

## Detection of *Leptospira* Species by Polymerase Chain Reaction (PCR) in Urine of Cattle

Burhan ÇETİNKAYA, H. Basri ERTAŞ, Hasan ÖNGÖR, Adile MUZ  
Department of Microbiology, Veterinary Faculty, University of Firat, 23119 Elazığ-TURKEY

Received: 08.03.1999

**Abstract:** This study was carried out to investigate the prevalence of leptospirosis in the urine of cattle slaughtered in three major abattoirs in the east of Turkey. A polymerase chain reaction (PCR) based on a pair of genus-specific primers was used to detect leptospiral DNA in the urine samples of 473 cattle, 284 of which were from Elazığ, 112 from Malatya and 77 from Diyarbakır. The detection limit of the method was determined to be approximately five bacteria per ml of urine. In the examination of urine samples, 4.02% (19/473) (95% confidence intervals [CI] 2.4-6.2) were found to be positive by PCR. The prevalence of disease was determined to be 3.6% (9/250) (95% CI 1.7-6.7) in males and 4.5% (10/223) (95% CI 2.2-8.1) in females, but the difference according to sex was not statistically significant ( $P=0.79$ ). When the prevalence was calculated in different abattoirs, the highest proportion was obtained in Elazığ with 4.9% (14/284) (95% CI 2.7-8.1), and the lowest in Malatya with 1.8% (2/112) (95% CI 0.2-6.3). However, the difference between the abattoirs was not significant ( $P=0.35$ ).

**Key Words:** *Leptospira*, cattle, urine, prevalence, PCR

### Sığır İdrarlarında *Leptospira* Türlerinin Polimeraz Zincir Reaksiyonu (PZR) ile Saptanması

**Özet:** Bu çalışma Elazığ, Malatya ve Diyarbakır illerindeki üç kesimhanede kesilen sığırlardan toplanan idrar örneklerinde Leptospirozisin prevalansını tespit etmek amacıyla gerçekleştirildi. Elazığ'dan 284, Malatya'dan 112 ve Diyarbakır'dan 77 olmak üzere toplam 473 sığırdan elde edilen idrar örneklerinde *Leptospira* DNA'sını saptamak amacıyla bir çift cins spesifik primerle kombine edilen bir Polimeraz Zincir Reaksiyonu (PZR) kullanıldı. Yapılan sensitive testinde, metodun 1 ml idrarda yaklaşık olarak 5 bakteriyi tespit edebildiği belirlendi. İdrar örneklerinin PZR'de değerlendirilmesi neticesinde, %4.02 (19/473) (%95 güven aralığı [GA] 2.4-6.2)'sinde pozitif sonuç elde edildi. Hastalığın prevalansının erkek hayvanlarda %3.6 (9/250) (%95 GA 1.7-6.7) ve dişilerde ise %4.5 (10/223) (%95 GA 2.2-8.1) olduğu, ancak cinsiyet bakımından elde edilen prevalans değerleri arasındaki farkın istatistiksel olarak önemli olmadığı belirlendi ( $P=0.79$ ). Leptospirozisin prevalansı kesimhaneler dikkate alınarak hesaplandığında en yüksek oran %4.9 (14/284) (%95 GA 2.7-8.1) ile Elazığ'da ve en düşük oran ise %1.8 (2/112) (%95 GA 0.2-6.3) ile Malatya'da elde edildi. Ancak bu oranlar arasındaki farklılık da istatistiksel olarak önemli bulunmadı ( $P=0.35$ ).

**Anahtar Sözcükler:** *Leptospira*, Sığır, İdrar, Prevalans, ZR

### Introduction

Bovine leptospirosis is a worldwide zoonotic disease of cattle associated with the *Leptospira interrogans* infection. It causes significant economic losses in the cattle industry worldwide due to abortion, reduced milk production and infertility. There have been some disputes over the classification of *Leptospira* species in recent years. Although *L. borgpetersenii*, *L. noguchii*, *L. santarosai*, *L. weilii* and *L. kirschneri* have been proposed as pathogen species in addition to *L. interrogans* in light of DNA hybridisation-based studies (1), the term *L. interrogans* is still widely used in reference to pathogenic leptospires. *L. interrogans* serovar *hardjo* (*L. hardjo*) is the primary causative agent of bovine leptospirosis throughout the world and responsible for most of the losses attributable to the disease (2).

