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The first molecular screening of equine leptospiral infection in Iran

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Abstract *Leptospira* is one of the spiral-shaped Gram-negative bacteria, which includes pathogenic (*Leptospira interrogans*) and non-pathogenic (*Leptospira biflexa*) species. This bacterium is zoonotic and is scattered all over the world. It infects a wide range of hosts, including humans, domestic animals and, rodents. Investigation and identification of *Leptospira* in horses is often neglected or less addressed. However, this infection in horses can cause significant economic losses, such as the loss of expensive foals of rare breeds. Leptospirosis in horses is often subclinical and shows fewer clinical symptoms, especially in adults, but in acute cases, it is associated with jaundice, hemoglobinuria, renal dysfunction, lethargy, periodic ophthalmia and, abortion in mares. Therefore, it is necessary to investigate *Leptospira* infection in horses. For this purpose, 100 blood samples with anticoagulants were taken from the jugular veins of clinically healthy horses in Kerman province, Iran. Then, using the DNA extraction kit, according to the manufacturer's instructions, the DNA of the samples was extracted. In the following, a PCR technique using specific primers of the 16SrRNA gene of *Leptospira interrogans* was used to trace *Leptospira* bacteria. No positive samples were found. Based on these results, it can be concluded that Leptospira bacteria likely do not exist in the horses of Kerman City.

Introduction

Leptospirosis is an acute febrile disease caused by spirochetes of the genus *Leptospira*. This disease affects all domestic animals and humans, and its symptoms include fever, hemoglobinuria, jaundice, abortion, mastitis, decreased milk production, reproductive disorders, and death [1]. The clinical manifestations of leptospirosis exhibit a wide range of presentations, including acute, subacute, and chronic infection [2]. In the majority of cases, horses serve as incidental hosts. The majority of *Leptospira* infections in horses are asymptomatic; however, clinical syndromes such as fever, anorexia, jaundice, and lethargy have been documented in affected horses [3]. *Leptospira* infections in horses appear to exhibit a preference for specific organs, namely the kidney, eye, and reproductive tract of mares. Other clinical syndromes of leptospirosis include acute renal failure in young horses and recurrent uveitis in adult horses. Infection of pregnant mares may result in placental inflammation, fetal infection, and abortion, or neonatal jaundice and weakness in the newborn foals. Acute renal failure, hemolytic anemia, and hematuria may manifest individually. The rapid diagnosis of *Leptospira* is an essential element in the

management of this zoonotic disease. The inability of serological methods to detect this bacterium during the initial week of the disease (due to the absence of specific antibodies) and the very slow growth of the bacterium in culture have rendered the polymerase chain reaction (PCR) a suitable method for diagnosis due to its high sensitivity and accuracy. Given the high prevalence of acute and chronic forms of the disease in Iran, rapid diagnosis of *Leptospira* is essential for the development of effective control strategies and eradication programs [4]. The objective of this study was to evaluate the prevalence of leptospirosis in Iranian horses.

Materials and Methods

Study population

In the current study, 100 blood samples were randomly collected from clinically healthy horses in Kerman province, Iran during the period between August and November 2021. Samples were collected from horse racing clubs and private horse owners in Kerman province, Iran. In addition, a detailed questionnaire including age, breed, sex, contact with other animals, and herd size was collected from each horse owner to investigate the risk factors associated with the disease (Table 1).

Table 1: The demographical characterization of horses used in this study.

Type		Herd size		Breed		Gender	
	No.		No.		No.		No.
A	82	1-5	18	Tur	18	Male	65
B	3	6-10	16	Ara	9	Female	35
C	15	10 <	66	Dar	69		
				Kur	1		
				Pon	3		

*A: Breeding, B: Racing, C: Breeding and Racing *Tur: Turkmen, Ara: Arabian, Dar: Dareshuri, Kur: Kurd, Pon: Pony

Sample collection

Blood was collected from each horse via the jugular vein using anticoagulant tubes. Subsequently, the samples were promptly preserved and transported to the laboratory of the Pathobiology Department at the Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, for subsequent analysis. Subsequently, the blood samples were stored at a temperature of -20°C until further analysis. All horses were clinically healthy and exhibited no clinical symptoms of leptospirosis at the time of blood collection.

