

Short Paper

Molecular detection of *Leptospira* spp. in the urine of cattle in northern Iran

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Summary

Leptospirosis is a zoonosis of worldwide distribution, caused by *Leptospira interrogans* and is considered as an emerging global public health problem. Transmission usually results from direct or indirect exposure to the urine or other body fluids of leptospiruric animals which may become a source of infection for human or other animals. Having a humid climate with plenty of annual rainfall, Guilan province is a suitable environment for maintaining *Leptospira* spp. Hence, early detection of *Leptospira* spp. in the host prompts control and protection, and the polymerase chain reaction (PCR) is a suitable method. The present report aimed to demonstrate the PCR analysis of bovine urine for detection of leptospiral DNA. A total of 98 urine samples were randomly collected from cattle bladder in Rasht abattoir of Iran and the presence of leptospiral DNA was assayed by PCR amplification of *rrs* (16S rRNA) gene and the results confirmed by nested PCR. Out of 98 urine samples in 42 samples leptospire DNA was identified with the frequency of 43%. The high presence of the organism in the urine of carriers is a serious threat to the dairy farms and to the public health which requires an effective control measure in the north provinces of Iran.

Key words: *Leptospira* spp., Nested PCR, Urine, Cattle, Rasht

Introduction

Leptospirosis is a zoonosis of worldwide distribution, caused by *Leptospira interrogans*. Leptospirosis causes economic loss to the cattle industry (Abdollahpour *et al.*, 2009). *Leptospira interrogans* serovar Hardjo type Hardjobovis is the primary cause of acute and chronic leptospirosis in cattle, and in addition causes persistent infection of kidneys and female reproductive tract (Morey *et al.*, 2006). Non-specific signs and symptoms of leptospirosis are often mistaken for viral illness, and this wide spectrum of clinical symptoms that characterize leptospirosis makes its diagnosis easily mistaken for other febrile disease (Vado-Solis *et al.*, 2002). For this reason, the diagnosis should be made by laboratory confirmation. The polymerase chain reaction (PCR) has come into increasing use for the diagnosis of infectious diseases caused by slow-growing or fastidious microorganisms like *Leptospira* spp. and can be used as a tool for rapid diagnosis as well as for large-scale epidemiological studies on leptospirosis (Wagenaar *et al.*, 2000). The PCR has also been used to detect *Leptospira* spp. in urine samples from cattle experimentally infected with *Leptospira interrogans* serovar Hardjo subtype Hardjobovis (Wagenaar *et al.*, 2000). A PCR to detect *Leptospira* spp. in the urine of naturally infected cattle using genus-specific primers has

been reported by Talpada *et al.* (2003). A nested PCR with primers derived from the *LipL32* sequence has been reported by Nassi *et al.* (2003) using DNA of reference *Leptospira* spp., and by Jouglard *et al.* (2006) and Bomfim *et al.* (2007) using DNA from clinical samples, including urine and serum.

The 16S rRNA gene proved to be a good molecular epidemiological marker for defining infected species in leptospirosis suspected humans and animals (Merien *et al.*, 1992).

As the north of Iran, especially Guilan province, has a humid climate with plenty of annual rainfall, it is a suitable environment for maintaining *Leptospira* spp. (Abdollahpour *et al.*, 2009). Carrier animals are a potential zoonotic risk for the slaughter house workers, meat inspectors and farmers. Humans become infected via urine of carriers directly or indirectly by contaminated water or soil (Adler and Moctezuma, 2010). The aim of this study was to detect leptospiral DNA in the urine samples of the cattle that referred to the Guilan industrial abattoir by PCR assay as a rapid diagnosis of the reservoirs.

Materials and Methods

Between May and August 2009, a total of 98 urine samples were randomly collected from cows (n=59) and

bulls (n=39) in abattoir. Urine samples were directly taken from the bladder using sterile disposable syringes. All samples were transferred to the Microbiology lab and were kept at -20°C until used.

A modification of the method reported by Gerritsen *et al.* (1991) was used for DNA extraction from urine samples and using DNA Extraction Kit (BIONEER) according to the protocol of the manufacturer.

