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Overview of laboratory methods to diagnose Leptospirosis and to identify and to type leptospires

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Summary. Leptospirosis is a virulent zoonosis with a global distribution. Pathogenic spirochetes of the genus *Leptospira* are responsible for this disease, and the primary animal reservoirs are rodentvvvs. Direct and indirect contact with infected urine constitutes the main route of transmission. Renal failure and advanced abortions are frequently observed in animals affected by leptospirosis, causing serious problems for farms. In humans, there is a high rate of mortality (10 percent), and farmers and persons in contact with water are frequently exposed. However, vaccines and strict prevention measures confer protection against leptospirosis. Serological tests facilitate the detection and identification of leptospire strains. Such tests are based on specific surface antigen recognition and are used for clinical analyses. To determine which serovars circulate in the environment, leptospires must be typed. Molecular methods, such as restriction enzyme-based techniques and the sequencing of specific regions, permit serovar identification. Unfortunately, although there are numerous techniques, they are not very efficient, and thus, new methods must be developed. With the advent of genomic sequencing, a substantial amount of information regarding leptospire genomes is now available, facilitating the selection of regions to discriminate between strains. Typing is important for both epidemiologic purposes and clinical analyses.

Keywords: Leptospirosis · zoonosis · methods · diagnosis

Introduction

Leptospirosis is a worldwide zoonosis [28] and is considered a re-emerging disease [9]. Leptospirosis affects a large variety of animal species, including humans [9, 28], and is caused by leptospires [2, 28], which are bacteria that belong to the order *Spirochaetae*. The primary reservoirs are rodents, particularly rats [2, 28, 38, 54]. Generally, these pathogens are carried asymptomatically in the kidney or liver. However, leptospires cause internal injuries to rats, such as lymphoplasmocytic inflammatory infiltration and cell hyperplasia, which have been observed in kidney tubules [5, 33, 49]. Various wildlife species, such as coypus and small mammals (hedgehogs, badgers, etc.), [58] contribute to the environmental persistence and dissemination of leptospires. Many studies have investigated the leptospire carrier status of small mammals [45, 50]. Animal reservoirs accumulate leptospires in their kidneys before excreting them into urine. Contamination-sensitive animals and humans primarily acquire leptospires via indirect contact with infected urine in water and in the environment [9]. However, direct contamination may also occur. The most frequently exposed people are farmers and those who professionally practise aquatic leisure activities. In other populations, the incidence of acquired leptospirosis has begun to decrease.

Leptospirosis presents a wide array of symptoms [28], ranging from benign to major disorders and infections. In animals, leptospires provoke symptoms such as abortion or milk-drop syndrome, which result in significant economic losses for

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breeders. Human infections are characterised by fever, renal failure or hepatic failure. In some cases, meningitis and pulmonary haemorrhages occur. Overall, the global mortality rate is estimated at approximately 10%, with a maximum of up to 25% in developing countries. Outbreaks are frequently observed in tropical regions, particularly in India [25] and Brazil [32]. Furthermore, the incidence of this disease is increasing in certain tropical regions, such as Malaysia [8]. The World Health Organisation (WHO) estimates the number of severe human cases at approximately 1,000,000 per year [14, 21]. Among European countries, France is the most heavily affected with 600 cases per year, which is the highest number in overseas territories.

The control of leptospirosis is complicated due to the large number of serovars, infection sources and variable transmission conditions. Furthermore, control is dependent on local environmental conditions (moisture, temperature, etc.). Control is achieved by regulating reservoirs or reducing infection in animal reservoir populations, such as in dogs or livestock, in addition to human vaccination. To achieve vaccine efficiency, the serovars circulating in a particular region must be identified. Currently, there are only 2 commercially available inactivated human vaccines. The Cuban vaccine (Vax Spiral®) contains whole bacterial cells of the Canicola, Icterohaemorrhagiae and Mozdok serovars, whereas the French vaccine (Spiroleptc®) contains only inactivated Icterohaemorrhagiae cells. In animals, vaccination is available for livestock and dogs, and the composition of commercially available vaccines depends on locally circulating serogroups and regulatory administration. In Europe, the available formulations for livestock contain the unique serovar Hardjo. In the United States, vaccines used on pig farms contain either the single serovar Hardjo or five serovars including Canicola, Grippotyphosa, Icterohaemorrhagiae and Hardjo. In New Zealand, vaccines are composed of 2 serovars, Hardjo and Pomona, or 3 serovars, comprising Hardjo and Pomona as well as Copenhageni. Dog vaccination includes 2 (Canicola and Icterohaemorrhagiae), 3 (Canicola, Icterohaemorrhagiae, and Grippotyphosa), or 4 serovars (Canicola, Icterohaemorrhagiae, Grippotyphosa, and Pomona or Australis depending on the continent). A recently manufactured horse vaccine derived from Pomona bacterin is currently available in the United States. Generally, Bratislava, Pomona and/or Tarassovi are included in commercially available swine vaccines, which are part of the recommended vaccination programmes for pig farms in the United States, Australia and New Zealand.

