View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Directory of Open Access Journals

DOI: 10.5433/1679-0359.2018v39n3p1125

Serological diagnosis and molecular characterization of *Leptospira* spp. in the blood and urine of bovine females from refrigerated slaughterhouses

Diagnóstico sorológico e caracterização molecular de *Leptospira* spp. em sangue e urina de fêmeas bovinas de matadouros-frigoríficos

Aliny Fernanda de Oliveira[1]*; Roberta Torres Chiderolli[2]; Luciano Seraphim Gasques[3]; Arianne Peruzo Pires Gonçalves[1]; Érica Dourado Neves[4]; Bruna Paula Martins Ferreira[1]; Lucienne Garcia Pretto Giordano[5]; Julio Cesar de Freitas[5]; Ulisses de Pádua Pereira[5]; Daniela Dib Gonçalves[6]

Abstract

Leptospirosis is an important socioeconomic disease in humans, as well as in domestic and wild animals, being caused by *Leptospira* spp. Bovine animals are considered reservoirs of this disease, because they intermittently disseminate the bacteria into the environment through their urine. In this way, the cattle an important source of *Leptospira* infection. The objective of this study was to detect *Leptospira* spp. antibodies and DNA in bovine females from two refrigerated slaughterhouses in the microregion of Umuarama, Paraná, Brazil. In particular, blood and urine samples from 52 crossbred bovine females older than 36 months from the two slaughterhouses were used. The microscopic agglutination test (MAT) was used to detect leptospiral antibodies, and the polymerase chain reaction (PCR) and subsequent sequencing were used to detect *Leptospira* DNA. The MAT yielded 22 (42.3%) serum samples considered reagent, while the nested PCR test resulted in one amplified sample (1.9%) of 289 bp. This single sample was then amplified again using primers for the *SecY* gene (549 bp). Sequencing of this gene characterized the bacteria as *L. borgpetersenii* that were similar to the serovar Hardjo of the genotype Hardjobovis. This is the first molecular confirmation of Hardjobovis-like *L. borgpetersenii* in the urine of crossbred bovine females older than 36 months from slaughterhouses in the microregion of Umuarama. This study's results show that it is important to combine serological and molecular diagnosis in the detection of *Leptospira* spp. Therefore, both methods were used to improve our understanding of the epidemiology of this disease in bovine animals from the microregion of Umuarama. In addition, the analysis informed the subsequent adoption of preventive measures and educational One Health actions to prevent economic losses related to the herd, as well as social losses related to workers and the environment.

Key words: Bovine. Occupational disease. Refrigerated slaughterhouse. Leptospirosis. One Health.

[1] Médicas Veterinárias, Discentes, Programa de Pós-Graduação em Ciência Animal, Universidade Paranaense, UNIPAR, Umuarama, PR, Brasil. E-mail: alinyfer@hotmail.com; arianneperuzo@hotmail.com; bp_ferreira@hotmail.com

[2] Médica Veterinária, Discente de Doutorado, Programa de Pós-Graduação em Ciência Animal, Universidade Estadual de Londrina, UEL, Londrina, PR, Brasil. E-mail: robertaa_tc@hotmail.com

[3] Biólogo, Discente, Comitê de Ética em Experimentação Animal, UNIPAR, Umuarama, PR, Brasil. E-mail: sgasques@unipar.br

[4] Médica Veterinária, Discente, UNIPAR, Umuarama, PR, Brasil. E-mail: erika_neves20@hotmail.com

[5] Médicos Veterinários, Drs., Laboratório de Leptospirose, UEL, Londrina, PR, Brasil. E-mail: luciennegiordano@gamil.com; freitasj@uel.br; upaduapereira@gmail.com

[6] Médica Veterinária, Profª, Programa de Pós-Graduação em Ciência Animal com Ênfase em Produtos Bioativos, UNIPAR, Umuarama, PR, Brasil. E-mail: danieladib@unipar.br

