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Seroprevalence of Bovine Leptospirosis in Kayseri, Turkey and Detection of Leptospires by Polymerase Chain Reaction

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Abstract: This study was carried out to determine the seroprevalence of bovine leptospirosis in Kayseri, Turkey and to detect the leptospires in blood and urine of clinically suspect animals by serologic and molecular methods. A total of 2395 blood samples were collected from slaughterhouses located during 12 months (from May 2005 to April 2006) determine the seroprevalence. In addition, blood and urine samples were collected from 500 clinically leptospirosis suspect cattle. Microscopic Agglutination Test (MAT) and Enzyme Linked Immunosorbent Assay (ELISA) were used as serological tests. Polymerase Chain Reaction (PCR) was used for molecular examinations. In the serological analysis of 2395 blood samples collected from slaughterhouses, 609 (25.42%) and 433 (18.07%) samples were found to be positive by the MAT and ELISA, respectively. Seven (1.40%) out of 500 leptospirosis suspect cattle were found to be infected by the MAT and ELISA and *Leptospira interrogans* serovar Hardjo and *L. kirchneri* serovar Grippotyphosa were the predominant serovars. And also leptospires were detected in the urine samples of these 7 cattle (1.40%) by PCR. No agent was detected in the blood of suspect animals by PCR. The results of this study have shown that leptospirosis is highly prevalent and predominantly caused by *L. interrogans* serovar Hardjo and *L. kirchneri* serovar Grippotyphosa in this region. Because PCR can compete to the serological tests for detecting the leptospires in urine samples of suspect animals, the molecular analysis may contribute to the diagnosis of the infection.

Key words: Bovine leptospirosis, ELISA, MAT, PCR, seroprevalence

INTRODUCTION

Leptospirosis, a bacterial zoonotic infection caused by spirochetes of the genus *Leptospira*, affects human beings, all domestic and wild mammals and it is seen worldwide (Levett, 2001). The disease is characterized by icterus, anemia, haemoglobinuria, septicaemia, petechial haemorrhages in organs and tissues, interstitial nephritis, abortions and mastitis in animals of any ages (Quinn *et al*., 2002). *Leptospira interrogans*, which can cause the infection in human and animals, consists of 23 serogroups and over 240 serovars (Collins, 2006). The serovars Hardjo, Pomona, Grippotyphosa, Icterohaemorrhagiae, Automnalis, Sejroe, Canicola, Bratislava, Hebdomadis, Tarassovi, Javanica and Poi are the most commonly detected serovars (Srivastava and Kumar, 2003; Collins, 2006). Although, cattle are the host of serovars Hardjo, they can be infected by several other *Leptospira* serovars. Cattle are important for the transmission of the infection to human because they excrete live leptospires in their urine for prolonged periods (Levett, 2001). Because the disease can be confused with several other diseases exhibiting similar clinical symptoms, the clinical examinations and necropsy findings are not sufficient for the diagnosis of leptospirosis in cattle.

Using rapid, reliable, sensitive and inexpensive tests are the main issues in the laboratory analysis. Laboratory diagnosis of leptospirosis is based primarily on either isolation of the *Leptospira* sp. from the samples collected during clinical examinations or necropsy procedure or demonstration of the antibodies in serum

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(Quinn *et al*., 2002). The culture of the agent is laborious, time consuming, expensive and may give false negative results in the animals treated with antibiotics. Therefore the bacterial culture procedure has a number of disadvantages (Van Eys *et al*., 1989).

The Microscopic Agglutination Test (MAT), in which the live leptospires are used as antigen, was accepted as a gold standard. It is the most frequently used diagnostic test for the diagnosis of Leptospirosis (Alonso-Andicoberry *et al*., 2001; Bajani *et al*., 2003; Genovez *et al*., 2006). Enzyme Linked Immunosorbent Assay (ELISA) is also among the most preferred indirect serological tests (Bajani *et al*., 2003; Mariya *et al*., 2006; Croda *et al*., 2007). In recent years, the interests are focused on Deoxyribonucleic Acid (DNA) based molecular methods for the classification of the leptospires. Because the live and dead bacteria can be detected by Polymerase Chain Reaction (PCR) has been used for detecting the reservoir animals and typing the strains isolated from tissue and urine samples (Levett, 2001; Bomfim *et al*., 2008). This method was also used for epidemiological studies.

