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**Original article**

## Serodiagnosis and molecular survey on leptospiral abortions from sheep farms in Tabriz and surrounding areas

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### ABSTRACT

Leptospirosis is presumed to be the most widespread zoonosis in the world. The purpose of this study was to determine of seroprevalence of leptospirosis among the aborted ewes and in the same time, detection of bacterial DNA in aborted fetal tissues by the PCR protocol. A total number of 70 aborted fetuses and related placentas were submitted to the large animal clinic at the University of Tabriz, from October 2010 to March 2011. Tissue samples were collected from several fetal organs including liver, brain, kidney, lung, spleen, heart, stomach fluid and placenta, then separately pulverized under liquid nitrogen and finally stored at -20°C until DNA extraction. DNA extraction from frozen tissues samples was performed using a commercial kit (AccuPrep Genomic DNA Extraction Kit, Bioneer, S. Korea) following the manufacturer's instructions. Of 70 submissions, 6(8/57%) samples were diagnosed positive to the leptospira Interrogans by the PCR. In contrast 7(10%) out of 76 dam's sera were diagnosed as positive to leptospirosis by the microscopic agglutination test (MAT). One out of 70 animals was seropositive to serovar canicula, two animals to serovar pomona, two animals to grippotyphosa, one animal to serovars: grippotyphosa, pomona, hardjo and one animal to serovars: grippotyphosa, pomona,

icterohaemorrhagiae. However, statistical difference was not observed between two diagnostic techniques ( $P \geq 0/05$ ). In conclusion, both pomona and grippotyphosa serovars induced abortions were determined to be more common leptospiral abortions in sheep flocks in Tabriz (north-west of Iran).

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## **1. Introduction**

Leptospirosis is a common disease of livestock, pet animals and wildlife throughout the world. Sporadic cases and outbreaks of the disease have been reported from USA, U.K, Australia, New Zealand, USSR and countries of Europe and Asia. The disease is common in cattle, buffalos, sheep, goats, dogs and equines and causes fever, jaundice, nephritis, reproductive disorders and death. In dairy animals, loss of milk and mastitis may be observed (Srivastava, 2006, Hassapour, et al., 2009).

In the recent serological investigations carried out in the Leptospira Research Laboratory of veterinary faculty of Tehran University, it was revealed that the people or animals living in the Tehran, Guilan, Azarbaijan, Khorasan, Khozestan, Isfahan, Chaharmahal bakhtiary and Boushehr provinces are 25-42% seropositive to the leptospira spp. respectively (Haji Hajikolaei, et al., 2005, Ebrahimi, et al., 2003, Sakhaee, et al., 2011, Abdollahpour, 2011) .

Among sheep most of the outbreak goes unnoticed due to lack of proper clinical signs and they usually react asymptotically to the infection (Kingscote, 1985).

The pathogenic leptospira can colonize in the kidneys and shed in the urine for prolonged periods. The clinical manifestation of leptospirosis ranges from mildness to sever life threatening disease with jaundice, renal failure or abortion during last month of pregnancy. Sheep may acquire the disease from contaminated urine of rodents, cattle or other farm animals (Shivaraj et al., 2009) Serological studies suggest that most prevalent sero groups associated with sheep worldwide are autumnalis, grippotyphosa and pomona (Faine et al., 1999). Due to the enormous losses that the disease causes to sheep industry of Iran (primarily due to abortion in the last month of gestation) the correct and prompt diagnosis is important in controlling and eradicating the disease in region.

Efficient diagnosis requires a complete diagnostic protocol associated with submission of appropriate specimens and clinical history.

Leptospirosis diagnosis based only on bacteriological culture from aborted fetus samples is not frequently successful, because the fetal expulsion occurs 24-48 hours after its death, and it is already contaminated with ubiquitous and faster growing bacteria.

MAT is considered as a specific test for diagnosis of the infecting serovar or closely antigenically related serovar, thus the high sera prevalence associated to one of the leptospiral serovars could be indicator of abortion cause. Although it has been proved that PCR is able to detect minimal quantity of DNA from any microorganism in all kind of biological sample, the processing is critical and must be adjusted to the tissue, fluid and species being tested (Genovez et al., 2006).

Considering the potential of PCR for etiological diagnosis of infectious ovine abortion, the objective of this study was to use PCR protocol as a tool for identification of pathogenic Leptospira spp. in tissues from aborted ovine fetuses and simultaneously MAT as a complementary test for detection of leptospiral antibodies in sera of dams subjected to abortion. This study included dam's sera and frozen tissues from the same aborted fetuses and placentas.

## **2. Materials and methods**

### **2.1. Samples**

From October 2010 to March 2011, 70 blood and tissue samples were collected from ewes and their aborted fetuses (n=70) immediately after abortion at the sheep farms located in the Tabriz vicinity. Blood samples were centrifuged and sera harvested and kept at -20°C. Tissue samples were collected from several fetal organs including liver, brain, kidney, lung, spleen, heart, stomach fluid and placenta, then pooled together pulverized

under liquid nitrogen and finally stored at -20°C until DNA extraction. All of the aborted fetuses had 4-5 months age (aborted in last month of gestation).

