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Cross-sectional study of 13 *Leptospira* serovars in cows in a Colombian dairy region¤

Estudio transversal de 13 serovares de Leptospira en vacas en una región lechera colombiana

Estudo transversal de 13 sorotipos de Leptospira em vacas em uma região leitera colombiana

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Abstract

Background: Leptospirosis in cattle is recognized as a major cause of reproductive loses due to abortions, early embryonic death, and infertility. The type of *Leptospira* serovars that are currently circulating in Colombian herds is largely unknown due to the lack of national reports. **Objective:** The prevalence of antibodies specific to 13 *Leptospira* spp serovars was investigated in unvaccinated cows from 26 herds in San Pedro de los Milagros (Province of Antioquia) dairy region. **Methods:** Microscopic agglutination test was used on serum samples, following WHO recommendations. **Results:** A total of 112/900 (12.4%) of the animals had antibody titers of 1:100 or greater to at least one *Leptospira* serovar. Of those positive, 34/900 (3,8%) animals seroconverted to two or more serovars. In order of decreasing prevalence, the serovars detected were: *hardjo prajitno*, *pomona*, *grippotyphosa*, *tarassovi*, *copenhageni*, *canicola*, *cellodeni* and *bratislava*. There were 28 animals seropositive

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to serovar *hardjo*. **Conclusion:** Our results indicate that leptospirosis should be included as a differential diagnosis for various reproductive problems in this region.

Keywords: *dairy cattle, leptospirosis, MAT, prevalence.*

Resumen

Antecedentes: La leptospirosis es la mayor causal de pérdidas reproductivas debidas a abortos, muerte embrionaria temprana e infertilidad. Se desconocen los serovares de *Leptospira* que actualmente se encuentran circulando en el rebaño colombiano debido a la falta de reportes nacionales. **Objetivo:** Se investigó la prevalencia de anticuerpos específicos para 13 serovares de *Leptospira* spp en vacas no vacunadas de 26 rebaños en la región lechera de San Pedro de los Milagros (Provincia de Antioquia). **Métodos:** La prueba de aglutinación microscópica fue empleada en muestras de suero de acuerdo a las recomendaciones de la OMS. **Resultados:** Un total de 112/900 (12,4%) de los animales presentaron títulos de anticuerpos de 1:100 o superiores para al menos un serovar de *Leptospira*. Entre los positivos, el 34/900 (3,8%) de los animales presentaron seroconversión para dos o más serovares. En orden decreciente de prevalencia, los serovares detectados fueron: *hardjo prajitno*, *pomona*, *grippotyphosa*, *tarassovi*, *copenhageni*, *canicola*, *cellodeni* y *Bratislava*. En total, 28 animales fueron seropositivos al serovar *hardjo*. **Conclusión:** La leptospirosis debería ser incluida como diagnóstico diferencial en problemas reproductivos en esta región.

Keywords: *ganado lechero, leptospirosis, MAT, prevalencia.*

Resumo

Antecedentes: A leptospirose em bovinos é reconhecida como uma das principais causas de perdas reprodutivas devido a abortos, morte embrionária precoce e infertidade. Os sorotipos de *Leptospira* que estão atualmente em circulação no rebanho colombiano não se conhecem devido à falta de reportes nacionais. **Objetivo:** Neste estúdio foi investigada a prevalência de anticorpos específicos para 13 sorovares de *Leptospira* spp em vacas não vacinadas de 26 rebanhos leiteiros da região de San Pedro, Antioquia. **Métodos:** Foi empregado o teste de aglutinação microscópica (MAT) em amostras de soro de acordo com as recomendações da OMS. **Resultados:** Um total de 112/900 (12,4%) animais apresentaram títulos de anticorpos de 1:100 ou superiores para pelo menos um sorotipo de Leptospira. Dos animais positivos, 34/900 (3,8%) soroconverteram para dois ou mais sorotipos. A fim de diminuir a prevalência, foram detectados os sorotipos: *hardjo, prajitno, pomona, grippotyphosa, tarassovi, copenhageni, canicola, cellodeni e Bratislava.* Um total de28 animais foram soropositivos para o sorotipo hardjo. **Conclusão:** Os resultados indicam que a leptospirose deveria ser incluída nos diagnósticos diferenciais para o diagnóstico diferencial de problemas em doenças reprodutivas nos bovinos de Antioquia.

