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Serological and molecular findings in diagnosis of leptospirosis serovar hardjo in a dairy bovine herd

Acompanhamento sorológico e molecular do sorovar hardjo no diagnóstico de leptospirose em um rebanho bovino leiteiro

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Abstract

The cattle are considered hosts of the Hardjo serovar, causing economic damages due to the reproductive failures like abortions and infertility. The serovar Hardjo usually remains in the reproductive tract and also in the renal tubules where it is eliminated intermittently in the urine for months. Placental remnants, the aborted fetus and contaminated urine promote the permanence of this bacterium within the herd for years. Thus, the objective of this study was to monitor for prolonged period, cows naturally infected with *Leptospira* ssp. through microbiological culture, serological examination and DNA detection of the pathogen in the urine. The dairy herd was composed of 50 breeding cows with a history of abortion and infertility, without leptospirosis vaccine and located in the northern region of Paraná. Blood and urine samples were collected and laboratorial diagnosis were performed five times at intervals of four months. Blood samples were collected from the all 50 animals and the serum was submitted to the microscopic agglutination test (MAT) for the detection of anti-leptospira antibodies. Of the total cows, 20 showed antibody titres ≥ 1: 100 in MAT and urine samples were collected from only those animals with higher titers to perform nested-PCR (n-PCR) and bacterial isolation per culture. In addition, two urine samples from five animals with antibody titers < 1: 100 were collected in MAT for n-PCR. Serovar Hardjo was considered the most frequent during the serological monitoring of the animals evaluated. The n-PCR technique was able to detect leptospiral DNA in the urine of animals with MAT ≥ 1: 100 antibody titers and urine from animals whose titers were < 1: 100. Sequencing of the leptospiral amplicons shared 100% nucleotide sequence identity with the *Leptospira interrogans* species. Positive n-PCR results from animals with titers of < 1: 100 suggest that the cut-off of MAT is could be not sufficient to detect renal carriers, so it is also important to use n-PCR as an additional diagnostic tool for identify infected animals with Hardjo serovar and whose serology was negative.

Key words: Epidemiology. Leptospira. Microscopic agglutination test. Renal carriers.

Resumo

Os bovinos são considerados hospedeiros do sorovar Hardjo, causando prejuízos econômicos devido aos problemas reprodutivos como abortos e infertilidade. O sorovar Hardjo costuma permanecer no trato

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reprodutivo e também nos túbulos renais onde é eliminado de forma intermitente na urina por meses. Restos placentários, o feto abortado e a urina contaminada favorecem a permanência dessa bactéria dentro do rebanho por anos. Assim, o objetivo deste estudo foi monitorar por período prolongado, vacas naturalmente infectadas por *Leptospira* ssp. através de cultura microbiológica, exame sorológico e detecção de DNA do patógeno na urina. O rebanho leiteiro estudado era constituído por 50 vacas reprodutoras com histórico de abortos e infertilidade, sem uso de vacina contra a leptospirose e localizada na região Norte do Paraná. As amostras de sangue e urina foram coletadas e submetidas a análises laboratoriais em cinco vezes em intervalos espaçados de quatro meses. As amostras de sangue foram coletadas dos 50 animais e o soro foi submetido ao teste de soroaglutinação microscópica (SAM) para detecção de anticorpos anti-leptospira. Do total vacas, 20 demonstraram títulos de anticorpos ≥ 1: 100 na SAM e foram coletadas amostras de urina apenas destes animais com maiores título para realizar a nested-PCR (n-PCR) e isolamento bacteriano por cultura. Adicionalmente, foram coletadas duas amostras de urina de cinco animais com títulos de anticorpos < 1: 100 na SAM para n-PCR. O Sorovar Hardjo foi considerado como sendo o mais frequente durante o monitoramento sorológico dos animais avaliados. A técnica de n-PCR foi capaz de detectar DNA leptospiral na urina de animais com títulos de anticorpos ≥ 1: 100 e na urina de animais cujos títulos eram < 1:100. O sequenciamento dos *amplicons* leptospíricos compartilhou 100% de identidade da sequência de nucleotídeos com a espécie *Leptospira interrogans*. Os resultados positivos da n-PCR dos animais com títulos < 1: 100 sugerem que a diminuição do ponto de corte da SAM pode não ser suficiente para detectar os portadores renais, por isso também é importante usar a n-PCR como uma ferramenta diagnóstica adicional para identificar os animais infectados com serovar Hardjo e cuja sorologia foi negativa.
Palavras-chave: Epidemiologia. Leptospira. Portadores renais. Soroaglutinação microscópica.

