Epidemiol. Infect. (2013), **141**, 33–35. © Cambridge University Press 2012
doi:10.1017/S0950268812000416

SHORT REPORT
The role of horses in the transmission of leptospirosis in an urban tropical area

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Received 17 October 2011; Final revision 27 January 2012; Accepted 16 February 2012; first published online 15 March 2012

SUMMARY

The objective of this study was to demonstrate the presence of leptospires in equine urine, as evidence for a potential role of horses in transmission of this organism. Thoroughbred horses (aged 2–5 years, $n=276$) from Rio de Janeiro, Brazil, were studied. After a severe storm, the premises of the animals remained flooded for 72 h. Blood samples for serology were collected on days 20 and 35 (day of storm = day 0). On day 20, 132 (47·8%) horses were seroreactive (titre $\geqslant 200$) and, of these, 23 (31·0%) had increased antibody titres on day 35. Furthermore, 34 urine samples (for PCR and culture) were collected from seroreactive horses on day 35. Copenhageni was the most frequent serovar (88·8% of reactive titres). Although none of the urine samples were culture positive, 12 (35·2%) were PCR positive. This is apparently the first report of evidence of leptospires in urban horses. Furthermore, we suggest that these animals can play a role in the transmission of leptospirosis in urban areas.

Key words: Horses, carriers, urban tropical area, *Leptospira* spp.

Leptospirosis is a widespread zoonosis caused by spirochaetes of the genus *Leptospira* spp. These organisms are water-borne pathogens; extensive flooding and seasonal rainfalls are known risk factors for exposure to contaminated water, mainly in urban areas of tropical countries [1, 2]. Various animal species are considered maintenance hosts of *Leptospira* spp., e.g. rodents and dogs [2–5]. Although horses have rarely been reported as being important for the transmission of this agent to other animals and humans [3, 6], seroreactivity is common in horses, and titres to several serovars have been reported, mainly to Bratislava and members of the Icterohaemorrhagiae serogroup [3, 6–8]. The latter serogroup is also responsible for the majority of human infections in urban tropical areas worldwide [2].

The clinical syndrome of leptospirosis in horses is characterized by uveitis, abortion, stillbirth, premature foals, and renal and hepatic dysfunction. Common clinical signs include haematuria, fever, jaundice, anorexia, and respiratory distress [9]. Nevertheless, not all infected animals have acute disease, and subclinical infections are very common in endemic regions [7, 9].

Serology is the most common method to diagnose leptospirosis in many species [3]. Nevertheless, results can be quite difficult to interpret in animals previously

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vaccinated or coming from endemic areas [4, 6]. Therefore, direct evidence of the presence of the agent (bacterial culture) or its DNA (PCR) are required for differentiation of exposed animals from those that are acting as carriers of the bacterium and shedding it in their urine [2, 5].

Although the role of rodents and dogs in the transmission of leptospirosis in urban areas is well known [2, 4, 5], the role of urban horses has very rarely been addressed; only serological studies have been reported, which are more a measure of exposure than active infection [6, 7]. To our knowledge, direct evidence of shedding of leptospires (by culture or PCR) has never been reported in urban horses. Therefore, the objective of this study was to demonstrate the presence of the agent in equine urine, as evidence for a potential role in transmission.

During April 2010, an abnormally severe storm occurred in Rio de Janeiro, Brazil. Consequently, the premises of the horses kept at the Brazilian Jockey Club in the city remained flooded for 72 h. A total of 276 thoroughbred horses, a random selection of approximately 40% of the horses at the flooded premises, were subsequently studied. These horses ranged from 2 to 5 years old. Despite being in an endemic area, none had been vaccinated for leptospirosis. Blood samples were collected (jugular venepuncture) on day 20 (day of storm = day 0). On day 35, another blood sample was collected (for paired serology) from horses that had titres $\geqslant$200 on day 20. In addition, on day 35, urine samples were collected from 34 randomly selected seroreactive horses and subjected to bacteriological culture as well as PCR.

For detection of anti-*Leptospira* antibodies, a microscopic agglutination test (MAT) was used with a complete panel (28 serovars of 11 serogroups), according to international standards [3]. The serovar with the highest titre was deemed infective. Since it is an endemic area, samples with titres $\geqslant$200 were considered reactive.

A detailed clinical examination of each horse was conducted, including mental attitude, nutritional status, appetite, presence of ectoparasites, nasal secretions, skin turgor, eyeball retraction, palpation of superficial lymph nodes, rectal temperature, pulse and capillary refill time, auscultation of the digestive tract, heart and lungs, and a thorough clinical examination of the musculoskeletal system.

