

PREVALENCE OF *LEPTOSPIRA* ANTIBODIES IN HORSES FROM ROMANIA

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Abstract

Leptospirosis, a zoonotic disease with a worldwide distribution, can affect horses causing reproductive disorders such as abortion, embryonic absorption and stillbirths. The aim of the current study was to determine the prevalence of Leptospira antibodies in mares and stallions belonging to a breeding farm in the southern region of Romania. Blood samples were collected from 91 horses and were analysed by the microscopic agglutination test (MAT) using 12 distinct live antigens. To determine if acute infection was present, the positive individuals were retested after 14 days. The results showed that Leptospira antibody prevalence was high, with 59.34% of samples testing positive. Of the 91 samples included in the study, 40 tested positive for one serotype, 11 samples for 2 serotypes, 2 samples for 3 serotypes and 1 sample reacted positive with 5 serotypes.

Key words: equine leptospirosis, horses, MAT, reproduction, serological survey.

INTRODUCTION

Leptospirosis is a zoonotic disease with a wide global distribution. The economic impact of leptospirosis on society stems from the expenses associated with farm animals and public health, including mortality, declining productivity, and abortions (Gurău & Drăgan, 2018). With over a million cases and almost 60,000 deaths annually from human infection, it is a major but underappreciated cause of morbidity and mortality (Costa et al., 2015); however, considering the nonspecific symptoms that many individuals present with, this number probably represents a severe underestimate of the disease burden (Sykes et al., 2022). The persistence of the aetiological agent in the environment is dependent on the interactions between humans, animals and ecosystems. The natural reservoir of *Leptospira* is wild animals, particularly rodents, which play an important role in the long-term maintenance and circulation of the causative agent in outbreaks (Burlacu et al., 2020). In many cases, wild, stray or domestic animals can carry the pathogen without exhibiting any noticeable symptoms. The stealthy carrier status facilitates dissemination of the bacteria in the

environment, contaminating water sources and soil (Constantinescu et al., 2015).

Although horses have rarely been implicated in the spread of leptospirosis to humans or other animals (Hamond et al., 2013), seroreactivity is common in this species, and titres to multiple serovars have been reported, such as Copenhageni in Brazil (Hamond et al., 2013), Pomona in the USA (Timoney et al., 2011), and Icterohaemorrhagiae and Bratislava in North and South America, Europe and Asia (Turk et al., 2013; Verma et al., 2013).

While horses may present all the classical symptoms of leptospirosis (fever, anorexia, jaundice, anaemia, haemorrhages on the mucosa and depression) (Verma et al., 2013), a more common presentation is the subclinical chronic form, in which leptospiral colonization of the reproductive tract takes place. Also called “silent leptospirosis”, it is characterized by low fertility, oestrus repetition and sometimes, late-term abortions. Often, this type of infection is caused by strains belonging to the Australis serogroup, particularly serovar Bratislava, which has been considered a non-pathogenic serovar, adapted to horses. (Di Azevedo & Lilenbaum, 2022).

The current study was performed to determine the prevalence of *Leptospira* antibodies in an equine breeding farm in order to gain insight into the possible reasons for reproductive underperformance exhibited by multiple mares.

MATERIALS AND METHODS

A total of 91 horses, 5 males and 86 females used for breeding, were included in the study. In the female group, both donors and recipients were tested. None of the horses had a history of vaccination against leptospirosis. During the clinical examination, no classic symptoms of leptospirosis were recorded in any of the horses; however, two of the recipient mares had suffered recent mid-term abortions. A large number of the mares included in the study presented with oestrus repetition and low fertility.