* Author for correspondence

Received: Oct. 06, 2017 - Approved: Feb. 20, 2018

Resumo

A leptospirose é uma importante doença sócio-econômica acarretada pela *Leptospira* spp. que afeta homens e animais domésticos ou selvagens. Os bovinos são considerados reservatórios desta enfermidade, sendo importante fonte de infecção por eliminar a bactéria pela urina de forma intermitente no meio ambiente. O objetivo deste trabalho foi detectar anticorpos e DNA de *Leptospira* spp. em fêmeas bovinas provenientes de dois matadouro-frigoríficos da microrregião de Umuarama, Paraná, Brasil. Neste trabalho foram utilizadas amostras de sangue e urina de 52 fêmeas bovinas mestiças com idade superior a 36 meses provenientes de dois matadouros-frigoríficos. Para detecção de anticorpos anti-*Leptospira* spp. foi realizada a prova de Soroaglutinação Microscópica (SAM), e para a detecção de DNA foi realizada a reação em cadeia pela polimerase (PCR) e posterior sequenciamento. Na SAM, 22 (42,30%) amostras de soro foram consideradas reagentes e na nested PCR uma (1,92%) amostra amplificou 289 pb, e posteriormente, a mesma amostra amplificada novamente para o gene *sec* Y com 549 pb. O sequenciamento do gene *Sec* Y caracterizou o produto obtido como *L. borgpetersenii* semelhante ao sorovar Hardjo genótipo Hardjobovis. Esta é a primeira confirmação molecular semelhante ao genótipo Hardjobovis pertencente à espécie *L. borgpetersenii* em urina de fêmeas bovinas mestiças com idade superior a 36 meses proveniente de matadouros-frigoríficos localizados na microrregião de Umuarama no estado do Paraná. Os resultados deste trabalho evidenciam a importância da associação do diagnostico sorológico e molecular para a detecção de *Leptospira* spp. Isto é importante para o entendimento da epidemiologia desta enfermidade em bovinos da microrregião de Umuarama e adoção de medidas de prevenção e ações de educação em saúde na esfera da Saúde Única, evitando assim perdas econômicas relacionado ao rebanho e sociais relacionadas a trabalhadores e meio ambiente.

Palavras-chave: Bovinos. Doença ocupacional. Frigorífico. Leptospirose. Saúde *única*.

Introduction

Leptospirosis is an important disease in the concept of One Health and it is caused by bacteria of the genus *Leptospira*, which cause economic and social losses involving domestic animals, wild animals, humans, and the environment (GROOMS, 2015; MONTE et al., 2015). Leptospirosis is an infectious disease that is considered occupational, since exposure to infected animals during work can infect slaughterhouse and rural workers, veterinarians, among others (GONÇALVES et al., 2006, 2013; SIMÕES et al., 2016). In the context of occupational disease, workers often lack knowledge about leptospirosis, resulting in failure to use, or misuse of, personal protective equipment (PPE). This makes the workers more susceptible to infection (CORRÊA et al., 2013).

Beef and dairy cattle breeding play a key role in the epidemiology of bovine leptospirosis, since bovine animals intermittently excrete *Leptospira* spp. in their urine, thus contaminating the environment and infecting humans and animals (LOUREIRO et al., 2013; SHAFIGHI et al., 2014). Similarly, leptospirosis persists in bovine herds through reproductive tract infections in both male and female animals. Thus microorganisms can be disseminated into the environment via post-abortion uterine discharge, fetuses, placenta, uterine infections, and infected semen (ELLIS, 1994).

After the 1980s, when antigens to detect the serovar Hardjo were included in the microscopic agglutination tests (MATs) of Brazilian laboratories, researchers were better able to understand the prevalence and subsequent epidemiology of this serovar in Brazilian herds. At this time, the serovar was already known in bovine species in Australia, Europe, North and South America (LILENBAUM, 1996; BOLIN; ALT, 1999; FAINE et al., 1999; CHIDEROLLI, 2016).

Epidemiological investigations of leptospirosis in bovine animals must take into account clinical signs, type of breeding management, and breeding site, among other variables. Nonetheless, serological diagnosis using a MAT to detect antibody, and

molecular diagnosis to detect *Leptospira* DNA by PCR are also essential (FAINE et al., 1999; WHO, 2003; HAMOND et al., 2014; GROOMS, 2015).

According to the International Life Saving Federation of the World Health Organization (2003), the MAT is considered the gold standard method for the diagnosis of leptospirosis because it has high serovar and serogroup specificity. For this reason, it is the most common method used by researchers worldwide. On the other hand, different molecular techniques have been developed for DNA detection and subsequent identification of *Leptospira* spp. These are often used when it is necessary to work with fast, high-sensitivity, high-specificity methods (MAGAJEVSKI, 2002; ANZAI, 2006).

The advantage of these molecular methods is that they require minimal amounts of *Leptospira* DNA for amplification. Thus, different biological samples can be used, such as serum, cerebrospinal fluid, urine, feces, and tissues, and clinicians can obtain an early diagnosis of leptospirosis in both humans and animals. That is, the disease can be confirmed before antibody titers can even be detected, or when they are still low (BAL et al., 1994; ANZAI, 2006).

The northwest region has the largest bovine herd in the state of Paraná, with 2,084,593 animals (IBGE, 2015). Despite this, the data from this region are scarce regarding bovine leptospirosis, its relationship with public health, and its molecular characteristics. Thus, the objective of the present study was to detect *Leptospira* spp. antibodies and DNA in bovine females from two refrigerated slaughterhouses in the microregion of Umuarama, Paraná, Brazil.