For bacteriological culture, urine samples were transferred (serial dilution technique to 10^{-2} and 10^{-3} dilutions) into semi-solid medium (Fletcher medium, BD Difco, USA) containing 300 mg/l 5-fluorouracil and 20 mg/l nalidixic acid, and incubated for 24 h at 30 °C. After 24 h, aliquots from diluted tubes were transferred to both Fletcher medium and Ellinghausen–McCullough–Johnson–Harris (EMJH) liquid medium without antibiotics, incubated at 30 °C and examined weekly (dark-field microscopy) for 20 weeks [4].

The PCR was performed by extraction of DNA with the Promega Wizard SV kit genomic DNA Purification System® (Promega, USA). The primers [10] were LipL32-45F (5′-AAG CAT TAC CGC TTG TGG TG-3′) and LipL32-286R (5′-GAA CTC CCA TTT CAG CGA TT- 3′). For amplification reactions, 0·6 μm of forward and reverse primers were used, as well as 1·0 U *Taq* polymerase, 10× reaction buffer [50 mM KCl, 75 mm Tris–HCl (pH 9·0), 20 mm $(NH_4)_2SO_4$], 2·4 μM $MgCl_2$, 0·3 mM dNTP, and 3·5 μl DNA (final volume, 25 μl). The reaction was incubated at 94 °C for 5 min for DNA denaturation, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, extension at 72 °C for 1 min, and a final extension period of 5 min at 72 °C. Thereafter, PCR reaction products were subjected to electrophoresis on 2% agarose gels and stained with ethidium bromide. A molecular-weight marker was included for assessing amplicon sizes, and both positive (DNA of *L. interrogans* serovar Copenhageni strain L1-130) and negative (Ultra-Pure™ DEPC-treated water; Invitrogen, USA) controls were applied. After electrophoresis, gels were examined on an ultraviolet light transilluminator and photographed.

From the 276 serum samples tested on day 20, 132 (47·8%) were reactive (titres ranged from 200 to $\geqslant$800). Serovar Copenhageni was by far the most frequent, detected in 117/132 (88·7%) of reactive samples, whereas 10 (7·5%) were reactive against serovar Hardjo and five (3·8%) against serovar Bratislava. From the 132 serum samples collected on day 35, all remained seroreactive (titres from 200 to 800), but only 23 (17·4%) had increased antibody titres when compared to day 0. It was noteworthy that no clinical signs consistent with clinical leptospirosis were detected in any of these horses.

None of the 34 urine samples collected on day 35 were positive by culture. Nevertheless, 12 (35·2%) were positive by PCR, eight of which were from horses with titres of 200 and four with titres $\geqslant$400. There was no apparent association between horses with increasing titres and those that were positive by

PCR. From the 23 horses that increased their titres on day 35, eight had their urine sampled, three of these samples were PCR positive.

The seroreactivity detected in the present study was not unexpected. The predominance of serovar Copenhageni was consistent with other studies conducted in horses from tropical areas which implicated Icterohaemorrhagiae or Copenhageni (members of the same serogroup) as predominant serovars [6, 7], not only in horses but also in dogs and humans [2, 9]. Based on the predominance of serovar Copenhageni in this study, we inferred that, as a result of the flooding, infection occurred when these horses were exposed to rat urine.

Although no leptospires were detected by culture, infection was confirmed on the basis of specific leptospiral DNA (detected by PCR). Culturing of leptospires has several limitations, including the fastidious growth of this organism in artificial media, contamination, and poor sensitivity [4]. Although PCR is very useful for diagnostic purposes, it does not identify the causative serovar, an extremely important limitation to study the epidemiology of the infection [10]. Therefore, based on the collective epidemiological, serological and molecular evidence, we strongly suggest that these horses were infected with *L. interrogans* serovar Copenhageni.

Other studies have suggested a role of horses in the transmission of leptospirosis [6, 9]; nevertheless, they were based exclusively on serological evidence, which cannot reliably differentiate animals that have only been exposed to the agent from those that are spreading the bacterium [2, 4] and may be playing a role in transmission of the infection. This is apparently the first report of identification by PCR of urban horses carrying leptospires, confirming that those animals were spreading the bacterium and possibly acting as carriers in this urban environment, with important implications for public health.

In conclusion, we suggest that horses can play a role in the transmission of leptospirosis in urban areas by spreading the agent in the environment.

ACKNOWLEDGEMENTS

This study was supported by CNPq and FAPERJ, Brazil. W.L. is a CNPq fellow. The authors are grateful for the contribution of R. Diniz and C. Pestana, from Fiocruz-RJ and to Professor J. Kastelic, from Lethbridge, Canada, for a critical review.

DECLARATION OF INTEREST

None.

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