First Isolation of *Leptospira Borgpetersenii* Serovar Hardjobovis in Guanacos (*Lama Guanicoe*) in South America

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Research Article

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Abstract

Background

Leptospirosis is the most widespread zoonotic disease in the world. It is caused by pathogenic spirochetes of the genus *Leptospira* spp. and is maintained in nature through chronic renal infection of carrier animals, being rodents and other small mammals the main reservoirs. This bacterial genus is highly heterogeneous and divided into three clades (pathogenic, saprophyte and intermediate). Presence of pathogenic strains in wildlife populations is essential to monitor the epidemiological status of this disease worldwide.

Methods

In this study, we characterize an isolated strain of a Guanaco (*Lama guanicoe*) using Multiple Locus Variable number tandem repeats Analysis (MLVA) (Variable Number Tandem Repeats-VNTRs: 4, 7, 10, Lb4 and Lb5). To confirm the identity of the isolated strain, partial 16S rRNA sequencing was carried out. Phylogeny was constructed using Neighbor- joining.

Results

The pathogenic leptospiral strain isolated from *Llama guanicoe* had the genetic profile identical to *L. borgpetersenii* serovar Hardjobovis reference strain Sponselee.

Conclusions

To the best of our knowledge, this is the first isolation and genetic characterization of a pathogenic leptospiral strain in Guanacos in South America.

Background

Leptospirosis is a zoonosis of worldwide distribution in tropical and subtropical countries. The etiological agent is of bacterial origin and belongs to the order Spirochaetales and the genus *Leptospira* spp. This disease is endemic in Argentina and in addition to its public health importance, it is responsible for huge economic losses to the agroindustry. Indeed, 10% of the abortions in bovines caused by bacteria are estimated to be due to infections with pathogenic leptospiral strains [14], these are disseminated into the environment through urine of infected animals and reservoirs and have the ability to survive in the ecosystem (water bodies, mud and sand) during days, weeks and even months, these strains mainly infect mammals, but they can also be found in reptiles and amphibians. [1, 7,8]

In a recent study of genus wide *Leptospira* spp. core genome MLST (cgMLST), 35 species are reported (13 species belonging to the pathogenic clade, 11 to the intermediate and saprophyte likewise)[13].These clades are divided regarding their virulence and manifestation of symptoms, being the pathogenic clade the most virulent. In Argentina, several studies on taxonomy and molecular typing have been done, reporting in some cases new hosts (reservoirs) and new strains isolated from wildlife [2,5,9,11,12], domestic animals [10,12, 14] and also from water samples. [8, 21] These studies allow to conclude that the pathogenic strains of *Leptospira* spp. present in Argentina include: *Leptospira interrogans* of the serovars Pomona (four genotypes: A,B.C.D), *L. interrogans* serovar Canicola Hond Utrecht IV, and serovar Portlandvere MY1039, *L. interrogans* serovar Icterohaemorrhagiae RGA, and Ictero I, serovar Coppenhageni M20, *L. borgpetersenii* serovar Castellon Castellonis and serovar Hardjobovis [5,10,12,14,17]

The Guanaco (*Lama guanicoe*) is a South American Camelid (SAC) that is either wild or raised under silvestry conditions in Patagonia and other regions of Argentina and shares pastures with bovines and other production animals [3]. The SACs are composed also by *Lama glama, Lama pacos* and *Vicugna vicugna*. In the last decade, studies on its biology and ecology have intensified to impulse the development of alternative regional economies. Previous studies on serosurveys of SAC in the region (Perú), show positive titres (1:100 to 1:1600) to *L. interrogans* serovar Icterohaemorrhagiae and *L. interrogans* serovar Pomona in Alpacas and lower titres (1:100 to 1:400) in Vicuñas reacting to the same serovars.[19] Serosurveys done on SACs in Argentina, show 60% of seroprevalence (Buenos Aires Province) being the serovars Icterohaemorrhagiae, Pomona and Grippotyphosa most reactive[3]. In another study (Jujuy Province) 96,2% show seroreactivity being serovars Copenhagni (serogroup Icterohaemorrhagiae), and Castellonis (belonging to the serogroup Ballum) the most reactive serovars[16].

