YYU Veteriner Fakultesi Dergisi, 2012, 23 (3), 137 - 139
ISSN: 1017-8422; e-ISSN: 1308-3651

ORIGINAL ARTICLE

Determination of Prevalence of Pathogenic Leptospira spp. by Real-Time PCR in Cattle in Diyarbakır

Simten YESILMEN[1] Neval Berrin ARSERIM[1] Nurettin ISIK[2] Hasan ICEN[3]

[1] *Dicle University Veterinary Faculty, Department of Microbiology, Diyarbakır, Türkiye*
[2] *Laboratory of Research, Diagnosis and Control of Animal Diseases, Microbiology, Diyarbakır, Türkiye*
[3] *Dicle University Veterinary Faculty, Department of Internal Medicine, Diyarbakır, Türkiye*

Received: 16.09. 2012 Accepted: 05.10.2012

SUMMARY

In this study, we aimed to investigate prevalence of *Leptospira* at blood and urine samples from cattle slaughtered in Diyarbakır slaughterhouse with methods of real-time PCR. Urine and blood samples collected from 96 cattle in three major abattoirs formed the material of this study. The existence of pathogenic *Leptospira* in these samples was investigated with which based on the segment *hap1* specific of pathogenic *Leptospira*. In 9 (9.4%) of urine samples of 96 suspected cattle's, *hap1* gene was found. The positive results weren't obtained in serum samples collected from same cattle. Therefore, the early identification of carrier animals is crucial to prevent the spread of leptospiral infection to other animals and humans.

Key Words *Leptospira, Real-Time PCR, Hap 1, Cattle*

Diyarbakır Bölgesindeki Sığırlarda Patojenik Leptospira spp Prevalansının Real-time PCR Yöntemi ile Tespiti

ÖZET

Bu çalışmada Diyarbakır'daki kesimhanelerde kesilen sığırların kan ve idrar örneklerinde real time PCR yöntemiyle patojenik *Leptospira* prevalansının ortaya konulması amaçlanmıştır. Bu çalışmanın materyalini üç büyük kesimhanedeki sığırlardan 96 sığırdan toplanan idrar ve kan örnekleri oluşturdu. Bu örneklerde patojen Leptospiraların varlığı real-time PCR yöntemiyle araştırıldı. Patojenik *Leptospiraları* etkenlerini varlığını ortaya koymak için *hap 1* geninin tespit etme temeline dayanan yöntem kullanılmıştır. Leptospira şüpheli 96 sığırdan elde edilen idrar örneklerinden 9 (%9.4) adetinde *hap1* geninin varlığı tespit edilmiştir. Aynı hayvanlardan alınan kan örneklerinde pozitifliğe rastlanılmamıştır. Bu nedenle, taşıyıcı hayvanların erken teşhisi, leptospiral enfeksiyonun diğer hayvanlara ve insanlara bulaşmasının önlenmesi bakımından son derece önemlidir.

Anahtar Kelimeler *Leptospira, Real-Time PCR, Hap 1, Sığır*

INTRODUCTION

Leptospirosis is a zoonotic disease that can pose an occupational risk to veterinarians in small and large animal veterinary practices (Baer et al, 2010). Leptospirosis is caused by a spiral-shaped bacterium known as a spirochete. There are many strains of *Leptospira*, and most bovine cases are caused by *Leptospira hardjo*, *L. pomona*, and *L. grippotyphosa*. Leptospirosis is carried to a susceptible animal by contaminated water, rodents, wildlife, and domesticated animals (Adler and Moctezuma 2010). Infections may occur when the organism contacts the mucosal surfaces (mouth, eyes, nasal passages, etc.) or an injured area on the skin of a susceptible animal. Once an animal is infected, it sheds the bacteria in the urine, semen, vaginal secretions, or in the placenta and fetal tissues (Gazyagci et al, 2010). The demonstration of leptospires in blood, urine and milk of animals showing clinical signs suggestive of acute leptospirosis is considered to be diagnostic. However, isolation from blood is not often successful because bacteremia is transient and not always accompanied by clinical signs (Taylor et al, 1997; Lilenbaum et al, 2003). These cows do not show obvious clinical signs and therefore are difficult to identify and remove from the herd.