In both humans and dogs, penicillin and doxycycline are used to treat leptospirosis, but their efficacy is low if administered late during disease. Streptomycin is recommended by the World Organisation for Animal Health (OIE) for the treatment of leptospirosis in horses, cattle and other farm animals.

Leptospires must be detected quickly due to the risks of infection and for epidemiologic studies. However, there is no rapid detection method for leptospires. It is possible to perform serologic tests on blood samples obtained during the first week of infection, but testing rarely occurs. Thus, the number of infections induced by leptospires is likely underestimated.

Leptospires, the causative agents of Leptospirosis. Weil described leptospirosis as severe jaundice in 1886; Weil's disease was characterised by renal failure and sever haemorrhage [4, 41]. However, he did not isolate or identify the causative agent.

Several years later, in 1914, Inada and Ido were the first scientists to identify leptospires after they inoculated a guinea pig liver with the blood of a patient suffering from jaundice and observed a spirochete, naming it *Leptospira haemorrhagae*. In 1915, they published a paper describing their discovery and suggested this spirochete as the causative agent of Weil's disease [26].

Leptospires are long and motile bacteria. They have a diameter of 0.1 μm and are 6 to 20 μm in length. The ends of the bacteria are hooked. In the periplasmic space, two flagella are responsible for motility. The flagella are composed of the FlaA and FlaB proteins [2]. Additionally, leptospires have a double membrane structure: an outer membrane that envelops the cytoplasmic membrane and a peptidoglycan cell wall [15]. The outer membrane is composed of lipopolysaccharides (LPS), which are the primary leptospire antigen. Many of the structural and functional proteins found in this membrane are lipoproteins (LipL32, LipL21 and LipL41), integral membrane proteins and the type two secretion system (T2SS) protein secretin.

Leptospires are obligate aerobes and grow optimally at 30°C in medium containing vitamins B1 and B12, ammonium salts and long-chain fatty acids as the sole carbon source. These acids are metabolised via beta-oxidation [18]. Ellinghausen-McCullough-Johnson-Harris (EMJH) medium containing oleic acid, bovine serum and polysorbate is often used in culture.

One-percent sodium hypochlorite solutions, 70% ethanol, iodine-based and quaternary ammonium disinfectants, 10% formaldehyde, detergents and acids are used to disinfect and inactivate leptospires. Leptospires are sensitive to moist heat (121°C for a minimum of 15 minutes) and are killed by Pasteurisation according to a guideline from the Centre for Food Security and Public Health (CFSPH).

Leptospires are detectable in urine and tissues using culture, dark field microscopy (DFM), immuno-staining or PCR techniques [9, 18, 28]. However, leptospirosis diagnosis is difficult due to the wide diversity of symptoms associated with the disease. The quality of diagnosis depends on the analytical parameters. The degree of precision of specific antibody detection tests such as immuno-specific enzymatic assays (ELISAs) or micro-agglutination tests (MATs) represents an important bias because greater or diminished sensitivity determines the relevance of the results; therefore, the choice of test is an important parameter that must be considered. PCR assays to detect the 16S rRNA gene are efficient during early infection [31].

Classifications: nomenclature. In 1907, Stimson discovered a spirochete in the kidney of a patient that died from yellow fever. He named the bacteria *Spirochaeta interrogans*. Until 1989, leptospires were classified as one of two species: saprophytic (*Leptospira biflexa*) and pathogenic (*Leptospira*

interrogans) [28]. These 2 species were distinguished by serological classification based on their LPS structure and strain reactivity against antibodies; LPS carbohydrate fragments give rise to antigenic diversity. A serogroup includes serovars with overlapping antigenic factors [17]. Cross agglutination absorption test (CAAT) analysis, which involves the recognition of antibodies with associated antigens, facilitates the identification of a strain serovar or the comparison of two or more strains. Two strains with more than 10% heterogeneity are considered associated with different serovars. More than 300 serovars have been identified to date [53]. The serovar Icterohaemorrhagiae is most frequently implicated in human infections. Serovars have been grouped into 24 leptospire serogroups. Both serogroups and serovars are determined using a serology reference test and a MAT. These methods will be discussed in detail in this article.

To identify circulating serovars in different regions, different typing methods are employed. It is important to characterise serovar distribution for epidemiologic purposes and vaccine design.