This study was performed to determine the seroprevalence of bovine leptospirosis in Kayseri province and to diagnose the infection with MAT, ELISA and PCR using the blood and urine samples collected from clinically suspect cattle.

MATERIALS AND METHODS

Sample collection: A total of 2395 blood samples were randomly collected from either public or private slaughterhouses located in Kayseri province that is located in central Anatolia in Turkey during 12 months (from May 2005 to April 2006).

In addition, blood and urine samples were collected from 500 cattle suffering from haemoglobinuria, icterus, fever, loss of appetite and dullness. The animals were reared either at the center of Kayseri or in 8 districts. The age, breed, sex, vaccination status of the animals and the climatic conditions of the districts where the animals reared were recorded.

Cultures of standard bacteria: The cultures of reference strains of *L. interrogans* serovar Australis (Bratislava Jez Bratislava strain), *L. interrogans* serovar Canicola (Hond Utrecht IV strain), *L. kirchneri* serovar Grippotyphosa (Moskva strain), *L. interrogans* serovar Hardjo (Hardjoprajitno strain), *L. interrogans* serovar Icterohemorrhagiae (RGA strain) and *L. interrogans* serovar Pomona (Pomona strain) (supplied by Leptospira Laboratory, Central Veterinary Control and Research Institute, Etlik, Ankara, Turkey) were used for the preparation of the MAT and ELISA antigens.

The microscopic agglutination test: The MAT was performed in microtiter plates by Faine (1982) with 4-14 days old *Leptospira* cultures in EMJH medium. Approximately, an inoculum of $1\text{-}2\times10^8$ cfu mL^{-1} was used as the antigen. Sera were initially screened at a dilution of 1:100 and when the agglutination was in excess of 50%, the samples are defined as seropositive then the 2 fold dilutions of the sera were made in saline. The MAT titer was taken as the highest dilution giving at least 50% agglutination by direct counting under dark field microscopy. Reference positive and negative sera were included each time MATs were carried out.

Enzyme linked immunosorbent assay: Briefly, ELISA antigens were prepared with 10-12 days old *Leptospira* cultures in EMJH medium. Sterile Tween 80 was added at a final concentration of 1:10 to the culture medium after 4-5 days of incubation to enhance the growth and then leptospires were killed with 0.5% formalin.

The killed leptospires were boiled, centrifuged and the supernatant was used as the antigens. ELISA plates were coated with these antigens. Antigen-coated plates were used in the tests. After completing the procedure the results were interpreted as indicated by Terpstra *et al*. (1985).

Polymerase chain reaction

Primers: The primers that were specific for *L. interrogans* serovar Canicola were used in the PCR. The sequences of primers are presented in Table 1.

DNA extraction: DNA from the urine samples was extracted and PCR amplified with the methods originally described by Gerritsen *et al*. (1991) and modified by Cetinkaya *et al*. (2000). DNA from the blood samples was extracted using the genomic DNA purification kit (blood, tissue, bacteria) (Fermentas, Lithuania) following the manufacturer's protocol for blood samples.

Statistical analysis: The analyses of the data were performed by SPSS package release 13.0 (SPSS Inc., Chicago, III., USA). Chi-square test was performed to determine the impact of age, breed and sex of the suspect animals as well as the districts, months and seasons on the seroprevalence. The Mann-Whitney U-test was used to determine the differences in median values of the age of infected and uninfected cattle.

Table 1: The sequence of primers used in PCR

Primers	Oligonucleotide sequence (5'-3')	Reference
A	GGCGGCGCGTCTTAAACATG	Merien *et al.* (1992)
B	TTCCCCCCATTGAGCAAGATT	Merien *et al.* (1992)

RESULTS AND DISCUSSION

Effects of sex, breed, age, season and district on leptospirosis: One hundred and eight of the 500 leptospirosis suspect animals were bulls and the remaining were cows. Of 6 (1.53%) cows out of 392 and 1 (0.92%) bull out of 108 were infected. Of 249, 175 and 76 animals were Holstein, Montafon and Simmental, respectively. The infection was detected in 6 (2.40%) of Holstein and in 1 (0.06%) of Montafon. On the other hand, none of the Simmental cattle had infection. Considering the age of these animals, 55, 201, 233 and 11 animals were between 1-2, 3-4, 5-6 and 7-8 years old, respectively.