Serovar Hardjobovis (belonging to the species *L. borgpetersenii*) is the most adapted serovar to bovine hosts and is the most prevalent serovar in bovines worldwide [1,7]. Also, this serovar is not distinguishable using serological tools within serovar Hardjo (*L. interrogans* strain Hardjoprajitno). However, some molecular tools can be used to differentiate these serovares. In this study the typing scheme of Multiple Locus Variable Number Tandem Repeats Analysis (MLVA) [20] and sequencing of partial 16S rRNA was used to genotype this strain to confirm this genotype. In a recent study, this serovar was molecularly typed using MLVA from clinical cases in bovines in Argentina.[14]

To the best of our knowledge this is the first isolation of a pathogenic leptospiral strain in Guanacos in South America. The purpose of this study was to molecularly characterize the isolated strain from kidney subsamples of Guanacos (*L. guanicoe*) from Argentina.

Methods

Sample used in this study: The laboratory of Leptospirosis (OIE Reference Centre) belonging to the Institute of Pathobiology of the National Institute of Agricultural Technology (INTA), received kidney samples of two dead found adult Guanacos (*Llama guanicoe*) obtained from a Natural Reserve located in Salta Province (Argentina). These samples were frozen until processing. Once the samples were

received, a small sub sample (5mm of tissue) was cultivated in Fletcher (semisolid) and EMJH (liquid) media (Difco Laboratories,USA) [12]. The cultures were incubated at 28°C until development was determinated, every 15 days the cultures were observed under dark field microscopy.

DNA templates: The reference strains and the isolated strain were growing in semisolid Fletcher media (Difco Laboratories,USA) at 28ºC. To extract DNA from the isolated strain was obtained from the Fletcher medium, 20*ul* of the dinger ring was used. The templates were obtained after nucleic acid purification using the Chelex-100 resin (Bio Rad, USA) protocol.[12]

Molecular characterization:

Multiple-Locus Variable-number tandem repeats Analysis (MLVA): was used to characterize the isolated strain with 1 set of oligonucleotides specific for the pathogenic strains of *L. interrogans*, *L. kirschneri* and *L. borgpetersenii*, following loci were used, VNTRS: 4, 7, 10, Lb4 and Lb5 [20]. The final volume (50µl) of each reaction mixture contained PCR Buffer (20mM Tris-HCL,pH 8.4;50mM KCL), 200µM 119 deoxinucleoside triphosphates,2µM each corresponding primer, 2mM MgCl2, 1.25U Taq DNA polymerase (Invitrogen, USA) and 5µl DNA template. PCR was carried out in a Thermo Scientific PxE0,2 Thermal Cycler as follows: 94ºC for 5 min, followed by 35 cylces of denaturalization at 94ºC for 30s, annealing at 55ºC for 30s and extension at 72ºC for 90s, with a final cycle at 72ºC during 10min. Amplified samples (15µl) were revealed by electrophoresis in a 2% agarose gel in buffer TAE (40mM Tris-acetate, 1mM EDTA)with 0,2ul µg/ml of ethidium bromide at 100V for 50min. Amplified DNA bands were visualized upon UV light exposure (Uvi Tec transiluminator BTS-20.M). Amplicon sizes were estimated using a 100bp-marker (Embiotech, Argentina) and the GelAnalyzer 2010a program. To calculate the repeat copy number, the following formula was used: Number of repeat (bp) = [Fragment size (bp)-Flanking regions (bp)]/Repeat size (bp). Repeat copy numbers were rounded down to the closest whole numbers. If the copy number was less than one it was rounded to zero. [9,10,17,18]

