Genetics and Molecular Research

Isolation and molecular characterization of *Leptospira borgpetersenii* serovar Hardjo strain Hardjobovis in the urine of naturally infected cattle in Brazil

R.T. Chideroli[1], U.P. Pereira[2], D.D. Gonçalves[3], A.Y. Nakamura[4], A.A. Alfieri[5], A.F. Alfieri[5] and J.C. Freitas[1]

[1]Laboratório de Leptospirose, Departamento de Medicina Veterinária Preventiva, Universidade Estadual de Londrina, Londrina, PR, Brasil
[2]Laboratório de Bacteriologia, Departamento de Medicina Veterinária Preventiva, Universidade Estadual de Londrina, Londrina, PR, Brasil
[3]Laboratório de Medicina Veterinária Preventiva e Saúde Pública, Universidade Paranaense, Umuarama, PR, Brasil
[4]Medicina Veterinária, Universidade Estadual de Maringá, Umuarama, PR, Brasil
[5]Laboratório de Virologia Animal, Departamento de Medicina Veterinária Preventiva, Universidade Estadual de Londrina, Londrina, PR, Brasil

Corresponding author: U.P. Pereira
E-mail: upaduapereira@uel.br

Genet. Mol. Res. 15 (1): gmr.15018473
Received January 20, 2016
Accepted January 20, 2016
Published February 19, 2016
DOI http://dx.doi.org/10.4238/gmr.15018473

ABSTRACT. Most epidemiologic studies on bovine leptospirosis are based on serological tests that use antibodies against several serotypes, including the serovar Hardjo, which is widespread and considered to be the most adapted to bovine hosts. However, using only serological studies is not sufficient to identify and distinguish species of leptospires. The aim of this study was report the first isolation in Brazil of two strains serovar Hardjo obtained in urine samples from naturally infected cows in a small Brazilian dairy herd and find the genetic species and consequently the type strain

Hardjobovis by molecular characterization. Fifteen dairy cows with a history of reproductive failure, such as abortion and infertility, were selected. Urine samples obtained from each animal were immediately seeded in tubes containing Ellinghausen-McCullough-Johnson-Harris culture medium. The identification of the isolates was performed by Multilocus variable-number tandem-repeat analysis (MLVA) technique and phylogenetic analysis of partial sequence of gene *sec Y.* From the 15 urine samples evaluated, two *Leptospira* were found and identified as the Londrina 49 and Londrina 54 strains. The MLVA profiles and sequencing of gene *sec Y* characterized the isolates as *L. borgpetersenii* serovar Hardjo strain Hadjobovis because it has different genetic pattern of *Leptospira interrogans* serovar Hardjo strain Hardjoprajitno. Therefore, more studies are needed including isolation and molecular characterization from regional strains to obtain a better knowledge about epidemiology of serovar Hardjo in bovine which may assist in future strategies of prevention and control of bovine leptospirosis.

Key words: Bovine leptospirosis; MLVA; Diagnosis; Bacterial culture

INTRODUCTION

The infection caused by bacteria from the genus *Leptospira* is considered one of the major reproductive infectious disease in cattle worldwide (Adler and de la Peña Moctezuma, 2010). In Brazil, the infection is widespread in dairy and beef cattle herds (Favero et al., 2001; Hashimoto et al., 2012). Leptospirosis causes economic losses that are mainly associated with reproductive failure (abortion, infertility and embryonic death), decreased milk production and indirect costs of treatment (Ellis, 1994).

Despite its relevance in animal health, leptospirosis in cattle must still be studied further, particularly its etiology and epidemiology and the prevention and control of its infection (Lilenbaum and Martins, 2014). Most epidemiologic studies on bovine leptospirosis are based on serological tests that identify antibodies against several serotypes (Faine et al., 1999), including serovar Hardjo which is widespread and considered to be the most adapted to bovine hosts (Ellis, 1994).

