

Identification of *ompL1* and *lipL32* Genes to Diagnosis of Pathogenic *Leptospira* spp. Isolated from Cattle

Hernández-Rodríguez Patricia^{1,2,3*}, Gomez Ramirez Arlen^{2,4}, Baquero Mónica⁴, Quintero Gladys^{1,3}

¹Molecular Biology and Immunogenetics Research Group—BIOMIGEN, Bogotá, Colombia

²Health Research Center—CISVI, Bogotá, Colombia

³Department of Basic Sciences, Universidad de la Salle, Bogotá, Colombia

⁴Faculty of Agricultural Sciences, Veterinary Medicine Program, Universidad de la Salle, Bogotá, Colombia

Email: phernandez@unisalle.edu.co

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Abstract

Diagnosis of leptospirosis in Colombia is based on clinical history and serological testing. However, disease symptoms are nonspecific and there is no uniform criteria regarding the qualifications considered positive. Therefore, it is important to identify and characterize genes associated with pathogenicity in native strains for the development of new diagnostic tests and vaccine production. The aim of this study was to identify the *ompL1* and *lipL32* genes in *Leptospira* strains isolated from urine samples of cattle. Sixteen strains were obtained from urine samples and, DNA was isolated to perform two Polymerase Chain Reaction (PCR) tests which identified *lipL32* and *ompL1* genes. As positive control, a reference strain of *L. interrogans* was used. *L. biflexa* and *Escherichia coli* strains were used as a negative control. In 100% of the samples were identified amplicons of 960 bp and 423 bp corresponding to *ompL1* and *lipL32* genes respectively. Thus, the pathogenic property and conservation of genes in the isolated strains were confirmed. This study is presented as a contribution to the diagnosis of leptospirosis to use these genes as molecular markers of infection. The results of this study might provide clues for future clinical, epidemiological and molecular research leading to implement new diagnostic strategies and to expand knowledge of the pathophysiology of a disease of public health impact on human and animal.

Keywords

Leptospirosis, PCR, *ompL1*, *lipL32*

*Corresponding author.

1. Introduction

Leptospirosis is a worldwide distributed disease, affecting most of mammals (cattle, goats, sheep, pigs, dogs, horses, and rodents) in which it causes several signs including subclinical symptoms, abortions, stillbirths and mummification. These changes affect production cycles causing multiorgan complications that can lead to death [1]-[3]. The etiologic agent of the disease belongs to the genus *Leptospira*, which includes saprophytic and pathogenic species. Wild and domesticated mammals are the main sources of leptospires, which harbor in kidneys and excrete with urine [4]. Direct contact with contaminated urine or indirect exposures through contaminated water with urine is one of the ways of infection to humans [5].

Leptospirosis is considered as an important emerging global public health problem because of increasing incidence in both developing and developed countries, being recognized as a disease with epidemic proportions [6]. However, the impact of this disease in some regions and countries is unknown due to few epidemiological reports. Even so, the reported data infer a high incidence in human and livestock industry worldwide. Serovar Hardjo of *Leptospira interrogans* is the most frequently isolated agent in infections with *Leptospira* in cattle [7]-[9] and it is one of responsible for abortions in bovines [8]. Bovine leptospirosis has an impact in livestock industry due to economic losses for reproductive and non-reproductive causes such as abortions and septicemia respectively [1].

There are three recognized stages of the disease: acute, subacute and chronic. Acute disease is the less common stage, it is generated by accidental serovars and it is usually the most severe and mortal in calves with clinical signs as pyrexia, hemoglobinuria, hematuria, anemia, pulmonary congestion, and meningitis. On the other hand, cows present agalactia and abortions due to pyrexia [10]-[12]. Subacute stage is common and the less severe; cows present pyrexia and agalactia. Milk has calostrum appearance and the udder is soft [12]-[14]. Chronic leptospirosis is the most common stage; pregnant cows present abortions, weak calves and placental retention. This stage is associated with serovar Hardjo [12] [15] [16]. Molecular classification indicates that within serovar Hardjo there are two important species in bovine leptospirosis: *Leptospira interrogans* serovar Hardjo (hardjoprajitno) and *Leptospira borgpetersenii* serovar Hardjo (hardjobovis). Epidemiological studies have demonstrated that *Leptospira borgpetersenii* serovar Hardjo (hardjobovis) is the most common cause of bovine leptospirosis worldwide. *Leptospira interrogans* serovar Hardjo (hardjoprajitno) has been isolated mostly in cattle from United Kingdom [2]. However, Pomona and Grippotyphosa serovars are also associated with reproductive losses [17] [18].