Sequencing 16S rRNA (partial gene): A PCR targeting the 16S rRNA gene was carried out for bacterial identification after sequencing. The following primers were used: 5´GGCGGCGCGTCTTAAACATG and 5´GTCCGCCTACGCACCCTTTACG.[6] These primers have the ability to amplify all pathogenic and nonpathogenic species of *Leptospira* spp. PCR was performed as indicated in [6]. After verification of the amplicon by electrophoresis in an ethidium bromide-containing 2% agarose gel and visualization upon UV light exposure, PCR products were purified using a commercial kit (Embiotech, Argentina). The sample was sequenced by Macrogen, Korea.

Phylogeny: For alignment and construction of the phylogeny, the program MEGA version 6.06 was used [22]. The dendogram was constructed using Neighbor-joining, partial sequences of the16S rRNA gene were used.

Results And Discussion

MLVA: One the amplicons were obtained, the copies were calculated using the methodology detailed above. The copy numbers of this strain were (2,0,1,5,4) VNTR 4: 2; 7:0; 10:1;Lb4:5;Lb5:4). This results represent a genetic profile identical to *Leptospira borgpetersenii* serogroup Sejore serovar Hardjobovis strain Sponselee (2, 0, 1,5,4) [20](Figure 1).

In Brazil, *L. borgpetersenii* serovar Hardjo strain Hardjobovis was isolated from naturally infected cattle and characterized this strains using MLVA and the partial sequence of secY[4], representing the first isolation of this serovar in Latin America in naturally infected cattle. In the same study, only two out of 50 urine samples evaluated were positive by culturing. The gold standard method to confirm the presence of a leptospiral strain is the isolation, this is a major bottleneck since this bacterium is difficult and laborious to isolate [4,7,14]. Recently in Argentina, strain Hardjobovis was also isolated from a clinical case in bovines from urine samples and molecularly typed with MLVA. [4]

16S rRNA sequencing: BlastN was run with the sequence obtained using Genbank. The strain Guanaco is 100% homologue with *Leptospira borgpetersenii* serovar Hardjobovis 16S ribosomal RNA gene (partial sequence), belonging to the serogroup Sejroe. The sequence was deposited in Genbank under the Accession NumberKP901271. BlastN results can be visualized under https://go.usa.gov/xmvMh. Figure 2 shows the Neighbor-joining phylogenetic tree of 16S rRNA of strain Guanaco (***) this phylogenetic analysis based of 16S rRNA includes 19 representative species of *Leptospira* spp.

Conclusions

Reports of new possible hosts for this zoonosis are relevant to better understand the complex epidemiology of this disease. SACs are either wild or raised under silvestry conditions in Patagonia and other regions of Argentina and share pastures with bovines and other production animals[15]. Further studies on epidemiology of leptospirosis in SACs have to be made in Argentina to evaluate possible risk of dissemination within other animals that cohabit the same pasture. This report represents, to the best of our knowledge, the first isolation of *L. borgpetersenii* serovar Hardjo Bovis strain Sponselee in Guanacos (*Llama guanicoe*) in Argentina and in South America.

Abbreviations

OIE: World Organization for Animal Health

EMJH: Ellinghausen McCulough Johnson y Harris Media

VNTRs: Variable Number Tandem Repeats

bp: base pairs

MEGA: Molecular Evolutionary Genetics Analysis

SACs: South American Camelids

M: Marker (100bp)

Declarations

Ethics approval and consent to participate. Not applicable

Consent to publish: Not applicable

Availability of data and materials: Data sharing is not applicable to this article as no datasets were generated or analysed during the current study

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

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Authors' Contributions: BB isolated the strain from Guanaco kidney subsamples. SV contributed with MLVA typing. MM contributed writing and editing this manuscript. WO performed PCR for sequencing. HM permorfed DNA extraction and purification of the strain. GLS carried out MLVA typing, sequencing and phyolgeny in this study and was a major cotributor in writing the manuscript. All authors read and approved the final manuscript.

