Araştırma Makalesi/Original Article
Makale Dili "İngilizce"/Article Language "English"

Türk Hijyen ve Deneysel Biyoloji Dergisi

Molecular detection of leptospirosis from genital system in mares

Kısrakların genital sisteminden leptospirosisin moleküler tespiti

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ABSTRACT

Objective: Leptospirosis is a worldwide zoonotic disease and well recognized infectious disease of horses. Equine leptospirosis is to cause the birth of weak foals, neonatal deaths and abortion after pregnancy period. Studies on leptospirosis in horses are generally investigations of urine samples and serological studies. Although leptospirosis causes reproductive disorders, genital sample studies that may be a source of infection have been ignored. The aims of this study were to study the prevalance of *Leptospira* by PCR using vaginal swab from apparently healthy horses.

Methods: A total of 92 vaginal swab samples were collected and transferred to the bacterial diagnosis laboratory of Selcuk University Veterinary Faculty. The vulva and vagina were cleaned before swab samples were taken. All samples were stored in the refrigerator at -20°C and taken to the laboratory for processing. These samples were sent to the laboratory under cold chain conditions. DNA was extracted from suspicious samples and conventional PCR was used to detect Leptospira spp. Specific primers were selected and PCR was finalized for *Leptospira* spp.

ÖZET

Amaç: Leptospirosis, dünya çapında zoonotik olan ve atların iyi bilinen bir enfeksiyöz hastalığıdır. Atlarda leptospirosis, zayıf tayların doğumuna, yenidoğan ölümlerine ve gebelik sonrası abortlara sebep olmaktadır. Atlarda leptospiroz ile ilgili çalışmalar genellikle idrar örneklerinin araştırılması ve serolojik çalışmalardır. Leptospirosis, reprodüktif bozukluklara yol açmasına rağmen, enfeksiyon kaynağı olabilecek genital örnek çalışmaları göz ardı edilmiştir. Bu çalışmanın amacı, herhangibir klinik semptom göstermeyen kısraklardan alınan vajinal sürüntü örneklerinde PCR ile *Leptospira* prevalansını incelemekti.

Yöntem: Toplam 92 adet vajinal sürüntü örneği toplanarak Selçuk Üniversitesi Veteriner Fakültesi bakteriyel tanı laboratuvarına aktarılmıştır. Sürüntü örnekleri alınmadan önce vulva ve vajina temizlenmiştir. Tüm örnekler -20°C'de buzdolabında saklanmış ve işlenmek üzere laboratuvara götürülmüştür. Bu numuneler soğuk zincir koşullarında laboratuvara gönderilmiştir. Şüpheli örneklerden DNA ekstrakte edilmiş ve *Leptospira* spp.'yi saptamak için spesifik primerler seçilmiş ve konvansiyonel PCR kullanılmıştır.

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Geliş Tarihi / Received : 14.12.2022
Kabul Tarihi / Accepted : 08.05.2023

DOI ID : 10.5505/TurkHijyen.2024.01212

Karataş Yeni D, Balevi A, Gök A. Molecular detection of leptospirosis from genital system in mares. Turk Hij Den Biyol Derg, 2024; 81(3): 253 - 258

Results: As a result of the study, it was found that out of 92 mare's vaginal swab samples 7 (7.6%) were positive for *Leptospira* spp. This study is the first report from our country due to the detection of Leptospira spp DNA in asymptomatic mares.

Conclusion: It revealed that the Leptospira PCR positive mare were not showing any signs and symptoms. When the results and observations were evaluated, it was thought that Leptospira PCR positive mares could play a role as a carrier in the transmission of leptospirosis. Our study is one of the rare studies on mares carrying the possible causative agent. Detection of leptospirosis by PCR can be considered as a reliable method for early detection of *Leptospira* shedding in asymptomatic animals. In addition, molecular studies from vaginal swab samples were observed as a rapid and definitive diagnostic option, considering the difficulty of isolation of the *Leptospira* agent and possible contaminations. According to the results of our study, it is recommended to reevaluate the control measures against the disease and to carry out molecular characterization and vaccination studies in risky areas.

