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MOLECULAR BIOLOGY AS A DIAGNOSTIC TOOL FOR DETECTION OF *Leptospira* spp. IN COWS OF A BORDER REGION – CASE REPORT

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Abstract

The reproductive efficiency of livestock is the basis for the success of livestock, dairy or beef, and having high reproductive performance depends on several factors within the production system and the presence of infectious diseases of the reproductive sphere in the herd is one of the factors that can compromise that efficiency. The aim of this study was to use molecular biology as a diagnostic tool for the detection of *Leptospira* spp. DNA in cows with reproductive disorders on a rural property in the municipality of Boca do Acre, Amazonas, Brazil. Vaginal mucus was collected from nine Nelore breeding cows with a history of abortion and birth of weak calves submitted to DNA extraction and nested-PCR technique for 16S gene amplification at the bacterial genus level. Of the nine samples analyzed, five (55.55%) amplified a product of 331bp. The municipality of Boca do Acre is bordered by Peru and Bolivia, and knowledge of the prevalence of the disease, serovars, and circulating *Leptospira* species is essential for the adoption of measures related to animal husbandry, as well as health education for ranchers and their workers to avoid a possible occupational infection since this disease is considered an important zoonosis. New molecular studies using primers that allow the identification of the *Leptospira* species and mainly pathogenic species should be conducted in this region in order to elucidate the possible species of this etiological agent and the possible reservoirs of the disease to begin the understanding of the epidemiology of this disease in cattle in this region of border.

Keywords: Acre. Bovinae. Herd. Leptospirosis. PCR. Reproduction. Vaginal Mucus.

1. Introduction

The reproductive efficiency is the base for success in meat or dairy cattle farming. Several factors within the production system contribute towards high reproductive performances and the presence of reproductive diseases in the herd is one of the factors that may jeopardize such efficiency, since these diseases may be caused by several infectious agents (Embrapa 1999; Oliveira et al. 2018).

One of the agents involved in reproductive issues is leptospirosis, an infectious-contagious disease affecting animals and humans alike, caused by any pathogenic species of the *Leptospira* spp. Genus (OIE 2008). In cattle, *Leptospira* spp. is mainly transmitted by the presence of sick or asymptomatic animals eliminating the bacteria through urine. It can also be transmitted by vaginal discharges, placenta, or infected aborted fetuses (Faine et al. 1999; Barragan et al. 2017).

Leptospirosis is considered one of the main reproductive diseases in cattle in Brazil and also in the world, with great epidemiological importance in the livestock scenario, where it can present different reproductive issues, such as infertility, abortion, the birth of weak calves, bloody mastitis and decrease in dairy production (Faine et al. 1999; Cervantes et al. 2002).

In order to diagnose this disease in cattle, epidemiological data must be collected, considering the clinical signs presented by the animal, the type of handling, the breeding location, vaccination, presence of rodents, geographical location, among other variables. However, laboratory diagnosis is also essential, both serological by using microscopic agglutination test (MAT) for detecting antibodies and molecular, using the polymerase chain reaction (PCR) for detecting the DNA of the etiological agent (Faine et al. 1999; WHO 2003; Hamond et al. 2014; Grooms 2015; Oliveira et al. 2016; Nally et al. 2020).

A molecular diagnosis is more advantageous when compared to serological techniques due to its capacity of amplifying minimum amounts of DNA from the etiological agent in biological samples such as serum, liquor, urine, feces, mucus, and tissue. Therefore, the disease can be diagnosed early with the possibility of confirming the diagnosis even before the appearance of antibody titers, or even if they are low (Bal et al. 1994; Anzai 2006; Oliveira et al. 2016; Oliveira et al. 2018).

The city of this study is located in the state of Amazonas, with approximately 210.000 cattle units (IBGE, 2018). Due to the scarcity of scientific data on bovine leptospirosis associated with reproductive problems in the Amazon region, this study aimed to use molecular biology as a diagnostic tool for the detection of *Leptospira* spp DNA. in cows with reproductive disorders on a rural property in the municipality of Boca do Acre, Amazonas, Brazil.

2. Material and Methods

This study was carried out in June 2017 in a rural property located in the municipality of Boca do Acre, state of Amazonas, Brazil (Latitude: -8.74069, Longitude: -67.38418° 44’ 26” Sul, 67° 23’ 3” Oeste). Of the 716 cows on the farm, 102 (14.24%) had a history of reproductive problems such as abortion at the end of gestation, retention of the placenta, and the birth of weak calves.

