Serodiagnosis and molecular survey on leptospiral abortions in the dairy cattle of Tabriz

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Summary

Leptospirosis is an important infectious disease of animals and humans caused by the pathogenic leptospires which are classified into one species of *Leptospira interrogans* containing over 212 serovars. This study was conducted to determine the prevalence of *Leptospira*-induced abortions in Tabriz (north-west of Iran) dairy herds and to determine the pathogenic *Leptospira* serovars responsible. From May 2008 through August 2010, 16 (21.05%) of 76 submissions (fetuses and placentas) to the Large Animal Clinic of the Veterinary Faculty at the University of Tabriz were diagnosed as positive to *L. interrogans* serovars by PCR. In contrast, only 9 (11.85%) of 76 dam's sera were diagnosed as positive to leptospirosis by the microscopic agglutination test (MAT). Two out of 9 animals were seropositive to serovar *pomona*, one animal to serovar *icterohaemorrhagiae*, two animals to *canicula*, three animals to both *pomona* and g*rippotyphosa*, and one animal to the both *canicula* and g*rippotyphos*. Moreover, the prevalence of *Leptospira*-induced abortions was high in the aged cows and advanced pregnancies (7-9 months). However statistical difference was not observed among these groups or different periods of pregnancy. In conclusion, serovar *pomona* induced abortions were determined to be more common leptospiral abortions in cattle in Tabriz and combination of PCR protocol with the MAT test would be more effective than the single test for etiological diagnosis of bovine abortions.

Key words: Leptospirosis, Cattle, Abortion, PCR, Serodiagnosis

Introduction

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; Lim, 2011).

It is a common disease of livestock, pet animals and wildlife throughout the world. Sporadic cases and outbreaks of the disease have been reported from the USA, the UK, Australia, New Zealand, the former 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; Jamshidi *et al*., 2008; Hassanpour *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 Tehran, Gilan, Azarbaijan, Khorasan, Khozestan, Isfahan, Chaharmahal Bakhtiary and Boushehr provinces are 25-42% seropositive to the *leptospira* spp. (Ebrahimi *et al*., 2003; Haji Hajikolaei *et al*., 2005; Haji Hajikolaei *et al*., 2007; Sakhaee *et al*., 2007; Sakhaee and Abdollahpour, 2011).

In the bovine family, leptospirosis is a worldwide distributed infection, responsible for great losses in bovine breeding due to abortion, stillbirth or weak calves, reduction in fertility rates and decrease in milk production (Faine *et al*., 1999) Leptospiral serovars that affect bovines more frequently

are *hardjo*, *pomona*, *canicola* and *icterohaemorrhagiae*. Nowadays, serovar *hardjo* is considered as the most frequent and important serovar for bovines (Ellis *et al*., 1982; Ellis, 1994; Dhaliwal *et al*., 1996).

Because of the enormous losses that the disease causes to dairy and beef cattle industries of Iran (primarily due to abortion in the second half of gestation) the correct and prompt diagnosis is important in controlling and eradicating the disease in the 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 often successful, because the fetal expulsion occurs 24-48 h after its death, and it is already contaminated with ubiquitary 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 with one of the leptospiral serovars could be an indicator of abortion cause. Although it has been proved that PCR is able to detect a minimal quantity of DNA from any microorganism in all kinds of biological samples, 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 bovine abortion, the objective of this study was to use PCR protocol as a tool for identification of pathogenic *Leptospira* spp. in tissues from aborted bovine 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.

Materials and Methods

Samples

From May 2008 through August 2010, 76 blood and tissue samples were collected from cows and their aborted fetuses (n=76) immediately after abortion at dairy 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, kidney, lung, spleen, heart, stomach fluid and placenta, then pooled together, pulverized under liquid nitrogen and finally stored at -20°C until DNA extraction.

Microscopic agglutination test (MAT)

The MAT was used to detect antibodies in dam's sera to *L. interrogans* serovars: *hardjo*, *grippotyphosa*, *pomona*, *ictero-haemorrhagiae* and *canicula*. 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-day-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 dark field 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.

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 17.5 µL proteinase K (20 mg mL^{-1}) and incubation at 60°C for 3 h, vortexing every 30 min. Three microliters of RNase A (4 mg mL^{-1}) were added, the samples were mixed and incubated at 37°C for 30 min. After cooling, 200 µL 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 600 µL of isopropanol, mixed, and centrifuged at 13,000 g for 3 min. The supernatant was

discarded and the pellet was washed with 600 µL of 70% ethanol, followed by a final centrifugation at 13,000 g for 3 min. Each pellet was dissolved in 100 µL of DNA Rehydration Solution by incubating at 65°C for 1 h.