Materials and Methods

Study population, sampling and collection site

Blood and urine from 52 bovine females older than 36 months were used. This study was carried out between July and September 2016 in two refrigerated slaughterhouses in the microregion of Umuarama, Paraná, Brazil. The study was conducted by the Federal Inspection Service (SIF) and the State Inspection Service of Paraná (SIP).

Sample collection

Blood samples were collected in 5 mL sterile tubes from the carotid artery and jugular vein during the bleeding stage of the slaughter line. Urine samples were collected shortly after the evisceration stage by direct puncture of the urinary vesicle using a sterile 5 mL syringe. After blood and urine collection, the samples were identified and conditioned in a refrigerated isothermal box; they were then immediately sent to the Molecular Biology Laboratory of the Animal Science Postgraduate Program with emphasis on Bioactive Products, Universidade Paranaense (UNIPAR), where they were processed, aliquoted into microtubes, and frozen at -20 °C.

Serological diagnosis

In the Leptospirosis Laboratory of the Department of Preventive Veterinary Medicine at the State University of Londrina (UEL), the sera were submitted to MAT with live antigens (FAINE et al., 1999) to detect leptospiral antibodies. Twenty reference serovars were used: Australis, Bratislava, Autumnalis, Butembo, Castellonis, Bataviae, Canicola, Cynopteri, Grippotyphosa, Hebdomadis, Copenhageni, Icterohaemorrhagiae, Panama, Pomona, Pyrogenes, Hardjo, Wolffi, Shermani, Sentot and Tarassovi. Antigens were maintained at 28 °C for 5 to 10 days in Difco™ Leptospira enrichment medium (DIFCO®, USA) modified by the addition of rabbit serum (GONÇALVES et al., 2006). A dilution of 1:100 was used as a cut-off point (MYERS, 1985).

Sera containing at least 50% agglutinated leptospires were considered reagent. The reagent samples were then geometrically diluted at ratios of 2:1 to determine the maximum positive dilution.

In the analysis of the results, we considered the serovar with the highest titer as the most probable serovar (VASCONCELLOS et al., 1997), and only sera that presented coagglutination at the highest dilution were considered reagent for *Leptospira* spp. (ALMEIDA et al., 1994).

Molecular diagnosis

The urine samples were subjected to molecular tests to detect *Leptospira* spp. DNA at the Molecular Biology Laboratory of the Animal Science Postgraduate Program, with emphasis on Bioactive Products, UNIPAR, and at the Laboratory of Leptospirosis, Universidade Estadual de Londrina (UEL).

To detect *Leptospira* spp. DNA samples were extracted immediately after each urine collection using the PureLink Genomic DNA Mini Kit (Invitrogen, USA). Subsequently, the DNA was subjected to nested PCR (n-PCR) using primers A (5'-GGCGGCGCGTCTITAAACATG-3'), B (5'-TTCCCCCCAT TGAGCAAGATT-3'), C (5'-CAAGTCAAGCGGAGTAGCAA-3'), and D (5'-CTTAACCTGCTGCCTCCCGTA-3'), as described by Mérien et al. (1992). The Platinum PCR SuperMix Kit (Invitrogen, USA) was used in all PCR reactions. The final product of the n-PCR amplification was subjected to electrophoresis in a 2% agarose gel containing ethidium bromide (0.05 μg/μL) and visualized using ultraviolet light in a transilluminator. The product's molecular weight was estimated by comparison with a 100 bp molecular marker.

Samples considered positive after n-PCR were subjected to new DNA amplification using primers specific for the *SecY* gene: F (5′-ATGCCGATCATTTTTGCTTC-3′) and R (5′-CCGTCCCTTAATTTTAGACTTCTTC-3′) to identify and confirm the genetic species (AHMED et al., 2006).

Amplification and sequencing of the *SecY* gene PCR (AHMED et al., 2006) were performed for samples and the products of gene amplification were purified with a Purelink Genomic DNA extraction kit (Invitrogen Life Technologies, Eugene, OR, USA), quantified by a QubitTM Fluorometer (Invitrogen Life Technologies, Eugene, OR, USA), and sequenced on a ABI3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using forward and reverse primers. The contig were obtained by CAP3 and sequence quality was analyzed by visually in BioEdit software v. 7.2.5 (http://www.mbio.ncsu.edu/bioedit/). The nucleotide similarity was compared with all the sequences that were deposited in non-redundant database of GenBank using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The alignment was created in the BioEdit program using clustalW package and phylogenetic tree built using the MEGA 7.0.18 program (KUMAR et al., 2016).