As the number of animals in the herd was high, samples of 10% of the animals were collected to show the prevalence of this infection in animals of this herd (Pellegrin et al. 1999). The animals were aged between four and seven years old and weighed between 400 and 550 kg.

It was verified that, in the rural property, the cows did not maintain contact with any other animal species, except for a few capybaras (*Hydrochoerus hydrochaeris*) that moved from the native forest and shared the water and also mineral salt together with the animals of the property.

During the visit of the veterinarian, ten animals were closed to perform the collection of vaginal mucus using an applicator cover (Tampax® Regular, Procter and Gamble, São Paulo, SP, Brazil) of Nelore breeding cows that had already had abortions and the birth of weak calves. However, it was only possible to collect biological samples from nine animals due to the high reactivity of one animal and the absence of adequate infrastructure to contain the animal, it was decided to discard this sample to preserve the physical integrity of the collection team.

That tampon was introduced into the vagina of cows for a period of ten minutes and then inserted in a sterile tube with a 20-mL PBS solution (Lilenbaum et al. 2008). The samples were stored in an isothermal box under refrigeration (about 1 or 2◦C), being immediately forwarded to the Laboratory of Animal Virology at State University of Londrina (UEL) for posterior molecular diagnosis (about 20 hours after). At the laboratory, the DNA was extracted from the vaginal mucus samples following the guanidinium isothiocyanate technique (Alfieri et al. 2006). The samples were later submitted to the nested-PCR (n-PCR) technique using the following primers*:* A, 5'-GGCGGCGCGTCTITAAACATG-3'; B, 5'-TTCCCCCCAT TGAGCAAGATT-3'; C, 5'-CAAGTCAAGCGGAGTAGCAA-3'; and D, 5'-CTTAACCTGCTGCCTCCCGTA-3' as previously described by Mérien et al. (1992), for 16S gene amplification at the bacterial genus level. Negative control (ultra-pure water) and positive control (strain Canicola) were also used in the reaction.

3. Results

The final product from the n-PCR amplification was submitted to 2% agarose gel electrophoresis containing ethidium bromide (0.05µg/µL), and visualization was performed in an ultraviolet transilluminator, where the positive sample aligned with 331bp. In n-PCR, five (5/9; 55.55%) samples amplified a 331pb product (Figure 1).

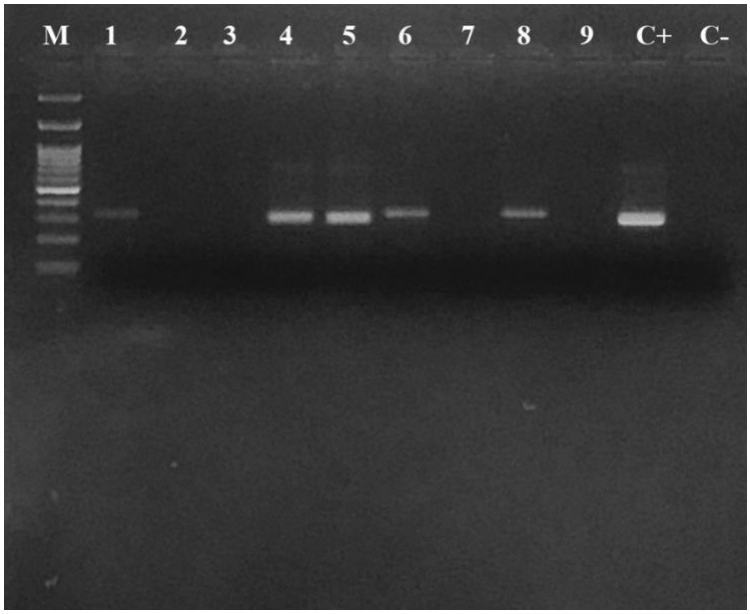

Figure 1. Nested-PCR for *Leptospira* spp. of the vaginal mucus samples from Nelore breeding cows with a history of reproductive problems of a rural property in the municipality of Boca do Acre state of Amazonas, Brazil. 2% Agarose Gel. Molecular Marker, Line 1 sample 1 (+), line 2 sample 2 (-), line 3 sample 3 (-), line 4 sample 4 (+), line 5 sample 5 (+), line 6 sample 6 (+), line 7 sample 7 (-), line 8 sample 8(+), line 9 sample 9(-), line 10 positive control and line 11 negative control. Source: Developed by the authors.

