

Molecular Study of the Prevalence of *Leptospira* spp.

Serovar *Hardjo* in Blood Samples of Iranian Cattle and Sheep

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Abstract

Leptospira is an emerging global public health problem which involves both animals and human. The aim of this study was to appraise the presence of *Leptospira* DNA in blood samples from cattle and sheep via Polymerase Chain Reaction (PCR) method in Chaharmahal VaBakhtiari and Isfahan provinces of Iran. One hundred and ninety five samples (92 blood samples of cattle and 103 blood samples of sheep) were collected randomly from clinical healthy animals. DNA was extracted from the blood samples and stored at -20°C before examination. PCR reaction was performed for detection of *Leptospira* DNA using specific primers for 16s rRNA gene and PCR products were visualized in a 1% agarose gel electrophoresis. Results showed that 18.63% of the blood samples were positive. Leptospiral DNA was found in 20 of 92 (21.73%) and 16 of 103 (15.53%) of cattle and sheep blood samples, respectively. Nine (9.78%) out of the 92 cattle sampled and 5 (4.85%) out of the 103 sheep sampled were positive for *Leptospira* spp. serovar *Hardjo*. This indicated that these cows and sheep's are reservoirs and dangerous for human health. Moreover, the PCR method is sensitive and specific for diagnosis of *Leptospira* in suspected cases.

Keywords: blood, cattle, Leptospirosis, PCR, prevalence, sheep

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Introduction

Leptospirosis is an important re-emerging zoonotic disease in tropical and subtropical areas and acute febrile infection and a conveyable bacterial disease of animals and humans caused by pathogenic spirochaetes of the genus *Leptospira*. Leptospirosis is a emerging global public health problem as it causes increased mortality and morbidity in different countries (Radostits, 2000; Adler and de la Pena Moctezuma, 2010; Baer et al., 2010; Shafighi et al., 2010; Doosti et al., 2011). These bacteria have the skill to outlive in a wide range of environmental reservoirs, including several mammalian hosts (domestic and wildlife animals) and humans. The causative organisms can be found in cattle, sheep, swine, horse and dogs and can cause chronic tubulointerstitial nephritis, uveitis, mastitis, myocarditis and hemolysis (Haji Hajikolaei et al., 2007; Radostits et al., 2007; Agampodiet al., 2010). In cattle this infection causes reproductive losses, abortion, barrenness, stillbirths or weak calves, decrease in milk production, and even death (Levett, 2001; Bharti et al., 2003; Dufour et al., 2008).

Most leptospiral infections in sheep and goat are asymptomatic, however they may result in high fever, abortion, birth of a dead fetus and prenatal death. Affected lambs and kids may manifest fever, hemoglobinuria and jaundice, which may also result in death and all of which cause an economic loss (Ciceroni et al., 2000; Aghaiypour and Safavieh, 2007; Lilenbaum et al., 2008). In human, a serious effect of leptospirosis is the high rate of case fatality because of its most severe complications: severe pulmonary haemorrhage syndrome (50%) (Gouveia et al., 2008) and Weil's disease (10%) (Bharti et al., 2003).

In Asia pacific area, Southeast Asia and in Middle East and Latin America it is highly prevalent and there has been a marked enlargement in the total of eruption and cases reported during the last twenty years (Victoriano et al., 2009; Zakeri et al., 2010). Illness is well kept via chronic carrier hosts that excrete the organism into the environment. Members of the genus *Leptospira* are conventionally grouped into 2 detached species based on pathogenicity. The pathogens are from the parasitic "interrogans" group, while the no pathogens are from the saprophytic "biflexa" group. Usually, *Leptospira interrogans*, not *Leptospira biflexa* can be extracted from the patient's blood, cerebrospinal fluid, and urine. All the pathogenic leptospires were previously classified as members of the species *Leptospira interrogans*, though the genus has lately been reorganized and pathogenic leptospires are now recognized in 17 named species and four genomospecies of *Leptospira*. There are over 200 distinct leptospiral serovars recognized and they are organized in 23 serogroups (Levett et al., 2005; Honarmand et al., 2009).

According to the report of Doosti et al. (2011), The frequency of Leptospiral DNA in liquid rennet samples of aborted bovine was 17 (14.16%) of 120. The results showed a high frequency of *Leptospira* infection in aborted bovine in Chaharmahal VaBakhtiari province (Doosti et al., 2011). Moreover, Momtaz et al.

