

Prevalence and risk factors associated with equine leptospirosis in an endemic urban area in Southern Brazil**Prevalência e fatores de risco associados com a leptospirose equina em uma área urbana endêmica no sul do Brasil**

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ABSTRACT

The burden of equine leptospirosis in Pelotas (RS) remains unclear due to underreporting. In this city, socially vulnerable families use these animals to collect food and recyclable material. In this context, this study aimed to know the seroprevalence of leptospirosis in urban working horse population in Pelotas, and to assess risk factors for equine leptospirosis in the study area. Serum samples from 119 horses were analysed by microscopic agglutination test (MAT). As results, 28.57% (n=34) animals were positive in the MAT with titres equal or above 400. Canicola was the most prevalent serovar in this study, followed by Hardjo, Bratislava, and Ballum. Exposure to risk factors was assessed and the occurrence of abortions ($p = 0.04$) and the occurrence of stillborn foals in the establishments ($p = 0.04$) were associated with the disease. Furthermore, abortion itself was associated with factors usually regarded as having leptospirosis risk, these were: Grazing on open pastures ($p < 0.01$); the presence of rodents in or around the house ($p < 0.01$); and forage being kept in sheds ($p = 0.03$). Conversely, fodder stored in plastic barrels (with lids) seems to reduce the risk of abortion ($p = 0.01$). Thus, urban working horses used to collect food and recyclable materials in different parts of the city may be potential spreaders of the disease to humans and other animal species. In addition, our study indicates that these animals may be viable sentinels in the research of circulating serovars in urban settings.

Keywords: equine, *Leptospira*, public health, neglected zoonosis.

RESUMO

A importância da leptospirose nos equinos em Pelotas (RS) não é clara devido à subnotificação. Nesta cidade, famílias socialmente vulneráveis usam esses animais para coletar alimentos e materiais recicláveis. Nesse contexto, este estudo teve como objetivo conhecer a soroprevalência da leptospirose na população urbana de cavalos de trabalho em Pelotas e revelar os fatores de risco para leptospirose equina na área de estudo. Amostras de soro de 119 equinos foram analisadas através da técnica de soroprecipitação microscópica (MAT). Como resultado, 28,57% (n = 34) dos animais foram reagentes no MAT com títulos iguais ou superiores a 400. Canicola foi o sorovar mais prevalente neste estudo, seguido por Hardjo, Bratislava e Ballum. A exposição a fatores de risco foi avaliada e a ocorrência de abortos ($p = 0,04$) e a ocorrência de potros natimortos nos estabelecimentos ($p = 0,04$) foram associadas com a enfermidade. Além disso, o aborto foi associado a fatores comumente considerados de risco para leptospirose, sendo eles: Pastagem em campos abertos ($p < 0,01$); presença de roedores

dentro ou ao redor da casa ($p < 0,01$); e forragem sendo mantida em galpões ($p = 0,03$). Por outro lado, as forragens armazenadas em barris de plástico (com tampa) parecem reduzir o risco de aborto ($p = 0,01$). Assim, os equinos de trabalho urbano usados na coleta de alimentos e materiais recicláveis nos diferentes pontos da cidade podem ser potenciais disseminadores da enfermidade para seres humanos e outras espécies animais. Além disso, nosso estudo indica que estes animais podem ser sentinelas viáveis na pesquisa de sorovares circulantes em cenários urbanos.

Palavras-chave: equinos, *Leptospira*, saúde pública, zoonose negligenciada.

1 INTRODUCTION

Leptospirosis is a global disease of animals and is an important zoonosis. In horses, leptospirosis can cause considerable economic consequences, especially in mares, through abortions, stillborn foals, embryonic absorption, perinatal deaths, and the birth of weak foals (Ellis 2015). Noticeable clinical signs in horses will usually include weight and performance loss, reproductive complications and recurrent uveitis, however, subclinical leptospirosis is much more common. In acute cases, fever, anorexia, anaemia, icterus, and petechial haemorrhaging of mucosae may occur, culminating in respiratory symptoms (Verma et al. 2013).

Pelotas is a subtropical city in southern Brazil placing it among the cities with the highest incidence of leptospirosis in the country (Schneider et al. 2015). In this city, horses are used to pull carts for transportation and, mostly, collection of recyclables, often venturing to different parts of a city in a single day. This increases the animal's chance of exposure to *Leptospira*, and offers a chance for those shedding the bacteria, to spread it (Hamond et al. 2013). The urban setting is ideal for the transmission of the disease, especially when associated with poor sanitary and social conditions (Vasconcelos et al. 2012). In southernmost Brazil, equine leptospirosis varies greatly. Studies that considered draft horses in urban settings show prevalences of 60% and 75% (Finger et al. 2014; Lasta et al. 2013).

