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Research Article

Determination of the Seroprevalence of Leptospirosis in Cattle by MAT and ELISA in Hatay, Turkey*

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Abstract: Five hundred and twelve serum samples collected from randomly selected cattle of different age and sex from the towns and central villages of Hatay during April-July 2003 were tested for antibodies against 3 different *Leptospira interrogans* serovars (*grippotyphosa*, *hardjo* and *icterohaemorrhagie*) using a microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA). Forty-five (8.8%) and 72 (14%) of the 512 serum samples tested were detected to be positive with MAT and ELISA, respectively. Of the 45 MAT positive serum samples, 26 (57.7%) were positive against serovar *grippotyphosa*, 9 (20%) were positive against serovar *hardjo*, 1 (0.2%) was positive against serovar *icterohaemorrhagie* and 9 (20%) were positive against serovar *grippotyphosa* + serovar *hardjo*. Of the 72 ELISA positive serum samples, 32 (44.4%) were positive against serovar *grippotyphosa*, 15 (20.8%) were positive against serovar *hardjo*, 2 (2.8%) were positive against serovar *icterohaemorrhagie* and 23 (31.9%) were positive against serovar *grippotyphosa* + serovar *hardjo*. The dominant serovar was *grippotyphosa*. Statistical analyses indicated a significant difference in seroprevalence among locations ($P < 0.05$); however, no significant differences in positivity rates were found with respect to the age or sex of the animals ($P > 0.05$).

Key Words: Leptospirosis, cattle, MAT, ELISA

Hatay İlinde Sığırlarda Leptospirosis'in Prevalansının MAT ve ELISA ile Saptanması

Özet: Hatay ili merkez köy ve ilçelerinde Leptospirosis'in seroprevalansını belirlemek amacıyla Nisan-Temmuz 2003 aylarında tesadüfi örnekleme ile değişik yaş ve cinsiyette toplam 512 adet sığırdan alınan kan örnekleri 3 farklı *Leptospira interrogans* serovarına (*grippotyphosa*, *hardjo* ve *icterohaemorrhagie*) karşı oluşan antikorlar yönünden mikroskopik aglutinasyon testi (MAT) and enzyme-linked immunosorbent assay (ELISA) ile incelendi. İncelenen serum örneklerinin 45'inde (% 8,8) MAT, 72'sinde (% 14) ELISA ile pozitiflik saptandı. MAT ile pozitif bulunan serum örneklerinin 26'sı (% 57,7) serovar *grippotyphosa*, 9'u (% 20) serovar *hardjo*, 1'i (% 0,2) serovar *icterohaemorrhagie* ve 9'u (% 20) da serovar *grippotyphosa* + serovar *hardjo* ile pozitif reaksiyon verdi. ELISA ile pozitif bulunan serum örneklerinin ise 32'si (% 44,4) serovar *grippotyphosa*, 15'i (% 20,8) serovar *hardjo*, 2'si (% 2,8) serovar *icterohaemorrhagie* ve 23'ü de (% 31,9) serovar *grippotyphosa* + serovar *hardjo* ile pozitif reaksiyon verdi. Dominant serovar olarak *grippotyphosa* belirlendi. Yerleşim yerleri arasında seroprevalans değerleri istatistiksel olarak önemli farklılık gösterirken ($P < 0,05$), yaş ve cinsiyetin pozitiflik oranları üzerine önemli bir etkisi görülmedi ($P > 0,05$).

Anahtar Sözcükler: Leptospirozis, sığır, MAT, ELISA

Introduction

Leptospirosis is a zoonosis of worldwide distribution, caused by pathogenic *Leptospira* species. The genus Leptospira is classified serologically into 2 species, the pathogenic species *Leptospira interrogans* and the saprophytic species *Leptospira biflexa*. Both *L. interrogans* and *L. biflexa* are divided into numerous serovars by agglutination after cross-absorption with homologous antigen (1). Although Leptospira was divided into several species (*L. borgpetersenii*, *L. noguchii*, *L. santarosai*, *L. weilii* and *L. kirschneri*) in addition to *L. interrogans* on the basis of DNA relatedness (2), the term *L. interrogans* is still widely used in reference to pathogenic leptospires.

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In cattle, leptospirosis causes abortion, infertility, stillbirths, birth of weak calves, and decreased milk production (3). Serovars causing infection in cattle have been classified into 2 groups: those adapted to and maintained by other cattle (serovar *hardjo*), and incidental infections caused by strains maintained by other domestic and free-living animals (4).

