

Molecular Detection of *Leptospira* spp. Isolated from Aborted Ovine Genital Swabs in Lorestan, Iran, 2019-2020

Shabnam Anari¹ , Amin Jaydari^{1*} , Nemat Shams¹ , Heidar Rahimi² 

1. Department of Pathobiology, Faculty of Veterinary Medicine, Lorestan University, Khorramabad, Iran
2. Department of Pathobiology, Faculty of Veterinary Medicine, Semnan University, Semnan, Iran

ABSTRACT

Background and Aim: Leptospirosis is a zoonotic bacterial disease caused by the genus *Leptospira*. This disease is observed in humans, domestic and wild mammals. Abortion is known as the most important side effect of *Leptospira* in infected sheep. Molecular tests such as PCR are considered for the early detection of disease which cannot trigger the production of species-specific antibodies. This study was designed to detect *Leptospira* spp. as a cause of abortion in sheep using PCR in Lorestan province, Iran.

Materials and Methods: In the current study a total of 150 samples of vaginal swabs were randomly collected from a sheep that had an abortion in some areas of Lorestan province, Iran. The collected samples were accurately investigated to trace the DNA of *Leptospira* spp. using specific primers.

Results & Conclusion: Based on results of *Leptospira* spp. *lipL32* gene, 2 positive samples (1.3%) were identified. Hence, it was concluded that there is no significant relationship between the abortion rate and Leptospirosis in Lorestan province, Iran. It must be mentioned that due to the zoonotic nature of this bacterium, rapid diagnosis of this disease in livestock can extremely decrease the outbreak of this disease in humans.

Keywords: Leptospirosis, Abortion, Sheep, PCR

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Corresponding Information:

Amin Jaydari, Department of Pathobiology, Faculty of Veterinary Medicine, Lorestan University, Khorramabad, Iran
Email: jaydari.a@lu.ac.ir



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1. Introduction

Leptospira are one of the most widespread bacteria which can infect both humans and animals (1). These bacteria are not the microbiota of the human and animal bodies. They are aquatic and mobile objects which live naturally in humid areas such as moist soil, freshwater, sludge, and vegetation for long periods. In humans, direct contact with infected animals is the most known transmission route of leptospira. Moreover, animals are usually contaminated via soil, water, and body fluids which constantly excrete bacteria through urine, genital secretions, milk, and infected aerosols. It has been

reported these bacteria in infected and asymptomatic animals are able to survive for a couple of months in the proximal tube of the kidney and are excreted in the urine, consequently, it can increase the contamination rate of the environment and transmit the disease to human or other animals (1-3).

Leptospirosis is currently known as one of the major health problems in the world. The commonality of this disease between humans and animals can highlight the importance of this disease (4). This disease not only can extremely threaten

public health but also causes major economic losses to the country's livestock industry (e.g., abortion, poor births, fertility...). It must be mentioned that abortion is considered the most prevalent symptom of leptospirosis in sheep (2, 4, 5).

Since sheep husbandry plays a vital role in the livestock industry of many zones of Iran, such as Lorestan province, hence abortion is able to directly impress the economy of these provinces. Given the issue's importance in public health and economic losses, planning and implementing scientific standards in line with the importance of abortion management factors can be very influential in reducing this issue and preventing the resulting economic losses. Therefore, the aim of this study was to investigate the presence of *Leptospira* spp. genomic DNA using PCR in the vaginal swab samples of sheep with abortion history in Lorestan province, Iran.

2. Materials and Methods

Sample Collection

In this cross-sectional descriptive study, a total of 150 samples of vaginal swabs were collected from different areas of Lorestan province. It is important to note that the abortion history of the sheep which were selected for sampling, was less than a month. Moreover, the sampling was randomly performed with the cooperation of veterinary offices of Khorramabad, Chegni, Boroujerd, Noorabad, Kuhdasht, and Poldakhtar cities during the spring and autumn seasons from May 2019 to June 2020. The collected samples were transferred to tubes containing phosphate-buffered saline (PBS) and stored at -20°C until performing the project.

DNA extraction and amplification

To extract *Leptospira* spp. genomic DNA of the collected samples, the DNA extraction kit (GeneAll® Exgene™ Cell SVmini-250, Korea), was employed. The DNA extraction procedure was performed based on the manufacturer's instructions. PCR as a robust molecular tool was applied to amplify *Leptospira* spp. DNA in the collected samples. Nucleotide sequences were selected for *lipL32* gene amplification. The amplification was conducted in 25 µL total volume using 12.5 µL of 2X master mix (Ampliqon Taq DNA Polymerase Master Mix RED), 0.5 µL of each specific forward and reverse primers (10nM) (provided by Takapou Zist Company, Tehran, Iran), and 5 µL of the extracted DNA (Table 1).