Results

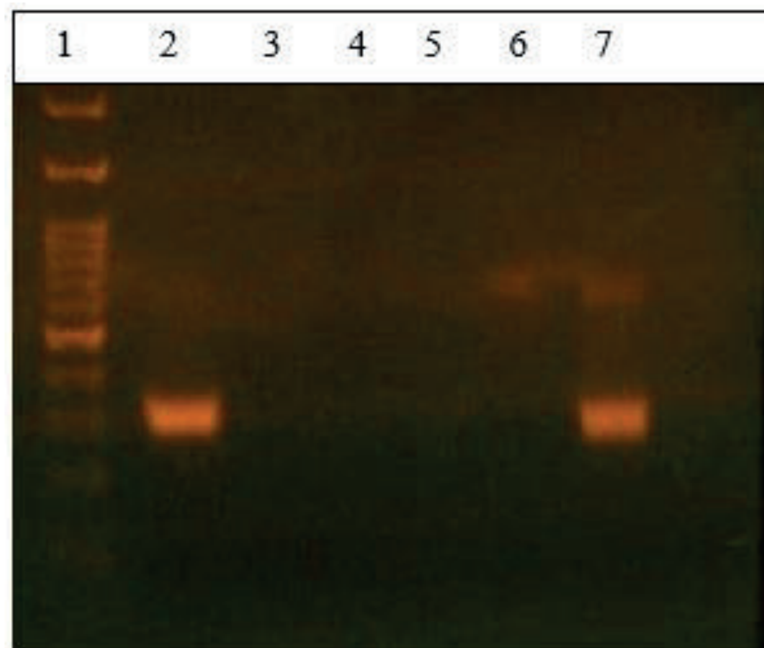
The MAT yielded 22 (42.3%) serum samples considered reagent, of which 18 (81.8%) samples contained antibodies against a single serovar Wolffi (15 samples; 68.2%), Pomona (two samples; 9.1%), and Pyrogenes (one sample; 4.54%) with titers from 100 to 3,200. In four samples (18.2%), antibodies against two or more serovars were simultaneously detected, with titers from 100 to 400. It was not possible to characterize the most probable serovars in these samples (Table 1).

Table 1. Most probable serovars and titers detected by the MAT in 22 seroreagent bovine female samples from two refrigerated slaughterhouses in the microregion of Umuarama, Paraná, Brazil, 2016.

	Serological Titers							
Most probable serovars	100	200	400	800	1600	3200	Total	%
Wolffi	4	5	3	1	1	1	15	68,18%
Pomona	1	1	-	-	-	-	2	09,10%
Pyrogenes	-	-	-	-	1	-	1	04,54%
Reagentes	2	1	1	-	-	-	4	18,18%
Total	31,81%	31,81%	18,20%	04,54%	09,10%	04,54%	100%	100%

In the n-PCR, it was possible to amplify a 289 bp product in only one urine sample (1.9%). This single sample was then amplified again using primers for the *SecY* gene (549 bp). Importantly, this sample was considered negative in the MAT (Figure 1).

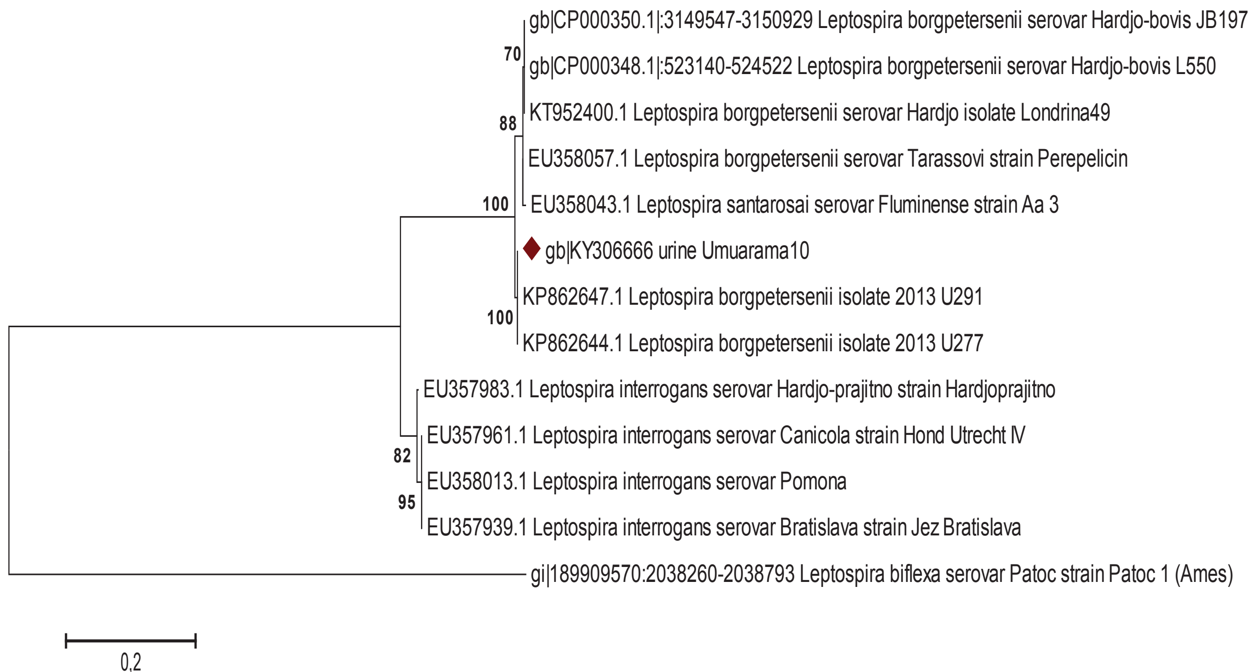
Figure 1. Nested PCR for *Leptospira* spp. in bovine female urine samples from two refrigerated slaughterhouses in the microregion of Umuarama, Paraná, Brazil in 2016.



