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

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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

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; Lilienbaum 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 microorganisms. 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...
and Cetin 2004; Gumussoy et al, 2009).

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

**MATERIALS and METHODS**

Blood and urine samples were collected from 96 cattle slaughtered in Diyarbakir 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 Institute of Diyarbakir 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, which 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. noguchii, L. santarosai, Linadai and L. kirschneri. The Adiavet® LEPTO REALTIME PCR kit only detects the 7 pathogenic species. Specificity 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 cattle 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; Fearney 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 (Gaziyagdi 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.

Leptospirosis has been reported worldwide the seroprevalence among cattle is 7.4%- 45% (Guan et al, 2001; Prapong et al, 2003; Jafari et al, 2011). The seroprevalence in Turkey is 8.04% however; the reported seroprevalence 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; Aslan, and Ozdemir, 2005) performed in Turkey. This proportion is higher than a more recent serosrpreferredemical study carried out on the cattle population of Diyarbakir, 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 Elazığ, 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.

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REFERENCES