However, using only serological studies to determine serovars prevalence is not sufficient to identify and distinguish the species of leptospires that are found in a particular host. Currently, large variability with respect to species and genotypes circulating in cattle has been reported in molecular studies (Hamond et al., 2015a). The species *Leptospira borgpetersenii* serovar Hardjo strain Hardjobovis (Salgado et al., 2014), as well as *Leptospira interrogans* serovar Hardjo strain Hardjoprajitno (Rinehart et al., 2012), is considered to be well adapted to cattle. Consequently, it appears that cattle worldwide are most commonly infected with the strain Hardjobovis because they are its natural host (Hanson, 1982).

Multilocus variable-number tandem-repeat (VNTR) analysis (MLVA) is a molecular technique that identifies DNA from many pathogenic bacterial species (Lindstedt, 2005) and has been used for typing different serovars (Salaün et al., 2006) and strains (Majed et al., 2005) of the genus *Leptospira*. Therefore, it may be a powerful methodology that contributes to epidemiological leptospirosis data (Slack et al., 2006). Together with MLVA, the partial sequencing of the gene *sec Y* is also effective in the molecular characterization of some species from the genus *Leptospira (*Ahmed et al., 2006*)*.

Once the strategies to prevent and control leptospirosis infections are directly related to knowledge of circulating strains in a specific host and region, bacterial isolation and correct

identification from genetic characterization will prove extremely important for understanding the etiology, epidemiology and pathogenesis of different leptospires species (Lilenbaum and Martins, 2014). Therefore, more studies with regional isolated strains are needed to fill these gaps.

Thus, the aim of this study was to perform the molecular characterization (in the genetic species and type strain) of two strains of *Leptospira* belonging to serovar Hardjo isolated from naturally infected dairy cows. In this work, we report the first isolation in Brazil and Latin America of *L. borgpetersenii* serovar Hardjo strain Hardjobovis confirmed by MLVA and phylogeny of gene *sec Y*.

MATERIAL AND METHODS

Selection of animals

Fifteen dairy cows with a history of reproductive failure, such as abortion and infertility, were selected for this study. The cows were from five small farms located in the northwest of Parana State, Brazil. The microscopic agglutination test (MAT), which was undertaken approximately 15 days prior to urine sample collection, showed titers of leptospira antibodies between 400 and 1600 for the serovar Hardjo for these animals (Faine et al., 1999).

Urine collection and seeding

A urine sample from each animal was obtained during a perineal massage and was immediately seeded in tubes containing Ellinghausen-McCullough-Johnson-Harris (EMJH) culture medium (Difco, USA) with the following antibiotics: 5-fluorouracil (400 mg/L; Sigma®, USA), chloramphenicol (5 mg/L; Sigma®), nalidixic acid (50 mg/L; INLAB®, BR), neomycin (10 mg/L; Sigma®) and vancomycin (10 mg/L; Acros®, USA) (Zacarias et al., 2008). After incubation at 28ºC for 24 hours, the cultures were seeded in duplicate using the same medium (but without antibiotics) and these tubes were evaluated weekly for six months with a dark field microscope (Olympus BX40 Model).

Extraction and amplification of DNA

For genetic characterization, DNA from leptospires cultures was extracted using the PureLink Genomic DNA Mini Kit (Invitrogen, USA). DNA from the leptospires strain isolates was amplified using the Platinum PCR SuperMix Kit (Invitrogen) according to the following conditions: 45 μL of each reaction containing SuperMix, 1 μL of each primer (10 nM) and 3 μl of DNA template. All products were analyzed by electrophoresis in a 2% agarose gel with ethidium bromide (0.5 g/mL) in 0.5X TBE buffer (89 mM Tris, 89 mM boric acid; 2 mM EDTA), pH 8.4, and visualized with ultraviolet light; molecular size was estimated by comparisons with a 100-bp ladder.

