DIAGNOSTIC METHODS FOR THE DETECTION OF *LEPTOSPIRA* SPP. IN BIOLOGICAL SAMPLES

Helio Langoni[1]
Leila Sabrina Ullmann[1]
Virgínia Bodelão Richini-Pereira[2]
Luciana da Silva Ruiz[2]
Gabriela Pacheco Sánchez[3]
Simone Baldini Lucheis[3]

ABSTRACT

Leptospirosis is a bacterial zoonoses distributed worldwide. It can undertake any animal species, maintaining the agent in the nature via renal chronical infection of carriers animals, which can secrete the organism through the urine into the environment, being able to infect other animals and the human. Regarding *Leptospira* spp., diagnostic techniques have helped understand important eco-epidemiological aspects such as environmental serovar distribution and new hosts, in addition to improve the clinical diagnosis of the disease. This review focuses on present diverse techniques used in leptospirosis diagnosis to biological samples, including some studies on practical applications for the molecular detection of *Leptospira* spp., reinforcing the importance of the knowledge and the choice of the correct technique in the diagnosis of the disease.

Keywords: *Leptospira,* leptospirosis, diagnosis.

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RESUMO

Leptospirose é uma zoonose bacteriana distribuída mundialmente. Pode acometer qualquer espécie animal, mantendo o agente na natureza via infecção crônica renal de animais carreadores, os quais excretam os organismos pela urina no meio ambiente, podendo infectar outros animais e humanos. Em relação à *Leptospira* spp., as técnicas diagnósticas têm auxiliado na compreensão de aspectos eco-epidemiológicos importantes tais como a distribuição ambiental dos sorovares e novos hospedeiros, além de aprimorar o diagnóstico na clínica da doença. A revisão tem como objetivo apresentar as diversas técnicas utilizadas no diagnóstico da leptospirose em amostras biológicas, incluindo alguns estudos sobre aplicações práticas da detecção molecular de *Leptospira* spp., reafirmando assim a importância do conhecimento e da escolha das técnicas corretas no diagnóstico da doença.

Palavras-chave: *Leptospira*, leptospirose, diagnóstico.

[1] Departamento de Higiene Veterinária e Saúde Pública – FMVZ-UNESP-Botucatu. Correspondência.
[2] Núcleo de Ciências Biomédicas, Centro de Laboratórios Regionais CLR Bauru II, Instituto Adolfo Lutz
[3] APTA - Agência Paulista de Tecnologia dos Agronegócios

MÉTODOS DIAGNÓSTICOS PARA LA DETECCIÓN DE *LEPTOSPIRA* SPP. EN MUESTRAS BIOLÓGICAS

RESUMEN

Leptospirosis es una zoonosis bacteriana distribuida en todo el mundo. Puede afectar a cualquier especie animal, manteniendo el agente en la naturaleza a través de la infección renal crónica en los animales portadores, que excretan los microorganismos en el ambiente mediante la orina y pueden infectar a otros animales y el hombre mismo. Acerca de *Leptospira* spp., técnicas de diagnóstico han ayudado en la comprensión de los aspectos eco-epidemiológicas importantes como la distribución ambiental de los serotipos y nuevos huéspedes, y mejorar el diagnóstico clínico de la enfermedad. Esta revisión tiene como objetivo presentar las diferentes técnicas utilizadas en el diagnóstico de la leptospirosis en muestras biológicas, incluyendo algunos estudios sobre las aplicaciones prácticas de detección molecular de *Leptospira* spp., reafirmando la importancia del conocimiento y la elección de las técnicas adecuadas en el diagnóstico de la enfermedad.

Palabras-clave: *Leptospira*, leptospirosis, diagnóstico.

INTRODUCTION

Leptospirosis is considered a zoonosis of worldwide distribution, present in all continents, except Antarctica (1), with great importance among the diseases considered as emerging and re-emerging (2). In addition, over 853,000 cases and 48,000 deaths are estimated to occur each year (3). It occurs endemically or as outbreaks affecting humans in several developing or developed countries, especially those of tropical and subtropical climate (4). In Brazil, leptospirosis is endemic and considered a serious public health problem, with 37,035 cases notified from 1999 to 2009 (5). The mortality rate of Weil's disease (severe form) and severe pulmonary hemorrhagic syndrome is >10% and 74%, respectively (6).

