Use of the Coding Region of Leptospira Sp. LigB C-terminus as a Marker for Diagnostics of Animal Leptospirosis

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Research Article

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Abstract

Background

The causal agent of leptospirosis, pathogenic strains of the genus *Leptospira* spp., has Outer Membrane Proteins (OMPs) on its surface which play a fundamental role in infection and pathogenesis. Studies on the genome showed that the LigB protein gene is conserved in different pathogenic species.

Methods

The aim of this work was to propose a new end point PCR based on the amplification of the LigB C-Terminal coding region (*ligb-ct*), never used before and conserved among pathogenic *Leptospira* spp.

Eighteen reference pathogenic, 2 intermediate and 2 no-pathogenic strains of *Leptospira* spp. were used. DNA from 10 other microorganism species were included in this study to determine the analytical specificity.

Results

We obtained 100% positivity for pathogenic *Leptospira* strains. We found no cross-reaction with intermediate and non-pathogenic strains or with other microorganisms, highlighting a high analytical specificity. Analytical sensitivity estimated on clinical samples was higher on serum than blood and urine (6-9 x 10^2 lept/ml and 6-9 x 10^5 and 6-9 x 10^6 leptospires/ml, respectively). Multiple sequence alignment of this region in positive *Leptospira* species confirmed a high degree of conservation, with only a few single nucleotide polymorphisms.

Conclusion

To the best of our knowledge, the LigB C-Terminal coding region has not been previously used for molecular diagnostic and could be used for early diagnosis of leptospirosis.

Background

Leptospirosis is an antropozoonotic endemic disease worldwide, mainly in underdeveloped countries with high levels of poverty. It is caused by pathogenic strains of *Leptospira* spp., characterized by fever and multi-organ failure in humans and animals. In addition, reproductive problems such as abortion and infertility in production animals as a consequence of these infections, result in important economic losse [1, 2].

The taxonomy of the genus was first based on serology, which distinguished almost 300 serovars. According to a more recent characterization using genetic molecular taxonomy, at least 64 species divided into 2 phylogenetic clades (P and S) and 4 subclades (P1, P2, S1 and S2) were described [3].
The pathogen has a variety of Outer Membrane Proteins (OMPs), such as LigA and LigB lipoproteins, on its surface, which mediate interaction with host extracellular matrix proteins, allowing the bacteria to colonize multiple host organs [4, 5].

LigA and LigB belong to the superfamily of bacterial immunoglobulin-like (Big) repeat domain proteins, shared with adhesins of other bacteria, such as intimin and invasin from enteropathogenic *E. coli* and *Yersinia* spp., respectively [6, 7].

Exposure to physiological osmolarity induces leptospires to express high levels of the Lig surface proteins and increases adhesion between leptospires and the host extracellular matrix and plasma proteins, such as collagens, laminin, fibronectin and fibrinogen [8]. Unlike LigA, LigB contains 12 Big domains followed by a long carboxy-terminal region, exposed to the extracellular medium and its are expressed early in the course of infection [9].

*Lig* genes are highly conserved (70–99% identity) among pathogenic species and virulent pathogenic leptospiral isolates [10, 11], however, the *ligB* gene is present in all pathogenic leptospirose, while the *ligA* gene is only found in *L. interrogans* and *L. kirschneri* strains, and both are absent in intermediate or saprophyte *Leptospira* spp. species [12, 13].

Despite its relevance, leptospirosis is one of the most under-diagnosed zoonotic diseases. The Micro Agglutination Test (MAT) is considered the gold standard and is the most used tool for serological diagnosis. However, it presents certain inconveniences such as the requirement of paired samples with intervals of 15–21 days to have a confirmatory result, and lack of detection in the acute phase of the disease due to the absence of detectable antibodies in blood [1].

As a complement to MAT, PCR has been lately used for the molecular detection of pathogenic *Leptospira* DNA using different target genes such as *secY* and *flab* [14], *ompL1* [15], *lipL32* [16, 17], and *ligB* [18,19]. However, few of the described PCR tests were subjected to rigorous validation analysis in the human and veterinary areas.

The aim of this work was to propose a new end point PCR based on the amplification of the LigB C-Terminal coding region (*ligb-ct*) as a novel diagnostic tool for leptospirosis.