Several studies have investigated about the pathogenetic mechanisms of leptospirosis, particularly host-parasite interactions and identification of virulence genes in different serovars of pathogenic and non-pathogenic leptospires [19] [20]. In this way, antigenic proteins expressed during infection have been identified. These have an important implication for the development of new techniques for diagnosis and vaccine production.

Spirochetes, including bacteria of the genus *Leptospira*, have a cytoplasmic membrane and outer membrane [21]. Identification and characterization of components of the outer membrane of *Leptospira* species are complex. Nevertheless, several transmembrane proteins, lipoproteins and peripheral membrane proteins have been determined [22]. By its strategic location, these outer membrane proteins (OMP's) are important in research because are responsible for the interaction between pathogen and host [23]. This fact is more important if it is assumed that the OMP's participate in mechanisms of evasion of the immune response as well as the persistence of spirochetes in the host [24]. Among isolated proteins from *L. interrogans* serovar Copenhageni are found: LipL32, LipL21, LipL45, LipL31, OmpL1, Flagelin/FlaB1, LipL36, LipL41, LipL71, and LigA [25]. Among these, LipL32 and OmpL1 proteins are candidates for designing new diagnostic techniques and producing immunogens.

First studies for identification of *Leptospira* were performed using techniques such as direct observation of the microorganism by dark field microscopy. Samples of blood, urine and tissues (kidney or liver) can be obtained for isolation of these bacteria by using biopsy or immediately after death in semisolid media such as fleetchers medium or EMJH. Microscopic agglutination test (MAT) is used as gold standard test. However, this serological test shows a low sensitivity and specificity. In addition, immunoenzymatic methods as enzyme-Linked Immuno Sorbent Assay (ELISA) and immunochromatography have been used. In the 80's, it was implemented the use of restriction enzymes for the taxonomic knowledge of some serovars of *Leptospira* [26] [27]. Polymerase Chain Reaction (PCR) has been used for diagnosis of leptospirosis in human. This technique offers:

- 1) Sensitivity, because this microorganism is detected in a sample with very small amounts of genetic material;

2) Specificity, because through strict conditions, the microorganism to be detected is capable of being amplified. 3) Speed, because it allows fast processing compared with other techniques to detect bacteria, which require more complex procedures. And 4) It can be mentioned that this test offers versatility because it is allowed to the diagnosis of various microorganisms [28]-[31].

Diagnosis of leptospirosis in Colombia is based on clinical history and serological testing. However, disease symptoms are nonspecific and there is no uniform criteria regarding the qualifications considered positive. Therefore, the aim of this study is to identify *lipL32* and *ompL1* genes associated with pathogenicity and antigenicity in native strains as molecular markers of infection with *Leptospira*.

2. Materials and Methods

2.1. Bacterial Strains

Sample size from 70 isolated strains of bovine urine characterized as pathogenic by PCR in a previous study was calculated by using the program Win Episcope 2.0. [39]. It was used a confidence level of 95% from which it was obtained a minimum sample size of 16. An ATCC reference strain of *L. interrogans* (23581) and a strain of *L. interrogans* donated by the Colombian Agricultural Institute (ICA—Instituto Colombiano Agropecuario) were used as positive controls. *Escherichia coli* and an ATCC reference of *L. biflexa* strain (23582) were used as negative controls.

2.2. DNA Extraction

DNA was isolated from 16 samples and controls. DNA was extracted from 1 mL of each culture of isolated strains and controls by using QIAamp® DNA Mini Kit (QIAGEN Inc., USA). The total concentration and purity of the DNA were quantified by spectrophotometry using absorption of light at 260 and 280 nm (A260/280). DNA was considered good quality with range = ~1.8 - 2.0.

2.3. Bioinformatic Analysis

Two pairs of primers: forward primer *lipL32* (5'-CGC TGA AAT GGG AGT TCG TAT GAT T-3') combined with the reverse primer *lipL32* (5'-CCA ACA GAT GCA ACG AAA GAT CCT TT-3') and forward primer *ompL1* (5'-TTG ATT GAA TTC TTA GAG TTC GTG TTT ATA-3') combined with the reverse primer *ompL1* (5'-AAG GAG AAG CTT ATG ATC CGT AAC ATA AGT-3') [32]-[34] were used to amplify DNA products of 423 bp and 960 bp of the *L. interrogans* *lipL32* and *ompL1* genes, respectively. Resources and database of the National Center for Biotechnology Information (NCBI) were used to confirm sequence, size, position genes and binding site of used primers in PCR.