Molecular typing of isolates

The MLVA technique was used to identify isolates with five primer pairs for the VNTR loci 4, 7, 10, LB4 and LB5, as previously described (Salaün et al., 2006). Reference strains of *L. interrogans* serovar Canicola strain Canicola Hond Utrecht IV, *L. interrogans* serovar Hardjo strain Hardjoprajitno and *L. borgpetersenii* serovar Hardjo strain Hardjobovis were used as positive controls for each of the five PCRs for the VNTR loci that were analyzed. Additionally, amplification and partial sequencing of *sec Y* were used to identify and confirm genetic species, as previously described (Ahmed et al., 2006).

The products of *sec Y* gene amplification were purified with a commercial kit, quantified by a QubitTM Fluorometer (Invitrogen) and sequenced on a ABI3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using forward and reverse primers. Sequence quality was analyzed by the Phred program (http://asparagin.cenargen.embrapa.br/phph/). The consensus sequences were obtained by CAP3 software (http://asparagin.cenargen.embrapa.br/cgi-bin/phph/cap3.pl), and identities were compared with all the sequences that were deposited in GenBank using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The identity matrix was created in the BioEdit program with the alignment and phylogenetic tree developed by the MEGA 6.06 program.

RESULTS AND DISCUSSION

From the 15 urine samples evaluated, two leptospires were found and were identified as the Londrina 49 and Londrina 54 strains from two cows that had MAT serological titers against serovar Hardjo of 400 and 800, respectively. According the Figures 1 and 2, the results of MLVA for samples Londrina 49 and Londrina 54 are identical to the result obtained for the reference strain *L. borgpetersenii* serovar Hardjo Hadjobovis. BLASTn software was used to compare the partial sequences of gene *sec Y*, and the sample sequences were found to be more similar to *L. borgpetersenii* serovar Hardjo strain Hardjobovis. Furthermore, in the phylogenetic tree, the isolate samples were grouped in the same cluster as the serovar Hardjo strain Hardjobovis GenBank reference (Figure 3).

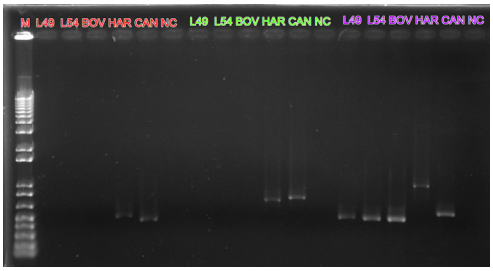

Figure 1. Banding patterns of VNTR visualized with an agarose gel. *Lane M* = molecular ladder bp, L49 (Londrina 49 strain) and L54 (Londrina 54 strain); BOV = reference sample serovar Hardjo strain Hardjobovis; HAR = reference sample serovar Hardjo strain Hardjoprajitno; CAN = reference sample serovar Canicola strain Hond Utrecht IV; NC = negative control. Locus colors: red (VNTR- 4), green (VNTR-7), and purple (VNTR-10).

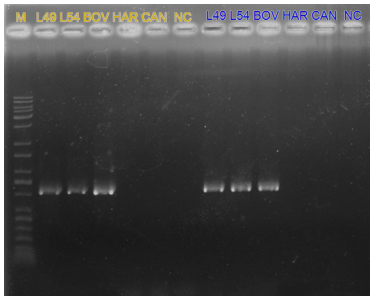

Figure 2. Banding patterns of VNTR visualized with an agarose gel. *Lane M* = molecular ladder bp, L49 (Londrina 49 strain) and L54 (Londrina 54 strain); BOV = reference sample serovar Hardjo strain Hardjobovis; HAR = reference sample serovar Hardjo strain Hardjoprajitno; CAN = reference sample serovar Canicola strain Hond Utrecht IV; NC = negative control. Locus colors: yellow (VNTR-Lb4) and blue (VNTR-Lb5).