Direct observation and/or isolation of *Leptospira* spp. is considered the best way to diagnose the disease, however, isolation may be time consuming and laborious (Ellis 2015). For epidemiological surveys, the microscopic agglutination test (MAT) is considered the ideal assay for both human and animal leptospirosis (Adler et al. 2010). In this light, this study aimed to know the prevalence of leptospirosis in urban working horse population in Pelotas, and to assess risk factors for equine leptospirosis in the study area.

2 MATERIAL AND METHODS

2.1 STUDY POPULATION AND SAMPLING

This study was developed in a veterinary outpost of the animal hospital of the school of veterinary medicine, Universidade Federal de Pelotas (UFPel). This outpost services the local population of a low income neighbourhood in the city of Pelotas, Rio Grande do Sul state, Brazil, the southernmost state in the country. Sample size was calculated considering a population size of 172 horses, this was the number of animals that were attended in the previous year (2015). Considering the population, a confidence interval of 95% (C.I. 95%), an expected prevalence of 50%, and 5% error (Thrusfield 2007), the necessary sample would be 119 animals. In our study, there were no exclusion criteria, nor resampling of the same animal. Blood samples were harvested from the jugular vein in 5mL vacutainer™ tubes. After harvesting, sera were separated and stored at -20 C until needed.

2.2 EPIDEMIOLOGICAL ENQUIRY AND STATISTICAL ANALYSIS

Prior to sample harvesting, the animal's caretaker/owner was asked to sign a consent form, and inquired regarding multiple possible risk factors or epidemiological cues that may be associated with leptospirosis in horses. A total of 38 variables were considered. All statistics were carried out on the Statistical Package for the Social Sciences (SPSS software), version 22.0; or on Epiinfo 7. A two tailed chi square test was used to compare exposure and outcome in 2x2 contingency tables. A power of 95% was used, with p values of 0.05 or less considered statistically relevant.

2.3 MICROSCOPIC AGGLUTINATION TEST (MAT)

The serological assay was executed with a panel of 13 antigens (12 pathogenic and 1 saprophytic) provided by the *Coleção de Leptospira (CLEP)/Laboratório de Referência Nacional para Leptospirose (LNRNL)*, FIOCRUZ-RJ. They were: Australis, Autumnalis, Bataviae, Bratislava, Canicola, Copenhageni, Grippothyphosa, Hardjo, Icterohaemorrhagiae, Pomona, Pyrogenes, and Patoc1. The assay was executed according to recommendations (WHO 2003). Briefly, *Leptospira* strains were cultured in liquid Ellinghausen–McCullough–Johnson–Harris (EMJH) medium (Difco laboratories) at a temperature of 29 °C, and sub cultured every seven days. For the MAT procedure, bacteria concentrations were adjusted to 2×10^8 leptospires/mL. Samples were initially diluted 1:200 in sterile saline, pH 7.2, and then challenged against the specific antigens at a proportion of 1:1, resulting in a test titre of 1:400, which was considered the cut-off titre for acute animal leptospirosis (Goris et al 2012). Those

samples that were seroreactive at this titre were then retested at serial two-fold dilutions until 1:6400. Sera were considered reactive when agglutination occurred >50% when compared to control.

2.4 ISOLATION PROCEDURE

In particular cases, when the owner of the animal has authorized, samples of blood, urine and tissues were inoculated in tubes containing 5 ml of EMJH (Difco Laboratories) liquid and semi-solid medium, for *Leptospira* isolation attempt. Cultures were incubated at 29°C and examined weekly, by dark-field microscopy. When growth was detected, successive transfers were made in liquid and semisolid media until growth was sufficiently abundant (Silva et al. 2010).

2.5 ETHICS

All procedures were carried out according to the Brazilian animal experimentation control council (*Conselho Nacional de Controle da Experimentação Animal - CONCEA*). The project was registered in the coordinator council for teaching and research of the Federal University of Pelotas (UFPe) (*Conselho Coordenador de Ensino e Pesquisa - COCEPE UFPe*), and approved by the ethics committee of this institution (*Comitê de Ética em Experimentação Animal - CEEA UFPe*, protocol number 4637-2015).