Introduction

Leptospirosis infection of cattle causes significant economic losses in the beef and dairy industries. Infected herds experience reproductive failures such as abortion, infertility, agalactia, stillbirth and weak calves. Furthermore, leptospirosis is one of the most important zoonosis in the world (ELLIS, 2015; NGBEDE et al., 2012).

Many serovars are involved in bovine leptospirosis; however, the Hardjo serovar is considered to be the most common serovar worldwide and causes the greatest impact on the reproductive health of cattle herds (CASTRO et al., 2008; FIGUEIREDO et al., 2009; HASHIMOTO et al., 2010; NGBEDE et al., 2012). In affected herds, the spread of leptospires occurs primarily by the presence of infected animals or asymptomatic carriers that eliminate the bacteria in the urine intermittently for long periods of time, thereby maintaining the pathogen within the herd (ELLIS, 2015). Furthermore, the Hardjo serovar is a weak immunogen that produces short-lived immunity as evidenced by the low binding and neutralizing antibodies titers in vaccinated cattle (BOLIN et al., 1989).

Among the laboratory methods used for the diagnosis of leptospirosis, the microscopic agglutination test (MAT) is the most widely used (FAINE et al., 1999). However, negative serological results obtained by the MAT do not exclude the possibility that an animal is a renal carrier; thus, it is necessary to use additional laboratory methods for better disease control (LANGONI et al., 2008). The isolation of *Leptospira* spp. is considered to be the definitive technique for the diagnosis of bovine leptospirosis, but this method is laborious and restricted to a few laboratories (ADLER; DE LA PENA-MOCTEZUMA, 2010). The PCR assay has increasingly been used for the diagnosis of bovine leptospirosis and allows the amplification of minimal amounts of microorganism DNA from different types of biological samples, including urine (HERNÁNDEZ-RODRÍGUEZ et al., 2011; OTAKA et al., 2012).

Ordinarily in the laboratorial routine, serum dilution starts in 1:100 (FAINE et al., 1999), but,

previous results of our team shown that negative animals in MAT were positive in n-PCR for pathogenic leptospires.

Thereby, the objective of this study was to evaluate if low titers against the serovar Hardjo may be used to infer animals with active elimination of the pathogen in the urine.

Materials and Methods

Animals

This study was conducted in the northern region of Paraná State, in the south of Brazil in a dairy cattle herd consisting of 50 Jersey females of reproductive age that were raised in an extensive breeding system. This herd was picked due previous serology results that shown a prevalence of 40% approximately, for serovar Hardjo (data not shown) and these animals had a history of abortions, infertility and absence of vaccine history against leptospirose.

Animal ethics and usage

All samples were collected under the supervision of veterinarians from the State University of Londrina, UEL. The study was conducted and approved **(n° - CEEA – 58/06)** following the guidelines as stated in the Code of Practice for Care and use of Animals for Scientific Purposes as stipulated by State University of Londrina.

Sample collection

Between November 2009 and April 2011, five blood and urine collections were carried out at regular four-month intervals.

For the MAT, blood samples were collected from 50 females of the herd. Among these animals, urine samples were collected from 20 cows with the highest MAT antibody titers for the nested-PCR (n-PCR) assay and bacterial isolation by culture. Additionally, two urine samples were collected, in intervals of for 4 months, from five animals with MAT antibody titers <1:100 in all collect for use in n-PCR.