## 2.2. Microscopic agglutination test (MAT)

The MAT was used to detect antibodies in dam's sera to *L. interrogans* serovars: hardjo, grippityphosa, pomona, icterohaemorrhagiae and canicola. The test was performed at the Leptospira Research Laboratory of veterinary faculty of Tehran University in microtitre plates as previously described by Cole et al. (1973) with 4 days old cultures of the standard strains (Prepared by the same laboratory). Serial dilutions (1/100 to 1/12800) of serum in phosphate-buffered saline (PBS) solution, each dilution being half of the previous one, were tested and the agglutinations were read under darkfield microscopy after three hours of incubation at 30°C. Individual sera were considered positive where agglutination was present at dilutions of 1/100 or more, using agglutination of 50% or more of the leptospira as the end point.

## 2.3. DNA extraction

DNA extraction from frozen tissues samples was performed using a commercial kit (Accuprep Genomic DNA Extraction Kit, Bioneer, S. Korea) following the manufacturer's instructions. Briefly, 100 µL of thawed homogenates of fetal tissues were mixed with 600 µL of Nuclei Lysis Solution and homogenized for 10 seconds. Samples were incubated at 65°C for 30 min, followed by the addition of 17.5µL proteinase K (20mg mL<sup>-1</sup>) and incubation at 60°C for three hours, vortexing every 30 min. Three microliters of RNase A (4mg mL<sup>-1</sup>) were added, the samples were mixed and incubated at 37°C for 30 min. After cooling, 200µL of Protein Precipitation Solution were added, followed by vortexing and centrifugation at 13,000 g for 4 min. The supernatant was transferred to a new microtube with 600µL of isopropanol, mixed, and centrifuged at 13,000 g for 3 min. The supernatant was discarded and the pellet was washed with 600µL of 70% ethanol, followed by a final centrifugation at 13,000 g for 3min. Each pellet was dissolved in 100µL of DNA Rehydration Solution by incubating at 65°C for one hour. DNA quality was assessed by spectrophotometry and samples that had not DNA concentration lower than 100ng µL<sup>-1</sup> as assessed by spectrophotometry were excluded from further analysis.

## 2.4. PCR

PCR was used for detection of pathogenic *Leptospira interrogans*. PCR reactions were performed using 13µL of a commercial PCR mix (Accupower PCR preMix, Bioneer, S. Korea), 0.75µL of a 25µM solution of each primer (Table 1), and 1µL of DNA (100 to 500ng per reaction). Parameters used were initial denaturation at 95°C for 5min, followed by 35 cycles of denaturation at 95°C for 1min, annealing for 1min, extension at 72°C for 1min and a final extension at 72°C for 7min. Positive control from ATCC strains: 23470, 23478, 43642, 23469 (*Leptospira* pathogenic, Genekam Co., Germany) and negative controls (in which DNA template was replaced by PCR-grade water) were included in all reactions. Furthermore, two primers PRL033 and PRL035 that target a part of the ovine prolactin gene were considered as an internal control. PCR products were resolved by electrophoresis in a 1% agarose gel stained with ethidium bromide.

## 2.5. Statistical analysis

Frequencies of positive results were compared between PCR and MAT tests by the McNemar Test and the statistical difference was not observed between two diagnostic methods ( $P \geq 0.05$ ).

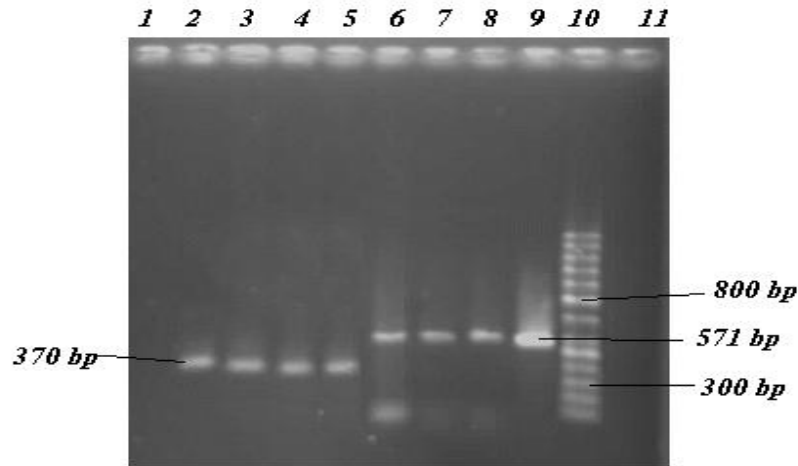
**Table 1**  
Primer sequences for pathogenic *Leptospira*.

