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FIRST ISOLATION OF *Leptospira kirschneri* SEROVAR CANICOLA AND *Leptospira interrogans* SEROVAR PYROGENES IN URINE SAMPLES FROM SLAUGHTERED CATTLE IN MIDWEST REGION OF SÃO PAULO STATE, BRAZIL

(Primeiro isolamento de *Leptospira kirschneri serovar canicola e Leptospira interrogans* serovar *pyrogenes* em amostras de urina de bovinos abatidos na região Centro-Oeste do Estado de São Paulo, Brasil)

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ABSTRACT: Leptospirosis is a zoonosis of great importance in public health. One hundred and four urine samples from slaughtered cattle in Midwest region of São Paulo State, Brazil were evaluated to detect Leptospira spp. The samples were kept in PBS and in cultures of EMJH and Fletcher. We observed growth of microorganisms in 13 samples of Fletcher medium, although it was not observed in EMJH medium. By PCR, 25 animals (samples) were positive with LEP1/LEP2 primers, which of them 16 samples were kept in PBS, six samples in Fletcher medium and three in EMJH medium. The 25 samples were submitted to PCR with the LipL32 gene to verify the pathogenicity. One sample amplified and by sequencing resulted in 99.77% of similarity to *Leptospira kirschneri* serovar Canicola and *Leptospira interrogans* serovar Pyrogenes. This is the first report of isolation of *Leptospira kirschneri* serovar Canicola and *Leptospira interrogans* serovar Pyrogenes from bovine urine sample in the Midwest Region of São Paulo state, Brazil. We concluded the importance of leptospirosis in the herd evaluated and the attention for the occupational risk, because of the possibility of contaminated urine in the pastures and the spread of infection to other animals and contacting persons.

Keywords: molecular diagnosis; slaughterhouses; leptospirosis.

RESUMO: A leptospirose é uma zoonose de grande importância em saúde pública. Cento e quatro (104) amostras de urina de bovinos abatidos na região centro-oeste do Estado de São Paulo, Brasil, foram avaliadas para detectar *Leptospira* spp. As amostras foram mantidas em PBS e em culturas de EMJH e Fletcher. Observamos crescimento de microrganismos em 13 amostras do meio Fletcher, embora não tenha sido observado no meio EMJH. Por PCR, 25 animais (amostras) foram positivos com os *primers* LEP1 / LEP2,

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sendo que 16 amostras foram mantidas em PBS, seis em meio Fletcher e três em meio EMJH. As 25 amostras foram submetidas a PCR com o gene *LipL32* para verificação da patogenicidade. Uma amostra amplificada e por sequenciamento resultou em 99,77% de similaridade com *Leptospira kirschneri* serovar Canicola e *Leptospira interrogans* serovar Pyrogenes. Este é o primeiro relato de isolamento de *Leptospira kirschneri* serovar Canicola e *Leptospira interrogans* serovar Pyrogenes em amostra de urina bovina na Região Centro-Oeste do estado de São Paulo, Brasil. Concluímos a importância da leptospirose no rebanho avaliado e a atenção para o risco ocupacional, devido à possibilidade de urina contaminada nas pastagens e disseminação da infecção para outros animais e pessoas contactantes.

Palavras-chave: diagnóstico molecular; frigorífico; leptospirose.

INTRODUCTION

Within the zoonotic diseases, leptospirosis is the most important and may attempt humans and wild, domestic and livestock animals, with global spread and endemic in Brazil. It is a disease with great importance in public health because it is an antropozoonotic disease, with high incidence and prevalence, neglected and mainly subdevelopment countries, with high level of poverty and in tropical and subtropical regions (Torres-Castro et al., 2016).

