Complete diagnosis of leptospirosis in tropical reproductive cattle

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Abstract

Leptospirosis is a worlwide zoonosis of great impact in both animal and public health. Bovine leptospirosis is commonly manifested by reproductive disorders, such as abortion, stillbirth and infertility; causing depletion of the economic balance of livestock farms, along with representing a health risk problem for farm workers. In view of these consequences, we aimed to evaluate the sanitary status of tropical cattle and their role as reproductive disseminators of leptospirosis. We analyzed blood and

semen samples from 11 brazilian herds by three diagnostic methods -Culture, Microscopic Agglutination Test and Polymerase Chain Reaction. All animals were negative for bacteriological culture in Fletcher's semisolid medium; 66% (264/400) animals were seropositive to at least one of 19 serovars (17 serogroups) of *Leptospira* spp. by MAT, given that 42.4% and 5.3% of animals presented titers against brazilians isolates Guaricura and Nupezo, respectively; furthermore, five animals were positive by PCR in blood and/or culture samples and three semen samples were positive by PCR (one of them also seropositive). These results highlight the coexistence of both disease's stages (acute and chronic) in the same environment, thus alert for venereal dissemination of leptospirosis, aggravating their sanitary condition and fomenting economic losses. We, authors, recommend the adoption of prophylactic measures, such as systemic vaccination, treatment of animals and improvement of hygienic-sanitary conditions.

Keywords: leptospirosis; diagnosis; culture; MAT; PCR; cattle

Introduction

Among reproductive diseases that affect livestock, leptospirosis has a high degree of importance, specially in tropical countries (1). This zoonosis is caused by members of genus *Leptospira* spp., which currently includes 22 species and more than 300 serovars (2). Bovine leptospirosis represents an animal health problem, given its common manifestation like chronic reproductive disorders, such as abortion, stillbirth and infertility (3,4). Clinical signs of the acute presentation and mortality are most frequent in calves (5). Furthermore, leptospirosis also represents a public health risk for farm workers along with decrease in economic balance of livestock farms (6). For diagnosis of leptospirosis, many current options are avaliable depending on sample type and clinical phase of disease, each method has its own advantages and disadvantages. Microscopic aglutination test – MAT, remains the reference test for

55 routine diagnosis (7,8). Concordance among positive MAT results and positive
56 outcomes by other techniques has proved that MAT is an efficient diagnostic test for
57 prediction of infecting serogroup (9); althought, another study proved that cattle may
58 not react against their own isolate by MAT, demanding caution when evaluating
59 negative MAT results for carrier status (10). Countries with strong livestock production,
60 like Brazil, have a particular concern on elucidate important features regarding
61 leptospirosis. Many research groups aim to explain the dissemination and risk factors
62 of this disease (11). Throughout the years, the interest on understanding leptospirosis
63 pathogenesis has led to studies envolving possible transmission routes. Venereal
64 transmission has attracted attention since the report of genital Hardjo infection in
65 naturally infected cattle (12), and presence of *Leptospira* spp. was demonstrated in
66 bovine semen by PCR (13), proving that the bacteria can also be established in
67 gonads, suggesting possible sexual transmission. These informations should be
68 considere when assessing disease's control programs, along with endemic serovars,
69 antibiotic and vaccination availability, besides epidemiological studies (14,15). In this
70 context, we aimed to evaluate the sanitary status of this zoonosis in clinically
71 asymptomatic cattle from a tropical region of Brazil.
72

73 ## Material and Methods

74 The area chosen for this study was the microregion of Bauru, which is a country town
75 of São Paulo, Brazil. Bauru is a tropical area known for its hot temperature, typically
76 from 59 F to 86 F; with extreme seasonal variation in rainfall, the least rain falls in
77 winter and the most rain falls around summer, encompassing up to 12 inches.
78 In order to estimate sample size for this study, we used the online program Epi Info^{TD}
79 (http://www.openepi.com), with confidence interval of 95% and based on a preview
80 study that found prevalence of 58.7% in the city of Bauru, years before (16). Sample
81 size calculated for disease frequency was 373 samples.