3 RESULTS

A total of 119 different horses sought health aid at the HCV outpost in Ceval. One blood sample was obtained from each animal (those that returned for subsequent appointments were not resampled). All the animals were over seven years old, breedless, draft horses (at the time of sampling, those that were not currently being used to pull carts were pregnant females). Of the sampled animals, 76 (63.87%) were male, and 43 (36.13%) were female.

Of the 119 samples obtained, 34 (28.57%; C.I.95% 21.22–37.26) were seropositive for at least one antigen in the MAT, at a trial titre of 1:400. Complete results regarding positivity, including reactive serovar and titre, can be found on Table 1.

Table 1: Microscopic agglutination test results according to the highest titre and corresponding serovar. Of the 119 horses sampled, 34 were positive (28.57%). Results presented regard only seroreactive animals

Serovars	Titre		Total (%)
	400	800	
Canicola	12	-	12 (35.29)
Bratislava	4	1	5 (14.71)
Hardjo	4	-	4 (11.76)
Ballum	2	2	4 (11.76)
Copenhageni	2	2	4 (11.76)
Australis	2	1	3 (8.82)
Grippotyphosa	2	-	2 (5.88)
Total	28	6	34 (100)

Practically all titres observed on table 1 are associated with infection, considering that only three (2.5%) horses were reported to have been vaccinated, and could be presenting vaccinal antibodies. Reactions in the MAT regarding possible risk factors are individually listed on tables 2, 3, and 4.

Table 2: Questions regarding the horses' environment, and their association with seroreactivity for leptospirosis in draft horses assessed in the city of Pelotas (RS)

Exposition	P.R.	C.I.95%	p
Other animals			
Cattle?	0.49	0.13-1.83	0.38
Sheep?	NA	NA	0.55
Pigs?	1.52	0.67-3.43	0.40
Dogs?	0.90	0.25-3.14	1
Cats?	0.44	0.18-1.06	0.09
Animals' water source			
Piped (city of Pelotas)?	1.23	0.36-4.12	1
Watering hole?	1.28	0.35-4.57	0.74
Stream?	0.61	0.06-5.69	1
Bog?	0.53	0.14-2.01	0.55
Where the animals stay			
At night. in stables?	2.16	0.44-10.4	0.50
At night. in a pen?	1.25	0.21-7.16	1
Are the pastures humid?	0.72	0.32-1.63	0.53
Are the stables humid?	1.02	0.45-2.32	1

P.R = Prevalence Ratio; C.I = Confidence Interval

Factors associated with positive reactions were, briefly: Food stored in a separated room was ($p=0.04$); Abortions ($p=0.04$); and the occurrence of stillborn foals in the establishment ($p=0.04$). Furthermore, abortion itself was associated with factors usually regarded as having leptospirosis risk, these were: Grazing on open pastures ($p<0.01$); the presence of rodents in or around the house ($p<0.01$); and forage being kept in sheds ($p=0.03$). Conversely, fodder stored in plastic barrels (with lids) seems to reduce the risk of abortion ($p=0.01$).

Table 3: Questions regarding the horses' feeding habits and the presence of rodents, and their association with seroreactivity for leptospirosis in draft horses assessed in the city of Pelotas (RS)

Exposition	P.R.	C.I.95%	p
Presence of rodents in animal fodder?	1.18	0.41-3.43	0.78
Presence of rodents in the home?	2.78	0.88-8.76	0.09
Do you promote rodent control?	1.25	0.45-3.49	0.80
Do other animals have access to the horse fodder?	1.70	0.27-10.7	0.62
Regarding animal fodder/feeding:			
Free grazing on fields?	1.25	0.45-3.49	0.80
Commercial horse feed?	0.71	0.24-2.12	0.60
Corn?	1.30	0.57-2.94	0.54
Silage?	NA	NA	0.55
Homemade horse feed?	1.92	0.77-4.78	0.16
Where is the food stored:			
Shed?	0.89	0.26-3.03	1
Separate room?	3.41	1.05-11.05	0.04
Inside the house?	0.42	0.08-2.00	0.34
Plastic barrel (with lid)?	0.65	0.28-1.50	0.40
Steel barrel (no lid)?	1.44	0.56-3.64	0.46

P.R = Prevalence Ratio ; C.I = Confidence Interval

An abortion occurred during a clinical examination in a mare at CEVAL. *Leptospira* isolation was obtained from excised kidney material from the fetus. Positive culture was sub-cultured into liquid medium and it was sent for virulence determination, serogrouping and molecular typing (data not shown).