The median age of infected 7 cattle was found as 4 (min 3-max 6). No sex, breed, age and district impact were found on the onset of the infection ($p>0.05$). Statistically significant differences were found between the seasons (Table 2) and months (Table 3) with respect to the seropositivity ($p<0.001$).

Samples from slaughterhouses: Of 609 (25.42%) out of 2395 sera collected from the slaughterhouses were found as positive with the MAT. The positivity was due to infection. The antibodies were detected solely against *L. interrogans* serovar Hardjo in 384 (63.05%), *L. kirchneri* serovar Grippotyphosa in 124 (20.36%), *L. interrogans* serovar Australis in 19 (3.11%), *L. interrogans* serovar Canicola in 8 (1.31%) and *L. interrogans* serovar Pomona in 2 (0.32%) of the positive sera. Antibodies against two or more serovars were determined in 11.85% of the positive sera by the MAT (Table 4).

ELISA revealed positive results in 433 (18.07%) sera collected from the slaughterhouses. Antibodies against solely *L. interrogans* serovar Hardjo, *L. kirchneri* serovar Grippotyphosa, *L. interrogans* serovar Australis and *L. interrogans* serovar Pomona serotypes were detected in 114 (26.32%), 70 (16.16%), 8 (1.84%), 8 (1.84%) of these positive sera, respectively, whereas antibodies against 2 or more serovars were found in the remaining sera by ELISA (Table 4).

Samples from suspect animals: Of 127 (25.40%) out of 500 leptospirosis suspicious sera were found to be positive by the MAT and the positivity in 120 (24%) of them was due to the vaccination, whereas in 7 (1.40%) was due to infection. Antibodies against solely one of the following serotypes *L. interrogans* serovar Hardjo, *L. interrogans* serovar Pomona, *L. kirchneri* serovar Grippotyphosa and *L. interrogans* serovar Australis were detected in 39 (30.70%), 34 (26.77%), 25 (19.68%) and 4 (3. 14%) of sera, respectively by the MAT. Antibodies were detected against 2 or more serovars in the remaining sera.

Seropositivity was determined in 99 (19.80%) out of 500 sera taken from the leptospirosis suspect animals by ELISA. *Leptospira interrogans* serovar Hardjo in 22 (22.22%), *L. kirchneri* serovar Grippotyphosa in 19 (19.19%), *L. interrogans* serovar Icterohemorrhagiae in 8 (8.08%) and *L. interrogans* serovar Pomona in 6 (6.06%) were detected as the solely serovars.

On the other hand, antibodies against >1 serovars were detected in 44.44% of the positive sera. The antibodies in 92 (18.40%) and in 7 (1.40%) out of 99 of the positive sera were resulted from vaccination and infection, respectively.

Molecular weight of the amplification products of positive strains was found as 331 bp. The DNA of *Leptospira* sp. was found in none of 500 blood samples. PCR revealed the positivity in seven urine samples (1.40%) that were also seropositive by MAT (Fig. 1). The sensitivity and specificity of ELISA and PCR was found as 100% when the MAT was taken as gold standard.

Table 2: The seasonal distribution of the seropositivity detected with the MAT in the sera of cattle collected from the slaughterhouses located in Kayseri, Turkey

Seasons	Positive	(%)
Spring (March, April, May)	256^{a}	42.04
Summer (June, July, August)	157^{b}	25.78
Autumn (September, October, November)	108^{c}	17.73
Winter (December, January, February)	88^{c}	14.45

$p<0.001$, $^{a-c}$Values within each row with different superscripts differ significantly

Table 3: The distribution of the positivity detected with the MAT in the sera of cattle collected from the slaughterhouses located in Kayseri, Turkey according to the months

Months	No. positive sera	(%)
May 2005	104^{a}	17.08
June 2005	49^{h}	8.05
July 2005	63^{g}	10.34
August 2005	45^{h}	7.39
September 2005	61^{g}	10.02
October 2005	28^{d}	4.60
November 2005	19^{cd}	3.12
December 2005	12^{c}	1.97
January 2006	35^{f}	5.75
February 2006	41^{h}	6.73
March 2006	67^{e}	11.00
April 2006	85^{b}	13.96

$p<0.001$, $^{a-h}$Values within each row with different superscripts differ significantly