The main site of infection in cattle is the kidney and genital organs. Infected cattle may not show any clinical signs of disease, but excrete the organisms in their urine, and therefore play an important role in spreading the infection not only to other susceptible animals, but also to the human populations at risk, such as farmers, veterinarians and others (3, 4). An incidence of 4% has been reported in farm workers (5). Some infected animals may not show any clinical signs of disease during their life span (4).

The identification of carrier animals is therefore crucial in tackling the leptospiral infection. However, the current methods used for diagnosis are insufficient for this purpose. The use of culture, which is the most reliable test, has been hindered by some disadvantages. It is slow, as it can take 3-6 months to grow leptospires in

vitro, and laborious (6). Leptospire remain viable in urine samples for only a few hours, and in tissues for several days (7). Therefore, the immediate processing of samples is essential in order to grow the organisms in vitro. Because of these disadvantages, immunological tests are more commonly used in the diagnosis of leptospirosis. The most frequently used serological test for this purpose is the microscopic agglutination test (MAT), which is still regarded as an international reference test (8). This test is of value in epidemiological studies as it provides information on the serogroups of the leptospire involved in the infection (9). In recent years, certain modifications of ELISA have been reported to be better than MAT in some respects (10, 11). Apart from these tests, the complement fixation (12), immunocomb (11) and immunofluorescence tests (13) have also been employed in various studies. However, all these tests lack sensitivity in identifying subclinically infected animals. Cross-reactions with other organisms such as *Borrelia burgdorferi* can also take place, also reducing the specificity (14).

Recent developments in molecular biology, particularly the introduction of polymerase chain reaction (PCR) (15), are promising for the diagnosis of leptospirosis, as for that of other slow growing microorganisms such as *Mycobacterium tuberculosis* (16). A number of PCR assays have been applied to various clinical specimens such as urine, blood, cerebrospinal fluids and semen in order to detect leptospiral DNA (17-21). These studies have proved that PCR is faster and more sensitive than the conventional tests.

Studies carried out in different parts of the world have shown that leptospirosis is widespread. Seropositivity against *L. hardjo* has been reported to vary between 30% and 76% in cattle in England (11, 22-24). Prevalence figures of 30% (25), 22% (26) and 9.2% (27) have been reported in the Netherlands, the United States and Belgium respectively.

The first isolation of leptospire in cattle population of Turkey was reported in 1954 (28). 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 (29-32). In a recent national serosurvey, *L. hardjo* and *L. grippotyphosa* were reported to be the commonest serovars, with the proportions of 82% and 18% respectively (32). In a more recent epidemiological study carried out in Elazığ, the seroprevalence of disease was estimated to be 2% in cattle (33).

The present study was carried out to estimate the frequency of *Leptospira* species by polymerase chain reaction (PCR) in the urine of cattle slaughtered at three major abattoirs in the east of Turkey.

## Materials and Methods

### Sample collection

Urine samples were collected from 473 cattle slaughtered in three major abattoirs in the east of Turkey (in Elazığ, Malatya and Diyarbakır) between October 1998 and January 1999. These abattoirs were chosen following telephone interviews with the abattoir managers according to the following criteria: geographical position in the region, a minimum daily throughput of 50 cattle, and the distance from the laboratories where the samples were to be processed. The abattoirs chosen were the most suitable of 12 abattoirs in this region. These abattoirs were receiving animals not only from well-distributed markets in the east, but also from the other regions of the country. Urine samples were directly taken from the bladder by sterile syringes. All samples were transferred to the laboratories immediately and were kept at -20°C until required.

### DNA extraction and PCR

A modification of the method reported by Gerritsen et al. (18) was used for DNA extraction from urine samples. In brief, 10 ml of urine samples collected from the bladders were treated with 2 ml of 0.1 M EDTA (pH 8.0) including 0.5% formaldehyde. The samples were centrifuged at 5,000 rpm for 15 min at room temperature. The supernatant was removed carefully so as to leave about 1 ml in the bottom of the tube. One ml of 1 mM EDTA was added to the pellet suspension, and then 1.5 ml of this mixture was transferred to an eppendorf and centrifuged at 13,000 rpm for 10 min. After the supernatant was removed, the pellet was suspended in 1 ml sterile distilled water, vortexed and centrifuged again at high speed for 10 min. The supernatant was removed so as to leave 100-200 µl in the bottom of the tube. The pellet suspension was boiled for 15 min and 5 µl from this suspension was used as a template in the PCR.