Blood was collected by jugular venipuncture using a sterile disposable needle and vacuum tubes, which were labelled for identification. Serum samples were stored in plastic microtubes and frozen at -20ºC. Urine samples were obtained after perineal massage and were stored in sterile glass vials. The first portion of the urine was discarded.

Serological test for Leptospira and others reproductive disease

For the detection of specific antibodies for *Leptospira* spp, all serum samples were tested by the MAT performed according to OIE (WORLD ORGANISATION FOR ANIMAL HEALTH, 2014) with a collection of antigens that included the following serovars: Australis, Bratislava, Autumnalis, Butembo, Castellonis, Bataviae, Canicola, Whitcomb, Cynopteri, Fortbragg, Grippotyphosa, Hebdomadis, Copenhageni, Icterohaemorrhagiae, Panama, Pomona, Pyrogenes, Hardjo, Wolffi, Shermani, Sentot and Tarassovi. The serum samples were screened at a dilution of 1:100, and those samples presenting agglutination levels of 50.0% or more were titrated using twofold dilutions. The titer was determined to be the highest dilution yielding a positive result. Samples that were not reactive at a dilution of 1:100 were also tested at dilutions of 1:20, 1:40 and 1:80. The analysis considered the most common serovar to be that which yielded the highest titer. Serum samples that were reactive for two or more serovars with identical titers were excluded from further analysis and considered seropositive for *Leptospira* spp.

Additionally, the serological diagnosis for brucellosis, infectious bovine rhinotracheitis (IBR) and bovine viral diarrhea (BVD) were performed according to OIE (WORLD ORGANISATION FOR ANIMAL HEALTH, 2014).

Culture of Leptospira

For isolation, the urine samples were immediately inoculated, on the farm, into tubes containing EMJH media (Becton-Dickinson Biosciences/ DIFCO, Detroit, USA) supplemented with the following antibiotics: 5-fluorouracil (400 mg/L, Sigma-Aldrich®, St Louis, USA), chloramphenicol (5 mg/L, Sigma-Aldrich®, St Louis, USA), nalidixic acid (50 mg/L; Inlab®, São Paulo, BR), neomycin (10 mg/L, Sigma-Aldrich®, St Louis, USA) and vancomycin (10 mg/L , Acros®, New Jersey, USA) (ZACARIAS et al.,2008) and were then transported to the laboratory and incubated at 28˚C for 24 h. After this period, each tube was subcultured in duplicate into EMJH media without antibiotics, and the cultures were evaluated weekly by dark field microscopy (Olympus System Microscope Model BX40) for up to six months.

Nested-PCR

After collection, the urine samples were aliquoted into 1.5 mL microtubes, stored immediately under refrigeration in coolers and transported to the laboratory. Subsequently, 1 mL of each urine sample was centrifuged at 12,000 x g for 10 min at 4°C. The pellet was resuspended in 300 µL of the supernatant, and DNA was extracted by the silica/ guanidine isothiocyanate technique, as described previously (BOOM et al., 1990). Negative (sterile ultrapure water) and positive (serovar Hardjo) control samples were included in all procedures.

The first PCR was performed using the primers *lip*L32 F: 5' CGCTTGTGGTGCTTTCGGTGGT 3' and*lip*L32R:5'CTCACCGATTTCGCCTGTTGGG 3', which amplify a 264 bp product between positions 73 and 336 of the region of the gene encoding the leptospiral major outer membrane lipoprotein (*lip*L32). The nested PCR (n-PCR) was performed using the primers *lip*L32 R1: 5'CTCCCATTTCAGCGATTACGG 3' and *lip*L32 F2: 5'TTCTGAGCGAGGACACAATCCC 3', which amplify a 183 bp product from the first amplification. The PCR and n-PCR procedures were performed as described (JOUGLARD et al., 2006). The reactions were performed in a thermocycler (Techne®, TC 312 model). The products obtained were then analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide (0.5 µg/ mL) in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.4) and visualized under UV light.