Leptospire genomic classification is based on DNA-DNA hybridisation and has permitted the separation of the two previously described species into 22 distinct genomospecies. The genus *Leptospira* is composed of 10 pathogenic species, 5 potentially pathogenic species (also called intermediate) and 7 saprophytic species [2, 11] (Table 1).

Leptospire species are geographically distributed. For example, in metropolitan France, leptospirosis in both humans and animals is attributable to strains belonging either to *Leptospira interrogans*, *Leptospira kirschneri* or *Leptospira borgpetersenii*. Genomic and serological classifications are independent and uncorrelated, but a species name and serovar name must be given for a characterised strain.

A large variety of methods have been used characterise the serologic and genomic diversity of leptospire strains. These methods are useful for epidemiologic purposes and to track outbreaks. The first techniques were developed to identify and type leptospiral isolates because the information provided by serological classification was insufficient. This review will list and

Table 1. Genomic species of genus *Leptospira* and associated reference strains

Categories	Species	Serogroup	Serovar	Type strain
Pathogenic	*L. interrogans*	Icterohaemorrhagiae	Copenhageni	Fiocruz LI-130
	L. kirschneri	Grippotyphosa	Grippotyphosa	Moskva V
	L. noguchii	Panama	Panama	CZ 214 K
	L. borgpetersenii	Sejroe	Sejroe	M84
	L. weilii	Celledoni	Celledoni	Celledoni
	L. santarosai	Tarassovi	Atlantae	LT81
	L. alexanderi	Manhao	Manhao 3	L60
	L. alstonii	ND	Sichuan	79,601
	L. kmetyi	ND	ND	Bejo-Iso 9
	L. mayottensis	-	-	200901116T
Intermediate	*L. wolffii*	ND	ND	Korat-H2
	L. licerasiae	ND	Varillal	VAR010
	L. inadai	Tarassovi	Kaup	LT64-68
	L. fanei	Hurstbridge	Hurstbridge	BUT6
	L. broomii	Undesignated	ND	5399
Saprophyte	*L. wolbachii*	Codice	Codice	CDC
	L. meyeri	Semaranga	Semaranga	Veldrat
	L. biflexa	Semaranga	Patoc	Patoc I
	L. vanthielii	Holland	Holland	WaZ Holland
	L. terpstrae	ND	ND	LT 11-33
	L. yanagawae	Semaranga	Saopaulo	Sao Paulo
	L. idonii	-	-	Eri-1 (T)

explain the diagnostic and typing methods used for leptospiral analysis and serovar discrimination because serovars are the taxonomic reference units.

Laboratory diagnosis of Leptospirosis

Direct observation. When leptospirosis is suspected due to symptoms such as renal insufficiency, patient blood, cerebrospinal fluid and urine samples are observed under a microscope to detect the presence of bacteria. Leptospires are easily and quickly detected, allowing serologic tests to be avoided. Furthermore, it is possible to administer specific treatments to patients in a short period of time. However, if the bacterial numbers are low, bacteria may not be detected in the samples, and thus, other test are required (culture, PCR, and serology). Notably, the direct observation of leptospires requires technical skill. Therefore, this method is not routinely employed.

Culture in specific medium. Leptospires replicate in media enriched with B1 and B12 vitamins, long-chain fatty acids and ammonium salts [2]. Usually, cultures in EMJH medium are performed to detect leptospires in fresh tissues, blood or urine, but leptospires must be cultured before antibiotic treatment is applied. Some contaminants are inhibited by 5-fluorouracil [24, 37], although its limited antibacterial spectrum permits other contaminants to grow [52, 59, 65]. Other antimicrobial agents are also applicable [18, 28]. Cultures are incubated for up to 13 weeks at 30°C and regularly examined by DFM to determine if a sample is negative for leptospires. As such, cultures are not useful as a routine diagnostic test for individual patients, but they allow strains to be isolated and analysed for epidemiological studies.

Serology.

MATs for routine analyses. To detect leptospirosis, specialised laboratories perform MATs on patient sera. MATs involve the detection of leptospire-specific patient antibodies that recognise antigens from known strains. This recognition results in agglutination that is observable by DFM. Several serogroups are routinely tested. The MAT test determines the serogroup but is not sufficiently precise to identify serovars because cross-reactivity frequently occurs between serovars within a given serogroup as well as between serogroups. As a serological reference test, the MAT test is highly sensitive and specific but requires live cultures of different serovars from specific geographical areas as controls. Furthermore, this technique does not discriminate between antibodies derived from infection or vaccination, and thus, it is important to know the vaccination history of the patient, including for the veterinary diagnosis of animal leptospirosis because vaccination is widely spread, particularly in dogs. Certain quality assurance programmes, such as th e International Leptospirosis MAT, are endorsed by the International Leptospirosis Society (ILS) to ensure reliability and standardisation between laboratories.