We analyzed blood samples of 11 herds from five farms (representing 400 animals); as to semen samples from two herds. Cattle were raised for reproductive purposes and shared water fountain and pastures among most herds studied. All farms presented historic of reproductive failures and no vaccination program installed for leptospirosis. Animals studied were clinically asymptomatic for leptospirosis. Approximately 5-10 ml of blood samples were collected using sterile syringes, by caudal tail vein venipuncture, and evacuated into one tube with anticoagulant and one without it. Semen samples were collected by electroejaculation (Electro-Ejaculator DUBOI), approximately 2 – 5 ml of semen were obtained from each animal. All samples were identified, refrigerated and transported to the Animal Sanity Laboratory of Paulista Agency of Agribusiness Technology, APTA, Bauru, Brazil. At the laboratory, blood and semen samples were inoculated in Fletcher medium for bacteriological isolation, employing a serial dilution technique with some modifications (17). From all dilutions, 0.5 mL were inoculated into a tube containing semisolid Fletcher's culture medium (Difco®) with 0.15% agar, supplemented with 100 µg of 5-fluorouracil/mL and 1% sterile rabbit serum and inactivated at 56 °C for 30 min. Cultures were incubated at 28-30 °C for 16 weeks. Dark-field microscopy evaluation of tubes was performed every two weeks. For detection of anti-*Leptospira* antibodies, Microscopic Agglutination Test (MAT) was used with a panel of 19 serovars (including two native isolates) representing 17 pathogenic and intermediate serogroups (table 1), according to international standards (18). Serum samples were considered reactive when reached titers ≥ 100 and the ultimate reactive serogroup was determined by election of the highest titer presented; when presence of coagglutinations, all serovars involved were considered as positive.

Table 1. Serovars used in Microscopic Agglutination Test (MAT).

SPECIES	SEROGROUP	SEROVAR	STRAIN
L. interrogans	Australis	Bratislava	Jez-Bratislava
L. kirschneri	Autumnalis	Butembo	Butembo

L. borgpetersenii	Ballum	Castellonis	Castellón
L. interrogans	Bataviae	Bataviae	Van Tienen
L. interrogans	Canicola	Canicola	Hond Utrecht IV
L. weilli	Celledoni	Whitcombi	Whitcombi
L. kirschneri	Cynopteri	Cynopteri	3552 C
L. interrogans	Djasiman	Sentot	Sentot
L. kirschneri	Grippotyphosa	Grippotyphosa	Moska V
L. interrogans	Hebdomadis	Hebdomadis	Hebdomadis
L. interrogans	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA
L. borgpetersenii	Javanica	Javanica	Veldrat Batavia 46/RA 94
L. noguchi	Panama	Panama	CZ 214 K
L. interrogans	Pomona	Pomona	Pomona
L. interrogans	Pyrogenes	Pyrogenes	Salinem
L. interrogans	Sejroe	Hardjo	Hardjoprajitno
L. santarosai	Shermani	Shermani	LT 821
L. santarosai	Sejroe	Guaricura	Bov G.
L. interrogans	Canicola	Nupezo	NUP-1

Serum samples were considered reactive when reached titers ≥ 100.

DNA from blood and culture samples (for confirmatory purposes) was extracted for Polymerase Chain Reaction (PCR) using the Illustra Blood Genomic Prep Mini Spin kit (GE Healthcare®), while DNA from semen samples was extracted using DNAzol® reagent, both according to manufacturers' recommendations. Primers employed amplify a fragment of 331 base pairs length (19): LEP 1 (5′ GGCGGCGCGTCTTAAACATG 3′) and LEP 2 (5′ TTCCCCCCATTGAGCAAGATT 3′). The final reaction volume was 25 µL, including 2.5 µL of PCR buffer solution (50 mM KCl and 10 mM Tris–HCl, pH 8.0), 0.75 µL of $MgCl_2$ (1.5 mM), 0.5 µL of dNTP solution (0.2 mM), 0.5 µL of ***Taq Platinum*** DNA (1 U) (Invitrogen®), 0.5 µL of each primer (10 pM), 17.75 µL of ultrapure water, and 2 µL of the DNA extracted from each sample. PCR reaction was conducted in a Mastercycler® gradient thermal cycler (Eppendorf), according to the protocol described by Merien et al., 1992, with modifications. The amplified products were visualized by electrophoresis in 1.5% agarose. Cultures of

122 *Leptospira interrogans* serovar Copenhageni in EMJH medium were used as positive
123 controls, as well as ultrapure water like negative controls.
124

125 ## Results

126 Cultures of all samples were performed to detect presence of *Leptospira* spp. in blood
127 and semen samples. All samples were negative to bacteriological culture, using
128 visualization by dark-field microscope. All eleven herds presented titers against at least
129 one pathogen serogroup of *Leptospira* spp. (minimum of 50% animals reactive per
130 herd); 66% (264/400) of animals were positive to MAT. Most reactive serogroups were
131 Sejroe, serovar Guaricura (local strain) with 112 positive animals and serovar Hardjo
132 with 102 positive animals; and serogroup Autumnalis with 77 positive MAT reactive
133 samples. Highest titers reached 3200 when tested against serovar Guaricura and
134 serovar Hardjo. Coaglutinations reached up to 37.1% of all MAT positive outcomes.
135 Analysis by PCR of blood samples showed some controversial results. Out of all
136 negative blood cultures, four animals turned out to be positive by confirmatory culture
137 PCR. When analyzing direct blood PCR results, we noted that three animals were
138 positive for presence of *Leptospira* spp.; perturbation aroused when confirming that out
139 of the positive samples, only one was positive both in confirmatory culture PCR as to in
140 blood PCR. Thus, confirming the conflicted scenario of having the same theoretical
141 sample positive in one test and negative in the other. None of these positive animals
142 had MAT positive outcomes (table 2).
143

144 **Table 2.** Molecular and serological outcome for bulls positive by PCR in semen
145 and/or blood samples.