Legend: 2% agarose gel. Line 1: molecular marker (100 bp). Line 2: sample 10 with DNA amplification. Line 3: sample 11. Line 4: sample 12. Line 5: sample 13. Line 6: negative control. Line 7: positive control.

BlastN software was used to compare partial sequences of the *SecY* gene, and the sample sequences were most similar to the genotype Hardjobovis of the species *L. borgpetersenii*, serovar Hardjo. The partial sequence of the *SecY* gene of sample 10 is deposited in the GenBank database under access number KY306666. In addition, the sample was grouped in the same phylogenetic cluster as Hardjobovis genotype of the serovar Hardjo and more phylogenetically distant from the genotype Hardjoprajitno (Figure 2).

Figure 2. Phylogenetic tree of *Leptospira* spp. strains. The position of the sequence obtained from the bovine urine sample is highlighted, showing its relative position within the genus *Leptospira*. This representation was based on GenBank sequences from partial *SecY* genes. The "maximum likelihood" statistical method was used, and the reliability of the branches was validated through the generation of 1,000 bootstrap repetitions. Evolutionary analyses were performed using MEGA 7.0.18 software, and the sequence obtained in the *SecY* gene sequencing was deposited in the GenBank database under the access code KY306666.



Discussion

Considering that the state of Paraná holds 9,314,908 bovine animals (IBGE, 2017), it is important that researchers ascertain whether leptospirosis is present in the region, and that they obtain information on circulating serovars and establish animal, human, and environmental health measures.

In the present study, 42.3% of the samples were reagent in the MAT, with titers ranging from 100 to 3,200. This confirms the presence of possible sources of acute and chronic infection in these animals (FAINE et al., 1999).

The disease persists and is disseminated within a population by infected animals or asymptomatic carriers that intermittently excrete the bacteria in their urine for extended periods. Therefore, to inform health prevention efforts and educational measures, it is important that researchers assess the prevalence of the infection in herds from refrigerated slaughterhouses. In this way, any possible occupational infections can be avoided (GONÇALVES et al., 2006; SIMÕES et al., 2016).

In the present study, antibodies against Wolffi (68.2%), Pomona (9.1%), and Pyrogenes (4.5%) were detected. The predominance of the antibody against the Wolffi serovar in this study corroborated the results of the state of São Paulo (SP) (LANGONI et al., 2000) and the state of Goiás (GO) (MARQUES et al., 2010). However, it conflicts with the research of the state of Maranhão (MA) (COELHO et al., 2014), the state of Rio de Janeiro (RJ) (HAMOND et al., 2014), and the USA (GROOMS, 2015), where the antibody against serovar Hardjo was detected with higher frequency. Thus, bovine animals are considered a maintenance host of the Hardjo serovar of *Leptospira* (FAINE et al., 1999).

There was no amplification of DNA in the urine samples that were considered reagents in the MAT, which suggests possible chronic infections, being only the establishment of the presence of antibody in the respective serum samples. However, in one of the non-reactive sera samples in MAT, it was possible to amplify DNA (549bp for the sec Y gene) in the urine, which characterizes that the respective animal was in the phase of leptospiruria, that is, eliminating live leptospiras by urine and may cause possible environmental contamination and make slaughterhouse workers susceptible to possible leptospirosis infection when not using or misusing personal protective equipment (FAINE et al., 1999; ANZAI, 2006).