2.4. PCR Amplification

Two PCR were performed to identify *lipL32* and *ompL1* genes associated with pathogenicity by using a modification of a procedure previously reported [35]. Two microliters of DNA extract were used for amplification in a total reaction mixture of 48 µL containing final concentrations of 16.25 µM Tris-HCl, 0.812 µM MgCl₂, 108.33 µM of each deoxynucleoside triphosphate (dNTP), 0.677 U of Taq polymerase and 2 pmol/µL of each of primers. The PCR was carried out as follows: 94°C for 5 min, forty cycles of 94°C for 15 s, 56°C for 35 s, 72°C for 40 s. The PCR products were separated on 2% agarose gel, stained with ethidium bromide and examined with ultraviolet light.

3. Results

3.1. Bioinformatic Analysis

The results obtained by using Nucleotide resource of NCBI indicate that the *lipL32* gene sequence has been reported only in pathogenic strains of *Leptospira* sp. The gene has 820 bp and it is located on chromosome 1 at position 5'-1'938, 935-1'939, 755 bp-3'. We identified 118 reports of this gene in pathogenic strains of *Leptospira* sp. *Leptospira* species in which the gene has been reported are: *L. interrogans*, *L. borgpetersenii*, *L. santarosai*, *L. kirschneri*, *L. noguchi* and *L. wali*. *ompL1* gene has been reported in the following pathogenic strains: *L.*

interrogans, *L. borgpetersenii*, *L. santarosai*, *L. kirschneri*, *L. noguchii* and *L. weilii*. This gene has 960 bp and it is located on chromosome at position 5'-686.212 - 687.174 bp-3'. The sequences of the *lipL32* primers were aligned with the genome of *L. interrogans*, *L. borgpetersenii*, *L. santarosai*, *L. kirschneri*, *L. noguchii* and *L. weilii* by using Blast program; while the sequences of the *ompL1* primers were aligned with the genome of *L. interrogans*, *L. borgpetersenii* and *L. noguchii*. This confirmed the alignment site of primers for *ompL1* and *lipL32* genes and that the selected primers were associated exclusively with pathogenic leptospires.

3.2. PCR Amplification

We identified the amplified products of 423 and 960 bp, corresponding to *lipL32* and *ompL1* genes respectively from 100% of the samples. The amplified product of 423 bp corresponding to *lipL32* gene is showed in **Figure 1**. **Figure 2** shows the amplified product of 960 bp of *ompL1* gene presents in pathogenic strains isolated from cattle. Positive and negative controls are showed in both figures.

4. Discussion

To contribute to a better understanding of molecular pathophysiology of leptospirosis, the identification of two OMP's genes was performed in strains of pathogenic leptospires. OMP's and lipoproteins are the main components of leptospiral surface [36]. These proteins play a role in signal transduction, nutrition uptake, and immunogenicity [37]. In this study, we identified *lipL32* and *ompL1* genes in strains of *L. interrogans* isolated from bovine urine. The bioinformatic analysis indicated that the *lipL32* gene is exclusively in pathogenic strains of