**Methods**

**Samples**

Twenty-two reference *Leptospira* spp. strains were used in this study, 18 of which were pathogenic, 2 non-pathogenic and 2 intermediate (Table 1). Four strains were isolated from clinical samples from dogs (serovar Canicola strain Hond Utrecht IV), bovines (serovar strain Pomona), and 2 from rodents (serovar Copenhageni strain M20 and serovar Fiocruz strain L1-130), from Argentina. Environmental samples were isolated from water from the Argentine localities of Puerto Iguazú, Misiones province (1 sample), and Añatuya, Santiago del Estero province (3 samples). Water was pre-filtered through Whatman filter
paper before filtration through 0.22 milipore size membranes. An aliquot (1 ml) of each filtered sample was inoculated in Fletcher medium supplemented with 5-Fluorouracil and Neomycin.

| Group | Leptospira spp. reference strain | LigBct | LipL32 |
|-------|---------------------------------|--------|--------|
| P     | 1- L. interrogans serovar Australis strain Ballico | +      | +      |
| P     | 2- L. interrogans serovar Bataviae strain Swart | +      | +      |
| P     | 3- L. interrogans serovar Canicola strain Hond Utrecht IV | +      | +      |
| P     | 4- L. interrogans serovar Djasiman strain Djasiman | +      | +      |
| P     | 5- L. interrogans serovar Icterohaemorrhagiae strain RGA | +      | +      |
| P     | 6- L. interrogans serovar Manhao strain Manhao | +      | +      |
| P     | 7- L. interrogans serovar Pomona strain Pomona | +      | +      |
| P     | 8- L. interrogans serovar Pyrogenes strain Salinem | +      | +      |
| P     | 9- L. interrogans serovar Wolffi strain 3705 | +      | +      |
| P     | 10- L. interrogans serovar Bataviae strain Bit | +      | +      |
| P     | 11- L. interrogans serovar Bataviae strain Swart | +      | +      |
| P     | 12- L. interrogans serovar Hardjo strain Hardjoprajitno | +      | +      |
| P     | 13- L. interrogans serovar Copenhageni strain Fiocruz L1-130 | +      | +      |
| P     | 14- L. borgpetersenii serovar Castellonis strain Castellon III | +      | +      |
| P     | 15- L. borgpetersenii serovar Hardjobovis strain Sponselee | +      | +      |
| P     | 16- L. weilii serovar Celledoni strain Celledoni | +      | +      |
| P     | 17- L. kirschneri serovar Grippotyphosa strain Moskva V | +      | +      |
| P     | 18- L. noguchi serovar Louisiana strain LSU 1945 | +      | +      |
| NP    | 19- L. biflexa Andamana strain CH-11 | -      | -      |
| NP    | 20- L. biflexa serovar Patoc strain Patoc I | -      | -      |
| I     | 21- L. fainei serovar Hurstbridge strain But6 | -      | -      |
| I     | 22- L. licerasiae serovar Varillal strain Var010 | -      | -      |

+ and – correspond to positive and negative amplification, respectively, as verified by horizontal gel electrophoresis

P: pathogenic leptospiral strains. NP: non-pathogenic leptospiral strains. I: intermediate leptospiral strains
The *Leptospira* spp. reference strains, and environmental and clinical samples used are part of the collection of the Laboratory of leptospirosis, Reference Centre of the OIE (World Organization for Animal Health), Institute of Pathobiology, National Institute Agricultural Technology (INTA Castelar), Buenos Aires, Argentina.

DNA extraction

For DNA extraction, *Leptospira* sp. were grown for 7 days at 30 °C in EMJH medium, as described by Ellinghausen and McCollough [20], following the modifications of Johnson and Harris [21] and bacterial growth was monitored under a dark-field microscope.

Genomic DNA extraction was performed using 20 µl sample and 150 µl Chelex-100 (Bio-rad USA) as described Hamer et al. [22], and incubated at 56 °C during 20 min followed by 8 min at 100 °C. Samples were centrifuged at 10,000 rpm for 5 s and 20 µl of the supernatant were collected and stored at -20 °C until use.

The integrity of the extracted DNA was analyzed by electrophoresis in 1% agarose gels. DNA quantities were estimated by absorbance measurements in a Nanodrop spectrophotometer.

PCR amplification

Primers LigBct-Fw (ATGCCTTACTTTGGCGGC) and LigBct-Rv (AGGCTCCCAGTTGTGTGATG) were manually designed to amplify a 726 bp segment of the 3' end of the LigB gene (*ligBct*), nt 5040 to 5764, based on the sequence of *L. interrogans* serovar Pomona (AF534640.1) (Fig. 1). Specificity of the primers was verified using Primer Blast.