Palavras chave: *gado leiteiro, leptospirose, MAT, prevalência.*

Introduction

Leptospirosis in cattle is recognized by reproductive loses associated with abortion, weak calves, still birth, and infertility (Grooms and Bolin, 2005; Radostitis *et al.*, 2007) as well as mastitis (Garoussi *et al.*, 2007; Radostitis *et al.*, 2007). There are no estimates of the prevalence of leptospiral infection in most Colombian herds. As with other infectious agents, the herd serological profile is frequently the only option available to evaluate the role of these agents in reproductive failure. Basic knowledge of the serovars circulating in a herd is necessary to understand the epidemiology of the disease before establishing control programs (Lilenbaum and Martins, 2014).

Infections with serovar *Hardjo* are typically considered host-adapted in cattle, and positive animals can be chronic carriers and persistent shedders through urine and genital discharges. Serovar *Hardjo* consists of two serologically indistinguishable but distinct species, *L. interrogans* serovar *Hardjo* (type hardjo prajitno) and *L. borgpetersenii* serovar *Hardjo* (type hardjo-bovis; Grooms and Bolin, 2005). While infection by this serovar is usually subclinical, it is the most commonly associated to sporadic abortions and infertility throughout the world (Grooms and Bolin, 2005). By contrast, other serovars are non-host adapted and acute infections can result in abortions (Grooms and Bolin, 2005). Serological testing is the most common procedure for diagnosing leptospiral infections and

the microscopic agglutination test (MAT) is the reference test for serological diagnosis. However, interpretation of serology can be complicated due to cross-reactivity with other serovars, vaccination status and antibody titers. An agglutinating antibody titer of ≥ 100 is considered significant by most laboratories and the World Organization for Animal Health (OIE, 2014). However, this cutoff level may not be reached with some serovar *Hardjo* infections. Therefore, a negative titer does not necessarily rule out infection. Additionally, the test does not indicate whether there is an active infection or a carrier or shedding status (Rajeev *et al*., 2010). Therefore, clinical cases should ideally be complemented with assays such as fluorescent antibody testing (FAT). A combination of these techniques helps to control leptospirosis by identifying urinary shedders to be treated and immunize with vaccines containing the circulating serovars (Libenbaun and Martins, 2014).

The aim of this study was to estimate the prevalence of 13 leptospiral serovars in cattle in a major dairy region of the Province of Antioquia, Colombia.

Materials and methods

A cross-sectional study was conducted in 26 dairy farms between May and June 2014. Farms were located in San Pedro de los Milagros (6°27′34′′N, 75°33′28′′W), at an elevation of 2,500 m.a.s.l. This study was part of a cross-sectional epidemiological survey on parasite and viral infections (Ramírez *et al.*, 2016). The sample size for each farm, number of farms, and ages within each farm was chosen to be proportional to the district population of 65,000 heads, with an overall expected prevalence of 50% for all pathogenes, an error margin of 3.1% (≤5%), and a 95% confidence interval. Out of 1,003 bovines sampled, only 900 animals from 26 herds that were unvaccinated for *Leptospira* spp were eligible to evaluate leptospirosis seroprevalence. In spite of this limitation on the number of animals to accurately estimate prevalence, this is the largest serological survey conducted so far in Colombia to evaluate seroprevalence of *Leptospira* spp in cattle.

The animals were chosen with age groups divided as follows: <1 year (n = 130), 1- <2 year (n = 105), 2-3 year (n = 159), >3 years (n = 506). All animals, except seven, were females. Breeds were Holstein (53.7%), Holstein-Jersey (21%), Jersey (8.37%), Jersey-Holstein (3.3%), others (14%). Farm size was assigned by the number of animals, as follows: smallholder herd (<50 animals), middle (50-100), and big (>100). According to this distribution, 133 (14.8%) animals belong to small size farms, 311 (34.56%) were in medium size, and 456 (50.67%) were in big size farms. Four farms allowed other livestock to trespass their premises, and 21 farms had dogs.

The management system in the area consisted of intensive rotational grazing on Kikuyu (*Penisetum clandestinum*) monoculture pastures with no confinement of adult cows, while calves were usually kept in separate paddocks between the ages of 3-9 months and then moved with replacement heifers until they were ≥ 15 months old. Paddocks were rested for approximately 30-40 days, and occupation days varied with animal grazing density.

Serum collection

Blood samples were collected by a Veterinarian between May and June 2014 from the coccygeal vein in vacutainer tubes with and without anticoagulant and transported to the laboratory within 12 h. Samples were then centrifuged at 1500 x *g* for 10 min to harvest serum within 24 h. Serum samples were stored at -20 °C until analysis. Antibodies against 13 *Leptospira* serovars (Table 1) were measured using MAT.

Current recommendations by the World Organization for Animal Health were followed to interpret the MAT results, such that titers ≥ 100 and <100 were classified as positive and negative, respectively (OIE, 2014). The reference strains and controls were obtained from the Royal Tropical Institute, Holland.