Key Words: Leptospirosis, mare, PCR

Bulgular: Çalışmanın sonucunda, 92 kısrak vajinal sürüntü örneğinden 7 (%7,6)'sinde *Leptospira* spp. pozitif olduğu saptanmıştır. Bu çalışma, asemptomatik kısraklarda Leptospira spp DNA'sının tespit edilmesi sebebiyle ülkemizden ilk rapordur.

Sonuç: Leptospira PCR testi pozitif bulunan kısrakların herhangi bir belirti ve semptom göstermediği belirlendi. Sonuçlar ve gözlemler değerlendirildiğinde, Leptospira PCR testi pozitif kısrakların leptospirosis bulaşmasında taşıyıcı olarak rol oynayabileceği düşündürdü. Çalışmamız, olası etkeni taşıyıcı kısraklar üzerine yapılan ender çalışmalardan biridir. Leptospirosisin PCR ile tespiti, asemptomatik hayvanlarda *Leptospira* saçılımının erken tespiti için güvenilir bir yöntem olarak kabul edilebilir. Ayrıca, *Leptospira* etkeninin izolasyon güçlüğü ve gelişebilecek kontaminasyonlar göz önüne alındığında, vajinal sürüntü örneklerinden moleküler çalışmalar, hızlı ve kesin bir tanı seçeneği olarak gözlemlendi. Çalışmamızın sonuçlarına göre, hastalığa karşı kontrol tedbirleri yeniden değerlendirilip riskli alanlarda moleküler karakterizasyon ve aşılama çalışmaları yapılması önerilmektedir.

Anahtar Kelimeler: Leptospirozis, kısrak, PCR

INTRODUCTION

Leptospirosis is a zoonotic infectious disease with multi-organ involvement caused by *Leptospira* spp., a gram-negative, non-sporeless, non-encapsulated and aerobic bacterium (1). Leptospiras are bacteria belonging to the Leptospiraceae family in the class Spirochaetales. Spirochetes are approximately 0.1 µm in diameter and 6-20 µm in length (1).

Leptospirosis is a zoonosis that has gained great importance in terms of public health. Transmission of the disease to humans occurs through animal reservoirs, contact with urine, and exposure to contaminated environments. In addition, floods that may occur as a result of natural disasters such as excessive precipitation and earthquakes contribute to the spread of the disease (2).

Diagnosis of leptospirosis is done by clinical signs, autopsy findings, microscopy, culture, animal experiments, serology and Polymerase Chain Reaction (PCR) (3). Microscopic Agglutination Test (MAT) is the most widely used serological test in reference laboratories of our country and known as the "gold standard" test (4). MAT has good specificity. However, the sensitivity of MAT in the early period is low (4). There is a high probability of false negative cases. Apart from these tests, complement fixation and immunofluorescence tests are also used in the

diagnosis of *Leptospira*. However, all these tests are not sufficient to identify subclinically infected animals (5). There is dire need for rapid diagnosis of the disease to detect carrier animals that do not show any symptoms at early stage of infection are possible with polymerase chain reaction (PCR). PCR is based on the molecular detection of amplified bacterial gene fragments found in pathogen. For the diagnosis of *Leptospira*, 16S rRNA target genes and real-time quantitative PCR has been used successfully (6).

This infection has been commonly observed in areas with tropical climates with a rainy season and high temperatures (7). Bacteria can survive in water or soil for days or months (8). The prevalence of infection is increasing due to flooding of many areas, poor hygiene and improper maintenance (8).

Leptospirosis can be controlled by adapting precautionary measures, e.g. stay away from animals and/ or areas contaminated with their urine. In addition, rodent control, treatment of carriers, occupational hygiene and routine vaccination of healthy animals are among the other recommended control methods (9). Doxycycline and penicillin options are the leading antibiotic treatment options and are considered to be more effective if the treatment is started within the first 3-4 days (9).

Leptospira spp. can causes reproductive disorders in animals. Especially, this disease symptoms have been observed in domestic animals such as cattle, sheep, pig and horse (10-12).

Molecular methods based on detection of DNA have higher sensitivity/ specificity rates compared to other diagnostic methods. Therefore, these methods are increasingly being used in the diagnosis of leptospirosis (13).