(2012) showed that out of 340 samples, 46 (20.9%) and 14 (11.66%) were recognized positive for *Leptospira* spp.

It is needed and required to improve diagnostic procedures for animal leptospirosis. Leptospiral isolation is costly, very hard and often failed (Lucchesi et al., 2004; Fearnley et al., 2008). Nowadays, for the isolation of bacteria and culture, serologic methods like microscopic agglutination test (MAT), immunofluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA) and slide agglutination test (SAT), in addition to molecular methods like Polymerase Chain Reaction (PCR), are used for detection of *Leptospira*. The standard laboratory diagnosis of leptospirosis is using the microscopic agglutination test (MAT). This method depends on antibodies to leptospiral antigens, is time consuming and complicated, and, requires trained personnel (OIE, 2000; Lucchesi et al., 2004; Ahmad et al., 2005; Bomfim et al., 2006).

Serologic examinations do not allow ahead of time leptospiral diagnosis, particularly in infections caused by serovar *Hardjo* type *Hardjo-bovis* (Bolin et al., 2003). Therefore PCR is applied as a diagnostic device for the partiality of pathogenic and non-pathogenic (Smythe et al., 2002).

In this study, researchers aimed to investigate prevalence of pathogenic *Leptospira* spp in blood samples from cattle and sheep with methods of PCR and report the development of PCR detection of *Leptospira* spp, without the need for prior isolation and culture.

Materials and Methods

Sampling and DNA extraction: In this research, from January 2013 to February 2013, 195 blood samples (via jugular vein) were collected randomly from clinically healthy animals from 92 cows and 103 sheep located in Chaharmahal VaBakhtiari and Isfahan provinces. Five cc of blood was collected from jugular vein of each sheep and cattle. Genomic DNA was extracted from blood specimens by CinnaGen DNA extraction kit (CinnaGen, Iran) as said by the industrialist's recommendation. The extracted DNA was quantified via spectrophotometric measurement at a wavelength of 260 nm as described by Sambrook and Russell (2001). The extracted DNA of each sample was kept frozen at -20°C until used and delivered to Biotechnology Research Center of Islamic Azad University of Shahrekord.

Table 1 Samples from cattle and sheep blood were positive for *Leptospira* spp. serovar *Hardjo*

Number of samples	<i>Leptospira</i> spp.	<i>Leptospira</i> serovar <i>Hardjo</i>
Cattle (n= 92)	20 (21.73%)	9 (9.78%)
Sheep (n= 103)	16 (15.53%)	5 (4.85%)

Gene amplification: Species-specific oligonucleotide primers Lp-F: 5'-GCGCGTCTTAAACATGCAAG-3' and Lp-R: 5'-CTTAACGTGCTGCCTCCCGTAG-3' designed from 16S ribosomal RNA gene of *Leptospira* (accession number: JF460977.1) were used for gene amplification.

Leptospira hardjo oligonucleotide primers Lep-haj-F: 5'-TAAGCAGCGATGTGATGATGG-3' and Lep-haj-R: 5'-CTACGCATTTCACCGCTACAC-3' (accession number: LBU12670) were used for gene amplification with Size of 237 bp.

PCR amplification was carried out in an entire volume of 50 μ l in 0.5 ml microtubes containing 2 μ g of template genomic DNA, 1 μ M of each primers, 2 mM MgCl₂, 200 μ MdNTP, 5 μ l of 10x PCR buffer and 1 unit of Taq DNA polymerase (Roche applied science, Germany). The following conditions of PCR were used for gene amplification: initial denaturation at 95°C for 5 min, followed by 32 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min. Then, a last extension at 72°C for 5 min was done at the end of the amplification. The PCR amplification products (15 μ l) were subjected to electrophoresis in a 1% agarose gel in 1X TBE buffer at 80 v for 30 min and stained with Ethidium Bromide. Then, they were images were obtained in UVIdoc gel documentation systems (Uvitec, UK). The PCR products were identified by 100 bp DNA size marker (Fermentas, Germany). A negative control (sterile water) and a positive control DNA from *Leptospira* ATCC\ 43642 strain were involved in each gel electrophoresis run.