Leptospirosis in bovine herds, as well in the most domestic animals, is a bacterium zoonosis, mainly associated with infertility and slow production. The economic and reproductive losses are associated with abortion, stillbirths, and low milk production. When the leptospirosis is in a herd in fact, the control is difficult, mainly because of the adaptation of the bacteria to the animal species, which can be the reservoir and/or the maintenance host (Guedes et al., 2019)

So, according to presented statements and due to importance of the productive chain of bovine meat, since the slaughter to manipulation hygiene, elaboration, prepare and conservation until the final product, and because the bacteria is the importance in public health, we researched *Leptospira* spp. in urine samples of bovine, from a slaughterhouse that export and with federal sanitary inspection, in the Midwest São Paulo State. Without this evaluation, the health of the workers´ frigorific industry and the meat commercialization, and the final consumer as well, may be in risk.

MATERIAL AND METHODS

Sample collection

One-hundred and four urine bovine samples were collected in a slaughterhouse from a Midwest region of São Paulo State, from June to July 2018.

The samples were collected once, in the moment of the slaughter. The choice of the animals was aleatory, with no discrimination of sex, breed, and age.

The molecular and isolation techniques were performed in Paulista Agency of Agribusiness Technology (APTA), Bauru-SP.

The urine samples were obtained by direct bladder puncture during the abattoir line, with sterile syringe of 5mL. The syringes with the urine were kept in aluminum (wrapped) paper to avoid the light and delivered to the laboratory to perform the cultures in the same day of the collects, in sterile conditions (Pinna et al., 2018).

Culture of urine in Fletcher and EMJH medium

Previously the technique of isolation, the syringes with urine were first kept in aluminum (wrapped) in sterile conditions about 30 minutes under U.V. light to eliminate possible external contamination. After that, 1 mL of urine was inoculated in tubes with 4.5 mL of sterile EMJH medium (Difco®) and in tubes with 5 mL of Fletcher semi-solid medium (Difco®) and immediately kept in bacteriological stoves under 28ºC to 30ºC for 16 weeks, with readings under dark field microscopy each 15 days, to evaluate the cultures.

Ten µL of each sample in culture medium was evaluated, considering positive the cultures when it was possible to see spirochaetes with motility under microscopy and/or with bacterium development with formation of opalescence ring (Dinger zone).

All the cultures (positive and negative) were kept in microtubes free of DNAses and RNAses at -20ºC, until to perform the extraction of the DNA and confirmation by Polymerase Chain Reaction technique to *Leptospira* spp., using the primers LEP1/LEP2 (Mérien et al., 1992). The positive samples were performed to PCR with the primers that detect the *LipL32* gene, that confirm the pathogenicity of the leptospire.

DNA extraction of urine samples

Each 1 mL of urine was transferred to sterile microtubes DNAse and RNAse free, containing 100µL of buffered saline solution pH 7.6 and after that were kept at -20ºC until the PCR will be performed as described to the cultures (Pinna et al., 2018).

Molecular analysis

Extration and DNA quantification

The urine DNA extraction was performed using the Illustra BloodTM genomic Prep Mini Spin (GE Healthcare®) kit protocol, with some changes. All the DNA samples were quantified by the NanoVue Plus®.

Leptospira interrogans serovar Pomona (ATCC BAA-1198) was used as a positive control and ultra-pure water as negative control.

Polymerase Chain Reaction – PCR - Amplification of DNA Leptospira spp. screening

The primers used were described by Mérien et al. (1992), LEP1/ LEP2 (LEP 1: 5′ GGCGGCGCGTCTTAAACATG 3′/LEP 2: 5′ TTCCCCCCATTGAGCAAGATT 3′). The size of amplified DNA fragment was 331 bp. The amplification was carried out in Mastercycler Pro Gradiente (Eppendorf®), under the following conditions: preincubation at 94ºC for 3 min, 30 cycles, each of 60 s at 94ºC (denaturation), 60 s at 63ºC (primers annealing), and 2 min at 72ºC (elongation), including more 10 minutes.

PCR for pathogenic Leptospira spp. - LipL32 gene

The primers used were described by Azizi et al. (2012), *LipL32_45F (*5′- ATCTCCGTTGCACTCTTTGC -3′) and *LipL32_286R (*5′- ACCATCATCATCATCGTCCA -3). The size of amplified DNA fragment was 474 bp, presenting 100% of specificity and 100% of sensibility. The amplification was carried out under the following conditions: preincubation at 94ºC for 3 min, 30 cycles, each of 60 s at 94ºC (denaturation), 90 s at 60ºC (primers annealing), and 2 min at 72ºC (elongation), including more 10 minutes.