Sequencing

The PCR products were purified by the using Illustra GFX PCR DNA and the Gel Band Purification kit (GE Healthcare, Little Chalfont, UK) and quantified with a QubitTM Fluorometer (Invitrogen Life Technologies, Eugene, OR, USA). Direct sequencing was then performed using the DYEnamic ET dye terminator cycle sequencing kit (GE Healthcare, Little Chalfont, UK) with n-PCR forward and reverse primers, in the 3500 Genetic Analyzer (Applied Biosystems, Carlsbad, USA). The obtained sequences were examined with the PHRED software (http://asparagin.cenargen.embrapa.br/phph/) for quality analysis by chromatogram readings. The sequences were accepted if the base quality was equal to or higher than 20. Consensus sequences were obtained by using the CAP3 software, and the sequence identity was compared with similar sequences deposited in the GenBank by using the nucleotide BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results and Discussion

The serological test showed serovar Hardjo to be the most common serovar in all of the serum samples evaluated, with titers between 20 and 1600. Table 1 presents the results of serological and molecular monitoring by n-PCR of the urine samples from the animals studied, comparing samples collected at five different periods.

Table 1. Results of MAT for serovar Hardjo and n-PCR assays on urine samples collected from reproductive-aged cows from a farm located in the Northern region of the Paraná State, Brazil.

Animal	1st collection (November/09)		2nd collection (March/10)		3rd collection (July/10)		4th collection (November/10)		5th collection (March/11)	
	MAT	n-PCR	MAT	n-PCR	MAT	n-PCR	MAT	n-PCR	MAT	n-PCR
1	400	+	100	+	200	+	800	+	400	-
2	800	-	100	-	100	-	400	+	100	+
3	800	-	100	-	200	-	400	-	400	-
4	400	+	100	+	Slaughtered	Slaughtered	Slaughtered	Slaughtered	Slaughtered	Slaughtered
5	800	+	200	+	100	-	Slaughtered	Slaughtered	Slaughtered	Slaughtered
6	1600	+	400	-	1600	-	800	+	800	+
7	800	+	100	+	200	-	200	+	Slaughtered	Slaughtered
8	400	-	100	+	100	+	200	-	Slaughtered	Slaughtered
9	400	-	80	-	200	+	100	+	100	-
10	800	+	200	-	800	+	1600	+	400	-
11	400	-	80	-	100	+	100	+	100	-
12	1600	-	200	-	400	+	800	-	800	-
13	200	-	100	+	200	-	80	-	200	-
14	400	-	80	-	200	+	100	-	100	-
15	1600	-	200	-	100	-	400	-	200	-
16	400	-	100	-	400	-	100	+	400	-
17	400	-	80	-	200	+	100	+	100	+
18	800	-	200	-	400	-	800	+	200	-
19	200	-	80	-	100	+	100	+	100	-
20	200	-	200	-	100	-	100	-	80	-
21	100		100		100		100		100	
22	200		100		100		100		80	
23	40		40		80		20		20	
24	80		80		80		100		100	
25	400		100		200		200		400	
26	80		80		100		100		100	
27	40		40		40		80		80	
28	80		80		80		100		80	
29	80		100		100		100		80	
30	40		40		40		100		80	
31	20		20		40		80		80	
32	200		100		400		100		200	
33	80		80		100		20		80	
34	200		200		800		100		100	
35	40		200		400		100		100	
36	100		100		100		100		80	
37	80		80		100		100		80	
38	200		100		100		100		80	

Continue...

Continuation...