Even though the antibody against the Hardjo serovar is less immunogenic in bovine species, DNA amplification showed that the respective animal was in the initial stages of infection. Therefore, the results of the present study show that it is important to combine serological and molecular diagnosis techniques (HASHIMOTO et al., 2017), because they complement one another. Thereby, even when no antibody was detected in the serum sample of one animal, there may be DNA in the urine sample of this animal. Thus, PCR is important in the early diagnosis of diseases, because it is sensitive in the initial stage and allows fast diagnosis, which in turn facilitates infection treatment and prevention (STODDARD, 2013; GALLOWAY; HOFFMASTER, 2015).

The sequencing of the *SecY* gene (VICTORIA et al., 2008) was one of a number of molecular techniques developed and used to characterize *Leptospira* isolates and biological samples known to be infected by *Leptospira* spp. In the present study, the sample was characterized molecularly as genotype Hardjobovis (Figure 2). This genotype belongs to the species *L. borgpetersenii*, which causes leptospirosis in production animals and is different from the genotype Hardjoprajitno since it has a smaller genome, and several genes related to survival in the environment, transport, and metabolite use have been lost. Consequently, researchers have concluded that these may be the reasons for the bacteria's strict dependence on the host-host transmission cycle in bovine species (ELLIS, 1994; CHIARELI et al., 2012; KOIZUMI; YASUTOMI, 2012).

Chideroli et al. (2016), evaluated 15 urine samples from bovine females with a history of reproductive failures. The animals came from a dairy herd in Paraná and demonstrated the molecular characterization of the strain *L. borgpetersenii* serovar Hardjo Hardjobovis. Thus, our research corroborated with the previous study and confirmed the presence and circulation of this strain in the north and northwest region of the state of Paraná. We hope that the present study will stimulate new serological and molecular studies in these regions to characterize the epidemiology of this genotype, as well as its possible consequences in affected herds.

This was the first molecular confirmation of Hardjobovis-like *L. borgpetersenii* species in the urine of crossbred bovine females older than 36 months from slaughterhouses located in the microregion of Umuarama, Paraná. The results of this work show the presence of antibodies and DNA of *Leptospira* spp. in biological samples of bovine females from slaughterhouses is important to establish preventive measures related to One Health health avoiding economic losses related to herd and social related to workers and the environment.

Acknowledgements

We thank the Coordination for the Improvement of Higher Education Personnel (CAPES) for the PROSUP scholarship and UNIPAR for research funding.

References

AHMED, N.; DEVI, S. M.; VALVERDE, M. L.; VIJAYACHARI, P.; MACHANG'U, R. S.; ELLIS, W. A.; HARTSKEERL, R. A. Multilocus sequence

typing method for identification and genotypic 1209 classification of pathogenic Leptospira species. *Annals of Clinical Microbiology and Antimicrobials,* Londres, v. 5, n. 28, 2006. Available at: https://ann-clinmicrob.biomedcentral.com/articles/10.1186/1476-0711-5-28. Accessed at: 05 mar. 2018.

ALMEIDA, L. P.; MARTINS, L. F. S.; BROD, C. S.; GERMANO, P. M. L. Levantamento soroepidemiológico de leptospirose em trabalhadores do serviço de saneamento ambiental em localidade urbana da região sul do Brasil. *Revista de Saúde Pública,* São Paulo, v. 28, n. 1, p. 76-81, 1994.

ANZAI, E. K. *Utilização da PCR para o diagnóstico da leptospirose em cães naturalmente infectados por Leptospira spp.* 2006. Dissertação (Mestrado em Ciência Animal) - Universidade Estadual de Londrina, Londrina.

BAL, A. E.; GRAVEKAMP, C.; HARTSKEERL, R. A.; MEZA-BREWSTER, J.; KORVER, H.; TERPSTRA, W. J. Detection of leptospires in urine by PCR for early diagnosis of leptospirosis. *Journal of Clinical Microbiology,* Washington, v. 32, n. 8, p. 1894-1898, 1994.

BOLIN, C. A.; ALT, D. P. Clinical signs, diagnosis, and prevention of bovine leptospirosis. *The Bovine Practitioner,* Ontário, v. 1, n. 33, p. 50-55, 1999.

CHIARELI, D.; COSAT, M. R. V.; MOREIRA, E. C.; LEITE, R. C.; LOBATO, F. C. F.; SILVA, J. A.; TEIXEIRA, J. F. B.; MARCELINO, A. P. Controle da leptospirose em bovinos de leite com vacina autógena em Santo Antônio do Monte, Minas Gerais. *Pesquisa Veterinária Brasileira,* Rio de Janeiro, v. 32, n. 7, p. 633-639, 2012.

CHIDEROLLI, R. T.; PEREIRA, U. P.; GONÇALVES, D. D.; NAKAMURA, A. Y.; ALFIERI, A. A.; ALFIERI, A. F.; FREITAS, J. C. Isolation and molecular characterization of *Leptospira borgpetersenii* serovar Hardjo strain Hardjobovis in the urine of naturally infected cattle in Brazil. *Genetics and Molecular Research*, Ribeirão Preto, v. 15, n. 1, p. 1-7, 2016.