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Figures

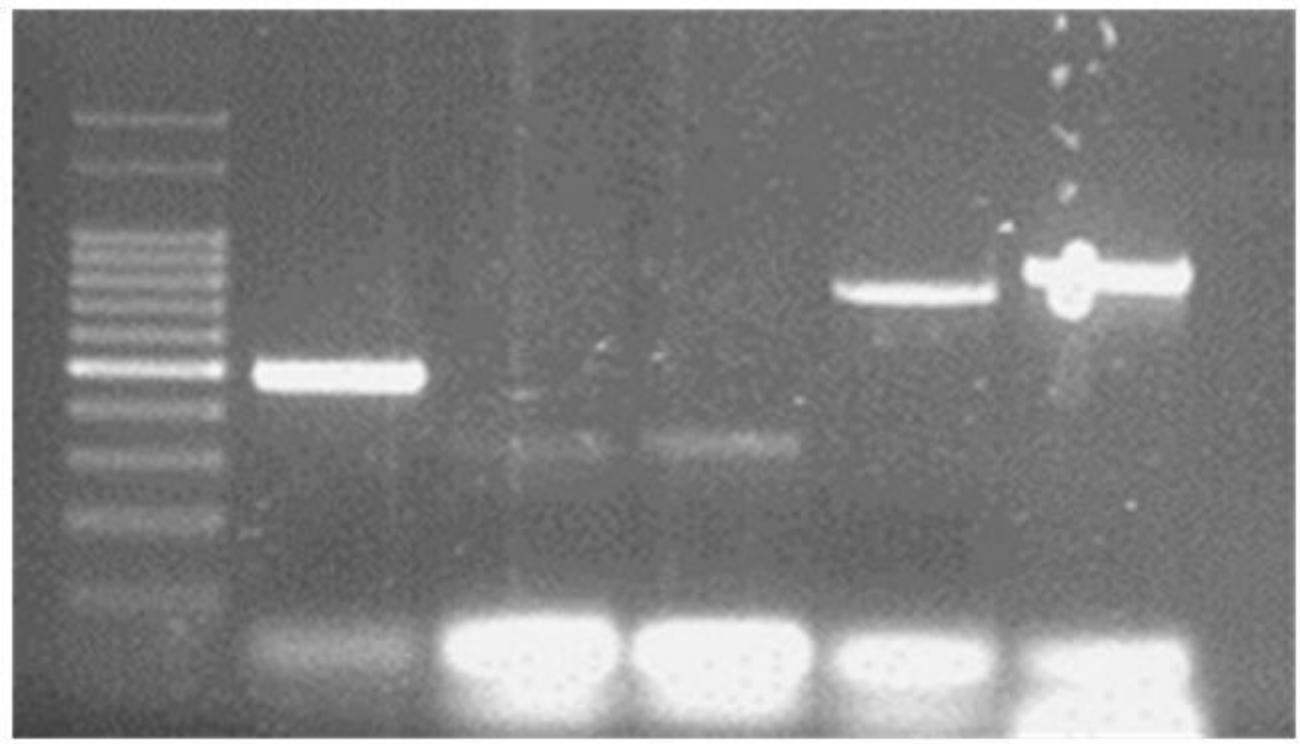

Figure 1

Gel at 2% of electrophoresis showing the genetic profile of the isolated strain obtained from the kidney sample of Guanaco (Llama guanicoe). (M: Marker 100bp, VNTRS: 4bis, 7bis, 10bis, Lb4, Lb5,bp= base pairs, copies= copy numbers (tandem repeats-VNTR)).