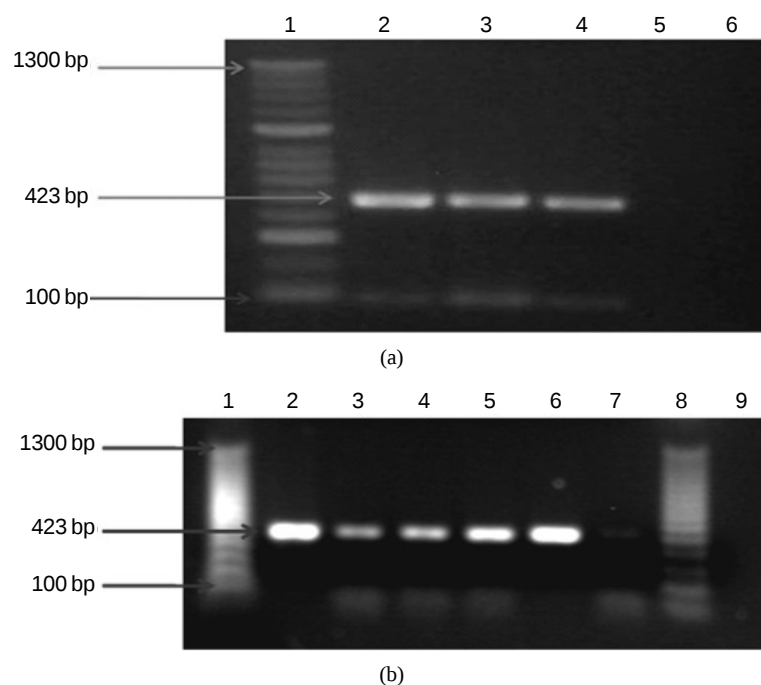


Figure 1. Agarose gel electrophoresis of polymerase chain reaction (PCR) analysis products for *lipL32* (423 bp) in pathogenic *Leptospira*. A 100-bp molecular weight marker was used. The PCR products were separated on 2.0% agarose gel, stained with ethidium bromide, examined with UV light, and visualized with a transilluminator (Bio-Rad). Analysis of amplified products, (a) Line 1: Molecular weight marker. Line 2: *Leptospira interrogans* (ATCC). Lines 3 and 4: Positive samples amplified from bovine urine (pathogenic leptospires-*lipL32*, 423 bp). Line 5: Negative control of PCR. Line 6: *Leptospira interrogans* (ATCC); (b) Line 1: Molecular weight marker. Line 2: *Leptospira interrogans* (ATCC). Lines 3-6: Positive samples amplified from bovine urine (pathogenic leptospires-*lipL32*, 423 bp). Line 7: *Leptospira interrogans* (ATCC). Line 8: Molecular weight marker. Line 9: Negative control of PCR.

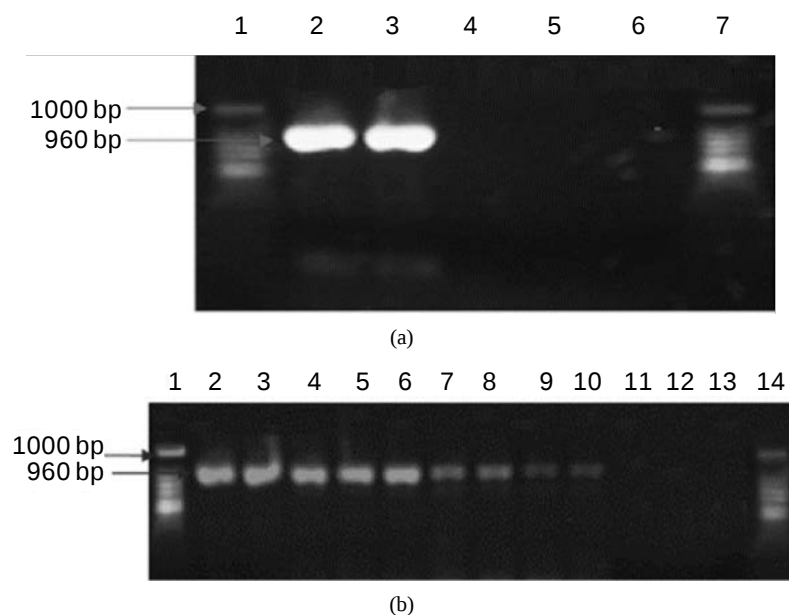


Figure 2. Representative polymerase chain reaction (PCR) for *ompL1* DNA in urine samples from bovine. The PCR products were separated on 1.5% agarose gel, stained with ethidium bromide, examined with UV light, and visualized with a transilluminator (Bio-Rad). Analysis of amplified products, (a) Line 1: Pathogenic *Leptospirainterrogans* (ATCC). Line 2: Positive samples amplified from bovine urine. Line 3: Negative control of PCR. Line 4: *E coli*. Line 5: Saprophytic *Leptospirabiflexa* (ATCC). Line 6: Negative control PCR. Line 7: Molecular weight marker; (b) Line 1: Pathogenic *Leptospirainterrogans* (ATCC). Line 2-10: Positive samples amplified from bovine urine. Line 11: Negative control of PCR. Line 12: *E coli*. Line 13: Saprophytic *Leptospirabiflexa* (ATCC). Line 14: Molecular weight marker.

Leptospira sp., which agrees with reports by various authors [22] [38] [39]. Similar to *lipL32* gene, *ompL1* gene has been reported only in pathogenic strains of *Leptospira* sp. [40] [41]. Both genes were identified in existing strains with public health impact. The primers used were obtained from reports in which it was confirmed the size of the amplified products [30] [31] [41]-[43]. The use of these primers for the identification of *lipL32* gen by real time or conventional PCR has been postulated to the development of diagnosis tests for leptospirosis in samples from patients who are in antibiotic therapy or in early stages of infection [30] [44]. Therefore, bioinformatic analyses are postulated as other useful tool for the study of *Leptospira*, which have become a significant method for researchers. During more than 20 years of taxonomic and phylogenetic studies, an important number of *Leptospira* sequences have been generated. All this information has been deposited in large databases such as GenBank (www.ncbi.nlm.nih.gov/Genbank/index.html) [45].