Statistical analysis

Statistical analyses were performed using Stata 12.0 (StataCorp, 2011). An initial descriptive analysis was followed with a logistic regression to calculate —from at least one of the animals (1/100)— the

Table 1. *Leptospira* reference serovars used as antigens in microscopic agglutination test for serological analysis of bovine leptospirosis in dairy cattle from San Pedro de los Milagros (Province of Antioquia).

Species	Serogroup	Serovar	Strain
L. interrogans	*Autumnalis*	*Autumnalis*	*Akiyami A*
L. interrogans	*Bataviae*	*L. interrogans*	*Bataviae*
L. interrogans	*Australis*	*Bratislava*	*Jez Bratislava*
L. interrogans	*Canicola*	*Canicola*	*Hond Utrecht IV*
L. weilii	*Celledoni*	*Celledoni*	*Celledoni*
L. interrogans	*Icterohaemorrhagiae*	*Copenhageni*	*Fiocruz L1-130*
L. kirschneri	*Cynopteri*	*Cynopteri*	*3522 C*
L. kirschneri	*Grippotyphosa*	*Grippotyphosatypemoskva*	*Moskva V*
L. interrogans	*Sejroe*	*Hardjotypeprajitno*	*Hardjoprajitno*
L. borgpetersenii	*Mini*	*Mini*	*Sari*
L. interrogans	*Pomona*	*Pomona*	*Pomona*
L. santarosai	*Shermani*	*Shermani*	*1342 K*
L. borgpetersenii	*Tarassovi*	*Tarassovi*	*Perepelitsin*

association between *Leptospira* spp seropositivity to the serovars and the predictors of interest (such as sex, age, number of animals, dogs in the farm, bull leasing, neighbors trespassing, and rodent control). All variables with a $p<0.05$ were considered significant although variables with a $p<0.1$ were also considered potentially associated to the response variable. Results are presented as odds ratio (OR) along with their 95% confidence interval (CI). To account for the clustering of the data (cows that were clustered within herds) an unconditional mixed−effects logistic regression model analysis with herd as group variable was conducted for selected variables. The Hosmer-Lemeshow criteria ($p<0.25$) were used to retain variables for the multivariable model.

Results

The number of animals with positive antibody titers to any of the 13 *Leptospira* serovars was 112 (12.4%) of the 900 samples analyzed. Serum samples with titers greater than 1:100 for each serovar are shown in Table 2. From the positive cases, 78, 24, 7, and 3 animals were positive to one, two, three and four serovars, respectively. No samples reacted beyond a 1:1600 dilution for any of the *Leptospira* serovars examined. No animals were positive to *Autumnalis, Bataviae, Cynopteri, Mini, or Shermani* serovars. Out of 112 seropositive animals, 69 (86.2%) were older than 3 years. Big herds had the highest positivity (41%), followed by small ones (35%) and middle ones (24%). The farm with the highest number of *Leptospira* spp seropositive cows (15 cows) had no animals seroconverting to *bratislava*, 3 to *hardjo*, 0 to *celledoni,* 3 to *copenhageni,* 6 to *grippothyphosa*, 3 to *hardjo prajitno,* 7 to *Pomona,* and 5 to tarassovi. As it can be observed, some cows had coinfection with several serovars.

The final output of the multivariable logistic regression model showed a tendency for increasing likelihood of positive antibody titers in older cows compared to younger ones. The odds of having a cow positive to *Leptospira* spp was 2.02 and 1.78, for cows between 2 to 3 years and cows older than 3 years, respectively ($p<0.1$) compared to animals younger than one year of age. Herd size showed association with positive antibody titers to *Leptospira*. The odds of having a cow positive to *Leptospira* was significantly lower in Medium (between 51 and 100 cows) and large-size herds (greater than 101 cows) with an OR of 0.17 and 0.21, respectively ($p<0.01$) compared with herds of less than 51 cows.

Table 2. Frequency of positive antibody titers (≥ 1:100) for seven *Leptospira* serovars in sera from 900 unvaccinated cows of San Pedro de los Milagros (Province of Antioquia).

