

DIAGNÓSTICO MOLECULAR E SOROLÓGICO PARA *Leptospira* spp. EM AMOSTRAS BIOLÓGICAS DE BOVINOS COLETADAS DURANTE A LINHA DE ABATE

Marcelo Augusto Orsi Dutra¹
Amanda Bezerra Bertolini¹
Suzane Manzini²
Livia Maísa Guiraldi²
Wesley José dos Santos²
Isabella Neves Aires³
Nassarrah Jabur Lot Rodrigues²
Thainá Valente Bertozzo²
Marcela Alexandrino²
Andresa Xavier Frade Gomes¹
Jackieline Sampaio Steinle¹
Maria Eduarda Cavalheiro²
Ana Carolina Yamakawa¹
Ana Paula Flaminio¹
Hélio Langoni¹
Antonio Carlos Paes¹
Maria Izabel Merino de Medeiros⁴
Vera Claudia Lorenzetti Magalhaes Curci⁵
Simone Baldini Lucheis^{1,2,4}

RESUMO

A leptospirose é considerada uma zoonose bacteriana de importância para a saúde pública. É comum em áreas tropicais, especialmente em países em desenvolvimento com escassos recursos de saúde e saneamento. Este estudo avaliou a presença de *Leptospira* spp. em bovinos abatidos em frigorífico da região Centro-Oeste de São Paulo, Brasil, e identificou animais positivos tanto por sorologia quanto em análise molecular. Amostras biológicas de sangue, fígado e rins de 150 bovinos foram investigadas pela técnica de Soroaglutinação Microscópica (SAM) e Reação em Cadeia da Polimerase convencional (cPCR). Os resultados sorológicos mostraram que dos 150 animais, 71 (47,3%) foram reagentes. Os resultados moleculares mostraram a presença de *Leptospira* spp. nos rins de 21 animais (14%), no fígado de 5 animais (3,3%), no fígado e rins em 2 animais (1,3%) e no sangue em 1 animal (0,7%). Esses resultados indicam um alerta sobre a saúde dos bovinos de corte devido à possibilidade desses animais serem fonte de infecção e a importância da característica ocupacional desta doença. Verificou-se também a importância de complementar as técnicas sorológicas e moleculares.

¹ Department of Animal Production and Preventive Veterinary Medicine. São Paulo State University – UNESP. dutramo@hotmail.com

² Department of Infectology, Dermatology, Diagnostic Imaging and Radiotherapy. Botucatu Medical School. São Paulo State University – UNESP. suzane.manzini@hotmail.com

³ Department of Biology. Faculty of Science. São Paulo State University – UNESP. aires.isabella@gmail.com

⁴ São Paulo's Agency for Agribusiness Technology (APTA/SAA). Bauru, São Paulo. maria.medeiros@sp.gov.br

⁵ São Paulo's Agency for Agribusiness Technology (APTA/SAA). Biological Institute, Araçatuba. vera.curci@sp.gov.br

Palavras-chave: Bovinos, Diagnóstico, Leptospirose, Zoonose.

MOLECULAR AND SEROLOGICAL DIAGNOSIS FOR *Leptospira* spp. IN BIOLOGICAL SAMPLES OF CATTLE COLLECTED DURING THE SLAUGHTER LINE

ABSTRACT

Leptospirosis is considered a bacterial zoonosis of public health importance. It is common in tropical areas, especially in developing countries with scarce health and sanitation resources. This study evaluated the presence of *Leptospira* spp. in slaughtered bovine in a slaughterhouse in the Midwest region of São Paulo, Brazil, as well as identified positive animals both in serology and by molecular analysis. Biological samples of blood, liver and kidneys from 150 cattle were investigated by the technique of Microscopic Agglutination Test (MAT) and conventional Polymerase Chain Reaction (cPCR). The serological results showed that of the 150 animals, 71 (47.3%) were reactive. The molecular results showed the presence of *Leptospira* spp. in kidneys of 21 (14%) animals, in liver of five (3.3%) animals, in liver and kidneys in two animals (1.3%) and in blood, in one (0.7%) animal. These results indicate a warning about the health of beef cattle due to the possibility of these animals being the source of infection and the importance of the occupational characteristic of this disease. It was also verified the importance of complementing serological and molecular techniques.

Keywords: Bovines; Diagnosis; Leptospirosis; Zoonosis.