DNA extraction

The DNA was extracted from each blood sample using a blood DNA extraction kit (Parstous, Iran) in accordance with the instructions provided by the manufacturer. The quality and quantity of the extracted DNA were assessed at wavelengths of 260 and 280 nm using a Nanodrop spectrophotometer (Epoc, BioTek Instruments Inc., USA). Subsequently, the DNA samples were stored at -20°C until the conduction of the molecular analysis.

PCR

PCR was performed in a 25 µL reaction mixture, comprising 12.5 µL of PCR Master Mix (Ampliqon, Denmark), 1 µL of each primer LP (0.4 µM) (Pishgam Biotech, Iran) (Table 2), 8 µL of nuclease-free water, and 2.5 µL of template DNA. The reactions were conducted using a Thermal cycler (Bio-Rad, USA) under the following conditions: The initial denaturation at 95°C for three minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. Distilled water was employed as the negative control, while commercial *Leptospira* DNA (Lepto PCR kit, Intron Biotechnology, Korea) was utilized as the positive control. The PCR products were visualized using 1% agarose gel electrophoresis, with staining performed using 10 µL of DNA Green Viewer (Parstous, Iran). The amplicon sizes were then compared against a 100 bp DNA ladder (Ampliqon, Denmark).

Table 2: The specific primers for *Leptospira interrogans* 16s rRNA gene.

Primer name	Primer sequence	Product size (base)	source
Lp-F	5'-GCGCGTCTTAAACATGCAAG -3'	307	[5]
Lp-R	5'-CTTAACTGCTGCCTC CCGTAG-3'		