This study was designed to detect molecular prevalence of Leptospirosis in mares. Suspected samples were collected from mares and PCR was used to detect the DNA of *Leptospira*. PCR is considered to be rapid, more specific and sensitive method as compare to the microbial culture, isolation and serological studies.

MATERIAL and METHOD

Sampling and storage

A total of 92 vaginal swab samples were collected and transported to the Selçuk Üniversity Faculty of Veterinary Medicine, bacterial diagnosis laboratory.. For this study, samples were taken from mares aged 2-5 years in individual horse farms from rural settlements in Konya. The vulva and vagina were cleaned before swab samples were taken. All samples were kept at -20°C in a refrigerator and taken to the laboratory for processing. These samples were sent to the laboratory under cold-chain conditions.

Polymerase chain reaction

DNA was extracted from all swab samples using the Wizard® Genomic DNA Purification Kit (Promega, USA) following the manufacturer's instructions. The DNA samples were stored at −20°C until PCR analysis. A total of 92 DNA extractions and positive control were used in PCR. As positive control, *L. interrogans serovar pomona* (Leptovac 5®/Vetal, Turkey) were used in PCR.

PCRs were performed for *Leptospira* spp. using specific primers (14). The thermal conditions were as follow; 95°C 15 min, 40 (94°C 1 min, 50°C 1 min, 72°C 1 min) and a final extension at 72°C 10 min. The detection limit was 10 ng/µL of extracted DNA in all reactions. The PCR assays were carried out according to the conditions listed in Table 1.

All PCRs were performed using 5 µL 5× FIREPol®Master Mix (Solis Biodyne, Estonia), 20 pmol of each primer, and DNA template (50 ng/µL), and 1 µL water (negative control). Positive control DNA was used in each PCR series. PCR products were analyzed by electrophoresis on 1.5% agarose gels at 60 mA for 1 h, stained with ethidium bromide and visualized under UV illumination. A 100 bp DNA ladder (Thermo Scientific, SM0373) was used for comparison of DNA sizes.

The study was approved by the SÜVDAMEK Local Ethics Committee (Date: 16.02.2021 and Number: 2021/07).

Table 1. PCR primers, cycle conditions, and product sizes

Microorganism	Target gene	Primer sequences (5'--3')	Termal conditions	Product (bp)	References
16S rRNA	*Leptospira* spp.	GGCGGCGCGTCTTAAACATG TTCCCCCCATTGAAGCAAGATT	95°C 15 min, 40 (94°C 1 min, 50°C 1 min, 72°C 1 min) 72°C 10 min	331	Tramuta et al., 2011

RESULTS

The vaginal swab samples were collected from apparently healthy mares. The mares (92) were observed/ examined clinically and no signs or symptoms of the disease were observed. DNA was extracted from vaginal swabs and PCR was performed to detect the asymptomatic mares. The primers used were derived from 16S rRNA target gene (16S) of *Leptospira* spp. *Leptospira* spp. were detected in seven vaginal swab samples (Figure 1). Results revealed that 7.6% mares (7/92) were positive as carriers and shedding pathogen (*Leptospira* spp.).

DISCUSSION

Leptospirosis is responsible for several chronic infection in domestic and wild mammals in many countries of the World. Colonization of *Leptospira* has been widely reported in various animals via the renal route. Particularly, canine leptospirosis is characterized by an acute or chronic illness. Some dogs are known as asymptomatic carriers by urinary excretion (15). It has been shown that the bacterium can also be found in the female genital tract in ruminant animals and can be transmitted sexually between animals by these means (16). Abortion, stillbirth and neonatal death has been