PCR products were stained with Syber Safe® (Invitrogen) 0,1µL/mL and visualized by 1.5 % agarose gel electrophoresis.

Sequencing

Sequencing was performed using the enzime ExoSap (GE) –USB ExoSap –IT® (USB Affymetrix, USA). The sequences were aligned by the Clustal program by MEGA Software (Molecular Evolutionary Genetics Analysis) version 7.0 for Windows (Kumar et al., 2018) and compared to the sequences deposited in the data bank NCBI (BLAST).

Statistical Analyses

The techniques of culture and PCR of urine were compared; it was calculated the kappa coefficient to verify the concordance between these tests. It was used the Excel for Windows program, version 2019, utilizing the Real Statistics Resource Pack to the statistical calculi of this study.

RESULTS

Microscopic observations in EMJH and Fletcher

During the period of 16 weeks, 58 samples of EMJH cultures and 12 samples of Fletcher medium presented contamination with fungi, and color alterations (dark or green color). It was observed by microscopy that 13 samples (12.5%) in Fletcher medium presented spirochaetes form, but do not observed in EMJH samples.

Molecular results

The results obtained by PCR in 104 urine samples in PBS 7.6 and 46 urine samples cultivated in EMJH medium and 92 urine samples cultivated in Fletcher medium, showed that 16 (15.4%) urine samples in PBS 7.6 were positive for *Leptospira* spp.; six samples (5.8%) of urine cultivated in Fletcher medium were positive; and three samples (2.9%) of urine in EMJH medium were positive. Only one sample cultivated in EMJH medium was positive to the gene *LipL32.*

Sequencing results

The urine sample that was positive in the EMJH culture and positive to *LipL32* gene, was sequencing and the DNA sequences generated were compared with the BLAST data base and showed 99.77% similarities with *Leptospira interrogans* serovar Pyrogenes (**accession number: AY609323.1**) e *Leptospira kirschneri* serovar Canicola (**accession number: AY461914.1**).

Statistical analysis

Between the culture and the molecular techniques, the agreement (Kappa coefficient) showed weak concordance.

DISCUSSION

The results concerning the observation of the urine cultures show the difficulty for the pure isolation of leptospires in Fletcher and EMJH medium, because of the development of other microorganisms and, due this, the high contamination in both culture medium, although the application of all sterile proceedings in the cultures, as observed by Soares et al. (2020). We verified that EMJH medium presented more conditions to development of contamination.

Probably the animals with PCR positive to *Leptospira* spp. in urine samples were in chronic phase of infection. These animals can contaminate the grass, and possibly infect other animals because of the liberation of the bacteria in urine. The occupational risk in leptospirosis is a concerning because the workers that are exposed to these animals and

in the environment of the slaughterhouse, by the manipulation and prolonged exposition to viscera and blood from infected animals.

The slow detection of DNA in buffered urine was observed, maybe due to inhibitors that difficult the visualization of amplifications in the electrophoresis. The contamination observed in more than a half of the samples cultured in EMJH medium do not allowed the comparison between the results obtained in the culture related to the molecular results.

We observed that the molecular results were different from the cultures, because of the difficulty in isolation and high contamination, which reduce the sensibility of the test, as observed by Guedes et al. (2019). In our results, the microorganisms were not visible in some cultures, although in the PCR test were positive.

The use of drugs to treatment of leptospirosis reduces the possibility of leptospires isolation, as observed by Stoddard et al. (2009). The animals evaluated were from good farms, and maybe were submitted to this treatment by veterinarians, which can explain the difficulty of the isolation.

The test of PCR was important to detection of positive animals and without clinical signs compatible with leptospirosis, mainly in slaughtered bovine and that can carry the bacteria in their kidneys, as observed by Pinna et al. (2018).