39	20	20	20	40	40
40	80	80	80	100	80
41	100	100	100	200	200
42	200	100	200	200	200
43	20	40	40	40	40
44	100	100	100	100	200
45	80	80	100	100	80
46	400	100	200	400	200
47	800	400	200	200	400
48	100	80	80	100	80
49	200	80	80	100	80
50	50	80	100	100	80

Although there was no successful leptospiral isolation from any urine sample after up to six months of incubation with weekly evaluations, the sequenced leptospiral amplicons shared 100% sequence identity with *Leptospira interrogans* which, together with the serological profile, indicates the serovar Hardjo as the most likely infected these animals. Table 2 shows that two of five animals presenting Hardjo-specific antibody titers <1:100 were positive in n-PCR and remain renal patients with intermittent shedding of these bacteria in urine.

Table 2. Results of n-PCR assays on urine samples, which presented MAT titers <1:100 for serovar Hardjo, collected from reproductive-aged cows from a farm located in the Northern region of the Paraná State, Brazil.

Animal*	1st collection (November/10)		2nd collection (March/11)	
	MAT	n-PCR	MAT	n-PCR
23	20	-	20	-
27	80	-	80	+
31	80	-	80	-
39	40	-	40	-
43	40	+	40	-

*Corresponding animals numbering of Table 1.

Reproductive disorders of infectious origin in cattle are considered to be multietiological because different microorganisms such as bacteria, viruses, fungi and protozoa may be responsible for the impaired reproductive performance of affected herds. Serological techniques have provided a substantial of information regarding the epidemiological profiles of cattle herds relative to certain etiologies (JUNQUEIRA; ALFIERI, 2006). In this study, the animals were found to be negative for brucellosis, IBR and BVD indicating no circulation of these pathogens in the herd. Serological studies in cattle have shown that *Leptospira* infections are widespread in Brazil (FIGUEIREDO et al., 2009; HASHIMOTO et al., 2010; HASHIMOTO et al., 2012; HERRMANN et al., 2012; SILVA et al., 2012). Hardjo is considered to be the most common serovar in this animal species and has been reported as a major cause of infertility and abortions in seropositive herds (LILENBAUM; MARTINS, 2014).

Monitoring the cattle for 540 days allowed serological profiling of the herd. The high frequency of seropositive animals and the fluctuations of antibody titers in the serum samples collected suggest that *Leptospira* serovar Hardjo may be considered responsible for the reproductive problems of the affected animals.

The isolation of *Leptospira* spp is considered to be the definitive technique for the diagnosis of leptospirosis and allows identification of the infecting serovar. This technique is considered to be laborious, requires weekly monitoring and can require months for the detection of any growth (FAINE et al., 1999). Recently in Brazil, *Leptospira* serovar Hardjo was isolated and molecularly characterized as genotype Hardjobovis (CHIDEROLI et al., 2016). In the present study, it was not possible to isolate *Leptospira* spp.; however, the results obtained by serological tests and n-PCR and amplicon sequencing confirmed the presence of the microorganism in the urine of the animals studied and that *Leptospira* spp. was the causal agent of reproductive problems in this herd.

Antibodies against serovar Hardjo were found to be the most common in the serological tests performed, suggesting that this serovar could be the circulating in the herd. Some animals were shown to have titers of 1:1,600 for this serovar (Table 1). These results agree with another author (KIRKBRIDE, 1990), which found that antibody titers against serovar Hardjo are generally low and, rarely exceed 1:800. Serovar Hardjo is considered to be an antigen of low immunogenicity that induces low antibody titers for a short period of time (TABATA et al., 2002; VERMA et al., 2012). MAT antibody titers < 1:100 against serovar Hardjo, as detected in this study, have also been reported. By experimentally monitoring heifers infected by serovar Hardjo, it has been demonstrated that significant MAT titers (≥ 1:100) were maintained for a week or two and then declined to titers of 1:10 or 1:30 (DHALIWAL et al., 1996). Antibody level fluctuations as observed in this study may be attributed to several factors, such as contact with other animal species, other existing serovars in the herds, and environmental and climate conditions, as well as management practices and opportunities for re-infections (ELLIS, 2015; LILENBAUM; SOUZA, 2003; LILENBAUM; MARTINS, 2014). Generally, antibody titers against *Leptospira* spp. decline quickly (BOLIN, 2003). The gradual increase observed in the titers in this study may be related to the management practices performed on the particular farm.