Target	Product size	Primer sequences	Name
16S rRNA	571 bp	5'-AGGGAAAAATAAGCAGCGATGTG-3'	Outer primer set
		5'-ATTCCACTCCATGTCAAGCC-3'	
16S rRNA	370 bp	5'-GAAAAGTGCAGGGCTCAAAC-3'	inner primer set
		5'-GCTCCACCGCTTGTGC-3'	
Exone 3	156 bp	5-CGAGTCCTTATGAGCTTGATTCTT -3	PRL35
		5-GCCTCCAGAAGTCGTTTGTG TTC-3	

## 3. Results

Six (8/57%) out of 76 samples (fetuses and placentas), were diagnosed positive to *L. Interrogans* serovars by the PCR test (Fig. 1, Table 2). In contrast, 7(10%) out of 70 dam's sera were diagnosed as positive to leptospirosis by the microscopic agglutination test (MAT). One out of 70 animals was seropositive to serovar canicula, two animals to serovar pomona, two animals to grippotyphosa, one animal to serovars: grippotyphosa , pomona , hardjo and one animal to serovars : grippotyphosa , pomona, icterohaemorrhagiae (Table3).

Amplification of the prolactin gene with primer pair (HL033 and HL035) gave product of ~ 156 bp in all fetal tissue samples.



**Fig. 1.** Representative results of PCR amplification of genomic DNA of pathogenic *Leptospira interrogans* in fetal tissues: Lane1: Negative control; Lane2&6: positive controls (Genekam Co., Germany; Lane 3, 4&5: PCR products with inner primers; Lanes 7, 8 & 9: PCR products with outer primers positive samples from aborted fetuses; Lane10: 100 bp molecular weight marker (Bioneer, S. Korea); Lane11: Non Template Control (NTC).

**Table 2**

Frequencies of abortions caused by *Leptospira* Spp. detected by MAT (in dam's sera) and PCR ( in fetal tissues) tests.

Test	Positive	Negative	Total
MAT	7 (ewes) (10%)	63 (ewes) (90%)	70 (100%)
PCR	6(fetuses) (8.57%)	64(fetuses) (91.43%)	70 (100%)

**Table 3**

Distribution of serovar specific antileptospirosis antibodies and their titration in seropositive aborted ewes.

Serovar	1:100	1:200	Total
Pomona	3	1	4(57.14%)
Icterohaemorrhagiae	-	1	1(14.2%)
Canicula	-	1	1(14.2%)
Grippotyphosa	2	2	4(57.14%)
Hardjo	-	1	1(14.2%)
Total	5	6	7 (100)*

\*Some of the ewes showed positive reaction against to more than a leptospiral serovars.

#### 4. Discussion

Leptospirosis is a worldwide zoonotic infection caused by pathogenic *Leptospira* spp, with a much greater incidence in tropical regions and has recently been identified as one of the emerging infectious diseases in Iran and many other countries (Levett, 2001, Abdollahpour, et al. 2009).

The source of infection in human and animals is usually either direct or indirect contact with the urine or uterine discharges of an infected animal (Everard, J. D. and Everard, C. O. R., 1993, Ratnam, 1994).

The etiologic agent is *Leptospira interrogans*, which has over 180 serovars in 19 serogroups. Each serovar is adapted to a particular reservoir host but can cause disease in any mammalian species. In sheep, the major serovars are *autumnalis*, *grippotyphosa* and *pomona* (Faine et al., 1999).

Sheep are not naturally maintenance hosts for some of the serotypes such as *pomona* or *hadjo* and are likely to have infections of relatively short duration, producing severe pathologic effect. However, persistent leptospiuria and high seroprevalence rates of the infection in sheep where no contact with cattle have occurred suggest that sheep may be a maintenance host (Radostitis et al., 2007).

Since the clinical signs of Leptospirosis have been infrequently seen in the sheep farms of Tabriz by clinicians; however, the most prevalent feature of disease in this area is abortion.

Signs in the aborted fetus are negligible. Focal tubular necrosis and interstitial nephritis may be seen. Autolysis of the fetus is common.

The serological results indicated that *Leptospira interrogans* serovars *pomona* and *grippotyphosa* are the most prevalent serovars in the sheep flocks of Tabriz. This is in accordance with the results obtained by other researchers in Iran and other countries (Tooloei et al., 2008; Faine et al., 1999; Haji Hajikolaei, et al., 2007), But in contrast with the results obtained by Hasanpour et al (2011) which emphasizes on the prominent role of serovar *Canicola* in prevalence of ovine leptospirosis (Hasanpour et al., 2011). This shows that the regional weather may affect the type of leptospiral serovar prevalence among the fields.

On the other hand, most of the leptospiral positive aborted fetuses in our study were belonged to the last month of gestation. This is in accordance with the above mentioned bacterial characteristics of *Leptospira* spp. in relation to the period of abortion in sheep.

Comparing the accuracy of MAT vs. the PCR protocol, it seems that there is no statistical difference between two methods but, the molecular tests are much more reliable than the serological tests, because the seropositive animals may abort by other pathogens and not necessarily by the *Leptospira* spp.

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