The animals positive to LEP1/LEP2 primers and negative to *LipL32* gene, presented the disease with a non-pathogenic bacteria, which can avoid a serious disease to another animals and contacting humans.

The *LipL32* gene is present in all pathogenic leptospire, so is a region highly conserved between the species. This gene is utilized in the leptospirosis diagnosis to detect pathogenic serovars (Azizi et al., 2012).

The urine sample that was positive in the EMJH culture and positive to *LipL32* gene, was sequencing and the DNA sequences generated were compared with the BLAST data base and showed 99.77% similarities with *Leptospira interrogans* serovar Pyrogenes (accession number: AY609323.1) and *Leptospira kirschneri* serovar Canicola (accession number: AY461914.1). To the best of the authors' knowledge, the presented study is the first report of isolation of *Leptospira kirschneri* serovar Canicola and *Leptospira interrogans* serovar Pyrogenes by the urine culture from bovine slaughtered in the Midwest São Paulo State, Brazil.

L. kirschneri is commonly related to wild animals, mainly small mammals, that maybe present this leptospire specie (Guedes et al., 2019). In EUA, *L. interrogans* serovar

Pomona and *L. kirschneri* serovar Grippotyphosa are associated with clinical cases in dogs (Grimm et al., 2020).

L. kirschneri can cause leptospirosis in humans, and *Leptospira interrogans* e *Leptospira borgpetersenii* as well. Cunha et al. (2016) related that *L. kirschneri* serogroup Pomona serovar Mozdok was found in human in Cuba and that this serovar is the causal agente of leptospirosis in canine specie, mainly in Europe. These authors describe that this serovar is endemic in Croacia, mainly in wild rodents; serovar Pomona is commonly found in Brazil, in Pelotas region. In the study of Cunha et al. (2016), the urine was submitted to EMJH culture and by molecular biology it was detected *Leptospira kirschneri.*

Leptospira kirschneri serogroup Pomona serovar Mozdok is in South and Southwest regions of Brazil during the last twenty years and probably is suitable to rodents as reservoirs, which can allow high virulence to humans, with risk to public health (Moreno et al., 2016).

Cunha et al. (2016) report that it was the first report of human and animal leptospirosis caused by *L. kirschneri* serogroup Pomona and serovar Mozdok in South of Brazil, in Pelotas region. In this study, the Midwest region of São Paulo State is a region that *L. kirschneri* was detected in a bovine from a rural property located in Santa Cruz do Rio Pardo, São Paulo State.

Balassiano et al. (2017) described the second report of human leptospirosis caused by *L. kirschneri* serovar Mozdok, in a rural area of Santa Vitória do Palmar, Rio Grande do Sul, Brazil, and also found the serovar Pomona, more frequent in animals than in humans. The authors report the importance of more studies to understand the epidemiological situation in the rural areas in Brazil.

The obtained data related to *L. kirschneri* serovar Mozdok is important to development of new vaccines; besides that, this serovar may be included to tests to rapid diagnostic and to the next molecular and serological studies. The authors report that serovars that are not frequent in Brazil is an important data that allows prevent and control the human and animal leptospirosis, which is benefic to the population (Cunha et al., 2016; Moreno et al., 2016; Balassiano et al., 2017).

The results obtained in this research agree with the study of Guedes et al. (2019) in Tocantins/Pará, Brazil, with female bovines, when only the molecular results in urine and kidneys were positive and not in the culture tests, finding the species *L. borgpetersenii, L. santarosai, L. interrogans* and *L. kirschneri.*

According to the authors Guedes et al. (2019), it is important to consider the contact between bovine and domestic and wild animals, and the deforestation can allow this contact and the transmission of the leptospires in the environment.

In this study, the identification of *L. kirschneri* agree with the study of Soares et al. (2020), that identified *L. kirschneri* serogroup Canicola and *L. kirschneri* serogroup Grippotyphosa in urine samples of bovine from Brazil, by serological and molecular methods, with *LipL32* gene. These authors report that, besides Brazil, this specie was also related in bovine from Zimbábue, Sri Lanka, Tanzânia and Bélgica.