Leptospirosis is caused by bacteria of the order Spirochetales, family Leptospiraceae, genus *Leptospira*, and they are classified in three groups according to their phylogenicity and pathogenicity (7). During the meeting of the Subcommittee on Taxonomy carried out in 2007 in Quito, Ecuador, the leptospire species were reclassified and divided into 13 pathogenic species (*L. alexanderi*, *L. alstonii*, *L. borgpetersenii*, *L. inadai*, *L. interrogans*, *L. fainei*, *L. kirschneri*, *L. licerasiae*, *L. noguchi*, *L. santarosai*, *L. terpstrae*, *L. wielli* and *L. wolffii*) and six saprophytic species (*L. biflexa*, *L. meyeri*, *L. yanagawae*, *L. kmetyi*, *L. vanthielii* and *L. wolbachii*) (1).

The different species are classified into serogroups composed of over 200 pathogenic and 60 non-pathogenic serovars. These serogroups are based on antigenic features (8) by the expression of epitopes exposed at the surface as a mosaic of LPS antigens, while the specificity of epitopes depends on the composition and display of their sugars (1). Serotyping has been recognized as an essential tool in clinical and epidemiological investigations and may indicate the reservoir involved in transmission (8).

This disease can be transmitted directly by the contact with blood or urine of infected animals or indirectly by the contact with water contaminated with the urine of carrier animals (9). It affects almost all domestic and wild mammals, as well as men, leading or not to clinical manifestation. Most wild species can become carriers and contribute to the spread of *Leptospira* in nature (10-12).

Serological studies have shown different wild synanthropic species of the orders Didelphimorphia and Rodentia as potential disseminators of different leptospire serovars in the

environment (13). Although some serovars are associated with a certain reservoir, all animals are susceptible to infection by any of the serological variants (8). That makes early diagnosis an important tool to treat ill animals, improving prognosis, and to identify wild carrier animals and sources of infection for domestic animals and humans.

LABORATORY DIAGNOSIS

The laboratory diagnosis of leptospirosis can be made by serological tests, isolation of the agent or molecular survey of the bacterial genetic material (14).

The currently available biomolecular technologies provide powerful tools for the detection and identification of leptospire species (pathogenic or saprophytic), in addition to the early diagnosis in situations of absence of immune response. These techniques have great advantages compared to the traditional approaches of culture and serological methods, such as rapidity, practicality and sensitivity; however, the classic methods cannot be substituted since they are the only means for identifying the serovar responsible for the infection (14), which constitutes important data in epidemiological studies.

IMMUNOLOGICAL METHODS

The microscopic agglutination test (MAT) or Martin and Pettit test was developed almost one century ago at the Pasteur Institute (15). This is the standard test in the laboratory diagnosis of leptospirosis. The principle of this technique is the reaction of agglutination between antibodies present in the sera of hosts and the antigen-O of lipopolysaccharides (LPS) at the membrane of leptospires (16). It is an indirect assay which does not differentiate antibodies resultant of infection from those of vaccination (1) nor the disease phase; and the antibody levels are detectable only between seven and ten days after infection, which may impair the patient prognosis. Confirmation of a case of leptospirosis by the MAT requires two samplings made two weeks apart, with sero-conversion or significant increase of the antibody titers (7).

Immunoenzymatic assay (ELISA), developed in the 1980's has been widely used in research and some screening programs. ELISA IgM methods are available for presumptive diagnosis, but they are not sufficient to diagnose a case of leptospirosis; it must be confirmed by MAT, PCR, or culture (14).

Several other tests may be used to screen antibodies including macro-agglutination, complement fixation reaction, indirect immunofluorescence, hemagglutination, and latex bead agglutination tests (17).

Recently, immuno-chromatographic strip tests "Lateral flow assays" have been developed in various laboratories; these tests use a membrane coated with total cellular extract or with a protein used to capture antibodies targeting leptospires when a drop of sampled blood is deposited. The antibody capture is visualized by a reaction with a colorimetric detection agent (a colloidal gold conjugate of protein-A) after migration of antigen–antibody complexes by capillarity (18). More recently in Thailand, one study demonstrated the use of anti Lipl-32 coupled with gold nanoparticles in an immuno-chromatographic test for detection of *Leptospira* interrogans (19). Another study performed in Japan developed this technique for antigen detection in *Leptospira* spp., which could be applied in areas where leptospirosis infection is endemic and is applicable for detecting antigen in urine samples (20).