Animal	Herd	PCR Blood		MAT
		Sample	Culture	Serum
31	2	-	+	-

33	2	-	+	-
170	6	+	-	-
191	7	+	+	-
327	10	-	+	-
387	11	+	-	-

Analysis by PCR of semen displayed some interesting results as well. Two bulls were positive for confirmatory PCR of negative culture (dark-field microscopy); three bulls were positive for semen PCR and for semen culture confirmation PCR; and other two bulls were positive solely for culture confirmation PCR, but not for direct semen PCR. Among these positive bulls, three were also positive by MAT (table 3).

Table 3. Molecular and serological outcome for bulls positive by PCR in semen and/or blood samples.

Animal	Herd	PCR Blood		PCR Semen		MAT
		Sample	Culture	Sample	Culture	Serum
5	1	-	-	-	+	+
7	1	-	-	+	+	-
27	2	-	-	+	+	-
29	2	-	-	-	+	+
30	2	-	-	+	+	+
31	2	-	+	-	-	-
33	2	-	+	-	-	-

Discussion

In order to evaluate sanitary state of the disease and not just merely exposure to the bacteria, we used serovars from pathogenic species of *Leptospira* spp., one serovar representative from each serogroup and also two native brazilian isolates, serovar Guaricura (*Leptospira santarosai* serogroup Sejroe) and serovar Nupezo (*Leptospira*

canicola serogroup Canicola). Serovar Nupezo showed reactivity when compared to serovar Canicola, supporting the inclusion of local strains for more sensitive MAT results. Serogroup Sejroe had most positive outcomes, however serogroups Autumnalis and Hebdomadis were also quite reactive, suggesting possible contact among cattle with wild animals, given that only serogroup Sejroe is known to be adaptive to cattle and the other two serogroups have been reported in wild animals (20,21). Several studies around Brazilian territory showed the magnitude and importance of leptospirosis in this tropical region, and despite variety of these studies' outcomes, the seriousness can't be disguised (table 4).

Table 4. Prevalence studies about bovine leptospirosis in Brazil.

Region	State	Prevalence	Animals	Frequent serogroups	References
North	Maranhão	35.94%	4832 cows	Sejroe	(22)
Northeast	Bahía	45.42%	10 823 bovines	Sejroe	(23)
Central-west	Mato Grosso do Sul	98.8%	1801 cows	Sejroe	(24)
Southeast	Santa Catarina	65.53%	3945 cows	Autumnalis and Sejroe	(25)
South	Rio de Janeiro	14%	120 cows	Sejroe and Shermani	(26)
	São Paulo	45.8%	2761 bovines	Sejroe	(16)

Considering that 66% (264/400) of cattle were seropositive, furthermore six animals were positive for PCR of blood and/or culture of blood, as three bulls were also positive by confirmatory PCR of semen cultures (one of them confirmed by semen PCR as well), we can suggest there is an imminent present exposure of cattle to the disease in

177 those farms, the same ones that constitute a representation of the extensive livestock
178 production system in this tropical region.
179 PCR outcomes also showed trustworthiness when compared to culture, given that
180 results that were negative by culture were positive by PCR, which was no surprise
181 considering difficultness of visualization of spirochetes by dark-field microscopy (7).
182 Surprise came when comparing PCR results between negative cultures and samples,
183 animals were positive on blood culture but not on whole blood or vice versa, suggesting
184 that there could be an inhibitor in blood that was diluted when cultured. Similar results
185 occurred with two semen samples, thus same explanation can be granted (27).
186 Some current studies have attempt to confirm presence of leptospiral DNA in
187 reproductive secretions. In cattle, two research group evaluated semen of seropositive
188 bulls with unsuccessful results (28,29)), but more recently another research group was
189 capable to detect Leptospiral DNA from vaginal fluid of cows (30). Other studies have
190 been executed in goats and sheeps, with positive results for vaginal fluids and semen
191 (31); and in mares and horses, which lead to detection of leptospiral DNA in vaginal
192 fluids, genital tract and semen (32,33). Thus, the results obtained in this study enhance
193 current knowledge of not only sanitary status of the disease, but to possible
194 participation of sexual transmission in farms representative of the extensive livestock
195 production farming in this tropical region.
196 These farms do not have an established surveillance program for leptospirosis,
197 therefore lack of prevention could be one of the main reasons for the serological and
198 molecular results. We, authors, strongly recommend the adoption of prophylactic
199 measures, such as systemic vaccination, treatment of animals and improvement of
200 hygienic-sanitary conditions, given the results obtained.
201

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