PCR was performed in a touchdown thermocycler (Hybaid, England) in a total reaction volume of 50 µl containing 5 µl of 10xPCR buffer (100 mM Tris-HCl, pH 9.0, 500 mM KCl, 15 mM MgCl<sub>2</sub>, 1% Triton X-100), 250 µM each of the four deoxynucleotide triphosphates, 2 U Taq DNA polymerase (Promega), 10 pg each of the

primers derived from the *rrs* (16S) gene of *L. interrogans*, primer A, 5'-GGCGGCGCGTCTTAAACATG-3' and primer B, 5'-TTCCCCCATTGAGCAAGATT-3' (19) and 5 µl of template sample DNA. The reactions were overlaid with 100 µl mineral oil and amplification was obtained with one cycle of denaturation at 94°C for 3 min., annealing at 63°C for 1.5 min and synthesis at 72°C for 2 min, followed by 29 cycles of denaturation at 94°C for 1 min., annealing at 63°C for 1.5 min and synthesis at 72°C for 2 min. A final extension at 72°C for 10 min was included at the end of the cycles.

The detection limit of this PCR was evaluated by amplification of DNA extracted from culture and PCR negative urine samples seeded with a dilution series (from  $5 \times 10^6$  to 5 bacteria) of *L. hardjo*. The number of leptospire in the bacterial suspension was estimated spectrophotometrically by comparison with McFarland standard tubes (Api, Basingstoke) at 550 nm. DNA was then extracted by the procedure described above and 5 µl of the sample was amplified in a 50 µl volume of PCR reaction mixture.

The amplified products were detected by ethidium bromide staining after electrophoresis in 1.5% agarose gels. Each well received 7 µl of sample with 3 µl loading solution (blue-orange dye). Tris-Boric acid-EDTA (TBE, pH 8.3) buffer was used for electrophoresis, which was carried out at 60 volts for one hour. Following electrophoresis, the gel was visualised with ethidium bromide (0.5 µg/ml) staining for 45 min at room temperature.

Possible cross-contamination during each step of the assay was checked. Cross-contamination of consecutive samples at the time of sample collection was checked by numbering samples. In order to assess the possibility of contamination during sample preparation, DNA extraction was carried out from 5 negative samples subsequent to a urine sample spiked with *L. hardjo*, and negative controls were always used during PCR. Consistently negative results suggested that each step of the assay was free of contamination.

#### Data Analysis

The sample size required was estimated from an expected prevalence of 50% with a 95% level of confidence, and a desired accuracy of 5%. A chi squared ( $\chi^2$ ) test was used to detect differences between proportions; a possibility of less than 0.05 was considered statistically significant. When appropriate, exact binomial 95% confidence intervals (CI) were calculated.

## Results

The primers used were derived from the *rrs* gene (16S) of *L. interrogans*. Six serotypes (*L. sejroe hardjo* Bakker, *L. pomona pomona* Mezzano, *L. icterohaemorrhagiae copenhageni* Wijnberg, and *L. grippityphosa grippityphosa* Moskva V) including two nonpathogenic serovars (*L. semaranga patoc patoc I* and *L. andaman andamana CH 11*) of *L. biflexa* used in the study produced positive signals with a molecular size of 331 bp in agarose gel electrophoresis. The PCR assay was determined to detect as few as five leptospire per ml of urine in ethidium bromide-stained agarose gels (Fig 1).

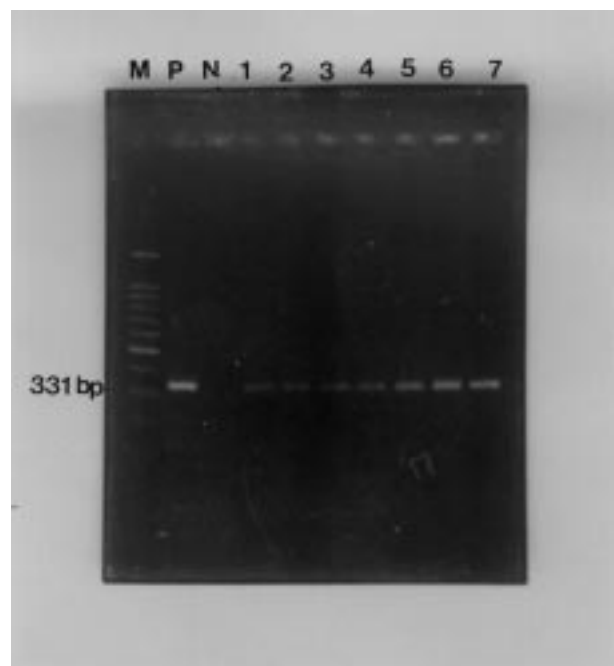


Figure 1. An ethidium bromide-stained agarose gel of PCR products that shows the sensitivity of the assay. M: DNA marker (100bp); P: positive control; N: negative control; 1-7: a negative urine sample seeded with a dilution series (from  $5 \times 10^6$  to 5 bacteria) of *L. hardjo*.

