected from suspect animals [16]. The development and adoption of standard efficient protocols for the extraction of nucleic acids, for example, are still an unmet need that has hampered the widespread routine use of DNA-based methods in the veterinary microbiology laboratory. PCR assays are sensitive, but quality control procedures and sample processing for PCR are critical and must be adjusted to the tissue, fluid and species being tested [21].

Finally, real-time PCR is an important technological PCR-based advance. Being faster than conventional PCR, it also shows improved sensitivity, robustness, reproducibility and a considerably reduced risk of carry-over contamination [18]. Due to these advantageous features, it has been taking an increasingly relevant role, by supporting and complementing the conventional diagnostics methods. Consequently, it is clear that real time PCR-based methods are definitely part of the future trends in *Leptospira* detection and identification. Rapid and reliable laboratory tests for the direct detection and identification of leptospiral infections in animals are in high demand, particularly to support suitable control measures. Recently, methodological challenges have emerged and chromatography- and mass spectrometry-based methods are also becoming much more frequently used [22]. Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) was proposed for the rapid identification and reliable identification of *Leptospira* isolates at the species level [23, 24] . This method is based upon the detection of highly abundant proteins assessing differences in the protein profile peak patterns by mass spectrometry in order to

identify specific peaks that would allow the discrimination of the serovars. Although promising, this method is limited by the fastidious growth of the *Leptospira*.

6.1.2. Molecular typing of leptospires

During the last four decades, molecular techniques had a tremendous impact in the *Leptospira* study, and especially in its typing. Typing of *Leptospira* organism presents several challenges, also related with the relatively current complexity of the taxonomy of this genus. Serological classification, as mentioned earlier in this dissertation (Chapter 1), claims the serovar as the basic taxon of *Leptospira*, which is usually incompatible with the molecular classification. Earlier DNA-DNA hybridization experiments and more recent sequencing-based advances, led to a major rearrangement of the taxonomic status of *Leptospira* serovars within the genus and this has proven to be very successful regarding the serogrouping of the *Leptospira* strains. A particular example is the retention of (sensu stricto) *L. interrogans* and *L. biflexa* as specific names in the genomic classification instead of (sensu lato) *L. interrogans* and *L. biflexa* in the phenotypic serological classification.

Many molecular techniques have been explored, alternatively, taking into account the current existing conventional serological characterization methods which are cumbersome and require a high level of technical expertise. Methods for *Leptospira* characterization that directly generate digital data have a major advantage over other methods. Among them, *Multilocus Sequencing Typing* (MLST) has been highlighted by its robustness and usefulness in assessing *Leptospira* serovars diversity. Indeed, MLST is considered to be the most robust, phylogeny-based typing method for *Leptospira* providing an online availability and analysis of data. These kind of typing methods, targeting multiple genomic targets, are especially important in order to limit the risk of misclassification due to a possible horizontal gene transfer already reported for *Leptospira*. In Chapter 4, a major effort was placed on the implementation and applying of such approach, focusing on the characterization of Portuguese *Leptospira* strains. A selection of isolates was genotyped with three MLST

schemes based on the sequence analysis of six or seven loci: 1) *adk; icdA; lipL32; lipL41; rrs2; secY* (6L) [25-27]; 2) *glmU; pntA; sucA; tpiA; pfkB; mreA; caiB* ($7L_{Boonsilp}$) [28]; and 3) *adk; glum; icdA; lipL32; lipL41; mreA; pntA* ($7L_{Varni}$) [29]. All the three MLST schemes revealed a similar discriminatory power for typing the Portuguese isolates, allowing the correct assignment of *Leptospira* species and, within each species, with some propensity to differentiate isolates according to their hosts. These findings were expected based on previous studies which reported associations between specific *Leptospira* STs, their hosts and geographic regions [28, 30]. Following the results obtained in our study, one of the schemes, based on the analysis of seven loci ($7L_{Boonsilp}$), is considered to be more useful, essentially due to its online database that comprises a large set of typed strains and thus capable to assign a sequence type to most of our isolates. Although the recent $7L_{Varni}$ scheme appears to be tempting and feasible in merging the most discriminative loci, it still needs a full database that encompasses a significant number of STs to provide a wide genotyping comparison of the *Leptospira* serovars. On the other hand, the 6L scheme makes use of *lipL41* and *lipL32* that are not housekeeping genes. Being part of different functional categories, encoding surface expressed proteins, they might be affected by selection pressure and hence lose their evolutionary neutrality. In summary, we believe this MLST study contributed to improve the knowledge about the genetic diversity of pathogenic leptospires circulating in Portugal, being also relevant for the enlargement of the relevant MLST online databases with the submission of a new set of allele and sequence type information regarding Portuguese, or European, isolates. However, the current variety of available schemes hampers an extensive agreement to achieve a single generally supported online database MLST scheme towards *Leptospira* genotyping. Additionally, requirements in highly skilled personnel and expensive equipment and reagents are also considered limiting for a wider use of this typing approach.