Blood samples were collected from each animal twice, 14 days apart. The samples were allowed to clot at room temperature for 4 hours, were centrifuged at 2000 rpm for 5 minutes, and the serum was collected in Eppendorf tubes and refrigerated until use. The serum samples were subjected to serological testing using the standard microscopic agglutination test (MAT). To detect the presence of *Leptospira* spp. antibodies, samples were tested against 12 reference serovars: Australis, Autumnalis, Ballum, Bataviae, Canicola, Grippothyphosa, Hardjo, Icterohaemorrhagiae, Pomona, Tarassovi, Sejroe and Wolffii. Prior to testing the equine serum samples, the identity of the antigens was confirmed against reference sera obtained from the Royal Tropical Institute in Amsterdam, The Netherlands. To reduce observer variability, the MAT was performed by one operator to limit inter-observer variations of the results.

Antigens were cultured in EMJH medium (Difco) for 7 days at 29°C, to a density of $2-3 \times 10^8$ CFU/ml. A screening test was performed using a 1/50 serum dilution, to which an equal volume of each antigen was added, making the final serum dilution 1/100. Testing was performed in microtitration plates, which were incubated for 2 hours at 29°C. The reactions were examined using a dark-field microscope, and samples showing $\geq 50\%$ agglutination at the 1/100 dilution were considered positive. For each positive sample, serial dilutions were

performed and tested against the respective antigen(s) to determine the final antibody titre. The endpoint was considered the final dilution showing 50% agglutination and 50% free *Leptospira* (WOAH, 2021).

RESULTS AND DISCUSSIONS

The first serological test revealed that 54 subjects (59.34%) were positive for *Leptospira* antibodies, including 20 recipient mares, 28 donor mares, 4 work horses and 2 stallions. Positive reactions were recorded for 10 of the 12 antigens included in the test. The majority of positive samples reacted to serovar Australis (83.33% of positive samples). The second most prevalent reaction was to serovar Icterohaemorrhagiae (22.22% of positive samples). The remaining serovars encountered lower prevalence: 7.40% for Grippothyphosa, 3.70% for Autumnalis, Bataviae, Canicola, Ballum and Sejroe, and 1.85% for Pomona and Tarassovi (Figure 1). No positive reactions were recorded for serovars Hardjo and Wolffii.

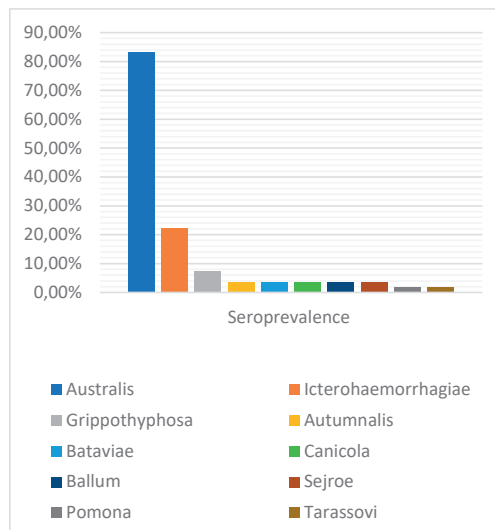


Figure 1. Prevalence of positive reactions to each *Leptospira* spp. serovar

The majority of the titres for most serovars were relatively low. Antibody titres of samples with positive reactions to Australis ranged from 1/100 to 1/1600. Samples positive for Icterohaemorrhagiae had titres of 1/100 to 1/200, as well as most of the samples with

positive reactions to the other serovars, only a few reaching 1/400. Table 1 presents the number of positive samples recorded for each serovar and their respective titres.

Table 1. Number of positive samples and antibody titres for each serovar

Number of samples/serovar										
Serovar/ Titre	Aus.	Ictero.	Gripp.	Aut.	Bat.	Can.	Ball.	Sej.	Pom.	Trs.
1/100	13	6	-	1	1	2	2	1	1	-
1/200	13	6	1	-	1	-	-	1	-	1
1/400	11	-	3	-	-	-	-	-	-	-
1/800	6	-	-	1	-	-	-	-	-	-
1/1600	2	-	-	-	-	-	-	-	-	-
Total	45	12	4	2	2	2	2	2	1	1

Legend: Aus. - Australis, Ictero - Icterohaemorrhagiae, Gripp. - Grippothyphosa, Aut. - Autumnalis, Bat. - Bataviae, Can. - Canicola, Ball. - Ballum, Pom. - Pomona, Trs. - Tarassovi.