Of the 473 urine samples, 250 were obtained from male and 223 from female cattle. Two hundred eighty-four of the samples were collected from Elazığ, 112 from Malatya, and 77 from Diyarbakır.

Positive PCR products with a molecular size of 331 bp were obtained from 19 samples, giving an overall proportion of 4.02% (19/473) (95% CI 2.4 to 6.2). No gross lesions were observed in the carcasses of animals, suggesting that they were apparently healthy. An example of PCR amplification of urine samples is shown in Fig 2.

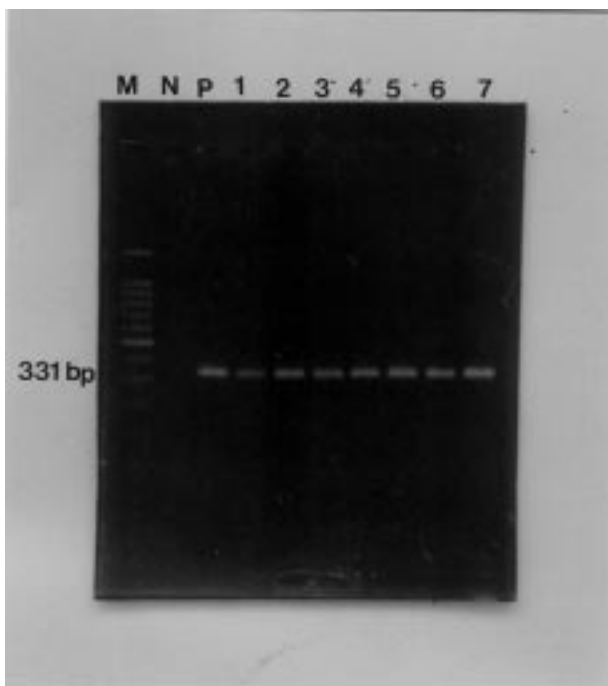


Figure 2. Gel electrophoresis of PCR products obtained from positive urine samples. M: DNA marker (DNA ladder: 100 bp); N: negative control; P: positive control (*L. hardjo*); 1-7: positive urine samples with molecular size of 331 bp.

The proportion of PCR positive samples was 3.6% (9/250) (95% CI 1.7-6.7) for males and 4.5% (10/223) (95% CI 2.2-8.1) for females. The difference between males and females was not statistically significant ( $P=0.79$ ). The proportion of PCR positive urine samples collected from three abattoirs ranged from 1.8% (2/112) (95% CI 0.2-6.3) in Malatya to 4.9% (14/284) (95% CI 2.7-8.1) in Elazığ. However, the differences between the abattoirs were not statistically significant ( $P=0.35$ ). The proportion of PCR positive samples at each abattoir is shown in Table 1.

Abattoir	Male		Female		Total	
	%	95% CI	%	95% CI	%	95% CI
Elazığ	4.8 (7/146)	1.9-9.6	5.1 (7/138)	2.1-10.2	4.9 (14/284)	2.7-8.1
Malatya	2.3 (2/86)	0.3-8.1	-- (0/26)	---	1.8 (2/112)	0.2-6.3
Diyarbakır	-- (0/18)	---	5.1 (3/59)	1.1-14.1	3.9 (3/77)	0.8-10.9
Total	3.6 (9/250)	1.7-6.7	4.5 (10/223)	2.2-8.1	4.02 (19/473)	2.4-6.2

Table 1. The proportion of PCR positive urine samples collected from cattle at each abattoir.

## Discussion

The ultimate aim of veterinary and medical disciplines is to control and eradicate diseases in populations. Early and accurate diagnosis is therefore important in developing effective strategies for this purpose. Another important point is the determination of contributing factors to the disease through large-scale epidemiological surveys. However, the absence of rapid and accurate diagnostic tests has been a major hindrance in carrying out such surveys.