During the course of this work, we also had the opportunity of serotyping by MAbs a large number of Portuguese strains at the WHO/FAO/OIE and National Collaborating Centre for Reference and Research on Leptospirosis, Royal Tropical Institute (KIT), Amsterdam, The Netherlands, according to the standard

serological methods used in this reference laboratory. Particular emphasis was given to the specific *L. kirschneri* serovar Mozdok type 2 str. Horse 112 [31], that may represent a special relevance for Portugal, since the serovar Mozdok type 2 was documented as being only isolated in Pancas, Portugal [32]. This reality drove the project's research effort to propose sequencing of the whole genome of that strain (Chapter 5), aiming to increase the knowledge on the genomic features of pathogenic leptospires, particularly of *L. kirschneri* serovar Mozdok types. The Horse 112 isolate has been first identified by REA and considered to belong to serovar Tsaratsovo [28], however, when typed by MAbs in the course of this study, it was identified as Mozdok type 2. The classification of Pomona serogroup serovars has always been difficult and the work of different authors**,** using several methods of identification, has given rise to controversial and sometimes contradictory observations [15, 33]. Several studies have reported that serovars Mozdok and Tsaratsovo are identical [33, 34], and it has been suggested that the designation of Tsaratsovo as a separate serovar should be abandoned. Terpstra et al. [35] found that Tsaratsovo, Dania and Mozdok were identical, by REA, although Tsaratsovo could easily be distinguished by MAbs analysis. However, in the REA study of Portuguese strains Horse 112 and Mozdok PT, performed by Rocha et al. [31], slight differences were observed between Tsaratsovo (Horse 112 and Tsaratsovo type strain B 81/7 reference strain) and Mozdok (Mozdok PT and Mozdok 5621 reference strain) serovars, contrarily to the previous REA observations of Terpstra et al. [35] on the same serovars. It is hoped that sequence-based approaches may bring further elucidation into the classification of Pomona group strains.

By 2009, when the current project started, the complete genome sequences of three *Leptospira* species were available in public databases, namely: *L. interrogans* serovars Lai [36] and Copenhageni [37], *L. borgpeterseni* [38]; and the saprophyte *L. biflexa* [39]. This available sequence information provided a good starting point for the use of bioinformatics to select novel markers for *Leptospira*. Since then, the number of available full genome sequences increased [40, 41] and is expected to continue increasing, with the sequencing of several genomes, already completed but not yet fully analyzed, achieved

through the ongoing *Leptospira* Genomics and Human Health Project (http://gcid.jcvi.org/projects/gsc/*Leptospira*/). In this context, the selection of *L. kirschneri* serovar Mozdok type 2 str. Horse 112 for genome sequencing and announcing (Chapter 5) provided an added challenge for this project. The complete genome sequences of other *L. kirschneri* strains, were not available for comparison when this project started.

Therefore, in Chapter 5 we described general features on the draft genome generated from *L. kirschneri* serovar Mozdok type 2 str. Horse 112. A generic comparison with other *L. kirschneri* whole-genome sequences made available during the last couple of years was also attempted: *L. kirschneri* serovar Mozdok types 1 and 3 (*L. kirschneri* serovar Mozdok str. Brem 166, serovar Mozdok str. B 81/7, serovar Mozdok 1 str. Vehlefans 3 and serovar Mozdok 1 str. Vehlevans 2). In the overall, by generating the first draft genome of a serovar Mozdok type 2 strain we were able to provide insights for a more detailed and comparative analysis to correlate serovars Mozdok characteristics and genomic sequences contributing to obtain, in a near future relevant information, about these pathogenic serovars (e.g. by the analysis of molecular determinants encoding virulence factors). In the coming years, there is no doubt that new-generation sequencing platforms will continue to undergo remarkable developments on the characterization of species-specific or serovar-specific leptospiral DNA, becoming more user-friendly and suitable to be applied in a diagnostic setting [42]. Advances on *Whole Genome Sequencing*-based tools will shape the transmission networks concerning novel diagnostic tools and molecular epidemiology studies (for surveillance and outbreak prevention and control) on leptospirosis both in the human and in the veterinary field.

6.2. Considerations for future developments in leptospirosis study and knowledge

The main expectation of this work is that it may contribute to the advance of the knowledge about leptospirosis and about the agent of this important disease. Original studies concerning the development and application of DNA-based approaches for the efficient detection, identification and typing of *Leptospira* strains, as a complement or alternative to conventional culture and serological approaches, were presented and discussed. It can be generally concluded that the studies performed were relevant in relation to its original aims. The following considerations should be highlighted to foster research and future advances in the *Leptospira* study field:

1. In Portugal, there is clearly a need of further research on leptospirosis towards the answer of questions, such as those related to (a) obtaining new local *Leptospira* isolates in different geographical regions; (b) the coverage of new possible host parasite relationships; and (c) the understanding of the impact of new Portuguese strains (e.g. *L. kirschneri* serovar Altodouro) in the international arena; Within a veterinary context, the emergence of new host parasite relationships should be covered by a permanent vigilance, which is important both economically and in the prevention of the disease importance;
2. Concerning molecular diagnostics, there is a need to balance the improvements in this field with the epidemiological and control requirements. The international reference centers have a key role in promoting the standardization of these methods as well as its validation and dissemination. Novel techniques capable of identifying the infecting strain in clinical specimens are imperative;
3. Due to the ambiguous nature of human leptospirosis symptoms, leading to its under-diagnosis, and being real-time PCR a very promising tool for the early diagnosis of the disease, the simultaneous DNA detection of other agents of febrile illnesses (e.g., besides leptospirosis, rickettsioses,

dengue, and other viral hemorrhagic infections) would represent a major improvement for the leptospirosis differential diagnosis;

4. There was evident research effort concerning the application of newly molecular strategies in *Leptospira* detection. Among the strategies for nucleic acid amplification worthy of note are those based on isothermal processes, such as the technology of loop-mediated isothermal amplification (LAMP) that may provide potentially more affordable tools, especially for the low-income countries;
5. In recent years, although the use of MLST approach is the current most robust method for typing *Leptospira* strains, the WGS-based tools are gaining increasing interest as they provide a meaningful data for selection of the most effective DNA markers to better define the serovar status and their evolutionary phyletic relationships; Thus, improvements on suitable software to achieve this goal can simplify *Leptospira* characterization.

6.3. References

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