Serovar	1:100	1:200	1:400	1:1600	No. of serum samples (% of 900)	Serovar frequency (% of 157)
Leptospira interrogans serovar *bratislava*	8	3			11 (1.23)	7
Leptospira interrogans serovar *canicola*	11	2			13 (1.45)	8,3
Leptospira interrogans group *icterohaemorrhagiae* serovar *copenhageni*	13	1			14 (1.56)	8,9
Leptospira kirschneri serovar *grippotyphosa*	23	3		1	27 (3.01)	17,2
Leptospira interrogans serovar *hardjo prajitno*	22	6			28 (3.12)	17,8
Leptospira interrogans serovar *pomona*	23	3	2		28 (3.12)	16,6
Leptospira borgpetersenii serovar *tarassovi*	24	1			25 (2.79)	16
Leptospira weilii serovar *celledoni*	13	0			13 (1.45)	8,3
Total	137	19	2	1	159 (17.7)	100

Table 3. Final logistic regression model assessing the effect of selected herd and cow variables on the probability for positive antibody titers to *Leptospira* spp in dairy cows of San Pedro de los Milagros (Province of Antioquia).

Variable	Odds ratio	SEM	p-value	95%CI
Age (years)				
<1	Referent			
1-2	1.04	0.52	0.98	0.4-2.8
>2 -3	2.03	0.81	0.07	0.9-4.4
>3	1.8	0.62	0.09	0.9 - 3.5
Herd size				
Small	Referent			
Medium	0.17	0.10	0.005	0.05-0.58
Large	0.21	0.12	0.008	0.07-0.66

SEM: Standard error of the mean. CI: Confidence interval. P-value: Statistical significance.

Discussion

Overall, the results showed little serological evidence of widespread exposure to either host-adapted (i.e. *hardjo*) or non-adapted leptospiral serovars in the cattle population examined. Only 28 of the 900 animals sampled had positive titers against *L*. serovar *hardjo* and titers for the other incidental serovars were very low, suggesting a chronic, convalescent or recovered state to previous leptospiral exposures. However, distinguishing between acute and convalescent states of the infection would have required doing titers in paired serum samples. Interpretation of individual titers is always difficult and subjective. In several diagnostic laboratory web sites, *hardjo* titers of 1:400 or more are considered strong evidence of recent infection in the absence of vaccination. For non-adapted serovars, titers in acute infections tend to be much higher than 1:400. In the present study, there were only three animals with a high level of seroconversion, two animals with 1:400 for *L. Pomona* and one animal with of 1:1600 for *L. grippotyphosa* that suggested a recent exposure.

There were 34 serum samples that tested positive to more than one serovar. It is known that different *Leptospira* serovars cross-react in MAT (Grooms and Bolin, 2005); in fact, some studies have shown

that the odds of a sample testing positive to other serovars can be as high as 10 times compared with a sample that tested negative to a former serovar (Peregrine *et al.*, 2006). From a practical standpoint, diagnostic laboratories generally assign the serovar with the highest titer to the infecting serovar, and interpret results in the context of clinical findings and vaccination history. In addition, because *hardjo*-infected cattle often have a poor agglutinating response to infection, a cutoff level of 1:100 may not be reached in some *hardjo* infections, yielding false-negative results. Obviously, these factors complicate the interpretation of the MAT results. Techniques aimed to identify the organism together with improvements in MAT sensitivity by including local strains in the usual reference-strain panels have been recommended to overcome some of these limitations (Pinto *et al.*, 2015).

The relationship between sero-status and a few risk factors was also examined. The tendency to increase the likelihood of positive antibody titers in older cows compared to younger animals ($p<0.1$) is consistent with a greater time of exposure from previous infections since vaccinations had not been used in these farms. This tendency for positive animals to increase with age has also been observed in other surveys (Prescott *et al.*, 1988; Peregrine *et al.*, 2006). Our study did not include whether *Leptospira* antibody status was associated with non-pregnancy. However, other studies have shown that increases in *Leptospira* titers for various serovars are associated with greater odds of non-pregnancy (Van De Weyer *et al.*, 2011).

None of the farms shared bulls for breeding, which is another known risk factor. Since natural breeding and direct contact with urine of carrier animals are the most common mechanisms of transmission, farms interested to establishing control measures should consider testing urine and genital tract secretions of chronic carriers with sensitive diagnostic tests such as polymerase chain reaction (PCR). Controlling this pathogen requires a combination of biosecurity, antibiotics, vaccination and culling. Transmission of *Leptospira* via contaminated urine could be prevented by treating animals with streptomycin or other long-acting adequate antibiotic (Gerritsen *et al.*, 1993). Ideally, this should be combined with a vaccination program to reduce the incidence of new infections. In conclusion, our results indicate that leptospirosis should be included as a differential diagnosis in cases of various reproductive problems in these farms; however, there is currently no information to propose leptospirosis control measures in Colombian herds. Further studies are necessary to identify if there are any health and production effects due to leptospirosis that would justify measures to control or limit any identified negative impacts.

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Conflicts of interest

The authors declare they have no conflicts of interest with regard to the work presented in this report.

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