The cows in this study were raised in an extensive breeding system but confined to a single small paddock during milking. The high concentration of animals in the paddock and the consequent accumulation of *Leptospira* spp in the environment, associated with management failures, could increase the chances of contact with the microorganism, allowing re-infections and increasing antibody titers of the animals.

Many authors consider n-PCR to be a sensitive and specific technique that has been used to detect leptospires in the urine of animals and humans suspected to be infected with leptospirosis (NASSI et al., 2003). However, the sensitivity of the assay may vary according to the primers used. The primers used for n-PCR in this study amplified a highly conserved fragment of the gene that encodes the LipL32 lipoprotein, which is considered to be the main outer membrane lipoprotein of pathogenic leptospires and an important virulence factor, absent in non-pathogenic species (JOUGLARD et al., 2006).

For each of the five sampling dates on which urine was collected, at least one animal was found to be positive by n-PCR (Tables 1 and 2). These animals could act as constant sources of leptospira, leading to infection of susceptible animals and maintenance of the infection in the herd. Probably, the high percentage of animals with positive n-PCR results is due to the unsatisfactory herd management.

Negative n-PCR results may be caused by the intermittent absence of leptospires in the urine (FAINE et al., 1999) or by the presence of inhibitory substances in the urine itself (SCHRADER et al., 2012). Additionally, freezing the sample prior to DNA extraction may cause lysis of the leptospires, and the DNA may subsequently be lost with the supernatant after centrifugation (LUCCHESI et al., 2004). Here, the urine samples were processed without freezing and within two hours after collected to prevent the degradation of the nucleic acid of the bacteria that may occur during freezing. These factors, combined with the intermittent elimination of leptospires in the urine, could explain the negative results obtained by n-PCR showed by several animals presenting high titers in MAT, suggesting recent infections. Another important aspect in leptospira infections is the long period of renal shedding of host adapted serovars, as serovar Hardjo in cattle, extending by several months, rearing new opportunities from infections in the herd (ZUERNER et al., 2011; ELLIS, 2015).

The n-PCR technique was able to detect leptospiral DNA in the urine of cattle with MAT antibody titers < 1:100. This finding demonstrates the importance of renal carriers, especially when these animals are introduced into new herds, which could shed and disseminate leptospires to other animals and potentially to humans, even in the absence of positive MAT titer. In the present study, among of all 17 results of titers < 1:100 two animals (11.7%) were positive in the n-PCR technique. According to Ellis et al (1982), MAT failed to detect antibody titers to serovar Hardjo in 19.60% of the animals suggesting the status of renal carriers occurs with a certain frequency. These data demonstrate that decreasing the cutoff point (<100) is not sufficient to detect renal carriers, so it is also important to use n-PCR as an additional diagnostic tool to detect those infected animals with serovar Hardjo whose serology was negative.

Epidemiological studies of leptospirosis in cattle herds are very important because they contribute to the monitoring of animal health, enabling the control of infection and minimizing losses. However, most studies focused on bovine leptospirosis are short-term, and the evolution of the infection within the herd is not monitored. In the present study, monitoring the herd for 540 days allowed clarification of the serological profile and provided evidence of leptospire shedding by urine even when the serology do not detect antibodies (cutoff ≥ 100).

Conclusion

The results suggested that the use of n-PCR should be considered (when possible) an additional diagnostic tool, particularly, for the identification of renal carriers. From these results, further studies should be carried out using a larger number of herds and animals in order to better infer a standardization of the MAT cutoff point for renal carriers.

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