DNA quality was assessed by spectrophotometry and PCR amplification of an internal control (prolactin gene). Samples that did not yield a prolactin amplicon nor had DNA concentration lower than 100 ng μL^{-1} as assessed by spectrophotometry were excluded from further analysis.

PCR

PCR was used for detection of pathogenic *Leptospira interrogans*. PCR reactions were performed using 13 µL of a commercial PCR mix (Accupower PCR preMix, Bioneer, S. Korea), 0.75 µL of a 25 µM solution of each primer (Table 1), and 1 µL of DNA (100 to 500 ng per reaction). Parameters used were initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 7 min. 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 bovine prolactin gene were considered as an internal control. PCR products were resolved by electrophoresis in a 1% agarose gel stained with ethidium bromide.

Results

Sixteen (21%) out of 76 samples (fetuses and placentas) were diagnosed positive to *L. Interrogans* serovars by the PCR test (Figs. 1 and 2, Table 2). In contrast, only 9 (11.84%) of 76 dam's sera were diagnosed as positive to leptospirosis by the MAT. Two out of 9 animals were seropositive to serovar *pomona*, one animal to serovar *icterohaemorrhagiae*, two animals to *canicula*, three animals to both *pomona* and *grippotyphosa*, and one animal to the both *canicula* and *grippotyphosa* (Table 3). Prevalence of the *leptospira*-induced abortions was high in the aged cow's groups vs. heifer's group (Table 4). On the other hand, abortions during the 7-9 months of pregnancy were more prevalent than the other months among the cows (Table 5).

Amplification of the *prolactin* gene with primer pair (*HL033* and *HL035*) gave

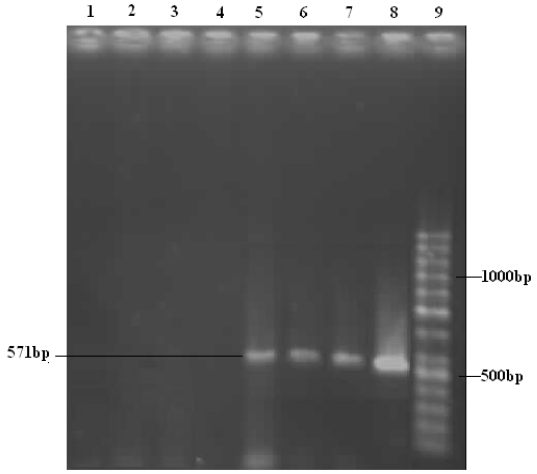

Fig. 1: Representative results of PCR amplification of genomic DNA of pathogenic *Leptospira interrogans* in fetal tissues. Lane 1: Non template control (NTC), Lanes 2, 3 and 4: negative samples from aborted fetuses, Lanes 5, 6 and 7: positive samples from aborted fetuses, Lane 8: positive control (Genekam Co., Germany), and Lane 9: 100 bp molecular weight marker (Bioneer, S. Korea)

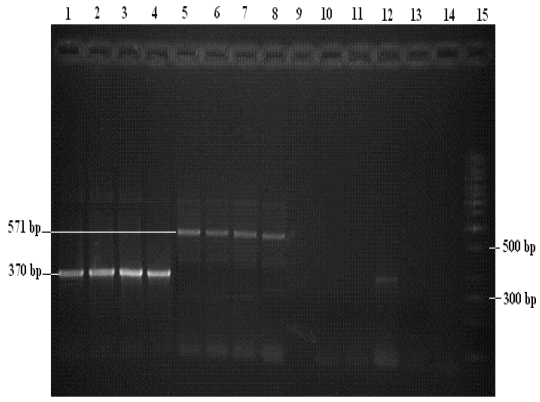

Fig. 2: Representative results of second run of PCR amplification of genomic DNA of pathogenic *Leptospira interrogans* in fetal tissues. Lane 1 to 4: positive samples from aborted fetuses in second round of Nested PCR, Lane 5 to 8: positive samples from aborted fetuses in first round of Nested PCR, Lane 9 to 13: negative samples from aborted fetuses, Lane 14: Non template control (NTC), and Lane 15: 100 bp molecular weight marker (Bioneer, S. Korea)