4. Discussion

Since the city of Boca do Acre, in the state of Amazonas, holds a herd of approximately 210.000 animals (IBGE 2018), the knowledge of the incidence of leptospirosis in cattle herds is of regional importance, to obtain information for establishing preventive measures that contemplate animal health, thus avoiding economic losses from production decrease.

There are several reports from farmers to veterinarians and technical experts on the area regarding cattle issues in the region. There are different possible causes for those problems happening in the field. However, the possibility of having a more assertive diagnosis of the etiological agent causing the problem is pivotal for the development of an effective sanitary plan that results in a better reproductive result for the rural properties.

According to Faine et al. (1999), cattle leptospirosis can be transmitted via urine, vaginal fluids, placenta, or aborted fetuses infected with *Leptospira* spp. and therefore, vaginal fluids were used for this research.

In this study, five (55.55%) of the tested vaginal fluid samples were considered positive in molecular diagnosis. Other studies using molecular biology as a diagnostic tool have also detected positive samples in sheep and cow fluids, however using primers that have the objective of detecting pathogenic leptospires (lipL32) (Director et al. 2014; Oliveira et al. 2016).

The primer used in this study and proposed by Mérien et al. (1992) uses a very preserved region in prokaryotes, gene 16S rRNA, a component of the lower ribosome subunit. It has previously been successfully used for the detection of Leptospira, not generating false negatives against other spirochetes. On the other hand, the amplification of the LipL32 gene codes a lipoprotein from the external membrane, a virulence factor that is absent in non-pathogenic species. Therefore, the LipL32 gene allows distinguishing saprophytic species from pathogenic ones, which is not accomplished with Mérien et al. (1992) (Stoddard et al. 2009).

The confirmation of Leptospirosis through culture is a long and labor-intensive process that does not provide a timely diagnosis. Polymerase chain reaction (PCR) does not require isolation and culture of the agent, being very useful in detecting fastidious or slow-growing bacteria. However, the products generated both by using Mérien and LipL32 do not allow species differentiation.

Attempts to develop species-specific primers are reported in the literature (Reitstetter 2006; Ferreira et al. 2014). However, they often use not only one, but sets of initiators in order to reach the expected result. Due to the diversity of serovars and the importance of leptospirosis in the context of human and animal infections, the development of new oligonucleotides for species differentiation is pivotal for faster diagnosis, increasing the knowledge of that pathogen.

This case report presents the probable cause of the problems related to pregnancy losses in the studied property, and, at the field level, it is possible to observe that the problem is real. However, in the other samples that *Leptospira* spp. we cannot rule out the possibility of infection from other infectious diseases such as bovine infectious rhinotracheitis (IBR), bovine infectious viral diarrhea (BVD), campylobacteriosis since they are also considered important infectious diseases that cause reproductive disorders in the bovine species.

A point to be considered is the location and difficult access in the rural properties of this municipality and the awareness of the rural producer by the veterinarians at the time of technical assistance (an infrequent situation) is important to encourage the rural producer to adopt this type of diagnostic technology for the detection of diseases of the reproductive sphere that will allow rethinking the best sanitary management to be adopted for the property in question.

In this region, many rural properties have a high population density of cattle and if these animals have DNA from *Leptospira* spp. in the vaginal mucus or even in the urine it will allow a differentiated sanitary handling of the animals avoiding environmental contamination and possible new infections. For this type of rural property, this molecular diagnosis is viable and accessible, and studies such as this should be expanded and disseminated to bring rural producers closer to academia, to stimulate research partnerships, in addition to contributing to regional development.

5. Conclusions

It is important to emphasize that the city of Boca do Acre is a border region, since it has its borders with Peru and Bolivia, and the knowledge of the prevalence of the disease, serovars, and of the circulating *Leptospira* species is essential for adopting measures related to the handling of the animals, as well as health education for farmers and their workers in order to avoid any likely occupational infection since this disease is considered an important zoonosis.

New molecular studies using primers that allow the identification of the *Leptospira* species must be developed in the region, both in cattle as in other domestic and wild animals, in order to clarify the possible species of the etiological agent and possible reservoirs for the disease to start understanding the epidemiology of the disease in cattle in this border region.

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