ELISA. ELISA tests involve the detection of leptospire-specific IgM and/or IgG in patient sera. This test is advantageous in that it does not require the maintenance of live cultures. Although ELISA tests detect leptospire-specific antibodies, results must be confirmed by MAT tests. Therefore, ELISA tests alone do not assure a definitive diagnosis. ELISAs have been developed for numerous antigen preparations and for leptospiral recombinant lipoproteins, such as LipL32 or LigA. IgM is detectable 5 to 7 days after infection; thus, ELISA assays must be performed at the proper time, resulting in a certain degree of difficulty [1, 7, 44, 47] (Table 2).

Table 2. Comparison of MAT, ELISA and Immunomigration sensitivity and specificity values in percentages

	Sensitivity		Specificity		Sensitivity		Specificity		Sensitivity	Specificity
Days	MAT	ELISA	MAT	ELISA	MAT	ELISA	MAT	ELISA	Immunomigration	Immunomigration
1-7	41	71,1		86,4	/	86,5				
8-30	82	88,2	/	85,8	48,7	48,7	97,3	97	98	93,5
>30	96	76,2		95,5	93,8	75				
Reference		[48]			[7]and the performance of each was compared with that of the current standard, the microscopic agglutination test (MAT				[28]	
Days	MAT	ELISA	MAT	ELISA	MAT	ELISA	MAT	ELISA		
1-7					30	52	99	95		
8-30	65,6 and 54,9	83 and 85,7	97,7 and 97,3	98,5 and 99,1	63	89	98	98		
>30					76	93	97	94		
Reference		[47]				[16]				

Immunofluorescence to detect leptospires. The principle underlying immunofluorescence is the recognition of a leptospiral surface protein, such as OmpL54 [40], by specific antibodies. Secondary antibodies coupled to a fluorescent stain then bind host-specific antibodies. When samples are observed under a fluorescence microscope, bacterial profiles are detectable. This assay facilitates the rapid identification of leptospires and requires the availability of leptospiral OmpL54-specific antibodies, which recognise surface-exposed protein epitopes. However, these antibodies may not recognise surface protein epitopes in the context of recombinant proteins.

Immunomigration. Rapid immunomigration tests permit the detection of IgM antibodies against pathogenic leptospires in dogs with suspected leptospirosis [27]. This test is performed on blood, plasma and serum samples. The samples and chase buffer are deposited on a test strip and migrate to a line where their antigens are impregnated. If there is sufficient anti-leptospira IgM present in the sample, antibody-antigen complexes accumulate, and a visible coloured band appears. The results are read in ten minutes. Clinical signs exhibited by a dog in association with test positivity indicate clinical leptospirosis. Notably, this test does not detect vaccine-induced antibodies. Furthermore, the test is advantageous in that it may be performed in vet clinics. However, the test may be negative during the very early stage of infection due to low antibody levels. Additional serological tests should then be performed. Importantly, immunomigration tests do not provide information on infecting serovars. Therefore, this test has no epidemiological value and should be reserved only for in-clinic utilisation.

PCR methods.

Classic PCR. A PCR assay involving the amplification of an *rrs* gene fragment has been developed, permitting the identification of bacterial strains from the genus *Leptospira*; DNA from other spirochetes, such as *Borrelia*, is not amplified. This method is applicable for blood, urine and tissue samples [31]. The PCR amplification of ribosomal RNA 16S subunit sequences identified by Mérien et al. in 1992 provides highly significant results for leptospire detection and species identification but not for serovar classification. To distinguish between pathogenic and non-pathogenic strains, the amplified DNA should be sequenced.

It is feasible to sequence a whole bacterial genome and compare it to a database to determine whether it is a leptospire, but this test is not used routinely.

Detection of leptospires by real-time PCR. Currently, it is possible to diagnose leptospirosis from paired serum samples by detecting seroconversion in conjunction with the MAT test. PCR assays were developed due to the need for a rapid and precise molecular diagnostic to detect pathogenic leptospires. Classic PCR analyses, particularly sequencing, are time consuming, which is problematic if rapid diagnosis is required. Medical and veterinary practitioners must be informed early in the case of infection. Thus, real-time PCR assays are specialised to target different genes to distinguish between pathogenic or non-pathogenic leptospires via simple curve interpretation. SYBR Green fluorescence assays utilising the *lipL32* gene, which encodes the outer membrane LipL32 protein, employ a target sequence of 423 base pairs [22] due to gene conservation among pathogenic serovars. Analytical tests are performed on human serum and urine [29] and animal urine, blood, serum and kidney samples. Assays employing the *secY* gene have also been performed; all 56 strains tested were amplified. The advantages of this method include its speed, quantitative results, minimal sample contamination, high sensitivity and specificity and standardisation. However, it is expensive and requires specific materials [34].