This study employed PCR combined with a pair of genus-specific primers in order to investigate the presence of leptospiral DNA in the urine of cattle slaughtered at three major abattoirs in the east of Turkey and revealed that approximately 4% of the apparently healthy animals were shedding leptospires in their urine. This proportion is higher than a more recent seroepidemiological study carried out on the cattle population of Elazığ, in which only 2% of the animals were found to be positive by MAT (33). There are several possible reasons for the difference between these studies. 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. The fact that the abattoir material would mostly represent unproductive and unthrifty animals may also have contributed to this. 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 (33). Sullivan (34) reported that there was no correlation between MAT results and leptospiuria. Because animals shed the leptospires in urine in the early days of infection, antibody secretion may not be at detectable levels by MAT.

Although MAT is recommended for use as a screening test at herd level, it has been reported to be unreliable for diagnosing infection at individual level (23, 24). MAT

lacks sensitivity also in detecting animals infected for more than two years due to the decline in agglutinating antibodies (6). The other important disadvantage of MAT is the inability to distinguish vaccinated animals from infected ones (12). The test is also laborious and time-consuming. The necessity for a live antigen to carry out the test poses a risk for the laboratory workers. In spite of all these disadvantages, MAT is still widely recognised as a reference test at both individual and herd levels due possibly to the determination of serogroups involved in an infection, which has epidemiological value because different serogroups may not be associated with a particular clinical form of leptospirosis. (9).

Other serological tests such as ELISA, Immunofluorescence and Immunocomb were employed in the diagnosis of leptospirosis (11), but their value in detecting carrier animals is yet to be evaluated. Another paucity of the serological tests is that antibodies start to appear 8-10 days after the onset of illness. Therefore, infection can not be detected by these means at the early stages. Ellies et al. (23) reported that a significant proportion (19.6%) of the carriers were not detected in serology, indicating the inadequacy of serological methods for diagnosing leptospiral infections.

Seroprevalence figures reported in other parts of Turkey, ranging from 8% to 30% (29-32), are much higher than the 4% of this study. The populations of these studies mostly consisted of herds that were suspected of having leptospirosis, whereas in the present study, sample collection was done randomly without consideration of the clinical status of the animals. In fact, the absence of any gross lesions in animal carcasses examined at the abattoirs suggested that they were free of clinical leptospirosis. Another plausible explanation for these differences is that leptospirosis may be intermittent, and due to the collection of single samples from each animal, the prevalence of disease might have been underestimated in the current study. Regional differences in climate, animal nutrition and husbandry, which have been reported in some European countries (25, 35, 36), may have also contributed to the difference between these studies.

The incidence of leptospirosis has been reported to vary significantly in different seasons, and to be higher in winter months (24). In the current study, the samples were collected between October and January, and the proportion obtained may not represent the actual prevalence of leptospirosis, as the seasonal fluctuations were noted. Seasonal fluctuations in the frequency of disease have been suggested to be due to changes in farm

management policies, high moisture levels in the environment, and an increased risk of contacting infected urine when the animals are housed in winter (37-39). However, outbreaks of leptospirosis have been reported in dry environments as well, suggesting that the incidence of disease is not necessarily rain-dependent (40, 41). Calving takes place mostly in the spring months in Turkey, and therefore the winter months are the most susceptible period of gestation, which has been suggested to influence the seasonal variability in the occurrence of the disease (37, 38).

The seroprevalence of disease in cattle populations in the UK has been reported to vary between 35% to 76% in different regions (22-24). A more recent study in the UK has reported seropositivity to be about 30% in cattle sera (11). The seroprevalence of disease has been reported as 30%, 23% and 9% in the Netherlands, Portugal and Belgium, respectively (25, 27, 42).

An important disadvantage of PCR is the lack of sensitivity when dealing with clinical specimens (e.g., faeces, milk) due to the presence of inhibitors. Robust sample preparation methods are needed in order to obtain successful results when PCR is employed for such purposes, the lack of which has until recently hindered the use of PCR in diagnosing leptospires directly from clinical specimens. In this study, urine samples were collected from the bladder by sterile syringes. This protected the samples from contamination, especially with faeces, which is known to have some unknown PCR inhibitors. Collection of urine from large populations may be somewhat difficult in the field, but some diuretics can be used for this purpose with some caution taken at the time of collection.