Results and Discussion

One hundred and ninety five cattle and sheep blood samples were examined for the presence of leptospiral DNA. Results of this research showed that 18.63% of the blood samples were positive. Leptospiral DNA was found in 20 of 92 (21.73%) cattle blood samples and Leptospiral DNA was found in 16 of 103 (15.53%) sheep blood samples. Nine (9.78%) out of the 92 cattle sampled and 5 (4.85%) out of the 103 sheep sampled were positive for *Leptospira* spp. serovar *hardjo* (Table 1). This showed that the cows and sheep were reservoirs in dairy herds of Chaharmahal VaBakhtiari and Isfahan provinces. The study of PCR products for the presence of 16S rRNA gene of *Leptospira* on 1% agarose gel revealed a 306 bp fragment (Fig 1). The existence of Leptospiral DNA was detected in the positive control. Moreover, also the positive control showed a target sequence on agarose gel electrophoresis, but no product was observed for negative control.

The results of this research display that 21.73% of the blood samples in of cows and 15.53% of the blood samples of sheep served as a reservoir of disease in dairy farms of Chaharmahal VaBakhtiari and Isfahan district. Consequently, it could be stated that the animal reservoirs increase the risk of potential spread of disease to other animals, especially humans, and this deserves special attention.

The prevalence of leptospiral infection in sheep based on serologic survey has been reported to be 16.8% in Greece (Burriel et al., 2002), 6.1% in Italy

(Ciceroni et al., 2000). Another study in Turkey reported that 44.77% of cattle and 8% of sheep reacted to one or more serovar of *L. interrogans* (Ozdemir and Erol, 2002).

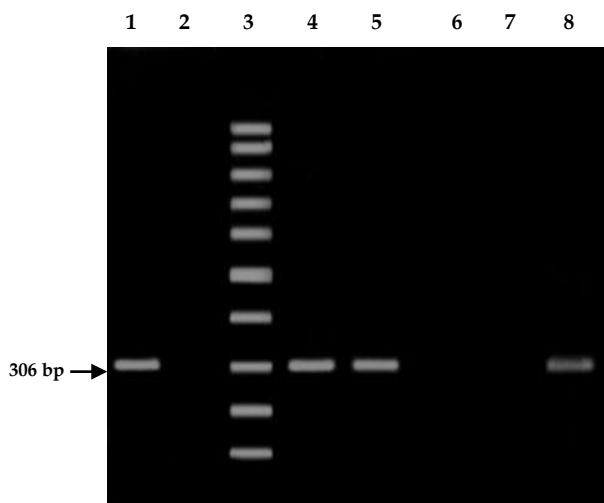


Figure 1 Agarose gel electrophoresis for identification of leptospiral DNA in the bloodsamples of cattle and sheep. Lanes 1 and 2: positive and negative controls, respectively; lane 3: 100 bp DNA ladder (Fermentas, Germany); lanes 4, 5 and 8: positive samples; lanes 6 and 7: negative samples

Rodrigues et al. (1999) detected *Icterohaemorrhagiae* and *Pomona* as dominant serovars in Brazilian cattle during the period of 1996 and 1997. Although earlier researches had shown that *Hardjo* and *Pomona* serotypes were predominant, these results propose that changes in the usual serovars in the district happened (Rodrigues et al., 1999). As said by studies showed in Ahvaz, the high prevalence of leptospirosis attributed to hot and humid weather of Khuzestan area and heat temperature was reported more important than moisture (Hajj Hajikolaei, 2007). *Canicola* and *hardjo* serovars were found in Gilan and Ahvaz, but in another research, three serovars were found in Shahrekord (*Icterohaemorrhagiae*, *Pomona*, *Grippotyphosa*) (Hajj Hajikolaei, 2007; Momtaz and Moshkelani, 2012).

In conclusion, even though the disease is seen in tropical nations, it could also be present in cold and mountainous regions for example Chaharmahal VaBakhtiari. Therefore, an animal with leptospirosis should be removed from the environment as it can be the cause of transmission of the disease to other animals and humans. Consequently, screening the serovars in all area frequently is important to prevent the spread of the disease and laboratory support is an important tool in the identification of the disease. Apparently, PCR is one of the best ways to detect and characterize *Leptospira* because it is a fast, less hazardous and sensitive method.

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References

Adler B and de la Pena Moctezuma A 2010. *Leptospira* and leptospirosis. *Vet Microbiol.* 140: 287-296.

Agampodi S, Peacock SJ and Thevanesam V 2010. The potential emergence of leptospirosis in Sri Lanka. *Lancet Infect Dis.* 9: 524- 526.