Serotyping.

Serotyping is important in epidemiology because it allows the identification of the serogroup or serovar carried by an animal reservoir [11]. MAT is used for epidemiologic purposes to identify unknown strains. In this test, a panel of serovar-specific antibodies produced in rabbit serum is tested against a sample to observe any agglutination. The strain in a sample belongs to the serovar demonstrating agglutination; if there is more than one agglutination reaction, the sample belongs to the serogroup demonstrating the highest antibody titres. This method requires the growth of bacteria to obtain sufficient concentrations for testing.

Molecular methods to type leptospires.

Restriction fragment length polymorphism (RFLP) techniques. The restriction endonuclease analysis (REA) method has been extensively used as a molecular typing method to differentiate between bacterial strains [19, 46]. Restriction enzyme methods allow the direct comparison of strain profiles in agarose or acrylamide gels. Briefly, total bacterial DNA undergoes endonuclease restriction by endonucleases followed by fragment separation on gels. It is difficult to detect polymorphisms in total genomes, and therefore, alternative methods, such as PCR amplification followed by restriction enzyme adjunction, have been developed. For example, *EcoRI* and *Hlal* have been used to type *Leptospira interrogans* [63]. DNA fragments are separated in an agarose gel, facilitating the detection of several zones associated with polymorphisms. The method is able to differentiate between 29 serovars [16, 43]; there are no differences between two strains belonging to the same serovar [63]. Furthermore, this method is used to characterise pathogenic species but not saprophytes. Advantageously, DNA sequences are not required, costs are lower than MAT, and results are easily reproducible [16].

RFLP analysis of rRNA genes is used for to identify and characterise leptospire species in sample isolates [23, 39], a

technique called ribotyping. Ribotyping permits determination at the species level but not the subspecies level, e.g., serovars [51]. Extracted DNA is digested by one or more restriction enzymes. Fragments are separated on an agarose gel, and the denatured DNA is hybridised to a membrane with a 16 rRNA probe. Variations are introduced by alternating the restriction enzymes, resulting in a bank of profiles for each enzyme. Ribotyping correlates well with the phylogenetic classification of 11 leptospire species. The use of three restrictions enzymes with PCR products generates patterns that permit serovar discrimination. However, this method does not distinguish between the Icterohaemorrhagiae and Copenhageni serovars, which is problematic in epidemiologic investigations [23]. Additionally, designed primers do not discriminate between pathogenic and non-pathogenic species of leptospires. Therefore, this assay does not detect sample contamination by saprophytic bacteria. The primers designed by Gravekamp et al. in 1991 do not amplify *Leptospira kirschneri* serovars. Therefore, two pairs of primers must be used to detect all leptospire serovars [12]. rRNA RFLP is performed to generate bacterial phylogenetic trees. It is possible to PCR amplify fragments of interest before performing RFLP to ensure protocol optimisation, thus permitting readable bands to be obtained.

Amplification fragment length polymorphism (AFLP) involves the PCR amplification of restriction fragments selected from total genomic DNA [55]. The protocol consists of three steps. First, DNA matrix restriction and adapter ligation are performed. Second, restriction fragment sets are amplified. Finally, amplified fragments are analysed on a gel to read the results. The adapter and restriction site sequences are target sites for primer annealing at the end of PCR amplification of restriction fragments, at which point the primers amplify only nucleotides in close proximity to restriction sites. Using this method, it is possible to study restriction fragments without knowing their nucleotide sequences. Many fragments undergo co-amplification, and approximately 50 to 100 restriction fragments may be analysed simultaneously on polyacrylamide gels depending on the capacity of the detection system. Samples are grouped by computers for analysis, although this approach requires large amounts of purified DNA. Additionally, this technique permits the study of DNA of various origins.

Pulsed-field gel electrophoresis (PFGE). PFGE is the standard method for molecular typing and involves the in-gel enzymatic digestion and electrophoretic separation of different DNA fragments. Both DNA liberation after cell lysis and DNA digestion are performed in a gel after embedding bacteria in agarose. Enzymes such as *NotI* cut rare DNA sequences, generating high-molecular-weight fragments, which are further separated by pulsed-field electrophoresis. This facilitates the simple comparison of profiles and therefore it is the reference method for typing. PFGE is a powerful method for species identification. To increase the efficiency of this technique, scientists have developed gel analysis software to compare strain profiles between laboratories using dendrograms [20]. However, this method does not work when there are fewer than 3 generated fragments. Additionally, approximately 10 percent of serovars cannot be identified [20]. Leptospires are difficult to culture, causing difficulty when an environmental strain must be isolated and cultured for PFGE. Furthermore, PFGE requires a whole bacterial genome to identify the serovar of a strain.