Table 4: The distribution of the serovars detected with the MAT and ELISA in the sera of cattle collected from the slaughterhouses located in Kayseri, Turkey according to the months

		Serological tests				
		MAT (%)		ELISA (%)		
Months	Serovars	Positive	Negative	Positive	Negative	Total sera
May 2005	Bratislava	9 (8.65)	94 (47.47)	8 (8.25)	101 (51.01)	198
	Grippotyphosa	33 (31.73)	-	8 (8.25)	-	-
	Hardjo	35 (33.65)	-	17 (17.53)	-	-
	G+B	4 (3.85)	-	22 (22.68)	-	-
	G+H	15 (14.42)	-	13 (13.40)	-	-
	G+H+B	4 (3.85)	-	14 (14.43)	-	-
	H+B	4 (3.85)	-	15 (15.46)	-	-
June 2005	Grippotyphosa	6 (12.24)	151 (75.50)	5 (25.00)	180 (90)	200
	Hardjo	42 (85.71)	-	7 (35.00)	-	-
	Pomona	1 (2.04)	-	8 (40.00)	-	-
July 2005	Bratislava	1 (1.59)	137 (68.50)	0 (0)	164 (82.00)	200
	Grippotyphosa	12 (19.05)	-	7 (19.44)	-	-
	Hardjo	41 (65.08)	-	23 (63.89)	-	-
	H+G	9 (14.29)	-	6 (16.67)	-	-
August 2005	Grippotyphosa	16 (35.56)	155 (77.50)	9 (28.13)	168 (84.00)	200
	Hardjo	26 (57.78)	-	8 (25.00)	-	-
	H+G	3 (6.67)	-	15 (46.88)	-	-
September 2005	Grippotyphosa	27 (44.26)	139 (69.50)	10 (23.26)	157 (78.50)	200
	Hardjo	32 (52.46)	-	10 (23.26)	-	-
	H+G	2 (3.28)	-	23 (53.49)	-	-
October 2005	Grippotyphosa	3 (10.71)	172 (86.00)	5 (29.41)	183 (91.50)	200
	Hardjo	25 (89.29)	-	5 (29.41)	-	-
	G+H	0 (0)	-	7 (41.18)	-	-
November 2005	Bratislava	3 (15.79)	181 (90.50)	0 (0)	183 (91.50)	200
	Grippotyphosa	5 (26.32)	-	2 (11.76)	-	-
	Hardjo	11 (57.89)	-	1 (5.88)	-	-
	G+B	0 (0)	-	1 (5.88)	-	-
	H+G	0 (0)	-	12 (70.59)	-	-
	H+G+B	0 (0)	-	1 (5.88)	-	-
December 2005	Bratislava	1 (8.33)	188 (94.00)	0 (0)	196 (98.00)	200
	Grippotyphosa	2 (16.67)	-	1 (25.00)	-	-
	Hardjo	7 (58.33)	-	1 (25.00)	-	-
	Pomona	1 (8.33)	-	0 (0)	-	-
	H+G	1 (8.33)	-	2 (50.00)	-	-
January 2006	Bratislava	5 (14.29)	163 (82.32)	0 (0)	186 (93.94)	198
	Canicola	8 (22.86)	-	0 (0)	-	-
	Grippotyphosa	4 (11.43)	-	0 (0)	-	
	Hardjo	18 (51.43)	-	5 (41.67)	-	-
	H+G	0 (0)	-	7 (58.33)	-	-
February 2006	Grippotyphosa	1 (2.44)	159 (79.50)	1 (3.33)	170 (85.00)	200
	Hardjo	38 (92.68)	-	17 (56.67)	-	-
	H+G	2 (4.88)	-	12 (40.00)	-	-
March 2006	Grippotyphosa	3 (4.48)	133 (66.50)	11 (17.46)	137 (68.50)	200
	Hardjo	57 (85.07)	-	11 (17.46)	-	-
	H+G	7 (10.45)	-	41 65.08)	-	-
April 2006	Grippotyphosa	12 (14.12)	114 (57.29)	11 (17.74)	137 (68.84)	199
	Hardjo	52 (61.18)	-	9 (14.52)	-	-
	H+G	21 (24.71)	-	42 (67.74)	-	-