The current study used standard *L. interrogans* genomic DNA donated by the *L. interrogans* Research Laboratory of the University of Tehran as a positive control (*L. interrogans* ATCC 43642). Also, all PCR reagents except extracted DNA were mixed and applied to prepare negative control. The PCR amplifications were performed by Bio-Rad thermocycler (Model T- 100, USA) under the following conditions: The initial step of 94°C for 2 min, followed by 35 cycles of 94°C for 30 s as denaturation, annealing at 59°C for 30 Second, extension at 72°C for 1 min, and followed by a final extension at 72°C for 5 min.

To investigate the PCR products, a 1.5% agarose gel (Merck, Germany) containing 2 µg/mL DNA-safe stain (Cinnagen, Iran) was used. After electrophoresis, the gel was placed in a Trans illuminator (E-Box, Iran) to visualize the specific bands. Finally, the PCR products with 242bp in length were compared with ladder and positive /negative controls.

Table 1. The sequence of specific primers used to amplify the genotype of *Leptospira* spp.

Target gene	Primer name	Primer sequence	Amplification Size (bp)	Reference
Lip L 32-45F	F ₁	5-AAG CAT TAC CGC TTG TGG TG-3	242	(6)
Lip L 32-286R	R ₁	5-GAA CTC CCA TTT CAG CGA TT-3		

3. Results & Discussion

The results of this study revealed that out of 150 samples of vaginal swabs collected from sheep with an abortion history in different areas of Lorestan province, generally, two samples (1.3%) were diagnosed as positive cases by PCR. All positive samples of the 242bp band were observed (Figure 1). The results of data analysis demonstrated that among the different areas which was selected for sampling pollution was traced only to Khorramabad city and the rest of the cities had no infection (Table 2).

Leptospirosis is one of the most important diseases with global spread which leads to significant economic damage to the country's livestock industry through the increase in mortality rate, a decrease in milk production, and an increase in reproductive disorders(e.g. abortion) (7, 8). Many records from the scientific centers demonstrated that this infection is one of the problems of the livestock industry in most parts of Iran. Due to the characteristics of this bacterium, it can properly survive in wet soil and

water, therefore this bacterium is mainly found in areas with hot and humid climates and heavy rainfall, such as the northern provinces and Khuzestan (9-12). Since no study has been conducted on the prevalence of this disease in sheep in Lorestan province, the current study was designed to

investigate the prevalence rate of this bacterium in sheep with an abortion history.

Table 2. Frequency and percentage of positive cases of *Leptospira* infection in vaginal swab samples of aborted sheep in Lorestan province

Sampling Location	Sampling Season	Number of Samples	Number of positive Cases	Number of negative cases
Khorrarnabad and Dore Chegni	Spring	89	2 (%2.3)	87 (7/97 %)
Borujerd	Fall	3	(0)0	3 (100)
Koohdasht	Fall	11	(0)0	11 (100)
Noor-Abad	Fall	17	(0)0	17 (100)
Poldokhtar	Fall	30	(0)0	30 (100)
Total		150	1.3 %	148 (98.7%)

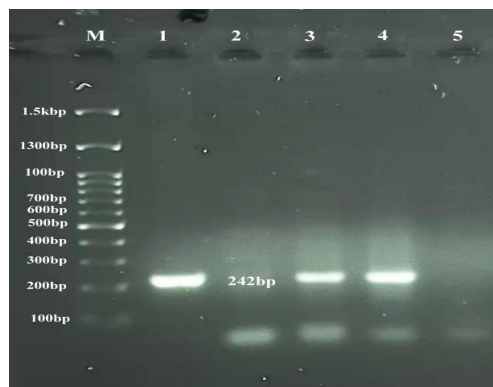


Figure 1. The PCR results of samples on agarose gel 1.5%. M; 100 bp DNA Ladder; Column 1: positive control; column 2: negative control; columns 3,4: positive samples; columns 5: negative sample. The desired band at this stage is 242bp