DIAGNÓSTICO MOLECULAR Y SEROLÓGICO PARA *Leptospira* spp. EN MUESTRAS BIOLÓGICAS DE BOVINOS RECOGIDAS DURANTE LA LÍNEA DE SACRIFICIO

RESUMEN

La leptospirosis es considerada una zoonosis bacteriana de importancia en salud pública. Es común en áreas tropicales, especialmente en países en desarrollo con escasos recursos de salud y saneamiento. Este estudio evaluó la presencia de *Leptospira* spp. en bovinos sacrificados en un matadero de la región Centro-Oeste de São Paulo, Brasil, así como animales positivos identificados tanto en serología como por análisis molecular. Se investigaron muestras biológicas de sangre, hígado y riñones de 150 bovinos mediante la técnica de Aglutinación Microscópica (MAT) y Reacción en Cadena de la Polimerasa convencional (cPCR). Los resultados serológicos mostraron que de los 150 animales, 71 (47,3%) fueron reactivos. Los resultados moleculares mostraron la presencia de *Leptospira* spp. en riñones de 21 (14%) animales, en hígado de cinco (3,3%) animales, en hígado y riñones en dos animales (1,3%) y en sangre, en un (0,7%) animal. Estos resultados indican una alerta sobre la salud de los bovinos de carne debido a la posibilidad de que estos animales sean fuente de infección y la importancia de la característica ocupacional de esta enfermedad. También se verificó la importancia de complementar las técnicas serológicas y moleculares.

Palabras-clave: Bovinos, Diagnóstico, Leptospirosis, Zoonosis.

INTRODUCTION

Leptospirosis is a global disease with an acute or chronic course that affects several species of domestic and wild animals and humans. This disease was discovered in Germany, in Heidelberg in 1870, by the physician Dr. Adolf Weil (1848-1916) who described four cases with symptoms of high fever, severe jaundice, enlarged spleen and liver. The disease was called Weil's Disease which at the time was fatal. It is also known as sugarcane fever and pigs fever (1).

Leptospirosis is considered a bacterial zoonosis of public health importance. It is common in tropical areas, especially in developing countries with scarce health and sanitation resources. As hosts and disseminators of leptospire in the environment there are rodents, cattle, swine and dogs, among others. This disease is commonly underestimated due to the fact that patients present febrile symptoms, similar to flu and several infectious diseases such as dengue and malaria, as well as limited access to laboratory diagnosis (2).

The etiological agent is the spirochete bacteria belonging to the genus *Leptospira* spp. that comprise saprophytic (*L. biflexa*) and pathogenic (*L. interrogans*) species. The taxonomic classification of the genus includes eight pathogenic *Leptospira* species and more than 200 pathogenic serotypes. For cattle, the serovars Hardjo, Wolffi, Pomona, Grippotyphosa, Icterohaemorrhagiae and Canicola are the most prevalent and importante (3).

It is a disease that remains neglected because it has a common incidence in areas where socioeconomic factors such as poverty, lack of water and sanitation, poor housing conditions; and environmental, such as heavy rains or floods, are crucial for the maintenance of leptospire and subsequent occurrence of the disease (4).

In cattle herds *L. interrogans* serovar Hardjo is directly associated with economic losses such as reproductive failure, including abortion and mastitis, causing productive problems in cattle farming (5).

Leptospira interrogans serovar Hardjo belonging to serogroup sejroe is the most relevant serovar in bovine herds worldwide. Sejroe serogroup is frequently identified by serology in Brazilian bovine herds. This suggests in the taxonomy of serovar Hardjo that there are two genotypes, Hardjobovis and Hardjoprajitno (3).

Zoonotic infection has become very widespread in the world with reports of up to one million cases per year and this estimate will probably increase as a result of climate change making leptospirosis a challenge for public health (1).

The World Organization for Animal Health (OIE) manual recommends the Microscopic Agglutination Test (MAT) for the serological diagnosis of leptospirosis (5).

Considering the importance of this zoonosis for the cattle herd and due to the reproductive problems associated with it, in addition to its importance due to economic losses and its relevance to public health, this study evaluated the presence of slaughtered bovine carriers in a slaughterhouse in the Midwest region of São Paulo, as well as identified positive animals both in serology and by molecular analysis.

MATERIAL AND METHODS

Sample collection took place between August and October 2017. Blood, liver and kidney samples were collected from 150 cattle during slaughter in a slaughterhouse located in the Midwest of São Paulo. The animals were of both sexes and aged between 4 and 7 years. They were healthy during ante-mortem observation without clinical signs and visible lesions at the carcass exam.

Blood aliquots were collected at the time of slaughter during the animals' bleeding. Ten (10) mL of whole blood were collected from each animal in two tubes, one tube with

anticoagulant (EDTA) for molecular biology (PCR) and another tube without anticoagulant for serology (Microscopic Agglutination Test - MAT), both were placed in a refrigerated thermobox. The 150 liver and kidney fragments, with approximately 10 grams, were collected aseptically, wrapped in aluminum foil and placed in a refrigerated cooler. Aliquots of blood and tissues were sent to the Animal Health Laboratory of Bauru (LASAB), located at the São Paulo's Agency for Agribusiness Technology (APTA/SAA) in Bauru/SP, to carry out serological and molecular analyses.