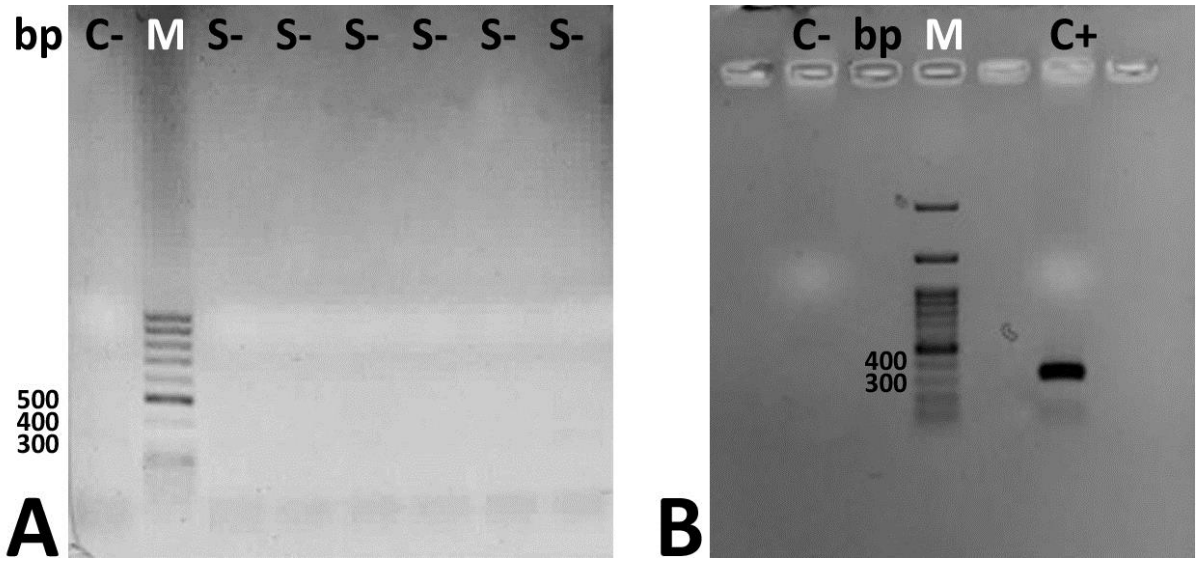

Fig 1. The electrophoresis image of PCR products for *Leptospira interrogans* (A): Lane C^- shows negative control; lane M indicates 100 bp DNA ladder; S^- are negative samples. (B): Lane C^- shows negative control; lane M indicates 100 bp DNA ladder; C^+ shows positive control.

Results

The electrophoresis results indicated that all blood samples in this study were negative for *Leptospira interrogans* (Figure 1A). Furthermore, the electrophoresis results of the negative control and the positive control substantiated the accuracy of the PCR technique (Figure 1B).

Discussion

The presence of *Leptospira* bacteria in horses has the potential to result in significant economic losses, including the loss of valuable foals from rare breeds. In cases of acute infection, the disease may present with jaundice, hemoglobinuria, renal dysfunction, and lethargy. Furthermore, the infection can result in abortion, stillbirth, and the birth of infected foals, which may exhibit severe clinical symptoms.

Another consequence of leptospirosis in horses is periodic ophthalmia, which affects the eyeball and is accompanied by pain and inflammation throughout the eyeball. Furthermore, leptospirosis in horses is frequently subclinical due to the zoonotic nature of this disease. This increases the risk of transferring bacteria from horses to humans and other animals, which is a significant concern [5].

The objective of this study was to detect the presence of *Leptospira interrogans* in horses in Iran. The results of this study demonstrate that none of the 100 horse blood samples examined exhibited evidence of infection with *Leptospira interrogans*. In 2016, Hajikolaei and colleagues in Iran conducted an investigation into the prevalence of *Leptospira interrogans* infection in sera of 152 horses belonging to seven horse-breeding units situated near Tehran. The microagglutination method revealed that 23 horses (15.13%) were infected with this bacterium [6]. In 2019, Hasanpour et al. conducted an investigation into the prevalence of common *Leptospira* serovars in traditional livestock farms in Tarem City, Iran. In this study,

200 blood samples were randomly collected from various locations in Tarem City and tested using the microagglutination method. The samples included 80 cow blood samples, 10 horse blood samples, 90 sheep blood samples, and 20 goat blood samples. The results demonstrated that 48 samples (24%) of the 200 tested, including 23 cows, 7 goats, 15 sheep, and 3 horses, exhibited evidence of infection with *Leptospira* bacteria (7). In South Carolina in 2007 and North Carolina in 2008, no species of *Leptospira* serovars were detected in 10 and 52 horses, respectively, which is similar to the results of the present study [8-9]. In 2013, Hammond and colleagues in Brazil conducted a meticulous examination of urine samples with the objective of identifying *Leptospira* species using the PCR technique. The objective of the study was to examine the efficacy of PCR as a diagnostic tool for leptospirosis in livestock.

A total of 512 adult animals (300 cattle, 138 horses, 59 goats, and 15 pigs) with reproductive problems in Brazil were subjected to study. Initially, a microagglutination test was employed to identify anti-*leptospira* antibodies present in the blood of the animals in question. Of the 512 serum samples tested, 43.5% (45.6% cow, 41.3% horse, 34% goat, and 60% pig) exhibited serological positivity. Subsequently, the PCR technique was employed to detect *Leptospira* DNA fragments in 32.4% of the samples (cow: 21.6%, horse: 36.2%, goat: 77.4%, and pig: 33.3%). The findings indicate that PCR is a highly accurate method for the detection of *Leptospira* [10]. In a study conducted by Errol et al. (2014), the diagnostic accuracy of three different tests was compared: real-time PCR, fluorescent antibody, and microscopic agglutination.