A study in France by Marquez et al. (2019) in a farm, with cases of human and animal leptospirosis, it was observed *L. biflexa*, *L. kirschneri* and *L. interrogans*, and in one rodent identified with *L. kirschneri* serogroup Grippotyphosa and *L. interrogans* serogroup Icterohaemorrhagiae as well.

The analyses of the identification of the species *L. kirschneri* and *L. interrogans* serovar Pyrogenes in this report can suggest a coinfection, with similarity of 99.77% by the sequencing. Besides that, the epidemiological conditions, explained by rodents in the same environment, and wild and domestic animals with these bovines, also observed by Marquez et al. (2019).

In Brazil, leptospirosis occurs asymptomatic in bovine by adapted serovars, specially from the Sejroe group, that include serovar Hardjo, and detected *L. kirschneri* in this report. Another study, Soares et al. (2020), verified that the serogroup Grippotyphosa presented high similarity with the reference strain Moskva V, highlighting the diversity of pathogenic leptospires that can cause leptospirosis in animals and the possibility of adaptation of these serovars to diferent animal species, such as in bovines.

Leptospira interrogans serovar Pyrogenes are commonly found in equine specie (Oliveira et al., 2013)], and in canine as well (Flores et al., 2017). Querino et al. (2003) report that Pyrogenes serovar is important to dogs, besides the human, because of its pathogenicity, which is relevant to the public health, and the wild animals as reservoirs of leptospires as a source of infection to dogs and humans. The serovar Pyrogens was isolated in Brazil from aquatic rats (*Nectomys squamipes)* in São Paulo region, and serologically detected in dogs of another studied areas (Querino et al., 2003).

In a study conducted by Gomes et al. (2017) in Uberlândia/Minas Gerais, Brazil, the authors report that the serovars Copenhageni and Pyrogenes were the most found serologically in Cerrado area, highlighting the need of the study in rodents to the control of the disease.

The study of Thibeaux et al. (2017) also reports the importance of the environment to the infection of bovines and contacting workers, by the detection of *Leptospira interrogans* serovar Pyrogenes in the environment and infected humans in Nova Caledonia, EUA. In this study, we detected *Leptospira interrogans* serovar Pyrogenes in a bovine urine sample, probably by the contamination of the water sources and the environment, as observed by Flores et al. (2017) and Gomes et al. (2017). In slaughterhouses, the contamination of the viscera and blood from infected animals can lead the disease to the workers as well. This is corroborated by Marquez et al. (2019), that report the contact with rodents and food sources to the animals in the farms, and the humans can be also infected, which characterizes the importance of the disease as a zoonosis.

In this work, the detection of *Leptospira kirschneri* serovar Canicola and *Leptospira interrogans* serovar Pyrogenes was a pioneer in the Midwest region of São Paulo and its detection represents that such serovars are circulating in the studied region. It denotes, therefore, the importance of carrying out future investigations to better understand these species of leptospires, as they are circulating in various environments among domestic, wild and livestock animals, with human beings inserted in this epidemiological context; hence the importance of further studies to understand the aspects of the disease in the ecosystem and for more effective control measures. The routine practice of vaccination in cattle is a measure that should not be neglected, but the need for knowledge of the diversity of leptospiral serovars present in different environments is also emphasized, in view of the ecological imbalance and the interaction of cattle with different species, mainly wild animals.

The cultivation of urine samples in EMJH and Fletcher media resulted in the contamination of many samples, mainly in EMJH media. The use of the molecular PCR technique was an important diagnostic tool in confirming samples positive to the culture, in view of the difficulty for their isolation, due to the possibility of contamination of the samples and the slow growth in culture medium.

CONCLUSION

It is concluded the importance of this zoonosis in the evaluated herds and the attention to the occupational risk in the slaughterhouse environment, by the direct contact with animal secretions, as well as in the studied farms, by the possibility of eliminating urine in the pastures and infection for other animals and people in contact;

such results suggest an alert for the implementation of health programs in search of improving the health of cattle herds.

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CEP: Protocol on Animal Use Ethics Committee – CEUA - 0063/2017.

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