The ability of detecting as few as five bacteria per ml of urine suggests that the PCR assay used in this study is very sensitive and compares favourably with culture, the drawbacks of which are well known (6). The sensitivity of the assay reported here is similar to the reports of other workers (17-19). The results of this study and other studies on the use of PCR indicate that PCR is more sensitive than conventional tests (17-19). The specificity of the primers used in the study has also been tested with a variety of microorganisms, including *Treponema* spp. and *Borrelia burgdorferi*, with which leptospires have a close antigenic relationship that causes false positive results in serology, and the absence of amplification with the DNA of these species has shown primers to be specific only for *Leptospira* (14, 19).

The primers used in this study were genus-specific and gave positive reactions with *L. biflexa* serovars as

well. Hence, some of the positive results might have been due to the presence of saprophytic leptospires in the urine of animals. In a few PCR-based studies, *L. hardjo* specific primers have been employed to detect leptospiral DNA in urine (17, 18, 43). Although *L. hardjo* is the most important serovar affecting cattle worldwide, other serovars affecting both animals and humans, such as *L. grippityphosa*, have been reported to be prevalent in Turkey (31, 32). Therefore, the genus-specific primers were the most appropriate for such an epidemiological survey. On the other hand, as the distinction of different serovars is important, PCR modifications such as arbitrarily primed PCR (AP-PCR), low stringency PCR (LS-PCR) and PCR-restriction endonuclease analysis (PCR-REA) may be used in order to clarify taxonomic contradictions (9, 44, 45).

In conclusion, 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 conventional diagnostic methods are not suitable for this purpose. The results of this study reveal that direct detection of leptospires in the urine of carriers was successfully accomplished by PCR with a remarkably high detection limit. The possibility of processing large samples simultaneously and with good sensitivity by PCR enables large-scale epidemiological studies to be carried out, as a result of which effective control strategies may be developed against leptospirosis.

### Acknowledgements

We wish to thank Mr. M Bazna for his help in sample collection, and Dr. V. Özdemir for kindly providing the *Leptospira* serovars used in this study.

### References

1. Yasuda, P.H., Stigerwalti A.G., Sulzer, K.R., Kaufmann, A.F., Rogers, F. and Brenner, D.J.: Deoxyribonucleic acid relatedness between serogroups and serovars in the family Leptospiraceae with proposals for seven new *Leptospira* species Bacteriol., 1987; 37, (4): 407-415.
2. Ellis, W.A., O'Brien, J.J., Cassells, J.A. and Hanna, J.: Bovine leptospirosis: microbiological and serological findings in aborted fetuses. Vet. Rec., 1982; 110: 147-150.
3. Thiermann, AB.: Experimental leptospiral infections in pregnant cattle with organisms of the hebdomadis serogroup. Am. J. Vet. Res., 1982; 43, (5): 780-784.
4. Ellis, W.A., Songer, J.G., Montgomery, J. and Cassells, J.A.: Prevalence of *Leptospira interrogans* serovar *hardjo* in the genital and urinary tracts of non-pregnant cattle. Vet. Rec., 1986; 118: 11-13.
5. Waitkins, S.A.: Leptospirosis as an occupational disease. Br. J. Indust. Med., 1986; 46: 721-725.
6. Smith, C.R., Ketterer, P.J., McGowan, M.R. and Corney, B.G.: A review of laboratory techniques and their use in the diagnosis of *Leptospira interrogans* serovar *hardjo* infection in cattle. Aust. Vet. J., 1994; 71, (9): 290-294.
7. Quinn, P.J., Carter, M.E., Markey, B. and Carter, G.R.: Clinical Veterinary Microbiology. Wolfe Publishing, Mosby-Year Book Europe Limited, Spain. 1994; 295-297.
8. Faine, S.: Guidelines for the Control of Leptospirosis. WHO offset publication, No: 67, Geneva, World Health Organization 1982; 20: 43.
9. Brown, P.D. and Levett, P.N.: Differentiation of *Leptospira* species and serovars by PCR-restriction endonuclease analysis, arbitrarily primed PCR and low-stringency PCR. J. Med. Microbiol., 1997; 46: 173-181.
10. Thiermann, A.B. and Garrett, L.A.: Enzyme-linked immunosorbent assay for the detection of antibodies to *Leptospira interrogans* serovars *hardjo* and *pomona* in cattle. Am. J. Vet. Res. 1983; 44, (5): 884-887.
11. Woodward, M.J., Swallow, C., Kitching, A., Dalley, C. and Sayers, A.B. *Leptospira hardjo* serodiagnosis: a comparison of MAT, ELISA and Immunocomb. Vet. Rec., 1997; 141: 603-604.
12. Hodges, R.T. and Day, A.M.: Bovine leptospirosis: the effects of vaccination on serological responses as determined by complement fixation and microscopic agglutination tests. NZ Vet. J., 1987; 35, (5): 61-64.
13. Dhaliwal, G.S., Murray, R.D., Dobson, H., Montgomery, J., Ellis, W.A. and Baker, J.R.: Presence of antigen and antibodies in serum and genital discharges of heifers after experimental intrauterine inoculation with *Leptospira interrogans* serovar *hardjo*. Res. Vet. Sci., 1996; 60, (2): 157-162.
14. Raoult, D., Hechemy, K.E. and Baranton, G.: Cross-reaction with *Borrelia burgdorferi* antigen of sera from patients with human immunodeficiency virus infection, syphilis, and leptospirosis. J. Clin. Microbiol., 1989; 27: 2152-2155.
15. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A.: Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science, 1988; 239: 487-491.