Due to technical problems of culture and isolation of the causative agent of this disease, such as time-consuming and low sensitivity, the major studies which are designed for the diagnosis of this disease are based on serological and molecular methods (13). Currently, serological tests are taken into account as the most common diagnostic test for the detection of this bacterium. MAT is the most important standard serological test used to detect anti-*Leptospira* antibodies in the serum. However, there are several limitations to applying this test, including the necessity of maintaining a wide range of *Leptospira* serovars to provide a live antigen source, the need for standard antisera and dark field microscope, the skill and expertise of laboratory technicians to have paired serum samples that delay diagnosis, and false-negative results, which are mainly due to not testing with all serotypes. However, interpretation of MAT is difficult due to the high rate of cross-reactions that occur among different serogroups, especially in the acute phase. Due to the mentioned points and limitations in the diagnosis of chronic infections with the MAT method, it seems that molecular methods

such as PCR can be reliably, and rapidly performed on a wide variety of samples such as urine, milk, liver, kidney, and vaginal secretions (14, 15). In this study, the PCR method was used to detect the presence of *lipL32* gene, which encodes one of the most important immunogenic proteins of the *Leptospira spp.* outer membrane. In this study, which was analyzed out of samples of vaginal swabs by the PCR method 1.3% of samples were positive for *Leptospira spp.* The results of this study were confirmed with the results of a study performed by Haji Hajikolaei *et al.* (11). The results showed a low percentage of infection in sheep in the region. According to the results of their study, the positive cases of the disease were related to the season (spring, which may be due to the favorable weather conditions and rainfall) and applying a traditional feeding system. Considering the mountainous climate of Lorestan province and considering that most of the studied areas are non-swampy environments and belong to cold and mountainous climates, the type of sample collection, the applied technique, and the low percentage of positive samples can be justified. Based on the

studies performed, the best sample to diagnose abortion because of leptospira is sampling from the abomasum of an aborted fetus, and if the fetus is not available vaginal sampling is important (16-18). In view of this point and the density of livestock population in the province, the traditional method of husbandry and raising sheep, and the climate of Lorestan province, it seems that the percentage obtained in this study cannot accurately justify the pollution situation in this province. Babakhani *et al.* (10) in a study of a total of 200 serum samples taken from Wisian paddy farmers in Lorestan province by MAT method, reported a 30% prevalence of serum contamination with *Leptospira* serovars. The most important reason for this difference can be related to the type of technique used and the selection of the sample under study.

Hamli *et al.* (12), in the study of *leptospira*-induced abortion in cows of Chaharmahal Bakhtiari province by PCR method, from 120 samples collected from the abomasum of an aborted fetus, reported 17 positive samples (14.16%) (12). The most important reason for the difference between the results of Hamlin's study and the current study is probably related to the type of sample studied and the animal species. According to sources, in cattle, unlike sheep, bacteria except in the liver, kidneys, lungs, placenta, and uterus of the cow can colonize in the vaginal secretions of cows. In the case of sheep, this bacteria is mainly located in the kidneys and can cause pyelonephritis, consequently, it is widely spread through urine in the surrounding environment (16, 17). Almeida *et al.* (3) sampled vaginal secretions from 15 ewes of a herd in Brazil that had no history of abortion or reproductive disorders, and their serum contamination had previously been confirmed by MAT. In the mentioned study, after PCR using *LipL32* gene primers, seven samples (46.7%) contained the *Leptospira* genome. Because *Leptospira* is mainly located in the kidneys of sheep and subsequently the bacteria can spread through urine in the environment, it is possible that the found DNA of the *Leptospira* in the present study and other researchers, such as Almeida *et al.* (3) contains bacteria due to vaginal contamination of urine. The results of this study and another study by researchers (16-18) indicate that whether the vagina is primarily infected by bacteria or secondarily by urine passage,

there is a possibility of disease transmission from female animals and the potential for infection from male sheep through sexual transmission. However, since *Leptospira* is traceable in semen and sexual secretions of male animals and sexual transmission from male sheep to ewes is well identified, this may be the reason for the infection of female sheep in this study.

A brief look at the mentioned studies shows that there are differences between various regions in terms of serum and molecular contamination with *Leptospira*. These differences can be related to geographical conditions, animal species, amount of stagnant and surface water in the area, location and method of livestock (traditional or industrial), quality of farms and the number of livestock, simultaneous maintenance of several types of livestock, herd health, vaccination, ambient temperature, and annual rainfall.

5. Conclusion

Unfortunately, few studies have been conducted on the small role of this bacterium in sheep abortion, it seems that the frequency of this crime in sheep herds in Lorestan province is more than the amount obtained in this study. Due to the zoonotic nature of this bacterium and the importance of diagnosing the disease in livestock to control the disease in humans, rapid diagnostic methods such as PCR and using serological tests with a sampling of the abomasum of an aborted fetus are used considered necessary.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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