MICROBIOLOGICAL METHODS

Culture is the definitive diagnosis of leptospirosis but is considered of low sensitivity, laborious and time consuming, and consequently is not useful for early diagnosis. The 5-

fluorouracil is frequently employed to culture leptospires, which aims to reduce contaminations risks (21).

Dark field microscopy can be used in the acute phase for total blood samples and in the chronic phase for urine samples, cavity liquids and tissues of recent dead animals. The sensitivity of darkfield microscopy is approximately 10^7 leptospires/mL. It is a direct technique of low sensitivity and low specificity, compared to molecular methods, since it is subjective (14,21).

MOLECULAR METHODS

Molecular biology tools have been important for the difficulties and limitations of serological and microbiological methods and are useful in leptospirosis diagnosis using biological samples from domestic and wild animals, which suggests that the rapid detection of leptospires by polymerase chain reaction (PCR) can proceed the detection of specific antibodies and shorten the time for the agent identification by isolation and culture.

Molecular techniques allow the detection of pathogens in several biological materials such as total blood (22), serum (23,24), urine (25), cerebrospinal fluid (26), feces, semen (27) and sputum (28).

The polymerase chain reaction (PCR) shows high specificity and sensitivity for the amplification of a certain pathogen-specific DNA fragment, presenting a great advantage for the early diagnosis of leptospirosis, and can be employed with specific primers (29,30). Those DNA fragments are universally present in bacteria as *gyr*B (31), *rrs* (16S rRNA gene) (32), *sec*Y (33); or restricted to pathogenic *Leptospira* spp. as *lip*L32, *lfb*1 (34) *lig*A, and *lig*B2 (35). Furthermore, the real-time PCR (qPCR) combines amplification and quantification of the number of microorganisms (36), i.e. the assessment of the present bacterial load in the same reaction vessel with excellent low contamination risk and can detect extremely low levels of leptopiral DNA (33).

Some molecular systems are sensitive enough to detect one copy of the leptospire genome/mL sample (23,24,35), but most systems show sensitivity of around 10 to 100 copies of leptospire genome/mL sample (36,37). In some cases, PCR is used in association with post-PCR hybridization techniques, which increases the system sensitivity (22-24).

Leptospira genome is between 3.9–4.6 Mb, composed of chromosomes containing the gene 5S, 16S and 23S rDNA (38). The genome region widely used in phylogenetic studies and population genetics is the ribosome gene (rDNA) that directly codifies the ribosomal RNA and is present at a number larger than 1000 copies which are arranged as long repeated series of the same basic unit at the same locus (39).

A genome region based on the ribosomal RNA 16S (rDNA 16S), which amplifies a 331 base-pair (bp) fragment, is widely used in the identification of the genus *Leptospira* (23).

The *lip*L32 gene codifies an outer membrane lipoprotein (LipL32) which is considered a virulence factor present in pathogenic leptospires, showing a high conservation degree. Thus, real time PCR using primers specific for this gene was developed in an attempt to differentiate between pathogenic and non-pathogenic leptospires (40).

Omps are membrane proteins which have important pathogenic compounds and are highly conserved in different serogroups and serovars of pathogenic leptospires. Analysis of the homology of the region of the gene *omp*L1 showed seven distinct clusters: *Leptospira borgpetersenii*, *Leptospira kirschneri*, *Leptospira santarosai*, *Leptospira weilii*, *Leptospira noguchii*, and *Leptospira interrogans* subgroup A (*L. interrogans* Wolffi, Grippotyhosa, Autumnalis, L1-130 and RGA) and subgroup B (*L. interrogans* Australis, Canicola, Hebdomadis, Paidian, Lai, 56601 and Pyrogenes, and *L. weilii* Manhao and *L. noguchii* Pomona) (40).

Other molecular methods have been developed for the detection of *Leptospira* spp. A study made in The Netherlands suggested the application of the Check-Points assay for this purpose. The work used the ligation-mediated amplification combined with microarray analysis to detect *Leptospira* and discriminate between saprophytic intermediate and pathogenic species (41).

The main genomic regions used for the design of primers for *Leptospira* diagnosis are listed in Table 1.

Table 1. Primers used in *Leptospira* detection.