The techniques, such as the enzyme linked immunosorbent assay (ELISA) and slide agglutination test (SAT), can detect different classes of antibody but may be subject to false positive reactions and require confirmation of these results by the MAT (Bomfim et al, 2005; Bomfim and Koury 2006). Among the DNA-based techniques, the polymerase chain reaction (PCR) has been used for the diagnosis of slowly growing or fastidious micro-organisms. With respect to bovine leptospirosis, several investigators have used PCR to detect *Leptospira spp.* in bovine blood and urine (Lucchesi et al, 2004; Sakhaee et al, 2007)

The conventional diagnostic methods are not suitable for the early identification of carrier animals. Direct detection of leptospires in the urine of carriers was successfully accomplished by PCR with a remarkably high detection limit (Cetinkaya et al, 2000).

In serological studies carried out in different parts of Turkey, the prevalence of disease has been estimated to vary between 8% and 30% in various animal species (Cetinkaya et al, 2000; Ozdemir and Erol 2002; Kocabiyik

Corresponding author: Hasan ICEN
Dicle Univ, Veterinary Faculty, Dept of Internal Medicine, Diyarbakır, Türkiye. e-mail: hasanicen@dicle.edu.tr

and Cetin 2004; Gummussoy et al, 2009).

In this study, researchers aimed to investigate prevalence of pathogenic *Leptospira* spp at blood and urine samples from cattle slaughtered in Diyarbakır slaughterhouse with methods of real-time PCR.

MATERIALS and METHODS

Blood and urine samples were collected from 96 cattle slaughtered in Diyarbakır slaughterhouse between January 2009 and February 2010. Serum samples were kept at -20 C until used for real–time PCR. All samples were analysed in Sanitation Instıtute of Diyarbakır with Real Time PCR. Put 1 mL of blood and 5 mL of EL buffer (Qiagen) in a 15 mL tube. Vortex and incubate 15 minutes on melting ice (about 0°C). Centrifuged 10 000 g during 30 minutes at 4°C. Discarded the supernatant. Add 180 µL of ATL buffer (Qiagen) and 20 µL of pK (Qiagen) to the pellet. Ten ml of urine sample in the transport media was used for PCR sample preparation. Centrifuge 10 mL urine at 10 000 g during 30 minutes at room temperature. Discarded the supernatant. Added 180 µL of ATL buffer and 20 µL of pK [Qiagen] to the pellet. The samples were used in the PCR reaction as described previously by Moinet (2008).

This qualitative PCR test enables the detection of pathogenic *Leptospira* from tissues, urine or blood. This test is based on the gene amplification of the DNA segment *hap1* specific of pathogenic *Leptospira*. The genetic classification, wich is based on DNA homology, divides leptospiral strains into four non pathogenic species: L. *biflexa, L. myeri, L. parva and L. wolbachii* and seven pathogenic species: L. *interrogans, L. borgpetersenii, L. weilii, L. nugochii, L. santarosai, L.inadai and L. kirschneri.* The Adiavet® LEPTO REALTIME PCR kit only detects the 7 pathogenic species. Specifity and sensitivity of the test has been evaluated on 23 strains of *Leptospira interrogans s.l*., 9 strains of *Leptospira biflexa s.l.* and 18 other bacterial trains. Specificity is of 100%.This test is based on enzymatic gene amplification or PCR technique. It uses primers and a TaqMan probe labelled by FAM, specific the of the *hap1* gene of *Leptospira*. DNA extraction is performed with a QIAamp DNA mini kit sold by QIAGEN (Hilden, Germany). A control DNA, referred as "internal control", is present in each reaction in order to validate each negative result. It is revealed with a TaqMan internal probe labeled with a fluorophore in the same spectra as VIC.