All urine samples extracted DNA were tested by PCR amplification using oligo nucleotid primers (A. 5'-GGC GGCGGTCTTAAACATG-3') (B. 5'-TTCCCCCAT TGAGCAAGATT-3') to amplify a 525 base pair fragment of *rrs* (16S rRNA) gene common to all leptospires (pathogenic and nonpathogenic) as described by Merien *et al.* (1992). A 25 µl reaction volume consisting of 1 µl of template DNA added to a tube containing 1 U of *Taq* DNA polymerase, 10 pM of each primer, 2.5 µl 10 x buffer containing 2 mM/L MgCl₂ and 0.2 mM/L dNTP was used. Amplification was performed in a TC512 thermal cycler with initial denaturation at 94°C for 6 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 2 min, extension at 72°C for 1 min 30 s and then a final extension at 72°C for 15 min. For PCR confirmation internal oligo nucleotid primers (C. 5'-CAAGTCAAGCGGAGTAGC AA-3' and D. 5'-CTTAACCTGCTGCCTCCCGTA-3') were used in a nested PCR assay using 1 µl of the PCR product as template. The same condition and program of amplification were followed as for PCR reaction to amplify an approximately 289 base pair fragment. Negative (no DNA template) and positive (DNA from *L. bataviae*) controls were also performed and aliquots were analyzed using 2% agarose gel electrophoresis, stained with Ethidium Bromide and images were obtained by UV transillumination.

Results

The results of study showed that out of 98 urine samples, 525 bp fragment of *rrs* gene was amplified in 42 (43%) samples including 19 out of 59 cows (32.2%) and 23 out of 39 bulls (59%; Table 1). The results of the study were analyzed using Statistical Package for Social Sciences, version 16. There was statistically significant difference between sex and PCR results (P=0.009). *Leptospira* positive samples were demonstrated (Fig. 1).

Table 1: Distribution of leptospiral infection in cattle stratified by sex

Sex	PCR results		Total
	Number of positive	Number of negative	
Cow	19 (32.2%)	40 (67.8%)	59 (100%)
Bull	23 (59%)	16 (41%)	39 (100%)
Total	42	56	98

In the nested PCR amplification which was performed for all PCR products using internal oligo nucleotide primers, the expected product of 289 bp was obtained in all 42 samples which confirmed the presence of *Leptospira* spp. DNA in these samples (Fig. 2).

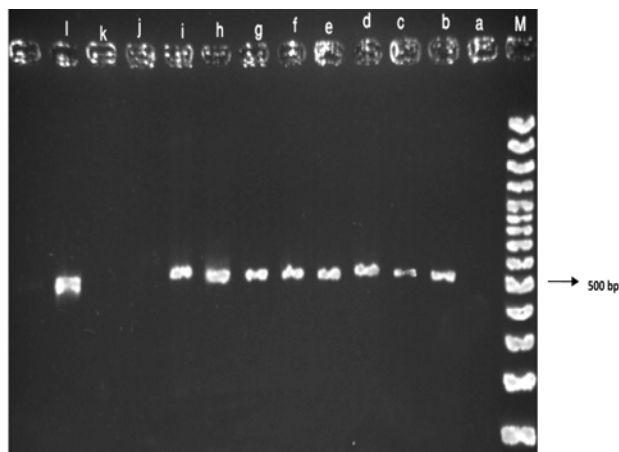


Fig. 1: Agarose gel stained with ethidium bromide showing PCR products. Lane M: Molecular size marker 100 bp DNA Ladder. Lane a: Negative control (without DNA), Lane b: Positive control (*L. bataviae*), Lane c, d, e, f, g, h, i, l: Positive samples, and Lane j, k: Negative samples



Fig. 2: Agarose gel stained with ethidium bromide showing nested PCR products. Lane M: Molecular size marker 100 bp DNA Ladder. Lane a: Negative control (without DNA), Lane p: Positive control (*L. bataviae*), Lane b, c, e, g, i, j, k, m, n, o: Negative samples, and Lane d, f, h, l: Positive samples

Discussion

The early identification of carrier animals and information on the shedding state are crucial to prevent the spread of leptospiral infection to other animals and humans. The aim of this study was to detect *Leptospira* spp. in the urine samples of the cattle that referred to the Guilan industrial abattoir using PCR assay as a rapid diagnosis of the reservoirs. Talpada *et al.* (2003) used PCR to detect *Leptospira* spp. in urine samples of naturally infected cattle using G1 and G2 primers. Nassi *et al.* (2003) and Jouglaud *et al.* (2006) used nested-PCR with primers derived from the *LipL32* sequence to amplify *Leptospira* spp. DNA from serum and urine samples of human and animals. The use of nested PCR primers derived from the genomic sequence of lipoprotein *LipL32* has been reported by Bomfim *et al.* (2008) to amplify pathogenic *Leptospira* spp. DNA from

the urine samples of naturally infected cattle. The results of this study showed that DNA was amplified from 26/30 urine samples taken from cattle with suspected leptospirosis. Studies carried out in different provinces of Iran using MAT, as a standard method for serological diagnosis of leptospirosis, indicated that *Leptospira interrogans* serovar Canicola is widespread in the cattle population (Haji Hajikolaei *et al.*, 2007; Abdollahpour *et al.*, 2009, Bahari *et al.*, 2011). Shafighi *et al.* (2010) investigated the seroprevalence of leptospirosis in slaughtered cattle in Guilan using MAT method. The results showed that 37.8% animals had a positive reaction against one or more serovars and the most prevalent *Leptospira* serovars was *Leptospira interrogans* serovar Pomona. Soltani Majd *et al.* (2012) determined epidemiological patterns of *Leptospira* spp. among slaughterhouse workers in Zanjan, Iran during 2011 using microscopic agglutination test. The results showed that from 98 samples, 34 samples (34.7%) were positive for different leptospiral serovars and the most prevalent *Leptospira* serovars was *Leptospira interrogans* serovar Hardjo (47.8%).