Table 1: Primer sequences for pathogenic *Leptospira*

Target	Product size	Primer sequences	Name
16S rRNA	571 bp	5'-AGGGAAAAATAAGCAGCGATGTG-3' 5'-ATTCCACTCCATGTCAAGCC-3'	Outer primer set
16S rRNA	370 bp	5'-GAAAACTGCGGGCTCAAAC-3' 5'-GCTCCACCGCTTGTGC-3'	Inner primer set
Exone 3	156 bp	5-CGAGTCCTTATGAGCTTGATTCTT -3 5-GCCTTCCAGAAGTCGTTTGTT TTC-3	PRL35 and 33

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	9 (cows) (11.85%)	67 (cows) (88.15%)	76 (100%)
PCR	16 (fetuses) (21.05%)	60 (fetuses) (78.95%)	76 (100%)

Table 3: Distribution of serovar specific antileptospiral antibodies and their titration in seropositive aborted cows

Serovar	1:100	1:200	Total
Pomona	2	3	5 (55%)
icterohaemorrhagiae	1	–	1 (11%)
canicula	2	1	3 (33%)
grippotyphosa	3	1	4 (44%)
hardjo	–	–	0 (0%)
Total	8	5	9 (100)*

*Some of the cows showed positive reaction against to more than a leptospiral serovar

Table 4: Frequencies of leptospiral abortions among the cows with different parity (number of parturition)

Parity number	1st (Heifer)	2nd	3rd	4th≤	Total
Number of cows aborted	3	3	4	6	16

product of ~ 156 bp in all fetal tissue samples.

Statistical analysis

Frequencies of positive results were compared between PCR and MAT tests by the McNemar test and the statistical difference was observed between two diagnostic methods (P≤0.05). Frequencies of abortions among the cows and among the different periods of pregnancy were compared by Chi-square test. However statistical difference was not observed among the parity or the period of pregnancy of cows (P>0.05).

Discussion

Leptospirosis is presumed to be the most widespread zoonosis in the world (WHO, 1999). 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 and Everard, 1993; Ratnam, 1994).

The etiologic agent is *Leptospira interogans*, 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 cattle, the major serovars are *hardjo* and *pomona*. The acute form of disease is most commonly manifested as mastitis in dairy cows. However, chronic infection is manifested as abortion, stillbirth, hydrallantois (Shanahan and Slovis, 2011) and the birth of premature and weak infected calves. Abortion may be the only manifestation of infection or may be related to an episode of illness, 6 (*pomona*) or 12 (*hardjo*) weeks earlier. Infection with *hardjo* is associated with infertility (early embryonic death) and abortion (4 months to term), while *pomona* infection is associated with abortion during the last trimester (Howard, 1993).

The clinical signs of leptospirosis have been infrequently seen in the dairy farms of Tabriz by clinicians. However, the most prevalent feature of disease in this area is

Table 5: Distribution of leptospiral abortions in different periods of pregnancy

Abortion time (days)	150-179	180-209	210-239	240-270	Total
Number of cows aborted	3	2	4	7	16

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 interogans* serovar *pomona* is the most prevalent serovar in the dairy herds of Tabriz. This is in contrast with the results obtained by other researchers in Iran and other countries, which emphasize the prominent roles of serovars *canicola* and *hardjo* in the prevalence of cattle leptospirosis (Ellis, 1994; Dhaliwal *et al*., 1996; Abdollahpour *et al*., 2009; Mineiro *et al*., 2011). This shows that the mountainous and cold weather of Tabriz city may affect the type of leptospiral serovar prevalence among the fields.

On the other hand, most of the leptospial positive aborted fetuses in our study belonged to the age group of 7-9 month gestation. This is in accordance with the above mentioned bacterial characteristics of *L. pomona* in relation to the period of abortion in cattle. Also, most of the aborted cows belonged to the aged cow's group, which is theoretically justifiable because the aged cows have a greater chance of being affected by leptospirosis disease than the heifers during the economical life.

Comparing the accuracy of MAT vs. the PCR protocol, it seems that the molecular tests are much more reliable than the serological tests, because in our study the positive rates resulted from the PCR was higher than the MAT. In other words, the PCR test has a greater ability to detect positive cases than the MAT test.

It is concluded that the serum antibody levels against the *Leptospira* spp. may be decreased at the time of abortion. Overall, we recommend more than one test for etiological diagnosis of bovine abortions and one of these professional tests could be the PCR protocol, which is a very important tool for detection of bacterial strains, particularly in the cases of infectious abortions caused by *Leptospira* spp.

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References

Abdollahpour, GR; Shafighi, T and Sattari Tabrizi, S (2009). Serodiagnosis of leptospirosis in cattle in north of Iran, Gilan. Int. J. Vet. Res., 3: 7-10.

