

Leptospirosis infection in domestic mares in North Queensland

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Abstract

Leptospira species are found worldwide, favouring tropical regions, and infect a wide range of animal species. Although renal persistence in infected individuals and excretion in urine is thought to be the primary mechanism of disease transmission, recent reports have suggested that persistence in the reproductive tract may be a feature in certain species, including the horse. The aim of this study was to investigate leptospiral infection, particularly within the reproductive tract, in healthy, non-breeding mares. Serum and endometrial swab samples were collected from 50 mares from the James Cook University Teaching Animal Herd, as well as, where possible, free-catch urine (n = 19). Sera were screened for antibodies to 24 *Leptospira* serovars, using the microscopic agglutination test (MAT). Endometrial and urine samples underwent real-time PCR testing, targeting the leptospiral *rrs* gene. Overall, the seroprevalence of leptospirosis was 48% (95% CI: 34–62%), with serovars Arborea, Bratislava and Australis detected most frequently. PCR positive results were obtained from 1/50 (2%) endometrial swabs and 2/19 (11%) urine samples. This is the first report of serovar Bratislava in horses in Australia.

Main text

Leptospirosis is a globally important disease of humans and domestic animals, caused by the spirochaete *Leptospira*. Classification of *Leptospira* is complex, with over 65 *Leptospira* species currently described, based on genetic sequencing. Alternatively, leptospiral strains are characterised by their serological reactivity, which correlates poorly to species identity, with multiple species contributing to some serovars. Serovars with similar serological reactivity are clustered into serogroups, for convenience.¹ At least 260 pathogenic serovars are currently recognised.²

Most species of *Leptospira* exist as free-living saprophytes. However, some species can cause acute to chronic infections in a wide range of animal species, with kidney tubules being a key site for leptospiral persistence and subsequent excretion into the environment. Leptospirosis infection generally occurs through direct contact of the skin or mucous membranes with

infected urine, or indirectly through exposure to contaminated water,² with warm, humid environments and flooding events favouring leptospiral survival and spread.³ Rodents are recognised as the principal animal reservoirs of *Leptospira*, contributing significantly to environmental contamination and transmission of leptospirosis to humans and other animals. However, other species such as dogs, pigs, bats and cattle can also contribute to maintenance and transmission.^{4,5} Cattle are considered maintenance hosts for *L. borgpetersenii* serovar Hardjo (Hardjobovis) and *L. interrogans* serovar Hardjo (Hardjoprajtino).¹ Recently, links between these serovars and reproductive disease in cattle, as well as the detection of leptospire in bovine reproductive tracts and semen, have led to the suggestion that serovar Hardjo may persist in the bovine genital tract, impacting reproductive success. Importantly, such chronic infections may be associated with low circulating antibody levels, with detection in urine being sporadic or absent, hampering diagnosis by conventional approaches.⁶

The horse has been suggested to be the maintenance host for serovar Bratislava,⁷ although this remains the subject of debate.⁸ Antibodies to this serovar have been widely detected in horses globally, with the exception of Oceania,⁹ although the serovar has been detected in Australian pigs.¹⁰ The occult persistence of serovar Bratislava in the equine genital tract and associated impacts on fertility, much like the impact of serovar Hardjo on cattle, has recently been suggested.⁹

The aim of the current study was to investigate leptospiral infection in domestic mares, with a focus on the reproductive tract as a potential site for leptospiral persistence following systemic infection. This study was approved by the James Cook University Animal Ethics Committee (A2879). In February 2023, approximately three weeks after rainfall in the area peaked at 160 mm in one day, whole blood and endometrial samples were obtained from 50 non-breeding mares from the James Cook University Teaching Animal Herd. Urine was collected from mares opportunistically, when voided spontaneously during sampling activities (n = 19). Mares consisted of 32 Standardbreds, 15 Thoroughbreds, 2 Quarterhorses and 1 Australian Stockhorse, with a mean age of 16.6 yrs (range 8–23 years) and mean weight of 497 kg (range 420–577kg). Endometrial samples were collected using guarded equine endometrial swabs (Minitube Australia, Smythesdale, VIC), under aseptic technique. Blood samples were collected by jugular venipuncture into plain vacutainer tubes with clot activator. Serum was aliquoted into cryovials and stored at -80°C until shipped to the Leptospirosis Reference Laboratory (Coopers Plains, QLD) for serological testing. Endometrial and urine samples were held at 4°C for up to 6 weeks prior to PCR testing.