B: Bratislava, G: Grippotyphosa, H: Hardjo

The seroprevalence of leptospirosis in cattle and dominant serotypes have been shown in many countries. In the screening studies conducted on cattle in Spain (Alonso-Andicoberry *et al*., 2001; Guitian *et al*., 2001), the prevalence of leptospirosis ranged from 18.3-42.8%. In several studies in India, 15.8, 18.44 and 21.18% of the sera were determined as positive (Srivastava and Kumar, 2003; Mariya *et al*., 2006; Srivastava *et al*., 2006). In Brazil, Lilenbaum and Souza (2003) detected the antibodies against *Leptospira* in 46.9% of the sera. On the other hand, a quite high seropositivity (95.3%) was determined by Aguiar *et al*. (2006). The antibodies against leptospires have been reported in 62.8% of cattle sera in Mexico (Segura-Correa *et al*., 2003).

A high seroprevalence (54.67%) was also found in cattle in Mongolia (Odontsetseg *et al*., 2005). In Turkey, in Elazig province, 395 cattle sera were screened for leptospiral antibodies by Cetinkaya *et al*. (1999) and 2.3%

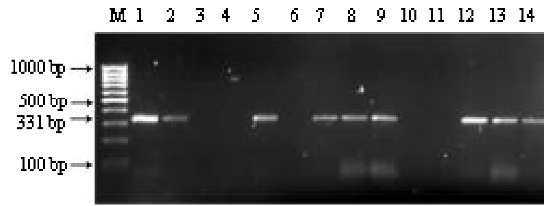

Fig. 1: *Leptospira* sp. amplicones (approximately 331 bp) determined by PCR. M. 100 bp DNA ladder, gene ruler™ 1: *L. interrogans* (RGA strain) positive control, 2: *Leptospira interrogans* serovar Borgpetersenii (Hardjoprajitno strain) positive control, 3: Negative control, 4: 6, 10, 11. Negative urine sample, 5: 7, -9, 12, -14. Positive urine sample

seropositivity was found by the MAT. In a large scale study conducted in Turkey by Ozdemir and Erol (2002), positivity was determined in 8.04% cattle sera collected from 71 provinces, between 1996 and 2000. In both studies, antibodies against *L. interrogans* serovar Hardjo and *L. kirchneri* serovar Grippotyphosa were prevalent.

In the present study, 609 (25.42%) of the cattle sera collected from the slaughterhouses were found to be positive by the MAT. In several countries different serovars were predominant (Alonso-Andicoberry *et al*., 2001; Guitian *et al*., 2001; Segura-Correa *et al*., 2003; Srivastava and Kumar, 2003; Odontsetseg *et al*., 2005; Mariya *et al*., 2006; Srivastava *et al*., 2006). In the present study, Hardjo and Grippotyphosa were detected predominantly in the sera collected both from slaughtered and from leptospirosis suspect cattle in Kayseri province. The previous surveys in cattle population in Turkey, also implicated that the most prevalent serovars Hardjo and Grippotyphosa (Ozdemir and Erol, 2002; Sahin *et al*., 2002) in consistent with the results of the present study.

It is reported that leptospirosis is widespread infection in countries located in tropical and subtropical regions (Levett, 2001). However, as in the present study, Lilenbaum and Souza (2003) claimed that geographical conditions had no direct effects on the occurrence of the infection. On the other hand, the seasons affect the onset of the infection. Guitián *et al*. (2001) determined the *L. kirchneri* serovar Grippotyphosa mostly in spring. In the present study, statistically significant differences ($p<0.001$) were detected with regard to the effects of seasons. High seropositivity was detected in the sera collected from slaughterhouse from April to September than the sera collected in the remaining months.

There have been studies investigating the effects of age and breed on the frequency of bovine leptospirosis and inconsistent results were reported. Segura-Correa *et al*. (2003) reported the highest seroprevalence in Zebu (0.63%) and the lowest seroprevalence in Holstein x Zebu (0.53%). However, Lilenbaum and Souza (2003) have claimed that breed is not a risk factor. Although, statistically significant differences were not determined with regard to impact of breed on infection, Holstein cattle were highly (49.80%) seropositive in the present study. This quite high seropositivity determined in Holstein may be due to the high density of the Holstein population in the region as well as the sensitivity of this breed to the infection.