16. De Wit, D., Steyn, L., Shomaker, S. and Sogin, M.: Direct detection of *Mycobacterium tuberculosis* in clinical specimens by DNA amplification. *J. Clin. Microbiol.*, 1990; 28: 2437-2441.
17. Van Eys, G.J.J.M., Gravekamp, C., Gerritsen, M.J., Quint, W., Cornelissen, M.T.E., Ter Schegget, J. and Terpstra, W.J.: Detection of leptospires in urine by Polymerase Chain Reaction. *J. Clin. Microbiol.*, 1989; 27: 2258-2262.
18. Gerritsen, M.J., Olyhoek, T., Smits, M.A. and Bokhout, B.A.: Sample preparation method for polymerase chain reaction-based semiquantitative detection of *Leptospira interrogans* serovar hardjo subtype hardjobovis in bovine urine. *J. Clin. Microbiol.*, 1991; 29: 2805-2808.
19. Merien, F., Amouriaux, P., Perolat, P., Baranton, G. and Saint Girons, I.: Polymerase chain reaction for detection of *Leptospira* spp. in clinical samples. *J. Clin. Microbiol.*, 1992; 30: 2219-2224.
20. Brown, P.D., Gravekamp, C., Carrington, D.G., Van de Kemp, H., Hartskeerl, R.A., Edwards, C.N., Everard, C.O., Terpstra, W.J. and Levett, P.N.: Evaluation of the polymerase chain reaction for early diagnosis of leptospirosis. *J. Med. Microbiol.*, 1995; 43: 110-114.
21. Masri, S.A., Nguyen, P.T., Gale S.P., Howard, C.J. and Jung, S.C.: A polymerase chain reaction assay for the detection of *Leptospira* spp. in bovine semen. *Can. J. Vet. Res.*, 1997; 61: 15-20.
22. Ellis, W.A. and Michna, S.W.: Bovine leptospirosis: a serological and clinical study. *Vet. Rec.*, 1976; 99: 387-391.
23. Ellis, W.A., O'Brien, J.J. and Cassells, J.: Role of cattle in the maintenance of *Leptospira interrogans* serotype hardjo infection in Northern Ireland. *Vet. Rec.* 1981; 108 (26): 555-557.
24. Hathaway, S.C., Little, T.W.A. and Pritchard, D.G.: Problems associated with the serological diagnosis of *Leptospira interrogans* serovar hardjo infection in bovine populations. *Vet. Rec.*, 1986; 119: 84-86.
25. Bokhout, B.A., Peterse, D.J., Koger, P.L. and Terpstra, W.J.: Het voortomen van hardjo-positieve melrunderen in Noord-Nederland. Een orientierend serologisch onderzoek (Preliminary serological survey for hardjo-positive cows in the northern Netherlands). *Tijdschrift-voor-Diergeneeskunde*, 1989; 114, (3): 123-130.
26. Behymer, D.E., Riemann, H.P., Utterback, W., D-Elmi, C. and Franti, C.E.: Mass screening of cattle sera against 14 infectious disease agents, using an ELISA system for monitoring health in livestock. *Am. J. Vet. Res.*, 1991; 52, (10): 1699-1705.
27. Dom, P.P., Haesebrouck, F., Vandermeersch, R., Descamps, J. and Van Ommeslaeghe, K.: Prevalence of *Leptospira interrogans* serovar hardjo antibodies in milk in Belgian dairy herds. *The Veterinary Quarterly*, 1991; 13 (2): 118-120.
28. Özgen, H. ve Tunus, M.: Türkiye'de ilk olarak *Leptospira bovis* suşunun kültürel geliştirilmesi. *Türk. Vet. Hekim. Derg.*, 1954; 24, (98-99): 1865-1874.
29. Vardar, T.: 1963-1974 yılları arasında yurdumuz evcil hayvanlarında görülen Leptospirosis olayları. *Etilik Vet. Bakt. Enst. Derg.*, 1976; 4, (5-10): 147-162.