All sera were screened against antibodies to 24 *Leptospira* serovars, using the microscopic agglutination test (MAT). Final titres represented the dilution at which at least 50% agglutination occurred in comparison to a positive control. A titre result of 1:100 or higher was considered evidence of previous exposure to leptospire.^{11, 12} For horses that recorded

multiple positive titres within single serogroups, titres lower than the highest titre in that serogroup were assumed to be cross-reactions and were disregarded.¹¹ For overall seroprevalence, a confidence interval was calculated based on a sensitivity of 92% and a specificity of 95%, as previously described.¹¹ Of the 50 mares sampled, 24 mares (48%; 95% CI 34–62%) showed evidence of exposure to one or more serogroups of *Leptospira* spp. In comparison, the seroprevalence of leptospirosis in horses in this region has previously been reported as 34% (16/32)¹³ and 35% (59/170).¹¹ For the latter two studies, the timing of sampling in relation to rainfall events was not reported. In the current study, a total of 13 serovars representing 9 serogroups were detected. Distribution of the MAT titres ranged from 100 to 1600 with the modal titre being 100 for 23 samples. Among the 13 serovars identified, serovar Arborea was the most common (10 mares), followed by serovar Bratislava (8 mares), and then serovar Australis (5 mares; Table 1). In a recent study in horses in this area, serovar Arborea was similarly serodominant.¹¹ In that study, exposure to serovar Arborea was linked to properties with high annual rainfall, as well as the presence of pigs and sugarcane fields in the area. In our study, both recent rainfall events and feral pigs in the area may have contributed to the high prevalence of this serovar. Notably, serovar Arborea has been recognised as an increasingly important cause of human leptospirosis in this region.³ This study is the first report of serovar Bratislava in horses in Australia.

Table 1: Distribution of microscopic agglutination test titres of *Leptospira* serovars in positive mares.

Number of sera with indicated MAT titres							
Serogroup*	Serovar	100	200	400	800	1600	Total (%)
Ballum	Arborea	10	0	0	0	0	10 (22.2)
Australis	Bratislava	6	2	0	0	0	8 (17.8)
Australis	Australis	1	2	0	2	0	5 (11.1)
Pyrogenes	Robinsoni	4	0	0	0	0	4 (8.9)
Cynopteri	Cynopteri	3	0	0	0	0	3 (6.7)
Pomona	Pomona	0	0	2	1	0	3 (6.7)
Tarrasovi	Topaz	2	1	0	0	0	3 (6.7)
Ballum	Ballum	2	0	0	0	0	2 (4.4)
Pyrogenes	Zanoni	2	0	0	0	0	2 (4.4)
Djasiman	Djasiman	2	0	0	0	0	2 (4.4)
Sejroe	Medanensis	0	0	0	0	1	1 (2.2)
Hebdomadis	Kremastos	0	1	0	0	0	1 (2.2)
Canicola	Canicola	1	0	0	0	0	1 (2.2)
TOTAL		33	6	2	3	1	45

Serovars (serogroups) included in the MAT panel but not detected at a dilution level of at least 1:100 included Bulgarica (Autumnalis), Bataviae (Bataviae), Celledoni (Celledoni), Grippytyphosa (Grippytyphosa), Icterohaemorrhagiae (Icterohaemorrhagiae), Copenhageni (Icterohaemorrhagiae), Javanica (Javanica), Szwajizak (Mini), Panama (Panama), Hardjo (Sejroe), Tarrasovi (Tarrasovi).

*For individual horses with more than one positive titre recorded within a single serogroup, titres lower than the highest titre in that serogroup were assumed to be cross-reactions and have been excluded. Where the highest titre was equal across two serovars in the same serogroup, neither was excluded.