COELHO, É. L. M.; CHAVES, N. P.; SÁ, J. C.; MELO, S. A.; SILVA, A. L. A. Prevalência de leptospirose em fêmeas bovinas abatidas em frigoríficos no município de São Luís, MA. *Revista Brasileira de Medicina Veterinária,* Rio de Janeiro, v. 36, n. 2, p. 111-115, 2014.

CORRÊA, J. M. X.; CARVALHO, F. S.; CARLOS, R. S. A.; WENCESLAU, A. A. Investigação molecular de *Leptospira* spp. em rins de bovinos. *Acta Scientiae Veterinariae,* Porto Alegre, v. 41, n. 1114, p. 1-6, 2013.

ELLIS, W. A. Leptospirosis as a cause of reproductive failure. *Veterinary Clinics North America Food Animal Practice,* Philadelphia, v. 10, n. 3, p. 463-478, 1994.

FAINE, S.; ADLER, B.; BOLIN, C.; PEROLAT, P. *Leptospira and leptospirosis*. 2th ed. Melbourne: Medi Science, 1999. 272 p.

GALLOWAY, R. L.; HOFFMASTER, A. R. Optimization of LipL32 PCR assay for increased sensitivity in diagnosing leptospirosis. *Diagnostic Microbiology and Infectious Disease,* New York, v. 82, n. 3, p. 199-200, 2015.

GONÇALVES, D. D.; BENITEZ, A.; LOPES-MORI, F. M. R.; ALVES, L. A.; FREIRE, R. L.; NAVARRO, I. T.; SANTANA, M. A. Z.; SANTOS, L. R. A.; CARREIRA, T.; VIEIRA, M. L.; FREITAS, J. C. Zoonoses in humans from small rural properties in Jataizinho, Parana, Brazil. *Brazilian Journal of Microbiology,* Rio de Janeiro, v. 44, n. 1, p. 125-131, 2013.

GONÇALVES, D. D.; TELES, P. S.; REIS, C. R.; LOPES, F. M. R.; FREIRE, R. L.; NAVARRO, I. T.; ALVES, L. A.; MULLER, E. E.; FREITAS, J. C. Seroepidemiology and occupational and environmental variables for leptospirosis, brucellosis and toxoplasmosis in slaughterhouse workers in the Paraná state, Brazil. *Revista do Instituto de Medicina Tropical de São Paulo, São Paulo, v.* 48, n. 3, p. 135-140, 2006.

GROOMS, D. L. *Bovine reproduction. Infectious agents*: leptospirosis. The cow: pregnancy wastage. Nova Jersey: John Wiley & Sons, 2015. 532 p.

HAMOND, C.; MARTINS, G.; LOUREIRO, A. P.; PESTANA, C.; LAWSON-FERREIRA, R.; MEDEIROS, M. A.; LILENBAUM, W. Urinary PCR as an increasingly useful tool for an accurate diagnosis of leptospirosis in livestock. *Veterinary Research Communications,* Amsterdam, v. 38, n. 1, p. 81-85, 2014.

HASHIMOTO, V. Y.; CHIDEROLI, R. T.; RIBEIRO, J.; ALFIERI, A. A.; COSTA, G. M.; FREITAS, J. C.; PEREIRA, U. P. Serological and molecular findings in diagnosis of leptospirosis serovar Hardjo in a dairy bovine herd. *Semina: Ciências Agrárias,* Londrina, v. 38, n. 5, p. 3155-3164, 2017.

INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA - IBGE. Números da Pecuária Paranaense

Ano 2016. Curitiba: SEAB/DERAL/DCA/PECUÁRIA, 2015. Disponível em: <http://www.agricultura.pr. gov.br/arquivos/File/deral/Prognosticos/bovinocultura_ de_ corte_2015_.pdf>. Acesso em: 2 out. 2016.

______. Números da Pecuária BR, PR, RS, SC e Região Sul - Ano 2018. Curitiba: SEAB/DERAL/DCA/PECUÁRIA, 2017. Disponível em: <http://www.agricultura.pr.gov.br/arquivos /File/deral/nppr.pdf>. Acesso em: 2 fev. 2017.

KOIZUMI, N.; YASUTOMI, I. Prevalence of leptospirosis in farm animals. *The Japanese Journal of Veterinary Research,* Sapporo, v. 60, n. 1, p. 55-58, 2012.

KUMAR, S.; STECHER, G.; TAMURA, K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, Chicago, v. 33, n. 7, p. 1870-1874, 2016.