The PCR amplification reaction was performed in a final volume of 50 µl using 5 µl purified DNA template (approximately 250 ng). The PCR mixture contained 1X buffer (500 mM KCl/100 mM Tris/HCl, pH 9.0), 3 mM MgCl₂, 0.5 µmol for each primer, 0.1 mM of each dNTP (Invitrogen, Carlsbad, CA, USA) and 1.25 U Taq polymerase (PBL, Buenos Aires, Argentina). The cycling program consisted on 3 min at 95 °C, followed by 35 cycles of 30 sec at 95 °C, 30 sec at 60 °C and 2 min at 72 °C, with a final extension period of 10 min at 72 °C. Amplification products were analyzed by electrophoresis in ethidium bromide-stained 2% agarose gels, using TAE Buffer, at 90 V during 60 min, followed by exposure to UV light. Amplicon sizes were estimated using a 100 bp ladder (PBL, Buenos Aires, Argentina).

The DNA integrity of negative samples was analyzed by LipL32 PCR followed by 1% agarose gel electrophoresis.

Analytical specificity and sensitivity

The analytical specificity of the LigBct PCR was evaluated using 10 DNA samples from other pathogenic organisms that produce similar clinical signs and/or are commonly present in animal clinical samples: *Brucella abortus, Campylobacter fetus fetus, Campylobacter fetus venearis, Neospora caninum,*
Infectious Bovine Rhinotracheitis Virus (IBRV-1 and 5), Bovine Viral Diarrhea Virus (BVDV), *Mycobacterium* spp., *Escherichia* spp., *Borrelia* spp. and *Salmonella* spp. Genomic DNA samples of these organisms were facilitated by researchers from the Center of Veterinary and Agronomic Research, INTA CICVyA, Argentina.

The analytical sensitivity was determined using serially diluted from *L. interrogans* serovar Pomona, in samples of bovine serum, blood and urine. The initial leptospiral concentration was adjusted by spectrophotometric measurements at 420 nm to approximately $6 - 9 \times 10^8$ bacteria/ml (corresponding to $OD_{420} = 0.365$).

**Sequencing and alignment**

After verification of the presence of amplicons by electrophoresis, 9 PCR products were purified and sequenced by Macrogen (Korea). The Clustal Omega program [23] was used to carry out a multiple sequence alignment to evaluate the degree of strain polymorphism. LigB C-terminal coding sequences were deposited in the GenBank under accession numbers MK976004 (*L. interrogans* serovar Pomona), MK976005 (*L. borgpetersenii* serovar Castellonis), MK976006 (*L. interrogans* serovar Canicola), MK976007 (*L. interrogans* serovar Copenhageni strain M20), MK976008 (*L. interrogans* serovar Hardjo), MK976009 (*L. interrogans* serovar Wolffi), MK976010 (*L. interrogans* serovar Pyrogenes), MK976011 (*L. interrogans* serovar Copenhageni strain Fiocruz L1-130), MK976012 (*L. borgpetersenii* serovar Hardjobovis)

**Results**

The end point LigBct PCR was performed under the conditions mentioned in Materials and Methods. Amplification of a 700–800 bp fragment, compatible with the expected 726 bp fragment, was observed in all of the pathogenic *Leptospira* spp. species used in this study, while no amplification was obtained in intermediate and non-pathogenic leptospires (Fig. 2). Also, a single band was observed for all clinical and environment DNA samples used in this study. DNA integrity of negative samples was controlled by agarose gel electrophoresis and LipL32 gene PCR (Table 1).

We found no amplification when DNA samples of the following organisms were used as template: *Brucella abortus*, *Campylobacter fetus fetus*, *Campylobacter fetus venearis*, *Neospora caninum*. Infectious Bovine Rhinotracheitis Virus (IBRV-1 and 5), Bovine Viral Diarrhea Virus (BVDV), *Mycobacterium* spp., *Escherichia* spp., *Borrelia* spp., *Salmonella* spp. (not shown). This indicates a high analytical specificity of LigBct PCR for pathogenic *Leptospira* spp.