Endometrial and urine samples were subjected to real-time PCR, targeting the *rrs* (16S) gene.¹⁴ Endometrial samples were resuspended in 1 mL of PBS, pH 7.4 for DNA extraction, while urine samples were used directly. The reaction volume in each of the microtubes used for PCR was 20 μ L. The PCR components included 2X Reaction Mix (QuantiTect Probe PCR Master Mix; Qiagen), Lepto-*rrs*-F (10 μ M) primer, Lepto-*rrs*-R primer (10 μ M), Lepto-*rrs*-P primer (10 μ M)¹⁴ and distilled water which were mixed by vortexing before adding the cDNA template. The PCR cycling protocol ran as follows: 95°C for 15 minutes, then 40 cycles of 94°C for 15 seconds and 60°C for 1 minute, followed by holding at 4°C. Samples with a cycle threshold (Ct) value less than 38 were considered positive. Two of 19 urine samples (11%) and one of 50 (2%) endometrial samples were positive on PCR. Both mares that were PCR-positive for urine were negative on serology. The mare with a PCR-positive endometrial sample was negative on urine, but positive for serovar Australis on serological testing. The endometrial sample obtained from this mare resembled a purulent exudate; medical records indicated that this mare was known to be suffering from recurrent endometritis. Two months after sampling for the current study, this mare deteriorated clinically and was euthanased. Post mortem findings included a severe, necrotising bacterial metritis and cervicitis. Culture of uterine fluid collected at necropsy revealed a mixed growth of *Fusobacterium nucleatum* and an anaerobic *Streptococcus* species.

Despite widespread serological evidence of leptospiral infection, none of the mares in this study showed overt signs of clinical disease at the time of sampling or since. This is consistent with other studies, suggesting that the horse is relatively resistant to leptospirosis.^{7, 11, 12, 15} Of concern is the detection of leptospiral antigens in the urine of two mares, which poses a zoonotic risk. Increased awareness of the possible risks of exposure to equine urine, particularly in tropical environments, is warranted. Interestingly, despite sampling approximately three weeks after a high rainfall event, few titres in the current study were at high levels consistent with a recent infection. This suggests that the *Leptospira* serovars detected in this study are endemic to this area.

In the current study, the detection of leptospiral DNA in the endometrial discharge of one mare is of uncertain significance and, regrettably, no further PCR testing was performed following her post mortem. Hamond et al.¹⁶ detected leptospiral DNA in 43/134 (32%) samples of vaginal fluid, collected from mares by retention of an absorbent tampon in the vagina for 15 minutes. Interestingly, all genetic analyses of vaginal fluid samples identified the serovar as Bratislava, while DNA analysed from urine samples identified the serovars Bratislava and Copenhageni. In a subsequent study by the same group, leptospiral DNA was detected in equine urine, vaginal fluid (collected using a cytology brush) and endometrial biopsy

samples from mares with reported reproductive abnormalities. All mares positive for leptospiral DNA on endometrial biopsy sample (7/38 mares) were similarly positive on the vaginal fluid sample, with some mares positive on vaginal fluid sample only,¹⁷ suggesting that leptospires localise in the vagina more than the uterus, or that endometrial biopsy is less sensitive for leptospiral detection than cytology of the vagina. In our study, we opted to collect endometrial samples using a commercially-available endometrial swab, as such swabs are commonly used in private practice and because leptospires remain mostly extracellular¹⁸ and may persist within a biofilm.¹ It is possible that the heads of these swabs, although absorbent, were too small to obtain enough material to detect leptospires. In the study by Hamond et al.¹⁷, only vaginal fluid and urine leptospiral DNA extracts were suitable for genetic sequencing; analyses of vaginal fluid DNA extracts favoured, but were not exclusive to, serovar Bratislava.¹⁷ In our study, none of the samples obtained from either urine or endometrial swab were suitable for further genetic analysis as the amount of *Leptospira* DNA in the samples was too low.

In conclusion, this study confirms that horses in Far North Queensland are exposed to a wide range of serovars of *Leptospira* spp., and is the first to test for and confirm the presence of serovar Bratislava in Australian horses. Limited evidence for the persistence of leptospires in the reproductive tract of mares was obtained from this study, however further study with a larger sample size is warranted. Moreover, given the possible role of venereal transmission in the spread of leptospires,¹⁹ and in particular, serovar Bratislava,²⁰ further study using mares bred by live cover is warranted.

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Conflict of Interest Statement

The authors have no conflict of interest to declare.

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