Aghaiypour K and Safavieh S 2007. Molecular detection of pathogenic *Leptospira* in Iran. *Arch Razi Inst.* 4: 191-197.

Ahmad SN, Shah S and Ahmad FM 2005. Laboratory diagnosis of leptospirosis. *J Postgrad Med.* 51: 195- 200.

Baer R, Turnberg W, Yu D and Wohrle R 2010. Leptospirosis in a small animal veterinarian. reminder to follow standardized infection control procedures. *Zoonoses Public Health.* 57: 281-284.

Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, Levett PN, Gilman RH, Willig MR, Gotuzzo E and Vinetz JM 2003. Leptospirosis a zoonotic disease of global importance. *Lancet Infect Dis.* 3: 757-771.

Bolin CA 2003. Diagnosis and control of bovine leptospirosis. Proceedings of the 6th Western Dairy Management Conference. Reno Nevada, p 155-160.

Bomfim MRQ and Koury MC 2006. Evaluation of LSSP-PCR for identification of *leptospira* spp. In urine samples of cattle with clinical suspicion of leptospirosis. *Vet Microbiol.* 118: 278-288.

Burriel AR, Vougiouka DM, Butsini S, Nomikou K and Patakakis M 2002. A serological investigation of some causes of reproductive failure among small ruminants in Greece. *Online J Vet Res.* 6: 57-63.

Ciceroni L, Lombardo D, Pinto A and Ciarrocchi S 2000. Prevalence of antibodies to *leptospira* serovars in sheep and goats in Alto Adige-South Tyrol. *J Vet Med.* 47: 217-223.

Doosti A and Hoveizeh Tamimian N 2011. Diagnosis of Leptospiral Abortion in Bovine by Polymerase Chain Reaction. *Global Veterinaria.* 7: 79-82.

Dufour B, Moutou F, Hattenberger AM and Rodhain F 2008. Global change impact, management, risk approach and health measures the case of Europe. *Rev Sci Tech.* 27: 529-550.

Fearnley C, Wakeley PR, Gallego-Beltran J, Dalley C, Williamson S, Gaudie C and Woodward MJ 2008. The development of a real-time PCR to detect pathogenic *Leptospira* species in kidney tissue. *Res Vet Sci.* 85: 8-16.

Gouveia EL, Metcalfe J, de Carvalho ALF, Aires TS, Villasboas-Bisneto JC, Queirroz A, Santos AC, Salgado K, Reis MG and Ko AI 2008. Leptospirosis-associated severe pulmonary hemorrhage syndrome, Salvador, Brazil. *Emerg Infect Dis.* 14: 505-508.

Haji Hajikolaei M, Ghorbanpour M, Gharibi D and Abdollahpour G 2007. Serologic study on leptospiral infection in sheep in Ahvaz, southwestern Iran. *J Vet Res.* 8: 333-336.

Honarmand HR, Mansour Ghanaei F and Heydarzadeh A 2009. Isolation and Serotyping of endemic leptospires of eastern part of flat area of Guilan province, Iran. *J Gorgan Uni Med Sci.* 11: 53-59.

Levett PN 2001. Leptospirosis. *Clin Microbiol Rev.* 14: 296-326.

Levett PN, Morey RE, Galloway RL, Turner DE, Steigerwalt AG and Mayer LW 2005. Detection of pathogenic leptospires by real-time quantitative PCR. *J Med Microbiol.* 54: 45-49.

Lilenbaum W, Vargas R, Brandão FZ, Cortez A, de Souza SO, Brandão PE, Richtzenhain LJ and Vasconcellos SA 2008. Detection of *Leptospira* spp. In semen and vaginal fluids of goats and sheep by polymerase chain reaction. *Theriogenology.* 69: 837-842.

Lucchesi PM, Arroyo GH, Etcheverria AI, Parma AE and Seijo AC 2004. Recommendations for the detection of *Leptospira* in urine by PCR. *Rev Soc Bras Med Trop.* 37: 131-134.

Momtaz H and Moshkelani S 2012. Detection and characterization of *Leptospira* spp. isolated from aborted bovine clinical samples. *Acta Vet Brno.* 81: 021-025.

OIE 2000. Manual of standards of diagnostic tests and vaccines, leptospirosis, part 2, section 22: chapter 2.2.4, Paris.

Ozdemir V and Erol E 2002. Leptospirosis in Turkey. *Vet Rec.* 150: 248-249.