DNA preparation

Total DNA from cattle blood and urine was prepared using QIAamp DNA Mini Kits (QIAGEN, Australia) according to the manufacturer's instructions. Single blood and urine samples were from 96 cattle with clinically suspected leptospirosis or from "at risk" area where the clinically seen before. These samples were tested by real time polymerase chain reaction (Real-time PCR). Real-time PCR kit is used in the study of specific pathogenic Leptospira pill 1 (hemolysis-associated protein1) gene detection is based on. Identification of gene-specific FAM-labeled TaqMan probe Leptospira hap1 and primers used.

RESULTS

We tested real-time PCR primer sets as reported by Moinet (2008). The presence of hap1 gene was determined in 9 (9.4%) of the urine samples obtained from 96 cattles in doubt about Leptospira. The positive results weren't obtained in serum samples collected from same cattle.

DISCUSSION and CONCLUSION

In this study real-time-PCR recognized pathogenic presence of hap1 gene was evaluated. Real-time PCR based assays are now used in some diagnostic and most reference laboratories for the detection of pathogenic leptospires in tissues and body fluids. The 423 bp target was amplified from pathogenic strains of Leptospira spp but not from non-pathogenic species, and not from a wide range of other clinically significant bacteria and yeasts (Moinet 2008). The analytical sensitivity of the assay was 3 genome copies per reaction in blood and approximately 10 genome equivalents per reaction in urine, comparable to a real-time assay which uses a 16S rRNA gene target (levvet 2005). A procedure for the preparation of urine samples for real-time PCR using hap1gen shows promise in enhancing the detection of pathogenic leptospires in urine (Branger et al, 2005; Fearnley et al, 2008).

The demonstration of leptospires in blood and milk of animals showing clinical signs suggestive of acute leptospirosis is considered to be diagnostic (Gazyagci et al, 2010). However, isolation from blood is not often successful because bacteremia is transient and not always accompanied by clinical signs (Bomfim et al, 2008). The failure to detect the agent in the blood of these animals was possibly due to the presence of the agents in the blood less than detectable quantity. To overcome this problem, sensitive methods are needed to detect the organism in urine or the genital tract of chronic carriers. A wide variety of serological tests, which show varying degrees of serogroup and serovar specificity, have been described (Cai et al, 2002). The majority of the Leptospirosis cases are diagnosed by serology and the reference standard assay is the microscopic agglutination test MAT. ELISA, IFAT, Dark Fiel Microscopy (DFM) and PCR methods are available for diagnosis (Bal et al, 1994; Ozdemir, 1994). In this study real-time PCR using hap1gen presently recognized pathogenic Leptospira species was evaluated.

Leptosipirosis has been reported worldwide the seroprevalence among cattle is 7.4%- 45% (Guitan et al, 2001; Prapong et al, 2003; Jafari et al, 2011). The seroprevelance in Turkey is 8.04% however; the reported seroprevelance rate in Kars and Ardahan Provinces was much higher 33.6% (Sahin *et al,* 2000; Kocabiyik and Cetin, 2004). In the present study, 9.4% were found to be positive real-time PCR using *hap1* gen. These results are consistent with the results of several studies (Ertas *et al,* 2002; Aslantas and Ozdemir, 2005) performed in Turkey. This proportion is higher than a more recent seroepidemiological study carried out on the cattle population of Diyarbakır, in which only 3.9% of the animals were found to be positive by PCR (Cetinkaya et al, 2000). However, the finding of this study is not consistent with the results of several studies the results of researchers (Ikiz and Ozgur, 2004; Sahin et al, 2000; Gumussoy et al, 2009) and higher than Cetinkaya et al, 1999; 2000).

The results of this study show that the urine samples in 9.4% of cattle served as a reservoir of disease in Diyarbakir district while they were negative in their blood samples. So it could be stated that the animal reservoirs increase the risk of potential spread of disease to other animals and especially humans, and this deserves special attention. 9.4% of the apparently healthy animals were shedding leptospires in their urine. There are several possible reasons for the difference between these studies. The sample population of this study cattle having clinical suspicion of leptospirosis were used as research material.