The majority of samples were positive to 1 serovar (40 out of 54); however, we encountered several samples with positive reactions to 2 or more serovars (Table 2). While exposure to multiple serovars is possible, especially in a farm surrounded by wildlife, there is also the possibility of cross-reactivity occurring between different serovars (Barwick et al., 1998).

Table 2. Number of samples with positive reactions to one or more serovars

Number of serovars	Number of positive samples
1	40
2	11
3	2
4	0
5	1

The results of our study showed that a large number of horses had been exposed to *Leptospira* antigens. To determine if the horses were undergoing an active *Leptospira* infection, blood samples from the same animals were collected 14 days after the initial test samples and were subjected to the same protocol in order to determine any increase in the antibody titres. A fourfold increase in antibody titres in paired serum samples, collected in the acute and the convalescent phases of the disease, represents a definitive criterion for diagnosis (WOAH, 2021).

The second assessment confirmed most of the results obtained in the first test. The majority of the animals maintained positive reactions to the same serovars as detected during the first trial and with similar antibody titres. Out of the 91

horses included in the study, 6 recorded a fourfold rise in antibody titres during the trial. All 6 individuals had initially reacted positively to serovar Australis with titres from 1/100 to 1/400, and after 14 days, the titres were 4 times higher. None of the animals with increased antibody titres had any leptospirosis symptoms or any sign of disease. Lower increases in antibody titres were also recorded in 19 other individuals; however, only two- or three-fold, and not conclusive for acute disease diagnosis.

The majority of the horses with negative samples in the first trial remained negative, with the exception of two mares, which were negative during the first trial and reacted positive to serovar Australis with a titre of 1/100 during the second trial (Table 3). The one mare which was positive to 5 serovars during the first MAT (Australis 1/100, Autumnalis 1/100, Ballum 1/100, Sejroe 1/100 and Tarassovi 1/200), during the second test reacted positive to only three serovars: Australis, with a titre of 1/400, Ballum 1/100 and Tarassovi 1/100.

Leptospirosis in Romania has been intensely studied over the years, especially in humans and dogs (Romaniuc et al., 2001; Manciu et al., 2018; Predescu et al., 2018; Sonea et al., 2024; Iorgoni et al., 2025), and in swine (Gurau and Dragan, 2018). However, in equids, there is very little to no information available on the prevalence of leptospirosis in our country. Equine leptospirosis is not only a potential cause of major economic losses but also an occupational hazard for the humans who come in contact with infected horses, as these animals

may be responsible for environmental contamination (Flores et al., 2017). Numerous studies have been conducted worldwide to assess the prevalence of

leptospirosis in equine populations. The reported prevalence rates vary considerably, reflecting differences in geographic location, environmental conditions, and study design.

Table 3. Number of positive samples and antibody titres for each serovar in the second test

Number of samples/serovar										
Serovar/ Titre	Aus.	Ictero.	Gripp.	Aut.	Bat.	Can.	Ball.	Sej.	Pom.	Trs.
1/100	10	6	-	-	-	-	1	1	1	1
1/200	8	5	3	-	-	1	-	-	-	-
1/400	16	1	1	-	-	1	-	-	-	-
1/800	6	-	-	1	-	-	-	-	-	-
1/1600	4	-	-	-	-	-	-	-	-	-
1/3200	1									
1/6400	1									
Total	46	12	4	1	0	2	1	1	1	1

Legend: Aus. - Australis, Ictero - Icterohaemorrhagiae, Gripp. - Grippothyphosa, Aut. - Autumnalis, Bat. - Bataviae, Can. - Canicola, Ball. - Ballum, Pom. - Pomona, Trs. - Tarassovi.