Cole, JR; Sulzer, CR and Pursell, AR (1973). Improved microtechnique for the leptospiral microscopic agglutination test. Appl. Microbiol., 25: 976-980.

Dhaliwal, GS; Murray, RD and Ellis, WA (1996). Reproductive performance of dairy herds infected with *Leptopira interrogans* serovar *hardjo* relative to the year of diagnosis. Vet. Rec., 138: 272-276.

Ebrahimi, A; Alijani, L and Abdollahpour, GR (2003). Serological survey of human leptospirosis in tribal areas of Farsan and Koohrang cities. Iranian J. Med. Sci., 28: 93-96.

Ellis, WA (1994). Leptospirosis as a cause of reproductive failure. Vet. Clin. Nor. Am.: Food Anim. Pract., 10: 463-478.

Ellis, WA; O'Brien, JJ; Neill, SD; Ferguson, HW and Hanna, J (1982). Bovine leptospirosis: microbiological and serological findings in aborted fetuses. Vet. Rec., 110: 147-150.

Everard, JD and Everard, COR (1993). Leptospirosis in the caribbean. Rev. Med. Microbiol., 4: 114-122.

Faine, S; Adler, B; Bolin, C and Perolat, P (1999). *Leptospira and leptospirosis*. 2nd Edn., Melbourne, Medisci. P: 272.

Genovez, ME; Del Fava, C; Castro, V; Gotti, TB; Dib, CC; Pozzi, CR; Arcaro, JRP; Miyashiro, S; Nassar, AFC and Cirillo, SL (2006). Leptospirosis outbreak in dairy cattle due to *Leptospira* spp. serovar *canicola*: reproductive rates and serological profile after treatment with streptomycin sulfate. Arq. Inst. Biol., São Paulo. 73: 389-393.

Haji Hajikolaei, MR; Ghorbanpour, M; Gharibi, D and Abdollapour, GR (2007). Serologic study on leptospiral infection in sheep in Ahvaz, southwestern Iran. Iranian J. Vet. Res., 8: 333-336.

Haji Hajikolaei, MR; Gorbanpour Najafabadi, M and Abdollahpour, GR (2005). Serological study of leptospirosis in cattle in Ahvaz. J. Fac. of Vet. Med., University of Tehran. 60: 7-14.

Hassanpour, A; Monfared, N; Abdollahpour, GR and Sattari Tabrizi, S (2009). Seroprevalence of leptospiral infection in horses in Tabriz-Iran. J. Bacteriol Res., 1: 97-100.

Howard, JL (1993). *Current veterinary therapy*

food animal practice. 1st Edn., Philadelphia, USA, W. B. Saunders Co., P: 787.

Jamshidi, Sh; Vandeussefi, GM; Dezfoulian, O and Selk Ghaffari, M (2008). Isolation of *Leptospira canicola* from a dog in Iran: first report. Iranian J. Vet. Res., 9: 291-294.

Levett, PN (2001). Leptospirosis. Clin. Microbiol. Rev., 14: 296-326.

Lim, VK (2011). Leptospirosis: a re-emerging infection. Malays. J. Pathol., 33: 1-5.

Mineiro, ALBB; Vieira, RJ; Costa, EA; Santos, RL; Goncalves, LMF; Carvalho, SM; Bofim, MRQ and Costa, FAL (2011). Serology, polymerase chain reaction and histopathology for leptospirosis in samples collected at slaughter from dairy cows of Parnaiba region, state of Piaui, Brazil. Pesq. Vet. Bras., 31: 859-866.

Ratnam, S (1994). Leptospirosis: an Indian perspective. Indian J. Med. Microbiol., 12: 228-239.

Sakhaee, E and Abdollahpour, GR (2011). Detection of leptospiral antibodies by microscopic agglutination test in north-east of Iran. Asian Pac. J. Trop. Biomed., 1: 227-229.

Sakhaee, E; Abdollahpour, GR; Blourchi, M; Hassani Tabatabayi, AM and Sattari Tabrizi, S (2007). Serologic and bacteriologic diagnosis of bovine leptospirosis in Tehran suburb dairy farms. Iranian J. Vet. Res., 8: 325-332.

Shanahan, LM and Slovis, NM (2011). *Leptospira interrogans* associated with hydrallantois in 2 pluriparous Thorobred mares. J. Vet. Intern. Med., 25: 158-161.

Srivastava, SK (2006). Prospects of developing leptospiral vaccines for animals. Ind. J. Med. Microb., 24: 331-336.

WHO (1999). Leptospirosis worldwide. Wkly. Epidemiol. Rec., 74: 237-242.