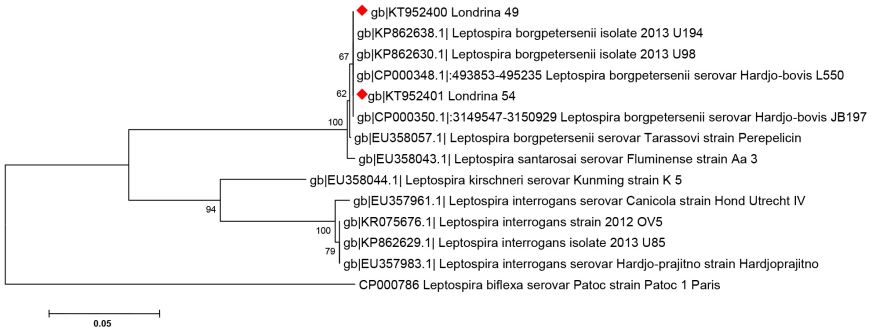

Figure 3. Neighbor-joining phylogenetic tree of *sec Y* gene sequences from two strains that were isolated from cows (Londrina 49 and Londrina 54) and other GenBank *sec Y* sequences of leptospires strains. Bootstrap percentages were based on 1000 replications. The sequences obtained through sequencing were deposited in the GenBank database with the following access code: Londrina 49- KT952400 and Londrina 54- KT952401.

In bovine, Hardjo is the leptospira serovar that is most prevalent worldwide (Levett, 2001). Although the species *L. borgpetersenii* serovar Hardjo strain Hardjobovis and the species *L. interrogans* serovar Hardjo strain Hardjoprajitno cause the same reproductive problems in cattle, they are epidemiologically different. The strain Hardjobovis cannot tolerate a lack of nutrients, and its transmission cycle is limited to direct contact; alternatively, the strain Hardoprajitno, after being eliminated in the urine, may survive months in aquatic environments until it infects a new host (Bulach et al., 2006).

L. interrogans serovar Hardjo strain Hardjoprajitno was initially isolated from cattle in the United Kingdom and is most commonly found in Europe (Bolin and Alt, 2001). However, the *L.borgpetersenii* serovar Hardjo strain Hardjobovis, which is considered to be the most common strain in cattle and the most prevalent in the world, was first isolated from a cattle herd in the United States of America (Bolin et al., 1989).

In Brazil, as well as Latin America, this is the first report that has isolated the species *L. borgpetersenii* serovar Hardjo strain Hardjobovis from naturally infected cattle. Before this study, only serological studies showed reagent animals with the serovar Hardjo in various countries (Favero et al., 2001; Hashimoto et al., 2012; Petrakovsky et al., 2014). However, this method not permit the differentiation between strains Hardjoprajitno and Hardjobovis.

Although bacterial isolation is the definitive diagnostic of leptospirosis, it is currently considered a major bottleneck for many research teams due to the difficult and laborious procedures used to obtain pure cultures of leptospires from the serovar Hardjo and to maintain them in a laboratory culture (Adler and de la Peña Moctezuma, 2010). Other studies in Brazil have used molecular methods to identify leptospira in cattle urine (Hamond et al., 2015a) and even in the reproductive tract of mares (Hamond et al., 2015b); however, none of these studies were successful in isolating the bacteria.

Antibody titers obtained in the MAT from both cows were only against the serovar Hardjo and did not distinguish between the strains Hardjobovis and Hardjoprajitno. The use of molecular methods, such as the MLVA, and genetic sequencing techniques allowed us to characterize the Londrina 49 and Londrina 54 strains that were isolated from the urine of two cows.

MLVA was a better molecular tool for differentiating most of the *Leptospira* serotypes, and the designs of the LB4 and LB5 primers made it possible to distinguish the species and strains of

L. borgpetersenii, *L. interrogans*, and *L. kirschneri* (Salaün et al., 2006). In this study, this technique allowed us to characterize the isolates (Londrina 49 and Londrina 54 strains) as belonging to serovar Hardjo strain Hardjobovis according to the molecular profile shown in VNTR loci-4, VNTR-7, VNTR-10, VNTR-LB4, and VNTR-LB5. However, the results did not fully correspond with that described by Salaün et al. (2006) because the two isolated strains (Londrina 49 and Londrina 54) and the Hardjobovis reference strain did not amplify the VNTR loci-4. Therefore, this is considered a poor discriminatory loci for the species *L. borgpetersenii*. Using the combination of VNTR loci-7, VNTR loci-10, VNTR loci-LB4 and VNTR loci-LB5 was sufficient to identify that Brazilian isolated strains were the same strain of Hardjobovis and different from the strain of Hardjoprajitno. To confirm the MLVA results, partial sequencing was followed by phylogenetic analyses of gene *sec Y*.