PRIMER	OLIGONUCLEOTIDE SEQUENCE	GENE	AMPLICON
	***Leptospira* spp. (23)**		
Lep1	GGCGGCGCGTCTTAAACATG	rRNA 16S	331bp
Lep2	TTCCCCCCATTGAGCAAGATT		
	Species-specific (40)		
Intergroup A	CTACTGGCGGCTTGATCAAC		396 bp
Intergroup A	CTGGATCTGTTCCGTCTGCGATC		
Intergroup B	CTTGATAGAACCACTGGTGGTGCC		406 bp
Intergroup B	CTGGATCGGTTCCATCGCTCAG		
Borgpeter	CTTGATAGAACAACAGGCGGCATCATC	*ompL1*	389 bp
Borgpeter rev	GCTAATAAGTTTGCAATGCTCGTAAC		
Kirschner	CGGTTTGATCAATGCGAGAAGCACC		389 bp
Kirschner rev	TTGGATCCGTTCCGTCTGCGATT		
Santarosai	CTTATCAATGCAAGATCTACCAAAGGT		408 bp
Santarosai	GCGGATATGTTCCCGAGTAGTAATC		
Noguchii fwd	GCGGATTTATCAATGCAAGAAGTACA		390 bp
Noguchii rev	CCGGATCGGTTCCGTCTGCGATCAG		
Weilii fwd	AGGCTGATATTGCAGGCTTC		277 bp
Weilii rev	CGGAATCGAATATGTTCACGAGTG		
	Pathogenic leptospires (42, 43)		
LipL32-45F	AAGCATTACCGCTTGTGGTG	*lip*L32	242 bp
LipL32-286R	GAACTCCCATTTCAGCGATT		
probe			Taq Man System(qPCR)
LipL32-189P	FAM-5′-AAAGCCAGGACAAGCGCCG-3′-		

New knowledge on molecular genetics has been described for studying the molecular epidemiology of *Leptospira*, including 16S rRNA sequencing and several PCR-based methods. Genotyping methods have been developed for a phylogenetic analysis of *Leptospira* species, but Multilocus Sequence Typing and High Resolution Melting (HRM) have the inability to distinguish certain isolates at the serovar level (44-46). Recently, multispacer sequence typing (MST) provides a method with a high discriminatory power to identify clinical isolates in correlation with the serovar profiles (47). The genotyping method is interesting for epidemiological applications and phylogenetic studies.

OPTIMIZATION IN THE PREPARATION AND PROCESSING OF BIOLOGICAL SAMPLES

Several factors can influence the quality of DNA extraction and inhibit PCR, yielding false-negative results.

The biological samples should be buffered and centrifuged to validate the technique since it has a large quantity of enzymatic inhibitors and must have the least possible contact with the atmosphere to prevent the oxidation of certain compounds, and consequently damaging PCR. A good option for neutralization is phosphate buffered saline (PBS) pH 7.2 (27).

Urine samples should be collected by cystocentesis or catheterization. The collected volume will vary according to the size of the species. The samples should be stored in sterile centrifuge tube with 15 mL capacity. Immediately after collection, the urine should be neutralized with sterile PBS 1X pH 7.2 at 1:1 proportion (500µL PBS 7.2 and 500µL urine) in microtube with 1.5 mL capacity. The samples should be kept at 4ºC for up to 24 hours, then centrifuged at 11,000 g for 5 minutes (spin) to eliminate urine residues, resuspended in 500 µL sterile PBS pH 7.2 in microtube with 1.5 mL capacity free of RNAse and DNAse, and frozen at - 80 °C until the molecular techniques are performed (48). Tissue samples should be neutralized with sterile PBS 1X pH 7.2, then centrifuged at 13,000g for 30 minutes at 4ºC (49), supernatant discarded and 50µL of sterile PBS 1X pH 7.2 added to the cellular sediment should be lysed with vortex and glass beads or maceration using liquid nitrogen (50).

A positive (DNA of the standard strain) and a negative control (sterile MilliQ water) should be included in all reactions, and the PCR detection threshold should be performed to determine the technique sensitivity.

FINAL CONSIDERATIONS

This review shows the importance of the diagnostic techniques in the research and clinic of leptospirosis. We presented several techniques used in the daily routine, as well as others that help in the investigation of the epidemiology and understanding of the disease. We also emphasize some steps for the good quality and correct diagnosis using biological samples.

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