The determination of *ompL1* and *lipL32* genes in reference strains of *L. interrogans* and *L. biflexa* used in this study agreed with reports related to the presence of these genes in pathogenic strains and their absence in saprophytic strains [19] [37] [41] [46] [47]. The identification of *ompL1* and *lipL32* genes specific bands of 960 bp [30] [44] [48] and 423 bp [31] [49] [50] respectively, indicates presence of these genes in native pathogenic strains isolated from bovine urine. This result suggests that OMP's are conserved proteins among pathogenic leptospires, which may have the potential of inducing comprehensive immunity and play an important role in virulence [5].

It is known that OMP's act as adhesions [23] [51]-[53], antibodies fixation points [54] [55], porins [56]-[58], receptors for soluble proteins such as siderophores [59], and complement proteins [60]. It is also possible that they are responsible for the interaction of pathogens with hosts due to their strategic localization [23]. It is presumed that OMP's participate in evasion mechanisms of immune response and the persistence of leptospires in the host [24].

lipL32 is a highly conserved gen in pathogenic leptospires during infection in mammals [61] and it has been

identified as an extracellular matrix-binding protein specifically to laminin, collagen I, and fibronectin [62]. It increases the cell permeability and accelerates the apoptosis process [63]. OmpL1 is a porin expressed in pathogenic *Leptospira* strains [33] [46] that allows the diffusion of hydrophilic solutes through the external membrane to the periplasm [22].

However, the function of the majority of OMP's and molecular pathogenesis remain almost unknown [5] [64], despite the above mentioned and the fact that several major OMP's express during infection have been potentially employed in the development of subunit vaccines and serologic test for leptospirosis diagnosis [31] [41] [65]-[67]. It is known that OMP's have an important role in the pathogenesis because they facilitate the adaptation and interaction between bacteria and host [68].

Leptospirosis is considered one of the most common zoonotic diseases worldwide [1]. However, it is neglected due to the fact that the endemicity of the disease is related to socioeconomic and climatic conditions of developing countries affecting impoverished populations [69] [70]. Leptospirosis has emerged as an important problem, especially where some risk factors are present such as inadequate sanitation that allows rat-borne transmission [3] [70] [71]. Actually there are over 500,000 reports of severe leptospirosis every year with fatality rates over 10% [72]. On the other hand, leptospirosis is a disease of livestock with an important economic impact due to reproductive losses. Abortions, stillbirths, embryo mortality and infertility are some of the factors involved in the presentation of leptospirosis in cattle [73]-[76].

According to several studies, bovine leptospirosis is an important disease that affects production systems in Colombia. Since 1991 researchers from different Colombian regions have been studying the seropositivity of the disease [16] [77]-[81]. Only one research established the presence of *Leptospira* spp. in bovine using PCR, and they determined a prevalence of 37% for Sabana de Bogotá [82]. It is evident the presence of *Leptospira* spp. in bovine production systems and the need of using new detection techniques. Due to the epidemiologic and economic relevance it is important that leptospirosis must be considered a disease with impact which should be studied in order to improve methods for diagnosis, prevention and treatment.

5. Conclusions

This study is presented as a contribution for the diagnosis of leptospirosis through the use of these genes as molecular markers of infection. The results of this study might provide clues for future clinical, epidemiological and molecular research leading to implement new diagnostic strategies and to expand knowledge of the pathophysiology of a disease of public health impact.

Both, *ompL1* and *lipL32* are considered key genes to be studied in order to develop new methods of diagnosis and prevention. For instance, molecular diagnosis based on the detection of these genes has been identified [30] [42] [83] [84]. Several OMPs have been evaluated as potential vaccine candidates [33] [85] [86]. Vaccines based in LipL32 include vectors such as *Mycobacterium bovis* [66] and adenovirus [34] expressing the LipL32 antigen. It has been shown that OmpL1 and LipL41 have immunoprotective effects on the hamster model [33]. The function and location of many proteins are not yet known in *Leptospira*. A recent study reevaluated the position of LipL32 in subsurface [87].

Further studies involving the identification of genes associated with the adaptation of leptospires to different hosts [88] and their expression profiles under several conditions should be under this way in an effort for a better understanding of molecular pathogenesis of this disease as well as for the development of strategies for control and prevention of leptospirosis as an emerging pathology with public health impact.

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Conflict of Interest Statement

The findings and conclusions in this report are those of the authors and do not necessarily represent the view of the funding agencies. The use of trade names and commercial sources are only for identification and it does not

imply endorsement by Universidad de la Salle in Bogotá D.C.-Colombia.

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