Analytical sensitivity analysis showed significant differences in a controlled assay in which bovine serum, blood, and urine were experimentally infected with serial dilutions of a *L. interrogans* serovar Pomona suspension. After DNA extraction and LigBct PCR, detection limits for serum, blood and urine were $6 - 9 \times 10^2$, $6 - 9 \times 10^5$ and $6 - 9 \times 10^6$ leptospires/ml, respectively.
Multiple sequence alignment of the amplified LigB C-terminus coding region was performed for 9 pathogenic leptospires belonging to *L. interrogans* serovar Pomona, *L. interrogans* serovar Canicola, *L. interrogans* serovar Copenhageni (strain M20), *L. interrogans* serovar Wolffi, *L. interrogans* serovar Hardjo, *L. interrogans* serovar Pyrogenes, *L. interrogans* serovar Copenhageni (strain Fiocruz L1-130), *L. borgpetersenii* serovar Castellonis, *L. borgpetersenii* serovar Hardjobovis. SNPs were observed at 17 positions. More than one possible nucleotide was observed at some of these positions when two samples of the same serovar were analyzed (Table 2).

| Serovar | Pomona | Canicola | Copenhageni (M20) | Hardjo | Pyrogenes |
|---------|--------|----------|-------------------|--------|-----------|
| SNP1    | 5      | C        | -                 | C/T    | C/T       |
| SNP2    | 8      | T        | -                 | T/A    | -         |
| SNP3    | 16     | A        | -                 | A/G    | -         |
| SNP4    | 17     | G        | -                 | -      | G/A       |
| SNP5    | 19     | A        | -                 | -      | A/T       |
| SNP6    | 26     | A        | -                 | A/C    | -         |
| SNP7    | 185    | A        | -                 | A/G    | A/G       |
| SNP8    | 186    | A        | -                 | A/G    | A/G       |
| SNP9    | 188    | T        | -                 | T/G    | -         |
| SNP10   | 203    | A        | -                 | A/G    | A/G       |
| SNP11   | 234    | G        | -                 | -      | G/A       |
| SNP12   | 385    | A        | A/C               | A/C    | A/C       |
| SNP13   | 418    | G        | -                 | -      | G/A       |
| SNP14   | 504    | C        | -                 | C/T    | C/T       |
| SNP15   | 535    | G        | -                 | G/A    | G/A       |
| SNP16   | 565    | C        | -                 | C/A    | C/A       |
| SNP17   | 672    | T        | -                 | T/G    | -         |

**Discussion**

Leptospirosis remains a significant public health problem in many countries. Initially, the diagnosis of leptospirosis was based on the isolation of leptospires from clinical samples or the demonstration of
seroconversion in serum samples [24]. Although these tools are still used, molecular methods based on DNA amplification such as PCR, also play an important role for diagnosis. PCR often proves to be superior to traditional methods for the detection of carrier and sick animals, due to its speed and high sensitivity and specificity [25, 26].

The first end point PCR to amplify Leptospira sp. DNA was developed by Gravekamp in 1993 using the secY and flaB genes of pathogenic leptospires as targets and was applied to blood samples [14]. Although this PCR was reported not to amplify the secY sequence of non-pathogenic species, Palaniappan amplified DNA from the non-pathogen L. biflexa, which could lead to false positive results in clinical samples [19].

The LipL32 protein gene is also widely used as target for diagnosis of leptospirosis in clinical samples. A Taqman qPCR developed by Stoddard [16] based on this gene can detect $10^1$ leptospires/ml blood and $10^3$ leptospires/ml serum, while a qPCR developed by Levett [17] has an analytical sensitivity of 3 genome equivalents per reaction and approximately 10 genome equivalents in human urine.

Studies of the genome of the Leptospira genus showed that the lipL32 gene is present not only in all species belonging to the pathogenic group, but also in recently described low-virulence species such as the intermediate group [27, 28, 12].

The ligB gene has also been used as PCR target. In 2017, Benacer et al [29] developed a duplex PCR targeting the 16S ribosomal RNA subunit gene (rrs) and different regions of the ligB gene. This assay detects pathogenic species DNA with a sensitivity of $10^3$ leptospires/ml of urine or water. Ali et al. [30] also worked with the ligB gene and developed a Loop Mediated Isothermal Amplification (LAMP) protocol, using six pairs of primers targetting a segment from nt 72 to nt 290. This LigB-LAMP assay was applied to urine samples from cattle and dogs and proved to be highly sensitive, detecting a minimum of 150 fg DNA. However, the cost of reagents for LAMP is higher than that of PCR, making LAMP more difficult to implement in some diagnostic laboratories. In 2018, Martinez et al. [18] proposed a typing tool based on the amplification of a 1044 bp fragment of the ligB gene, from 1158 nt to 2203 nt, which could discriminate some serovars.

In the present work, the LigB-C terminal coding region (ligb-ct) is used for the first time as PCR target, and proves useful for the diagnosis of animal leptospirosis. No cross-reaction with other microorganisms commonly found in clinical laboratory samples was observed, and no amplification was obtained with intermediate Leptospira species. This indicates that this tool is highly specific for pathogenic Leptospira species.