Radostits OM, Gay CC, Blood DC and Hinchcliff KW 2000. Leptospirosis: Veterinary Medicine, 9th ed., WB Saunders, London: 971-988.

Radostits OM, Gay CC, Hinchcliff KW and Constable PD 2007. Veterinary Medicine: A Textbook of the Diseases of Cattle, Horses, Sheep, Pigs and Goats. 10th ed. St. Louis, Saunders Elsevier 2160 pp.

Rodrigues CG, Müller EE and Freitas JC 1999. Bovine Leptospirosis, serology at dairy farms in Londrina region, Paran State, Brazil. *Cienc Rural.* 29: 309-314.

Sambrook J and Russell DW 2001. Molecular Cloning. A Laboratory Manual. 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Shafighi T, Abdollahpour G, ZahraeiSalehi T and Tadjbakhsh H 2010. Serological and bacteriological study of leptospirosis in slaughtered cattle in north of Iran (Rasht). *Afr J Microbiol Res.* 4: 2118-2121.

Smythe LD, Smith IL, Smith GA, Dohnt MF, Symonds ML and Barnett LJ 2002. A quantitative PCR (TaqMan) assay for pathogenic *Leptospira* spp. *BMC Infect Dis.* 8: 13.

Victoriano AF, Smythe LD, Gloriani-Barzaga N, Cavinta LL, Kasai T, Limpakarnjanarat K, Ong BL, Gongal G, Hall J, Coulombe CA, Yanagihara Y, Yoshida S and Adler B 2009. Leptospirosis in the Asia Pacific region. *BMC Infect Dis.* 9: 147.

Zakeri S, Sepahian N, Afsharparad M, Esfandiari B, Ziapour P and Djadid ND 2010. Molecular epidemiology of leptospirosis in northern Iran by nested polymerase chain reaction/restriction fragment length polymorphism and sequencing methods. *Am J Trop Med Hyg.* 82: 899-903.

บทคัดย่อ

การศึกษาความชุกทางอนุชีวโมเลกุลของเชื้อ *Leptospira* spp. serovar *Hardjo* จากตัวอย่างเลือดโคและแกะในประเทศไทย

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เชื้อ *Leptospira* เป็นสาเหตุของโรคอุบัติใหม่ทางสาธารณสุขที่โลกซึ่งเกี่ยวข้องกับสัตว์และคน วัตถุประสงค์ของการศึกษานี้เพื่อประเมินการตรวจ DNA ของเชื้อ *Leptospira* จากตัวอย่างเลือดโคและแกะจากจังหวัด Chalamahal VaBakhtiari และ Isfahan ในประเทศไทยร้านด้วยวิธี Polymerase Chain Reaction (PCR) ตัวอย่างทั้งหมดจำนวน 195 ตัวอย่าง (ประกอบด้วยเลือดจากโค 92 ตัวและแกะ 103 ตัว) ถูกเก็บแบบสุ่มจากสัตว์ที่แข็งแรง DNA ถูกสกัดจากตัวอย่างเลือดและเก็บที่ -20 องศาเซลเซียสก่อนการตรวจ ตรวจหา DNA ของเชื้อ *Leptospira* โดยใช้ primer ที่จำเพาะต่อ 16s rRNA gene ผลร้อยละ 18.63 ของตัวอย่างเลือดให้ผลบวกต่อ DNA ของเชื้อ *Leptospira* โดยพบในตัวอย่างโคจำนวน 20 ตัวอย่างจากทั้งหมด 92 ตัวอย่าง (ร้อยละ 21.73) และพบในตัวอย่างเลือดแกะ 16 ตัวอย่าง จากทั้งหมด 103 ตัวอย่าง (ร้อยละ 15.53) 9 ตัวอย่าง จากตัวอย่างโค 92 ตัวอย่าง และ 5 ตัวอย่างจากตัวอย่างแกะ 103 ตัวอย่าง ตรวจพบ *Leptospira* spp. serovar *hardjo* การศึกษานี้แสดงให้เห็นว่า โคและแกะเป็นแหล่งรังโรคและมีอันตรายต่อสุขภาพคน วิธี PCR มี ความไวและความจำเพาะในการวินิจฉัย *Leptospira* ในกรณีที่น่าสงสัย

คำสำคัญ: เลือด โค โรคนี้หนู PCR ความชุก แกะ

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