Insertion sequence (IS) typing methods. IS elements are useful for typing, particularly for epidemiologic purposes. The first element identified for pathogenic *Leptospira interrogans* was IS1500, which has been found in two closed serovars. Nucleotide sequence revealed a 1236-bp element surrounded by a 1159-bp region containing four open reading frames (orfA-orfD). This sequence has been found in all pathogenic strains but not in saprophytes such as *Leptospira biflexa* [10]. Therefore, it is valuable for the identification of pathogenic *Leptospira*. The second IS, IS1502, contains 19 ORFs [64]. IS1502 is found only in some strains. Additionally, IS1533 was identified in *Leptospira borgpetersenii* [61] and is used to identify leptospire serovars.

Arbitrarily primed PCR (AP PCR) [56] and randomly amplified polymorphic DNA (RAPD) [57]. These two methods are based on hazard priming PCR and allow the rapid identification of species as well as serovar comparison [13]. These techniques were used for epidemiologic studies in India [36]. Briefly, primers with arbitrary sequences that have few chances of undergoing auto-amplification and variable G-C percentages (between 40 and 80%) are chosen. For AP PCR, bacterial DNA is amplified by performing low stringency PCR, which, in contrast to high stringency PCR, consists of decreasing hybridisation temperatures and increasing concentrations of MgCl2 in the reaction mix. After the amplification of sequence targets by PCR with these primers, the results are read on an agarose or polyacrylamide gel. Forty-eight reference leptospiral strains have been classified using AP PCR [42]. For the RAPD method, one pair of primers is used to amplify random DNA fragments. Although they are highly efficient, these tools demonstrate weak value for serovar typing because of their low reproducibility.

Variable number tandem repeat (VNTR). The VNTR method was developed for epidemiologic studies to speculate on strain circulation between animal species in the environment. VNTR describes the profiles of 94 serovars based on the repetition of short sequences located in three to five genomic regions (Figure 1). These genomic regions consist of short patterns repeated different numbers of times depending on the locus, and the number of repetitions is serovar-specific. DNA extracted from samples is amplified by PCR with specific primer pairs for each locus. Then, the samples are run on an agarose gel using low voltage for four hours to ensure maximum precision. The profiles are read using UV and compared with known profiles in a database to deduce the infecting serovar. VNTR was used to define a new and unique group of *Leptospira interrogans* serovars, called Pomona, in California sea lions [62]. VNTR is reproducible, easily standardised, and permits the identification