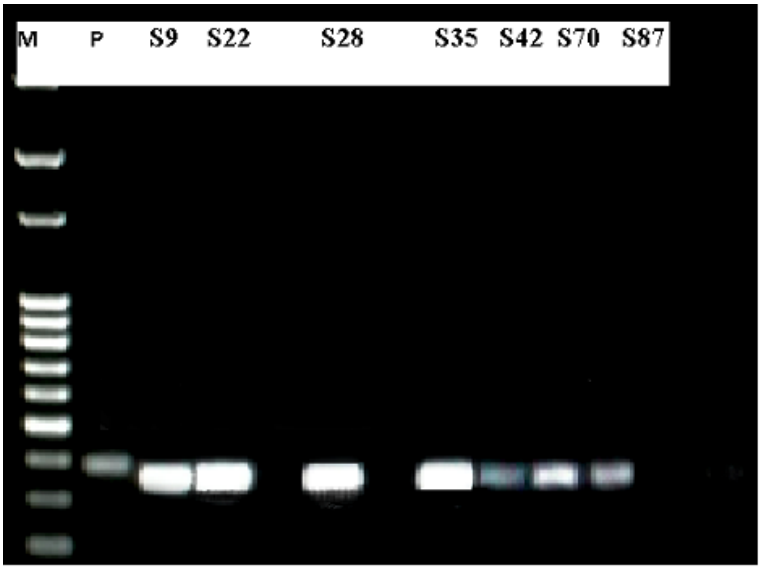

Figure 1. An ethidium bromide-stained agarose gel of PCR products that shows the sensitivity of the assay. M: DNA marker (100bp); P: positive control; S9-S22-S28-S35-S42-S70-S87:positive sample numbers)

noticed in mares with *Leptospira* infection in different studies (17-19). Till now, there have been no reports of evidence of *Leptospira* in the vaginal fluid of healthy mares (11). During this study DNA of leptospira spp was detected for the first time in vaginal fluid of apparently healthy mare.

"PCR is a very sensitive and specific test in the acute phase of leptospirosis, it detected 50% of cases that were negative with other serological tests. In the light of various studies, it has been shown that the PCR method has advantages over other serological tests in the early diagnosis of leptospirosis. Rapid diagnosis of Leptospirosis with a correctly applied molecular method is necessary for the efficient implementation of both animal and public health measures (10). Erol et al.compared the results of real time PCR, FAT and MAT in mare abortion cases and found that molecular methods are effective diagnostic method in the diagnosis of leptospiral abortion (20).

During this study we analyzed a total of 92 mare vaginal swab samples by conventional PCR assay using specific primers. Results revealed the presence of *Leptospira* especially in asymptomatic animals. During our study, none of the animals developed abortion while *Leptospira* spp. DNA was detected in seven samples.

The most important outcome of this study was evidence of leptospiral DNA in vaginal swab samples from mares with absolutely no clinical/ reproductive symptoms. Previously, It has been reported by researchers that leptospirosis can be transmitted between animals through direct or indirect contact with the urine of carriers. (16,21).

Therefore, detection of *Leptospira* spp. DNA in an apparently healthy mare's genital tract sample is a significant/ important finding. So, it is suggested that the carrier mares may be associated with spread of leptospiral infection through mucous membranes (11,22).

This study also revealed the possibility of sexual transmission of leptospirosis in horses (female to male). Early detection of asymptomatic carriers may play an important role in prevention and control of the disease. Di Azevedo et al. reported a systematic review of data on equine genital infection of leptospirosis (23). As a result of the study, it was reported that Leptospirosis can cause sexually transmitted "Equine genital leptospirosis" in horses. They emphasized the inadequacy of serological diagnosis for this disease and the contribution of molecular studies. This inference is consistent with our study. In order to adequately recognize this syndrome, clinical findings and molecular studies should be evaluated simultaneously, especially in mares.

Moreover, the results suggested that comprehensive further studies are required to investigate the disease in mares.

It was concluded that 7.6% apparently healthy mares were carriers of the leptospira, which is a great concern of animal and public health. Moreover, PCR was found to be a suitable method for early and rapid detection of leptospirosis in carrier animals. As a suggestion, genotypic characterization of *Leptospira* strains affecting mares by molecular methods should also be defined. At the same time, it will be evaluated in terms of public health and vaccinating horses in risky areas will make important contributions to protection and control measures.

ETHICS COMMITTEE APPROVAL

* The study was approved by the SÜVDAMEK Local Ethics Committee (Date: 16.02.2021 and Number: 2021/07).

CONFLICT OF INTEREST

The author declares no conflict of interest.

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