The sample population of the serological survey consisted only of cattle randomly selected in Elazıg, whereas in the current study, the abattoirs were receiving animals from a much wider geographical area. In addition, the use of different methodologies in the studies may have played role in the difference. In the serological study, MAT was carried out for a limited number of serotypes (Cetinkaya et al, 1999). Because animals shed the leptospires in urine in the early days of infection, antibody secretion may not be at detectable levels by MAT. Leptospiral antibodies appear within a few days of onset of illness and persist for weeks or months and, in some cases, years. Unfortunately, antibody titers may fall to undetectable levels while animals remain chronically infected. To overcome this problem, sensitive methods are needed to detect the organism in urine or the genital tract of chronic carriers (OIE 2008).

In conclusion, the findings of this survey indicate that leptospirosis is not much high in healthy cattle in Diyarbakır. Although the asymptomatic cattle should be considered as significant reservoir with regard to the spread of the disease. The infection is an important and continuing public health problem in rural areas. Beef and dairy producers and people employed on farms are at risk of contracting leptospirosis during normal cattle handling activities. In recent study the sero-prevalence of leptospirosis in workers at a slaughterhouse was 9.5% (31). Further investigation for this organism must be supported for studying and creating new preventive strategies.

ACKNOWLEDGEMENT

This study was supported by Head of Scientific Research Project of Dicle University (Project No: 06 VF-093).

REFERENCES

Adler B, Moctezuma A (2010). Leptospira and leptospirosis. *Vet Microbiol*, 140, 287–296.

Aslantas O, Ozdemir V (2005). Determination of the seroprevalence of leptospirosis in cattle by MAT and ELISA in Hatay, Turkey. *Turk J Vet Anim Sci*, 29, 1019-1024.

Baer R, Turnberg W, Yu D, Wohrle R (2010). Leptospirosis in a small animal veterinarian: reminder to follow standardized infection control procedures. *Zoonoses public health*, 57, 281–284.

Bal AE, Gravekamp C, Hartskeerl TRA, De Meza-Brewster J, Korver H, Terpstra WJ (1994). Detection of leptospires in urine by PCR for early diagnosis of leptospirosis *Journal Of Clinical Microbiology*, 32(8), 1894-1898.

Bomfim MRQ, Ko A, Koury MC (2005). Evaluation of the recombinant LipL32 in enzyme-linked immunosorbent assay for the serodiagnosis of bovine leptospirosis. *Vet Microbiol*, 109, 89–94.

Bomfim MRQ, 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.

Bomfim MRQ, Barbosa-Stancioli EF, Koury MC (2008). Detection of pathogenic leptospires in urine from naturally infected cattle by nested PCR. *The Veterinary Journal,* 178, 251–256.

Branger C, Blanchard B, Fillonneau C, Suard I, Aviat F, Chevallier B, Andre´-Fontaine G (2005). Polymerase chain reaction assay specific for pathogenic Leptospira based on the gene hap1 encoding the hemolysis-associated protein-1 *FEMS Microbiology Letters,* 243 437–445.

Cai HY, Hornby G, Key DW, Osuch MR, Maxie MG (2002). Preliminary study on differentiation of *Leptospira grippotyphosa* and *Leptospira sejroe* from other common pathogenic leptospiral serovars in canine urine by polymerase chain reaction assay. *J Vet Diagn Invest*, 14,164–168.

Cetinkaya B, Ertas HB, Muz A, Ongor H, Kalender H Ozdemir V (1999). Determination of seroprevalence of leptospirosis in cattle in Elazig. *Turk J Vet Anim Sci*, 23(3), 633-639.

Cetinkaya B, Ertas H.B, Ongor H, Muz A (2000). Detection of leptospira species by Polymerase Chain Reaction (PCR) in urine of cattle. *Turk J Vet Anim Sci*, 24, 123–130.