Currently, this is the first report that isolated the serovar Hardjo strain Hardjobovis from the specie *L. borgpetersenii* in urine from naturally infected dairy cattle in Latin America. Genetic analysis showed that the MLVA associated with the partial sequencing of gene *sec Y* allowed a rapid molecular characterization and typing of isolated cultures (Londrina49 and Londrina54). Although these molecular techniques have a moderate cost and are not frequently used in routine diagnosis of leptospirosis, they are indispensable tools for bacterial isolate characterization and are easy to standardize and execute. Despite the fact that bacterial isolation is time-consuming and laborious, it is an important tool in the diagnosis of leptospirosis because the inclusion of isolated strains in the reference antigens battery, used in the MAT, will be useful in future epidemiological studies. Another direction will involve proteomics, where studies will evaluate intraspecies and serotype protein differences, thereby helping us to better understand the pathogenicity of various bacterial strains and enabling the creation of new types of vaccines for cattle with regional strains in endemic areas.

ACKNOWLEDGEMENTS

We are grateful to Prof. Dr. Silvio Arruda Vasconcellos for the *L. borgpetersenii* serovar Hardjo strain Hardjobovis. Research supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) through the postgraduate program in Animal Science, Universidade Estadual de Londrina, Londrina, Paraná, Brazil.

REFERENCES

Adler B and de la Peña Moctezuma A (2010). *Leptospira* and leptospirosis. *Vet. Microbiol.* 140: 287-296. http://dx.doi.org/10.1016/j.vetmic.2009.03.012

Ahmed N, Devi SM, Valverde MdeL, Vijayachari P, et al. (2006). Multilocus sequence typing method for identification and genotypic classification of pathogenic *Leptospira* species. *Ann. Clin. Microbiol. Antimicrob.* 5: 28-44. http://dx.doi.org/10.1186/1476-0711-5-28

Bolin CA and Alt DP (2001). Use of a monovalent leptospiral vaccine to prevent renal colonization and urinary shedding in cattle exposed to *Leptospira borgpetersenii* serovar hardjo. *Am. J. Vet. Res.* 62: 995-1000. http://dx.doi.org/10.2460/ajvr.2001.62.995

Bolin CA, Thiermann AB, Handsaker AL and Foley JW (1989). Effect of vaccination with a pentavalent leptospiral vaccine on *Leptospira interrogans* serovar hardjo type hardjo-bovis infection of pregnant cattle. *Am. J. Vet. Res.* 50: 161-165.

Bulach DM, Zuerner RL, Wilson P, Seemann T, et al. (2006). Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential. *Proc. Natl. Acad. Sci. USA* 103: 14560-14565. http://dx.doi.org/10.1073/pnas.0603979103

Ellis WA (1994). Leptospirosis as a cause of reproductive failure. The Veterinary Clinics of North America. *Food Anim. Pract.* 10: 463-478.

Faine S, Adler B, Bolin C and Perolat P (1999). Leptospira and Leptospirosis. Medisci, Melbourne.

Favero M, Pinheiro SR, Vasconcellos SA, Morais ZM, et al. (2001). Leptospirose bovina - variantes sorológicas predominantes em colheitas efetuadas no período de 1984 a 1997 em rebanhos de 21 estados do Brasil. *Arq. Inst. Biol.* 68: 29-35.