The seroprevalence of leptospirosis in cattle has been reported to be 10.4% in Spain (5), 23.3% in Portugal (6), 3% in Germany (7), and 34.4% in Great Britain (8), and the most prevalent serovars were *hardjo*, *grippotyphosa*, *pomona* and *bratislava* in these studies. Studies carried out in Turkey showed that the seroprevalence of leptospirosis in cattle was 33.63% in Kars and Ardahan (9), 2.03% in Elazığ (10), and 17.8% in Eastern Turkey (11). In a national survey, the seroprevalence of leptospirosis in cattle was found to be 8.04% (12). Both in local studies and in the national survey, *hardjo* and *grippotyphosa* were the most prevalent serovars.

The diagnosis of leptospirosis is based on the isolation and identification of leptospires or the detection of anti-leptospiral antibodies. The isolation and identification of leptospires are time-consuming and necessitate specialised reference laboratories. Serological testing is the most widely used means for the diagnosis of leptospirosis, and the microscopic agglutination test (MAT) is the standard serological test (13). However, MAT has some disadvantages, such as the use of live antigen and subjective interpretation of test results. Enzyme-linked immunosorbent assays (ELISAs) (14-16) and other rapid serological tests based on whole-leptospiral antigen preparations (17) have been developed for use as an alternative to screen for leptospiral infection. ELISA has several advantages over MAT, including: i) killed antigen is used, which reduces the risk of infection of laboratory personnel; ii) IgG and IgM responses can be detected separately. A disadvantage of ELISA is that it requires a separate test for each serovar (14,15). Furthermore, DNA-based techniques have been used to demonstrate the presence of genetic material of leptospires in urine (18,19) and other body fluids (20).

The aim of his study was to investigate the seroprevalence of Leptospirosis using MAT and ELISA in the cattle population of the Hatay region.

Materials and Methods

Serum Samples

A total of 512 cattle sera were collected from different locations in Hatay. The age and sex of the animals were also recorded.

Leptospira Cultures

For the preparation of the antigens used in MAT and ELISA, reference strains (serovar *hardjo hardjoprajitno*, serovar *grippotyphosa*, and serovar *icterohaemorrhagie*) were obtained from the Royal Tropical Institute (Laboratory of Tropical Hygiene, Department of Biomedical Research, Amsterdam, the Netherlands).

Medium

EMJH leptospira medium was used for the preparation of antigens for MAT and ELISA.

Control Sera

Negative and positive control sera determined previously by MAT were also used as controls.

Microscopic Agglutination Test (MAT)

MAT Antigens: Live antigens required for MAT were grown in a Leptospira Laboratory (Etlik Veterinary Central Control and Research Institute, Ankara, Turkey). For this purpose, leptospira strains were incubated at 30-32 $^{\circ}$C for 4-14 days in EMJH medium. Approximately, an inoculum size of 1-2 x 10^8 cfu/ml was used as antigen. To determine the concentration of leptospira, the direct counting method was used under dark field microscopy (21).

Dilution of sera and MAT

First, to determine positive serum samples, 5 serum samples were pooled, and diluted 1:50 (4.5 ml of saline solution was added with 0.1 ml of each of the 5 serum samples). After that, 0.2 ml of 1:50 diluted serum samples was put into the wells of the MAT plates, and the same amount of antigen was added. Therefore, the final dilution of the serum samples was 1:100. This procedure was performed with 3 different serovars separately. After incubation at room temperature for 2-4 h, a loopful of each sample was examined under dark field microscopy for the presence of agglutination and lysis. For control purposes, negative and positive sera were included in each plate. Serum samples causing ≥50% of leptospires to agglutinate and/or lyse were considered positive. Each serum sample within a positive pool of sera was further

examined by diluting it 2-fold starting at 1:100 to determine antibody titres. The last dilution that gave a positive result under dark field microscopy was regarded as the antibody titre for leptospira (21).

ELISA

ELISA was performed as described by Terpstra et al. (22).

ELISA Antigens

Leptospira serovars were incubated in a shaking incubator at 30 °C for 10-12 days. To enhance the growth of leptospira, sterile Tween 80 was added at a final concentration of 1:10 to the culture medium after 4-5 days of incubation. After the complete consumption of Tween-80 and sufficient growth, 0.5% formalin was added to the culture medium to kill the leptospiras. Cultures were boiled in a waterbath for 30 min with stirring at 5-min intervals. Following centrifugation at 10,000 rpm for 30 min, the supernatant was used as antigen.

Coating ELISA Plates

A hundred microlitres of supernatant was put into each well of ELISA plates, which were then incubated at room temperature in a dark room until the plates became completely dry (1-3 days). These plates were stored at -20 °C until use.

ELISA Procedure

Antigen-coated plates were washed 4 times with PBS/Tween-20. Then 100 µl of 1:100 diluted (in PBS/Tween 20/BSA) serum samples was added to the wells, followed by incubation for 1 h at 30 °C. After washing, 100 µl of conjugate anti-bovine IgG diluted in PBS/Tween 20/BSA was added to the wells of the plates, which were then incubated for 1 h at 30 °C. The plates were washed 4 times, and after 100 µl of substrate 5-aminosalicyclic acid (5-AS) was added, they were mixed well and incubated at room temperature for 2 h. The reaction was stopped with 3 M H_2SO_4. The results were evaluated macroscopically by at least 2 persons.