Ertas HB, Çetinkaya B, Muz A, Ongor H, Ozdemir V, Yazioglu N (2002). Determination of the seroprevalence of leptospira in cattle by MAT and ELISA *Turk J Vet Anim Sci*, 26 1415-1420

Fearnley C, Wakeley PR, Gallego-Beltran J, Dalley C, Williamson S, Gaudie C, Woodward MJ (2008). The development of a real-time PCR to detect pathogenic Leptospira species in kidney tissue. *Research in Veterinary Science,* 85, 8–16

Gazyagci S, Yildirim M, Kaygusuz S (2010). Investigation on efficacy of a commercial vaccine for treatment of leptospirosis in cattle. *Journal of Animal and Veterinary Advances*, 9 (10), 1531 -1533

Gumussoy KS, Ozdemir V, Aydin F, Aslan O, Atabek E, Ica T, Doğan H O, Duman Z, A Ozturk (2009). Seroprevalence of bovine leptospirosis in Kayseri, Turkey and detection of leptospires by Polymerase Chain Reaction. *Journal of Animal and Veterinary Advances*, 8 (6), 1222-1229.

Ikiz S, Ozgur Y, (2004). Detection of *Leptospira interrogans* antibodies by ELISA and Microscopic Agglutination Test (MAT) in cattle in Trakya district and bacteriological studies on leptospirosis in slaughtered cattle. *Istanbul Univ. Vet. Fak. Derg*, 30, 99-111.

Jafari Dehkordi A, Shahbazkia HR, Ronagh N (2011). Evaluation of pathogenic serovars of *Leptospira interrogans* in dairy cattle herds of Shahrekord by PCR. *Iran. J. Microbiol*, 3 (3), 135-139.

Kocabiyik, AL, Cetin C (2004). Bovine leptospirosis in South Marmara region of Turkey: A serological survey. *Rev.Med. Vet*, 155, 606-608.

Levett PN, Morey RE, Galloway RL, Turner DE, 7. Steigerwalt AG, Mayer LW. (2005) Detection of pathogenic leptospires by real-time quantitative PCR. *J Med Microbiol,* 54, 45-49.

Lilenbaum W, Souza GN (2003). Factors associated with bovine leptospirosis in Rio de Janeiro, *Brazil Res.Vet. Sci*, 75, 249-251.

Lucchesi PMA, Arroyo GH, Etcheverría AI, Parma AE, Seijo AC (2004). Recommendations for the detection of *Leptospira* in urine by PCR. *Revista da Sociedade Brasileira de Medicina Tropical* 37(2),131-134.

Moinet M (2008). Étude comparative de la leptospirose chez le vison d'Europe [*Mustela lutreola*] et les autres petits carnivores sauvages du sud-ouest de la France. Thesis for Doctor of Veterinary Medicine, submitted to the University of Nantes, France, 1-108,

OIE Terrestrial Manual (2008). Leptospirosis. Chapter 2.1.9, 252-259.

Ozdemir V, Erol E (2002). Leptospirosis in Turkey. *Vet. Rec*, 23, 248-249.

Prapong S, Suwanchareon D, Tohmee N (2003). Genotyping Survey the Leptospira in Bovine Urine Samples in Thailand by PCR-Based Method: A Non Matching with the Antibody Titer by MAT. *The 11th International Symposium of the World Association of Veterinary Laboratory Diagnosticians and OIE Seminer on Biotechnology November*, 9-13

Sahin M, Aydin F, Ozdemir V, Genç O, Guler MA (2002). Serological survey of bovine leptospirosis in Kars and Ardahan provinces. *Turk J Vet Anim Sci*, 26, 17-25.

Sakhaee E, Abdollahpour GhR, Bolourchi M, Hasani Tabatabayi AM, Sattari Tabrizi S (2007). Serologic and bacteriologic diagnosis of bovine leptospirosis in Tehran suburb dairy farms. *Iranian Journal of Veterinary Research, University of Shiraz,* 8,4(21), 325-332.

Taylor MJ, Ellis WA, Montgomery JM, Yan KT, McDowell SWJ, Mackie DP (1997). Magnetic immuno capture PCR assay [MIPA]: detection of *Leptospira* borgpetersenii serovar hardjo. *Vet Microbiol*, 56(1-2), 135-145.