LANGONI, H.; MEIRELES, L. R.; GOTTSCHALK, S.; CABRAL, K. G.; SILVA, A. V. Perfil sorológico da Leptospirose bovina em regiões do Estado de São Paulo. *Arquivos do Instituto Biológico,* São Paulo, v. 67, n. 1, p. 37-41, 2000.

LILENBAUM, W. Bovine Leptospirosis in Brazil: a review. *Brazilian Journal of Veterinary Medicine*, São Paulo, v. 18, n. 1, p. 9-13, 1996.

LOUREIRO, A. P.; MARTINS, G.; THOMÉ, S.; LILENBAUM, W. Laboratorial diagnosis of animal leptospirosis. *Revista Brasileira de Ciência Veterinária,* Niterói, v. 20, n. 3, p. 119-126, 2013.

MAGAJEVSKI, F. *Avaliação da reação em cadeia da polimerase (PCR) na detecção de Leptospira interrogans sorovar hardjo em sêmen e urina de touros (Bos taurus) sorologicamente reagentes.* 2002. Dissertação (Mestrado em Medicina Veterinária) - Faculdade de Ciências Agrárias e Veterinária. Universidade Estadual Paulista, Jaboticabal, São Paulo.

MARQUES, A. E.; ROCHA, W. V.; BRITO, W. M. E. D.; FIORAVANTI, M. C. S.; PARREIRA, I. M.; JAYME, V. S. Prevalência de anticorpos anti-*Leptospira* spp. e aspectos epidemiológicos da infecção em bovinos do estado de Goiás. *Ciência Animal Brasileira,* Goiânia, v. 11, n. 3, p. 607-617, 2010.

MÉRIEN, F.; AMOURIAX, P.; PEROLAT, P.; BARANTON, G.; GIRONS, I. S. Polymerase chain reaction for detection of Leptospira spp in clinical samples. *Journal of Clinical Microbiology,* Washington, v. 30, n. 9, p. 2219-2224, 1992.

MONTE, L. G.; RIDIERI, K. F.; JORGE, S.; OLIVEIRA, N. R.; HARTWIG, D. D.; AMARAL, M. G.; HARTLEBEN, C. P.; DELLAGOSTIN, O. A. Immunological and molecular characterization of *Leptospira interrogans* isolated from a bovine foetus. *Comparative Immunology, Microbiology and Infectious Diseases,* Oxford, v. 40, n. 4, p. 41-45, 2015.

MYERS, D. *Leptospirosis*: manual de métodos para el diagnostico de laboratório. Buenos Aires: Centro Panamericano de Zoonosis, OPS/OMS, 1985. 30 p.

SHAFIGHI, T.; SALEHI, Z.; ABDOLLAHPOUR, G.; ASADPOUR, L.; H AKBAREIN, H.; SALEHZADEH, A. Molecular detection of *Leptospira* spp. in the urine of cattle in northern Iran. *Iranian Journal of Veterinary Research, Shiraz University,* Shiraz, v. 15, n. 4, p. 402-405, 2014.

SIMÕES, L. S.; SASAHARA, T. H. C.; FAVARON, P. O.; MIGLINO, M. A. Leptospirose - revisão. *Medicina Veterinária e Zootecnia*, Belo Horizonte, v. 10, n. 2, p. 138-146, 2016.

STODDARD, R. A. Detection of pathogenic Leptospira spp. through real-time PCR (qPCR) targeting the LipL32 gene. *Methods in Molecular Biology,* New Jersey, v. 943, n. 1, p. 257-266, 2013.

VASCONCELLOS, S. A.; BARBARINI JÚNIOR, O.; UMEHARA, O.; MORAIS, Z. M.; CORTEZ, A.; PINHEIRO, S. R.; FERREIRA, F.; FAVEIRO, A. C. M.; FERREIRA NETO, J. S. Leptospirose bovina. Níveis de ocorrência e sorotipos predominantes em rebanhos dos Estados de Minas Gerais, São Paulo, Rio de Janeiro, Paraná, Rio Grande do Sul e Mato Grosso do Sul, período de janeiro a abril de 1996. *Arquivo Instituto Biológico,* São Paulo, v. 64, n. 2, p. 7-15, 1997.

VICTORIA, B.; AHMED, A.; ZURNER, R. L.; AHMED, N.; BULACH, D. M.; QUINTEIRO, J.; HARTSKEERL, R. A. Conservation of the *S10-spc-α* locus within otherwise highly plastic genomes provides phylogenetic insight into the genus *Leptospira*. *PLoS One*, San Francisco, v. 3, n. 7, p. 1371-2752, 2008.

WORLD HEALTH ORGANIZATION - WHO. Human leptospirosis: guidancefor 941 diagnosis, surveillance and control. Geneva: World Health Organization*,* 2003.