The 150 blood serum samples were submitted to MAT in a battery of 17 *Leptospira* spp. specific for herbivores such as Bratislava, Castellonis, Canicola, Djasiman, Grippotyphosa, Copenhageni, Icterohaemorrhagiae, Pomona, Pyrogenes, Hardjo, Hardjoprajitino, Hardjomini, Hardjo CTG, Hardjobovis, Wolffi, Tarassovi, Guaricura, which were kept in liquid culture media Ellinghausen-McCullough-Johnson-Harris (EMJH), at 28°C to 30°C.

DNA extraction from blood samples was performed using the Illustra Blood™ genomic Prep Mini Spin kit (GE Healthcare®). DNA extraction from liver and kidney samples was used with the Illustra™ Tissue & Cells Genomic Prep Mini Spin Kit (GE Healthcare®). Subsequently, to assess the concentration of DNA present in each sample, quantification was performed using the NanoVue Plus® Quantifier equipment.

Primers LEP1 and LEP2 were used which amplify 331 bp (6) and the preparation was carried out in a Mastercycler Pro gradient thermocycler (Eppendorf®). Visualization of amplified products was performed using the electrophoresis technique. Results were visualized on the UV light transilluminator.

The research project was approved at a meeting of the Ethics Committee on the Use of Animals at the São Paulo State University Júlio de Mesquita Filho – Faculty of Veterinary Medicine and Animal Science – Campus Botucatu, under number 63/2017.

RESULTS

The serological results showed that of the 150 animals, 71 (47.3%) were reactive. The most frequent serovars were Djasiman in 40 (56.3%) animals, Hardjoprajitno in 29 (40.8%), HardjoCTG in nine (12.7%), Hardjo and Hardjobovis in eight (11.3%) respectively, Pyrogenes in seven (9.8%), Wolffi in six (8.4%), Castellonis in five (7.0%), Pomona in three (4.2%), Grippotyphosa in one (1.4%), Hardjomini in one (1.4%), Icterohaemorrhagiae in one (1.4%) and Bratislava in one (1.4%) animal. Thus, seropositivity was observed for 13 of the 17 serovars evaluated, with a prevalence for serovar Djasiman and non-reactive serovars were Canicola, Copenhageni, Guaricura and Tarassovi.

Concerning the titles observed in the MAT, 63 (88.7%) animals had titles between 100 and 200, ten (14.1%) with title 400, seven (9.8%) with a 800 title and 14 (19.7%) animals with title 1600. There was no response for titles 3200 and 6400.

The molecular results showed the presence of *Leptospira* spp. in five (3.3%) liver samples, in 21 (14%) kidney samples, two (1.3%) animals positive for liver and kidney, and, in blood, in one animal (0.7%). These results warn about the health of beef cattle, due to the possibility of these animals being the source of infection and the importance of the occupational characteristic of the disease. It was also verified the importance of complementing serological and molecular techniques for the diagnosis of this zoonosis.

DISCUSSION

The results demonstrate the need to implement zoosanitary management in rural properties, not only for the animals present in this study, but in all of them as a way to prevent the spread of leptospirosis not only to animals, but to the environment and human beings. This

zoonosis poses an occupational risk both on farms due to contamination of pastures, fomites and employees' contact with secretions such as urine, and in the slaughterhouse that employees are also exposed to blood, viscera and secretions (urine) of these animals in the slaughter line (7).

A study carried out with cattle slaughtered in a slaughterhouse in the state of São Paulo also reported the presence of *Leptospira kirschneri* serovar Canicola and *Leptospira interrogans* serovar Pyrogenes in the urine collected from these animals (7), which corroborates the present study.

Regarding the possibility of transmission of leptospirosis on farms, the proximity of farm animals close to homes on properties increases the risk of transmission of various zoonosis, including leptospirosis (8).

For serology, the titration in the reagent animals showed mostly titers of 100 and 200, which demonstrates the exposure to the agent and the chronicity of the infection (9). The highest titers were 1600 for the serovars Djasiman and Hardjoprattino. According to the literature, a titre of 50 indicates animal exposure to the agent, but titers of 100 or higher in the MAT are indicators of disease (5).

The high prevalence of serovar Djasiman, which are commonly found in wild animals, alerts to environmental imbalances and deforestation, which are factors that provide greater proximity between farm and wild animals (10).

A study carried out with 55 capybaras (*Hydrochoerus hydrochaeris*) from the states of São Paulo, Rio Grande do Sul and Paraná, through the MAT, found 23 (41.8%) seroreactive animals and about 13.04% of the animals were reactive to the serovar Djasiman (10). In another study, it was observed that of 35 wild animals and four of different wild species from the Pantanal, serogroups Javanica and Djasiman were the most prevalent (11).