Table 4: Questions regarding clinical and reproductive signs and their association with seroreactivity for leptospirosis in draft horses assessed in the city of Pelotas (RS)

Exposition	P.R.	C.I.95%	p
Vaccinated against leptospirosis?	NA	NA	0.55
Abortions in the establishment?	2.76	1.11-6.85	0.04
Stillborn foals in the establishment?	2.77	1.02-7.49	0.04
Return to estrus?	0.65	0.16-2.49	0.75
Clinical manifestations			
Anaemia?	1.70	0.27-10.7	0.62
Ocular manifestations?	1.18	0.41-3.43	0.78
Weight loss?	1.07	0.34-3.31	1
Anorexia?	1.68	0.26-10.5	0.62

P.R = Prevalence Ratio ; C.I = Confidence Interval

4 DISCUSSION

Horses pulling carts are a recurring practice in Brazilian cities. Leptospirosis is a well-known ailment of horses, with significant clinical and reproductive repercussions (Tadich et al. 2016). Our study shows that draft horses in urban settings are particularly exposed to the disease, with a seroprevalence of 28.57%, compared to other populations of the same region (Finger et al. 2014). To our knowledge, this is the first study in Brazil to focus on this population. Recyclers' horses are especially burdened by the disease due to the low income of

these workers, who are less likely to seek veterinary support, or to undertake preventive measures such as vaccination (in our study, only 2.5% of the caretakers claimed to have vaccinated their horses).

The population considered for this study was urban and, thusly, horses had to either be fed in stables, or taken to pastures where they could graze, often doing so with other horses, and/or farm animals. Mingling with other animals (horses or otherwise) is a likely reason for this, but the flat, lowland pastures where these animals were allowed to graze are very flood prone, and this probably also plays a role, not only in the positive association with abortion occurrence ($p < 0.01$), but also in the seroprevalence of the entire population (28.57%), relative to other studies (Finger et al. 2014; Oliveira Filho et al. 2014; Lasta et al. 2013; Braga et al. 2011; Lilenbaum 1998).

In our study, Canicola was the most prevalent serovar (35.29%), followed by Bratislava (14.71%), Hardjo (11.76%), and Ballum (11.76%) (Table 4). These were not unexpected, since most owners reported having dogs in the household (83.2%), associated with the habit of grazing in open fields with other species. Furthermore, these, along with *Icterohaemorrhagiae*, seem to be the most common serovars circulating in the city of Pelotas (Avila et al. 1998). Other studies in Brazil have found different results for horses, mostly in regards with *Icterohaemorrhagiae*, which is usually among the most frequent (Hamond et al. 2013; Linhares et al. 2005). Nonetheless, Pelotas is described as atypically harbouring a diverse number of serovars, causing human and animal leptospirosis (Cunha et al. 2016; Silva et al. 2010; Silva et al. 2009), contrary to the usual urban scenario, where most cases are caused by the same strain, usually *Icterohaemorrhagiae* (Martins et al. 2013).

Abortion, a frequent result of equine leptospirosis (Arent et al. 2015), was associated with free grazing as well ($p < 0.01$). Likewise, it was associated with the presence of rodents ($P < 0.01$) and their access to the horses' food supply (fodder kept in sheds, $p = 0.03$). Conversely, fodder being kept in barrels (with no access to rodents), was protective against abortions ($p = 0.01$). Although it cannot be said that leptospirosis is the cause of these abortions, it is the most likely scenario due to the association with rodents. The high overall prevalence is likely the reason we cannot see these associations directly with seroreactivity.

Isolation of leptospires is considered the gold standard for the definitive diagnosis of leptospirosis (Ellis 2015). Although it is a procedure considered difficult and requires time and technical experience, the isolation of leptospires allows identification of the causative serovar, which is important in epidemiological studies, and for the development of vaccines against

equine leptospirosis. In our report, the isolated was used to perform the virulence test in a susceptible animal model (Silva et al. 2010). All animals inoculated with the isolated showed clinical and pathological signs of the experimental disease (data not shown). This result demonstrates that the isolated has the potential to compose and testing experimental vaccines against animal leptospirosis.

The use of horses as draft animals in urban settings is common in Brazil, especially by people of vulnerable social standings, who use these as a means of income, through the collection of recyclables (Finger et al. 2014). These horses wander the cities, and, if shedding, can disseminate leptospires among different neighbourhoods, likewise, this practice offers greater opportunities for negative horses to become infected. The seroprevalence described here (28.57%) reveals that most of the horses circulating in Pelotas are, or have been at some point, infected, indicating that the disease is not being addressed in this population by policymakers. Moreover, these animals make excellent sentinels, revealing the circulating serovars in a city, which could, and should, be used by policymakers and researchers alike for this end.

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