The sensitivity of the LigBct PCR was higher for serum samples than for blood and urine samples. This could be because anticoagulants used in blood extraction can act as PCR inhibitors affecting the efficiency of PCR when a commercial extraction kit is not used [31].
Importantly, since serum samples are used in the MAT gold standard, LigBct PCR could be a complementary tool to detect traces of leptospiral DNA in this type of samples when there are positive cases with low antibody titers. In addition, the successful amplification of various species of pathogenic leptospires achieved, as shown in Table 1, indicates that the primers recognize conserved regions and can be used for diagnosis.

Multiple sequence alignment of the LigB C-terminus coding region of 9 strains of pathogenic leptospires showed the presence of 17 single nucleotide polymorphisms (SNPs) (Table 2). Since different nucleotides were observed at some of these polymorphic sites when two or more samples of the same serovar were analyzed, these SNPs cannot be used as a genotyping tool. The observed polymorphism within one serovar in the 3’ LigB-C terminal coding region may be due to the fact that the encoded protein segment is extracellular and exposed to the selection pressure of the host immune system, thus this region is not a marker of neutral selection.

We showed that the ligBct PCR could successfully amplify DNA of environmental samples, after isolating and cultivating leptospires under laboratory conditions. Future studies will be devoted to test whether this assay can be applied to amplify *Leptospira* spp. DNA from raw water samples without cross-reaction with other microorganisms present in the sample and not tested in the present work.

**Conclusion**

The analytical specificity and analytical sensitivity of a diagnostic PCR is given by the success of amplification on the pool of samples used. The results of this PCR targeting the 3’ region of the LigB-C terminal coding region are encouraging for the detection of leptospiral DNA in clinical samples in the initial phase of leptospirosis. LigBct PCR could be applied as a screening tool when a leptospire infection is suspected, or as a prophylaxis tool for early control in areas where there is a history of leptospirosis clinical cases. This PCR could also be a useful complement to MAT, the reference method worldwide, as it showed high sensitivity in bovine serum samples.

**Abbreviations**

OMPs
Outer Membrane Proteins.
Big
Bacterial Immunoglobulin-like.
MAT
Micro Agglutination Test.
*ligb-ct*
LigB C-Terminal coding region.
IBRV
Infectious Bovine Rhinotracheitis Virus.
BVDV  
Bovine Viral Diarrhea Virus.  
SNPs  
Single Nucleotide Polymorphisms.  

Declarations  

Ethics approval and consent to participate  
Not applicable.  

Consent for publication  
Not applicable.  

Availability of data and materials  
All relevant data are within the paper and its Supporting Information files  

Competing interests  
The authors declare that they have no competing interests.  

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Authors contribution  
VS conducted the molecular detection, sampling collection, data analysis, wrote the first draft, revisión and final manuscript. SGL, OW, MH, MM and BB contributed to study design and revisions. MFC contributed to revision, edition an drafted the manuscript.  
All authors read and approved the final manuscript.  

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Figures
Figure 1

Different serovars of L. interrogans (AF534640.1, CP011934.1, EU700271.1, CP001221.1 and CP006723.1) and L. kirscherii (AY190126.2) were used as template to design the primers to LigB C-Terminal coding region (ligb-ct).
Electrophoresis in a 2% agarose gel of amplicons by end point LigBct PCR. (2.a) All samples of pathogenic leptospires were positive with amplification of a 700-800 bp fragment, compatible with the expected 726 bp amplicon size. M: 100 bp DNA ladder. 1-L.i. serovar Pomona. 2-L.i. serovar Canicola. 3-L.i. serovar Copenhageni (strain M20). 4-L.i. serovar Wolffi. 5-L.i. serovar Hardjo. 6-L.i. serovar Pyrogenes. 7-L.i. serovar Copenhageni (strain Fiocruz L1-130). 8-L.b. serovar Castellonis. 9-L.b. serovar Hardjobovis. 10-L.biflexa serovar Patoc I. C-: nuclease free water. (2.b) M: 100 bp DNA ladder. 1-L.f. serovar Hurstbridge. 2-L.l. serovar Varillal. C+: L. fainei. C-: nuclease free water. C+: L.i. serovar Pomona Pomona. All samples of intermediate leptospires were negative. References: L.i.-Leptospira interrogans. L.b.-Leptospira borgpetersenii. L.l.-Leptospira licerasiae. L.f.-Leptospira fainei.