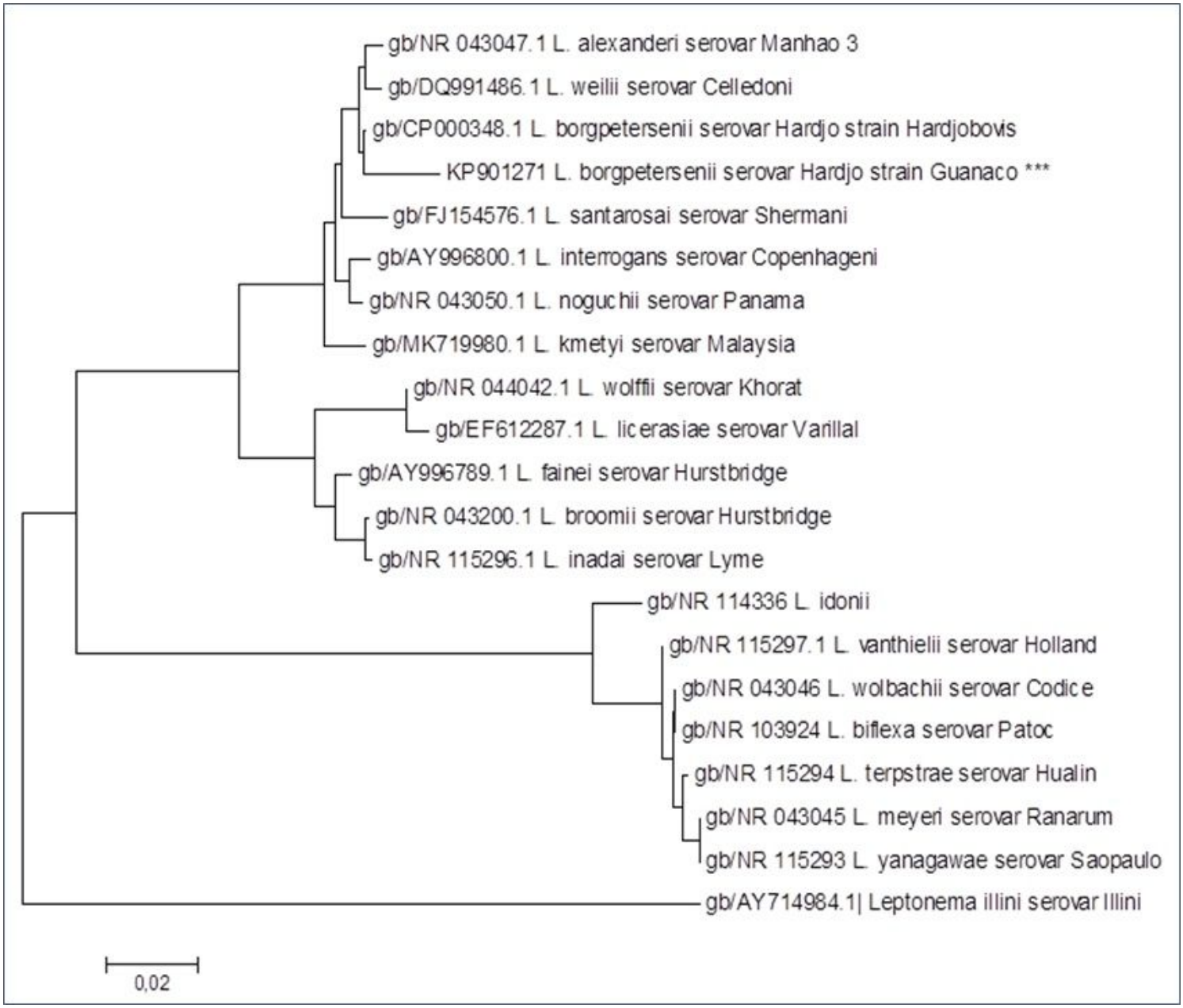

Figure 2

Neighbor-joining phylogenetic tree of 16S rRNA of strain Guanaco (***). Phylogenetic analysis based of 16S rRNA including 19 representative species of Leptospira spp. The corresponding sequence of Leptonema illini serovar Illini was used as outgroup.