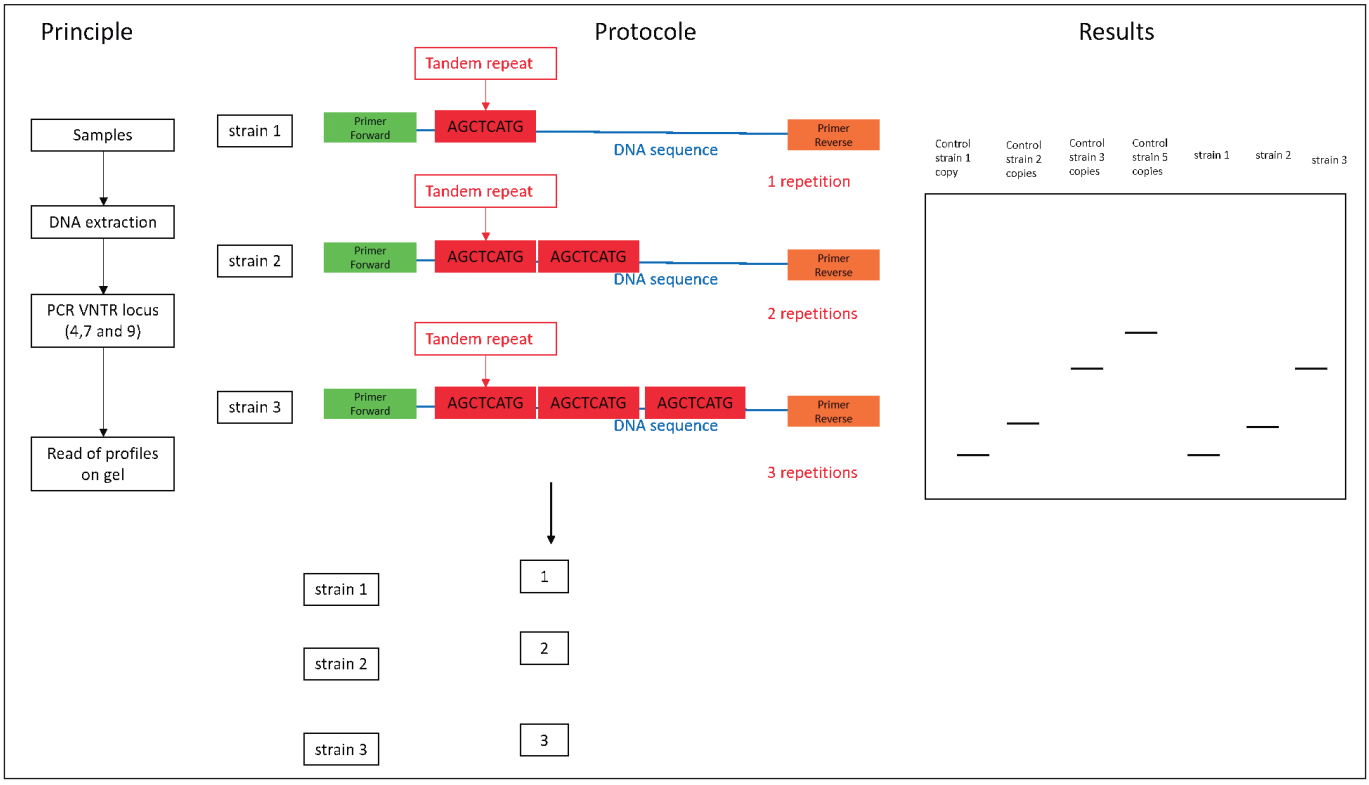

Fig. 1. Schema of Variable Number Tandem Repeat (VNTR).

of strains from three species, specifically *Leptospira interrogans*, *Leptospira borgpetersenii* and *Leptospira kirschneri*, which are the only species present in France according to a recent study of 28 wild species [6].

Multi-locus VNTR analysis (MLVA). MLVA was initially developed to study strains from the *Leptospira interrogans* serovar Australis in Australia. Scientists searched for tandem repeat sequences in the genome of the strain *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 [35] and evaluated them for diversity. They selected six loci for analysis and identified thirty-nine distinct patterns within thirty-nine reference strains. When applied to serovar Australis, three clusters were distinguished from different animal and human hosts.

MLVA was further applied in Argentina to analyse the relationships between leptospire infections over 45 years. Researchers studied genetic diversity in a collection of 16 strains of *Leptospira interrogans* serovar Pomona and analysed 7 loci using VNTR as described by Majed et al. [30]. The VNTR4 locus presented four different alleles that demonstrated the highest diversity within the tested group of loci. Clustering analysis permitted four new MLVA genotypes to be distinguished, one of which dominated over the other three. Like VNTR, this method is useful for both diagnostic and epidemiological analyses.

Multi-locus sequence typing (MLST). MLST is a method based on the PCR amplification of DNA sequences to study the allelic diversity of selected genes. There are two types of MLST. MLST was first developed to genotype leptospires based on the DNA sequences of 4 housekeeping genes and two gene candidates: *adk*, *icdA*, *lipL32*, *lipL41*, *rrs* and *secY*. Scientists analysed a set of 120 strains and 41 references from different locations and found the six most variable genes included *adk*, *icdA* and *secY* [3]. Another study identified *pntA*, *sucA*, *pfkB, tpiA*, *mreA*, *glmU* and *fadD* [48]. This method, which is used to identify clusters among outbreak isolates, does not require much purified DNA, offering an advantage. However, MLST does not differentiate between serovars Copenhageni and Icterohaemorrhagiae. Despite this shortcoming, it is useful as a routine technique to easily obtain and interpret results.

Multi-spacer typing (MST). MST was developed to type strains from the genomic species *Leptospira interrogans* and has been employed to identify four dominant serovars in France, specifically, Icterohaemorrhagiae, Australis, Canicola and Grippotyphosa [60]. This technique involves the sequencing of 3 intergenic regions with low selection pressure but with punctual mutations (Figure 2). A genotype number is assigned to the sequence for each region, and a profile is generated for each strain. MST can differentiate between the serovars Copenhageni and Icterohaemorrhagiae. Furthermore, it has been developed for 33 strains that each have been assigned a specific number for each genotype by region and is thus useful for epidemiologic analyses.