It is a fact that wild animals roam the properties and get close to farm animals in pastures (11). This allows these animals to become infected with contaminated urine released in the pasture as well as the opposite, that is, by the elimination of leptospires in the urine by these wild animals, which enables the infection of other contacting animals (7).

The presence of DNA from leptospires in the kidneys of 21 (14%) animals, liver in five (3.3%), liver and kidney in two (1.3%) and in blood in one (0.7%) animal, raises an alert to Public Health about the occupational risk of leptospirosis for workers in slaughterhouses.

There was one positive animal to PCR for *Leptospira* spp. in kidney, with serological titre 800 for serovar Djasiman. This result may demonstrate that at the time of collection, this animal was at the peak of antibody production, with a tendency to chronic infection, which could be verified if paired serology was performed, and observed the drop in antibody production in a second moment.

There were also two animals positive for liver and kidney, one of them being reactive for serovars Djasiman and Hardjoprattino, with a titer of 1600, which could mean the same situation for serovar Djasiman. In another animal, although the DNA of *Leptospira* spp. was detected, it was non reactive, suggesting an infection by another serovar not tested in this study, as well as a chronic infection.

On the other hand, one animal had *Leptospira* spp. in blood, but not reactive to serology. This demonstrates that the animal was also in the acute phase of the infection. Thus, contact with the blood of this animal may represent a risk for slaughterhouse employees, in view of the exposure, even when using personal protection equipment (7).

A study carried out in sheep meatpacking industries in New Zealand proved that in about 10,000 carcass handlers, around 276 can acquire the infection annually due to work in slaughterhouses, demonstrating a risk to Public Health. This fact reinforces the importance of using adequate Personal Protective Equipment (12). In the same way, it was proven that 20%

of cases of leptospirosis in humans are related to rural workers and workers exposed to feces, urine and blood from cattle (13).

Comparing serological and molecular results, it was noted that research with leptospirae has limitations in the detection of positive animals in the chronic phase of the infection, therefore PCR has been encouraged as an important tool for the diagnosis of leptospirosis (14). The use of the MAT showed a significant increase in sensitivity with 10% for the antigen (15). It is noteworthy that MAT is a method for diagnosing leptospirosis in herds, but not individually, it is serovar-specific and, in these cases, it is necessary to use direct methods such as PCR (5).

The high occurrence of anti-*Leptospira* spp. antibodies detected in the evaluated animals, indicates exposure to the agent and the chronicity of the infection. The presence of the agent's genetic material in blood and tissues indicates the need to implement animal health programs and the occupational character of this zoonosis in slaughterhouses. The complementation of the serological and molecular tests used in this study allowed for a more comprehensive and sensitive diagnosis, enabling better knowledge about the epidemiology of leptospirosis.

In tropical and developing countries, such as Brazil, bovine leptospirosis is considered a challenge for its control, which negatively impacts in Public Health. This information is present in this study that evaluated the aspects related to the serovars involved, the environment that offers maintenance conditions, in addition to human action (9).

It is important to emphasize that cattle slaughter has been the main focus of food security interventions. In the United States, beef accounted for the majority of foodborne outbreaks caused in the period 2009-2010, ranking as the third largest risk factor, followed by pork and poultry (16).

Brazil was considered one of the top 10 countries in 2012 in cattle raising, with beef production being the main pillar of the Brazilian economy. From 2007 to 2016, a total of 6,848 foodborne disease outbreaks were reported in Brazil to the Notifiable Disease Information System of the Ministry of Health of Brazil (17).

A key step in preventing foodborne diseases in beef is in the slaughter stage, with several antimicrobial interventions being applied to minimize pathogenic contamination of beef from beef hides and viscera. The fact that the animals were apparently healthy in the slaughterhouse in this study aggravates the risk for the final consumer, as they would normally go through inspection without any safer destination (16).

The consumption of raw beef is part of the cultural and traditional heritage of many countries and also characterizes new food trends. Consumer preferences place meat at high risk of transmitting an infection because raw or undercooked beef is often consumed compared to other animal species (18). Humans can acquire some infections such as toxoplasmosis (18) and taeniasis (19) with the habit of consuming raw or undercooked beef.

Thus, the results of this work suggest a warning about the health of bovine herds due to the possibility of these animals being sources of infection, both on the farms and in the slaughterhouse during the slaughter line, considering the direct and continuous contact with blood and viscera of animals, which corroborates that leptospirosis is an occupational disease. Therefore, a possible transmission of this zoonosis to humans is suggested, in view of the habit of eating raw or undercooked meat, which has been growing in Brazil. The importance of complementing serological and molecular techniques for the diagnosis of leptospirosis is also verified.

CONCLUSION

There is a high prevalence of leptospirosis in cattle slaughtered in the Midwest of São Paulo. This finding is of concern in One Health because of the leptospirosis poses risks to the

animal and public health due to its zoonotic character. There is a need to implement public policy to reduce this prevalence.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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