It has been suggested that the age of the animals is an important factor on the frequency of the infection (Sahin *et al*., 2002). Thompson *et al*. (2006) reported that seroprevalence was lower in the calf under 6 months old in comparison with the cattle aged 3 years. They also, found a negative relationship between the animals aged 19-30 months and over 30 months with respect to the seropositivity. However, in this study, no age effect was found as indicated by Lilenbaum and Souza (2003) and Aslantas and Ozdemir (2005).

Leptospirosis is diagnosed by identification of the serological types. The conventional diagnostic methods have both advantages and disadvantages. Accurate diagnosis of leptospirosis is not always possible by the available tests because of the low titers of antibody response, cross-reactivity and variable immune response of individuals (Reitstetter, 2006). The MAT, which is the standard reference test for serodiagnosis of leptospirosis, is complicated and time-consuming test and it also requires double samples. These disadvantages are the limitation of the MAT (Croda *et al*., 2007). Bajani *et al*. (2003) compared four tests by taking the MAT as a reference test and they found ELISA as a good choice. Srivastava *et al*. (2006) and Sahin *et al*. (2002) also compared the MAT and ELISA and they suggested that ELISA is less sensitive but more specific than the MAT.

In the present study, 609 (25.42%) out of 2395 screened sera were found to be positive with the MAT whereas, 433 (18.07%) sera were positive with ELISA. These results are not consistent with the results of several studies (Sahin *et al*., 2002; Ertas *et al*., 2002; Ikiz and Ozgur, 2004; Aslantas and Ozdemir, 2005) performed in Turkey. However, the results of the present study are consistent with the result of the study conducted by Odontsetseg *et al*. (2005) who tested bovine sera from three regions with the MAT and ELISA and obtained higher positivity with the MAT compare to ELISA.

Some molecular tests, like serological tests, allow the characterization of the *Leptospira* serovars and

serogroups and they also allow the demonstration of the regional species (Reitstetter, 2006). It has been reported that PCR can be used for determination of *Leptospira* serotypes in urine (Gerritsen *et al*., 1991; Cetinkaya *et al*., 2000; Talpada *et al*., 2003; Bomfim and Koury, 2006; Genovez *et al*., 2006; Bomfim *et al*., 2008), in semen (Masri *et al*., 1997) and in aborted fetus (Richtzenhain *et al*., 2002). In the present study, *Leptospira* sp. were detected in 7 (1.40%) of 500 urine samples collected from the suspected cattle with PCR, which is consistent with the results of serological tests.

In several previous studies, various primers have been used for the diagnosis of the leptospirosis with PCR (Bomfim and Koury, 2006; Bomfim *et al*., 2008). The primers used in the present study were initially reported by Merien *et al*. (1992). Each of *Leptospira* sp. detected in the urine samples developed specific bands which were consistent with the size of the bands demonstrated by the others using the same primers (Cetinkaya *et al*., 2000; Ricthzenhain *et al*., 2002; Genovez *et al*., 2006). The PCR was as sensitive as the MAT and ELISA as shown by the results of previous studies (Masri *et al*., 1997; Genovez *et al*., 2006).

There have been very limited studies using the blood as the sample in human (Kositanont *et al*., 2007). This is the first study testing the cattle blood for leptospires with molecular methods. Leptospires can be introduced to reservoirs' body directly or indirectly and transported to the blood circulation which results in the septicaemia (leptospiremia) within 4-6 days (Quinn *et al*., 2002). Taking into account the possibility of the presence of high number of bacteria in the leptospiremia phase, the blood samples, besides the urine samples, of suspected animals were tested with PCR in the present study. However, *Leptospira* sp. was detected in any of the 500 blood samples. In addition, no agent was detected by PCR in the blood samples of 7 urine positive cattle. 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 (10-100) (Van Eys *et al*., 1989).

CONCLUSION

The seroprevalence of bovine leptospirosis was found as 25.42%, but determination of 1.40% positivity in leptospirosis suspect cattle indicates that the positivity in the suspected animals was low and *L. interrogans* serovar Hardjo and *L. kirchneri* serovar Grippotyphosa were the prevalent serovars in Kayseri, Turkey. Polymerase chain reaction can compete to the serological tests for detecting the leptospires in urine samples of suspect animals and it can contribute to the early diagnosis of infection. Further studies on the application of highly sensitive-specific, cheap and rapid alternative tests are of value.

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