The objective was to determine the most effective method for diagnosing cases of equine leptospiral abortion. A total of 399 aborted horse fetuses were examined, of which 21 cases (19.6%) were diagnosed as leptospiral abortions. The results demonstrated that the PCR technique was able to successfully detect the presence of *Leptospira* DNA in all 21 aborted samples. While microagglutination and fluorescent antibodies identified 19 and 18 samples, respectively. Consequently, PCR represents an efficacious methodology for the identification of *Leptospira* [11]. In 2018, Savage and colleagues in Belgium conducted an investigation into the presence of *Leptospira* bacteria in horses with uveitis. In this study, 66 ocular samples from horses with uveitis were prepared and analyzed using the real-time PCR technique. The results demonstrated that the prevalence of *Leptospira* bacteria in horses with uveitis was 30.3% (20 out of 66 samples). The findings of this study indicate that leptospirosis represents a significant health concern in horses, with the potential for transmission via the eye cavity and associated fluids. This underscores the necessity for heightened attention and control measures to prevent further spread [12]. In 2020, Silva and colleagues in Brazil conducted a study to investigate the prevalence of leptospirosis and its impact on equine reproduction in southern Brazil. The researchers employed the microagglutination method to achieve this objective. In this study, 595 serum samples were prepared from horses from diverse locations in southern Brazil. The results demonstrated that among the 595 samples, 273 (45.9%) exhibited evidence of *Leptospira* infection [13]. In 2021, Haimbo et al. conducted a study to investigate the role of *Leptospira* species in the pathogenesis of uveitis in horses.

To this end, samples of blood serum and aqueous humor from horses exhibiting symptoms of uveitis were prepared and subjected to analysis via PCR testing. The findings of this study indicated that positive samples exhibited a notable correlation with uveitis [14]. In 2022, Paulin et al. conducted an evaluation of the serum prevalence of anti-*leptospira* antibodies in horses and workers related to them in Uruguay. This was achieved through the utilization of PCR and microagglutination methods. The findings of this study indicated that equines play a significant role as a reservoir for leptospirosis in the region [15].

In 2022, Jaeger and colleagues in Germany conducted an analysis of 1,840 horse intraocular fluid samples with the objective of identifying anti-leptospira antibodies and leptospira DNA. The presence of antibodies was identified in 83% of intraocular samples obtained from horses with

uveitis. Furthermore, the PCR method revealed the presence of Leptospira DNA in 72% of intraocular samples. These results, based on a very large number of intraocular samples, demonstrate the significant impact of leptospirosis and its association with uveitis in horses [16]. In 2022, Aziodo et al. conducted an investigation into a chronic reproductive syndrome in horses that was caused by leptospirosis in Brazil. In this study, the uterine samples were subjected to analysis using the PCR technique. The findings of this study substantiate the correlation between leptospirosis and chronic reproductive syndrome [17].

A review of the literatures reveals that the prevalence of Leptospira infection in horses varies across different geographical regions (8-18). Such discrepancies may be attributed to variations in climatic conditions across these regions, including fluctuations in temperature and humidity. Furthermore, it is pertinent to the diagnostic techniques employed to identify the *Leptospira* bacterium in horses. Additionally, the management and health factors present in the environment where horses are kept can also directly influence the rate of infection and contamination with this bacterium. It is also important to note that the simultaneous keeping of different animal species with each other, especially horses, in the vicinity of ruminants such as cows, has a significant and direct effect on the level of contamination of horses. In other words, studies indicated that the elevated prevalence of *Leptospira* infection in horses in a geographical area that cohabits with other animals, such as rodents, could be attributed to the fact that these infected animals have adapted to different serotypes, thereby facilitating transmission to horses.

Conclusion

The findings of the present study suggest that *Leptospira interrogans* is not a prevalent pathogen in horses in Kerman. Nevertheless, additional research is required to yield outcomes that are more definitive.

Conflict of interest

The authors declare that they have no competing interests.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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