Hamond C, Pestana CP, Medeiros MA and Lilenbaum W (2015a). Genotyping of *Leptospira* directly in urine samples of cattle demonstrates a diversity of species and strains in Brazil. *Epidemiol. Infect.* 16: 1-4.

Hamond C, Pestana CP, Rocha-de-Souza CM, Cunha LER, et al. (2015b). Presence of leptospires on genital tract of mares with reproductive problems. *Vet. Microbiol.* 179: 264-269. http://dx.doi.org/10.1016/j.vetmic.2015.06.014

Hanson LE (1982). Leptospirosis in domestic animals: the public health perspective. *J. Am. Vet. Med. Assoc.* 181: 1505-1509.

Hashimoto VY, Dias JA, Spohr KAH, Silva MCP, et al. (2012). Prevalência e fatores de risco associados á Leptospira spp. em rebanhos bovinos da região centro-sul do estado do Paraná. *Pesqui. Vet. Bras.* 32: 99-105.

Levett PN (2001). Leptospirosis. *Clin. Microbiol. Rev.* 14: 296-326. http://dx.doi.org/10.1128/CMR.14.2.296-326.2001

Lilenbaum W and Martins G (2014). Leptospirosis in cattle: a challenging scenario for the understanding of the epidemiology. *Transbound. Emerg. Dis.* 61 (Suppl 1): 63-68. http://dx.doi.org/10.1111/tbed.12233

Lindstedt BA (2005). Multiple-locus variable number tandem repeats analysis for genetic fingerprinting of pathogenic bacteria. *Electrophoresis* 26: 2567-2582. http://dx.doi.org/10.1002/elps.200500096

Majed Z, Bellenger E, Postic D, Pourcel C, et al. (2005). Identification of variable-number tandem-repeat loci in *Leptospira interrogans* sensu stricto. *J. Clin. Microbiol.* 43: 539-545. http://dx.doi.org/10.1128/JCM.43.2.539-545.2005

Petrakovsky J, Bianchi A, Fisun H, Nájera-Aguilar P, et al. (2014). Animal leptospirosis in Latin America and the Caribbean countries: reported outbreaks and literature review (2002-2014). *Int. J. Environ. Res. Public Health* 11: 10770-10789. http://dx.doi.org/10.3390/ijerph111010770

Rinehart CL, Zimmerman AD, Buterbaugh RE, Jolie RA, et al. (2012). Efficacy of vaccination of cattle with the *Leptospira interrogans* serovar hardjo type hardjoprajitno component of a pentavalent *Leptospira bacterin* against experimental challenge with *Leptospira borgpetersenii* serovar hardjo type hardjo-bovis. *Am. J. Vet. Res.* 73: 735-740. http://dx.doi.org/10.2460/ajvr.73.5.735

Salaün L, Mérien F, Gurianova S, Baranton G, et al. (2006). Application of multilocus variable-number tandem-repeat analysis for molecular typing of the agent of leptospirosis. *J. Clin. Microbiol.* 44: 3954-3962. http://dx.doi.org/10.1128/JCM.00336-06

Salgado M, Otto B, Sandoval E, Reinhardt G, et al. (2014). A cross sectional observational study to estimate herd level risk factors for *Leptospira* spp. serovars in small holder dairy cattle farms in southern Chile. *BMC Vet. Res.* 10: 126. http://dx.doi.org/10.1186/1746-6148-10-126

Slack AT, Symonds ML, Dohnt MF and Smythe LD (2006). Identification of pathogenic *Leptospira* species by conventional or real-time PCR and sequencing of the DNA gyrase subunit B encoding gene. *BMC Microbiol.* 6: 95. http://dx.doi.org/10.1186/1471-2180-6-95

Zacarias FGDS, Vasconcellos SA, Anzai EK, Giraldi N, et al. (2008). Isolation of *Leptospira* serovars Canicola and Copenhageni from cattle urine in the state of Paraná, Brazil. *Braz. J. Microbiol.* 39: 744-748. http://dx.doi.org/10.1590/S1517-83822008000400028