A study performed in Ukraine between 2007 and 2021 on 121,101 horses found that 10.8% tested positive for *Leptospira* antibodies. The dominant serovars were Copenhageni, with a prevalence of 32.8%; Bratislava - 16.1%; Grippothyphosa - 15.4%; and Canicola - 13.2%. Reactions to more than one serovar were found in 55.1% of positive cases (Ukhovskiy et al., 2023). In Switzerland, a study was performed on 615 horse sera against 15 *Leptospira* serovars. Results showed that 58.5% of samples were positive to one or more of the antigens. Pyrogenes had the highest prevalence, 22.6%, followed by Canicola (22.1%) and Australis (19.2%) (Blatti et al., 2011). Researchers from Croatia performed a 10-year-long study on apparently healthy horses and found that in the study period, seroprevalence of *Leptospira* antibodies varied between 5% and 15.94%. The dominant serovar was Pomona (41.98%), followed by Grippothyphosa (31.34%), Sejroe (8.03%), Icterohaemorrhagiae (7.05%), and Bratislava (6.47%) (Benvin et al., 2023). In Northern Italy, a study carried out on Bardigiano horses detected 67.2% seropositive animals, with the highest prevalence belonging to serovar Bratislava (41.8%), followed by Canicola, Tarassovi, Copenhageni, Pomona, and Hardjo (Vera et al., 2019).

In the present study, the most prevalent serovar was found to be Australis (83.33% of positive samples), followed by Icterohaemorrhagiae (22.22% of positive samples) and

Grippothyphosa (7.40% of positive samples). Similar results were obtained by researchers in the USA, in the states of Missouri and Nebraska in 2016-2017, with a prevalence of 30.5% for serovar Australis, 25.4% for Grippothyphosa and 17.8% for Icterohaemorrhagiae (Trimble et al., 2018). In Colorado state, 82% of equine serum samples analysed for *Leptospira* antibodies were positive to at least one serovar. All samples were collected from apparently healthy horses, and serovar Bratislava had the highest prevalence (Fagre et al., 2020).

In New Zealand, 25% of equines subjected to MAT had *Leptospira* antibodies, with positive reactions to serovars Pomona (13%), Ballum (12%), Hardjo (9%), Copenhageni (22%), and Tarassovi (15%) (Bolwell et al., 2020). However, in Australia, serovar Arborea was serodominant in horses from Northern Queensland (Wangdi et al., 2013).

A high prevalence of *Leptospira* antibodies was found in numerous studies on horses from Brazil, with positive MAT results ranging from 16.2% in Brejo Paraibano (de Oliveira Filho et al., 2014), and 45.9% in the Southern States (Da Silva et al., 2020) to 61.6% in the State of Goiás (Romanowski et al., 2023).

The variability, prevalence and predominant serovars across the world highlight the complexity of understanding the global epidemiology of leptospirosis in horses and underscore the need for continued surveillance and standardized research approaches.

CONCLUSIONS

During the first serological test, 59.34% of samples were positive for antibodies, with a cut-off titre of 1/100. Of the positive samples, 20 were collected from recipient mares, 28 from donor mares, 4 from work horses and 2 from stallions. Serovar Australis had the highest prevalence (83.33% of positive samples), followed by Icterohaemorrhagiae (22.22% of positive samples), Grippothyphosa (7.40%), Autumnalis, Bataviae, Canicola, Ballum and Sejroe (3.70% each), and Pomona and Tarassovi (1.85% each). Results of the second microagglutination test were highly similar to the first, with the majority of the animals maintaining similar antibody titres. Out of the 91 horses included in the study, 6 recorded a fourfold rise in antibody titres during the trial. The study highlights the presence of *Leptospira* antigen exposure in horses from our country. Given that infected horses often show no sign of leptospirosis, the findings of the current study underline the importance of routine testing and continued surveillance of equine populations, as these animals can serve as unnoticed carriers, posing a potential zoonotic risk.

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