The PCR and nested PCR assays in cattle urine samples used in the present study were genus specific and detect all leptospiral serovars (both pathogenic and non-pathogenic). The results of this study showed a high frequency (43%) of *Leptospira* spp. in the urine samples of cattle. Present research showed a high frequency (59%) in bulls (Table 1). Bulls may have a particularly high rate of infection due to their natural sexual behavior and are a common source of infection for the cows they breed. Jafari Dehkordi (2011) evaluated the pathogenic serovars of *Leptospira interrogans* in serum and urine samples of dairy cattle herds of Shahrekord by PCR and sequencing of PCR products. The results showed that 28% of urine samples and 23% of plasma samples were contaminated and most serovars were *L. interrogans* serovar Icterohaemorrhagiae (50%) and *L. interrogans* serovar Pomona (37.5%). Zakeri *et al.* (2010) demonstrated the presence of *L. interrogans* serovar Wolffii with 100% identity in clinical human samples and animals with suspected leptospirosis using nested-PCR/RFLP assay followed by sequencing analysis. Momtaz and Moshkelani (2012) characterized *Leptospira* spp. isolated from aborted bovine clinical samples by PCR-RFLP and their results suggested that *L. interrogans* serovar Hardjo has the highest prevalence and is a major pathogen causing bovine abortion in Chaharmahal va Bakhtiari and Isfahan provinces of Iran.

In conclusion, in agreement with similar studies, the results obtained from the present study revealed that direct detection of *Leptospira* spp. in the urine of carriers by PCR is useful in rapid identification of carrier animals. As contact with urine is probably the most common transmission route of *Leptospira* spp. (Morey *et al.*, 2006), the presence of the organism in the urine of carriers is a serious threaten to the public health in Guilan province, especially for individuals working on rice production farms and effective control and protection is needed. Also, the results of study showed

that there was statistically significant difference between sex and PCR results, and infection rate in bulls is higher than in cows and is a threat to the cattle industry. Because infected bulls should never be used for natural service or artificial insemination until they have been treated to eliminate shedding and be found free of disease.

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مقاله کوتاه: ردیابی مولکولی گونه‌های لپتوسپیرو در نمونه ادرار گاو در شمال ایران

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لپتوسپیروز همواره به عنوان یک بیماری مشترک و قابل انتقال میان انسان و حیوانات با گسترش جهانی مطرح بوده است که عامل ایجاد کننده آن سروواریهای مختلف باکتری لپتوسپیرو می‌باشند. در این بیماری انتقال معمولاً به دنبال تماس مستقیم یا غیر مستقیم با ادرار یا سایر مایعات بیولوژیک حیوانات لپتوسپیرویک رخ می‌دهد. بنابراین این حیوانات ممکن است به صورت منبع عفونت برای انسان و یا سایر حیوانات محسوب شوند. استان گیلان به دلیل داشتن آب و هوای معتدل و مرطوب و بارش سالانه فراوان محیط بسیار مناسبی برای بقاء لپتوسپیرو محسوب می‌شود. از اینرو تشخیص سریع لپتوسپیرو در بدن میزبان در کنترل بیماری و محافظت در برابر آن بسیار سودمند است. هدف از مطالعه حاضر تعیین حضور DNA لپتوسپیرویی در نمونه‌های ادرار گاوها با استفاده از روش مولکولی واکنش زنجیره‌ای پلیمرز است. از مجموع ۹۸ نمونه ادرار که به طور تصادفی از مthane گاوهایی ارجاعی به کشتارگاه رشت جمع‌آوری شد و مورد آزمایش واکنش زنجیره‌ای پلیمرز قرار گرفت، ۴۲ نمونه (۴۳٪) از نظر حضور ژن 16S rRNA مثبت بودند. نتایج حاصله با آزمون nested PCR تایید شد. با توجه به نتایج به دست آمده، حضور تعداد زیاد لپتوسپیرو در ادرار حاملین، تهدیدی جدی برای بهداشت عمومی محسوب می‌شود و نیازمند انجام اقدامات کنترلی موثر است.

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واژه‌های کلیدی: گونه‌های لپتوسپیرو، nested PCR، ادرار، گاو، رشت