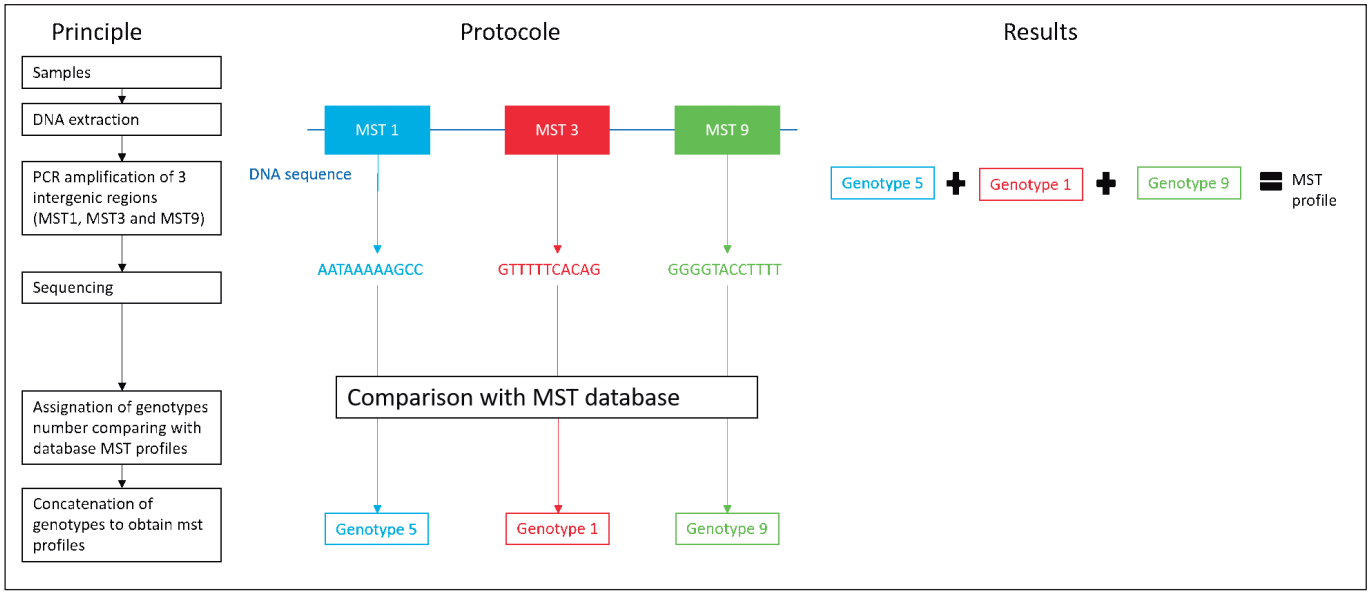

Fig. 2. Schema of Multi Spacer Typing.

Conclusions

Leptospirosis is an underestimated disease. With current global warming and climate change conditions, the expansion of leptospires appears more favourable. Infections by these bacteria are highly virulent, particularly in poor countries. Indeed, the development of suburbs and sanitary measures has brought rodents, such as rats, into close contact with humans. The subsequent exposure of populations to leptospirosis agents increases the risk of contamination. Wet weather and contact with puddles promote the cycle of contamination. As mentioned above, wildlife are important maintaining and contaminating of domestic animals, and studies have been conducted to elucidate their role in the leptospirosis cycle. Overall, this pathogen is of great concern and threatens all populations around the world. However, it is unclear whether we possess the tools to combat and prevent leptospirosis. Typing is very important for epidemiologic studies. Knowledge of the serovars circulating in different regions allows us to adapt prevention measures. It is essential to obtain this information to develop efficient vaccines. To prevent leptospirosis, vaccines are available for humans and domestic dogs, cattle and horses. Clinically, these vaccines hold no real value because antibiotics are used to treat all serovars. However, not all serovars possess equivalent virulence, and thus, it is important to characterise the pathogenicity of different serovars.

In conclusion, leptospirosis is an important pathological disease caused by a virulent bacterium. Leptospires colonise many animal species, and there is a large variety of potential hosts in which they can proliferate. Additionally, these bacteria can survive for long periods of time in water and affect many organisms over time. Infections are becoming more severe and are responsible for serious adverse outcomes, affecting persons and animals all over the world. In poor countries, humans come into contact with rats and are readily exposed to leptospirosis due to a lack of hygiene and proximity to waste. The diagnosis of the disease represents a significant problem because bacterial isolation is often required. This step presents real difficulties because leptospires are very fragile, and cultures derived from biological specimens, particularly from animals, may be contaminated. Therefore, molecular methods have attracted great interest in the past decade. However, all typing methods possess certain limitations, specifically the requirement for a large quantity of purified DNA, which is usually difficult to obtain from field samples. Thus, we must continue to develop the sensitivity of these tools.

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