30. Bulu, A.A. ve Ergün, O.: Yurdumuz sığır Leptospirosis'ine karşı etkin bir aşı hazırlanması üzerinde çalışmalar. *Etilik Vet. Mik06. Enst. Derg.*, 1987; 6, (1): 23-24.
31. Bulu, A.A. ve Dörterler, R., Özkan, Ö. ve Hoştürk, F.: Doğu Anadolu'nun bazı illerinde (Kars, Artvin, Gümüşhane, Erzurum) sığır ve koyunlarda Leptospirosis vakaları, yayılışı ve serotipleri üzerine araştırma. *Etilik Vet. Mikrob. Enst. Derg.* 1990; 6, (6): 49-60.
32. Özdemir, V.: and Türkiye'de Leptospirosis'in dağılımı ve serotiplendirmesi üzerine bir çalışma. I. Ulusal Veteriner Mikrobiyoloji Kongresi, 27-29 Eylül, Ankara, 1994; p 34.
33. Çetinkaya, B., Ertaş, H.B., Muz, A., Öngör, H., Kalender, H. and Özdemir, V.: Determination of seroprevalence of leptospirosis in cattle in Elazığ. *Tr. J. Vet. Anim. Sci.*, 1999 (in press).
34. Sullivan, N.D.: Further observations on *Leptospira hardjo* infections in pregnant cows. *Aust. Vet. J.*, 1972; 48: 388-90.
35. Corboz, L., Leisi, V., and Bertschinger, H.V.: Epidemiology of Leptospirosis in Switzerland-Regional prevalence of antibodies to *L. hardjo* in the bovine population. *Isr. J. Vet. Med.*, 1987; 43: 323-326.
36. Schonberg, A., and Brem, S.: Serological reactions in cattle and sheep to *Leptospira interrogans* serogroup sebroe. *Isr. J. Vet. Med.*, 1987; 43: 310-312.
37. Ellis, W.A. and Michna, S.W.: Bovine leptospirosis: infection by the Hebdomadis serogroup and abortion-A herd study. *Vet. Rec.*, 1976; 99: 409-412.
38. Little, T.W.A., Richards, M.S., Hussaini, S.N. and Jones, T.D.: The significance of leptospiral antibodies in calving and aborting cattle in south west England. *Vet. Rec.*, 1980; 106: 221-224.
39. Hathaway, S.C. and Little, T.W.A.: Epidemiological study of *Leptospira hardjo* infection in second calf dairy cows. *Vet. Rec.*, 1983; 112: 215-218.
40. Hoare, R.J. and Claxton, P.D.: Observations on *Leptospira hardjo* infection in New South Wales. *Aust. Vet. J.*, 1972; 48: 228-232.
41. Elder, J.K. and Ward, W.H.: The prevalence and distribution of leptospiral titres in cattle and pigs in Queensland. *Aust. Vet. J.*, 1978; 54: 297-300.
42. Collares-Pereira, M.: Bovine leptospirosis in cattle in Portugal: bacteriological and serological findings. *Vet. Rec.*, 1991; 128: 549-550.

43. Woodward, M.J., Sullivan, G.J., Palmer, N.M., Woolley, J.C. and Redstone, J.S.: Development of a PCR test specific for *Leptospira hardjo* genotype *bovis*. *Vet. Rec.*, 1991; 128: 282-283.
44. Damas de Caballero, O.L.S., Dias Neto, E., Koury, M.C., Romanha, A.J. and Simpson, A.J.G.: Low-stringency PCR with diagnostically useful primers for identification of *Leptospira* serovars. *J. Clin. Microbiol.*, 1994; 32: 1369-1372.
45. Savio, M.L., Rossi, C., Fusi, P., Tagliabue, S. and Pacciarini, M.L.: Detection and identification of *Leptospira interrogans* serovars by PCR coupled with restriction endonuclease analysis of amplified DNA. *J. Clin. Microbiol.*, 1994; 32: 935-941.