Statistical Analysis

A chi-square test was used to detect significant differences between proportions, and a probability of less than 0.05 was considered statistically significant (23).

Sensitivity and specificity of ELISA

The relative sensitivity and specificity of ELISA for the detection of anti-leptospiral antibodies in bovine sera were determined using MAT as a reference test (24).

Results

The seroprevalence of leptospirosis was determined to be 8.8% with MAT and 14% with ELISA (Table 1). Positive serum samples had antibodies against 1 or 2 of the serovars studied. Positive titres varied from 1:100 to 1:3200. Of the 45 MAT positive serum samples, 26 (57.7%) were positive against serovar *grippotyphosa*, 9 (20%) were positive against serovar *hardjo*, 1 (0.2%) was positive against serovar *icterohaemorrhagie*, and 9 (20%) were positive against serovar *grippotyphosa* + serovar *hardjo*. Of the 72 ELISA positive serum samples, 32 (44.4%) were positive against serovar *grippotyphosa*, 15 (20.8%) were positive against serovar *hardjo*, 2 (2.8%) were positive against serovar *icterohaemorrhagie* and 23 (31.9%) were positive against serovar *grippotyphosa* + serovar *hardjo*. The dominant serovar in both tests was *grippotyphosa*.

One hundred and eighty-two sera collected from 5 locations were found negative by MAT and ELISA. When positivity rates among different locations were compared, a significant difference was detected ($P < 0.05$). Of the 462 female cattle, 43 (9.3%) were positive. However, only 2 (4.4%) of the 50 male cattle were positive by MAT. Furthermore, only 1 of the 45 positive serum samples was positive for the group under 1 year old, 18 were positive for the group 1-3 years old, 17 were positive for the group 3-5 years old, and 9 were positive for the group over 5 years old (Table 2). Neither age nor sex had a significant effect on the frequency of leptospirosis ($P > 0.05$).

The sensitivity and specificity of ELISA were 82.2% and 94.2%, respectively, when MAT was used as the reference test.

Discussion

In this study, the seroprevalence of leptospirosis in cattle was determined to be 8.78% with MAT and 14.06% with ELISA. These rates were lower than those reported previously in local studies (9,11), but higher than that stated by Çetinkaya et al. (10). However, our results were similar to the seroprevalence rates found in a national survey carried out by Özdemir and Kaya (12).

Table 1. Seroprevalence of leptospirosis according to locations by MAT and ELISA.

Location	Number of samples	Serovars							
		grippotyphosa		*hardjo*		*icterohaemorrhagie*		*grippotyphosa* + *hardjo*	
		MAT	ELISA	MAT	ELISA	MAT	ELISA	MAT	ELISA
Kılıçtutan Village-Altınözü	32	1	-*	-	-	-	-	-	-
Kozkalesi Village-Altınözü	47	-	-	-	-	-	-	-	-
Çamsarı Village-Kırıkhan	60	18	20	2	5	-	-	8	17
Gültepe Village-Kırıkhan	17	-	2	1	3	-	-	-	-
Karapelit Village-Belen	52	-	-	-	-	1	1	-	-
Eğerci Village-Yayladağı	40	-	-	1	-	-	-	-	-
Güzelyurt Village-Yayladağı	21	-	-	-	-	-	-	-	-
Günyazı Village-Antakya	23	3	-	1	1	-	1	-	-
Aşağıoba Village- Antakya	41	1	8	2	5	-	-	1	5
Huzulu Village-Samandağ	39	-	-	-	-	-	-	-	-
Hıdırbey Village-Samandağ	23	-	-	-	-	-	-	-	-
Paşaköy- Antakya	17	3	2	1	1	-	-	-	1
Reyhanlı	52	-	-	-	-	-	-	-	-
Serinyol	48	-	-	1	-	-	-	-	-
TOTAL	512	26	32	9	15	1	2	9	23

*- indicates seronegativity

Table 2. Seroprevalence of leptospirosis according to sex and age by MAT.

Age Groups (year)	Number of Samples		Serovars							
			grippotyphosa		*hardjo*		*icterohaemorrhagie*		*grippotyphosa* + *hardjo*	
	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
<1	22	16	-	-	1	-	-	-	-	-
1-3	156	29	2	-	10	1	-	-	4	1
3-5	148	5	2	-	11	-	1	-	3	-
>5	136	-	5	-	3	-	-	-	1	-
Total	462	50	9	-	25	1	1	-	8	1

In those studies, *hardjo* was the commonest serovar. However, in our study *grippotyphosa* was the commonest serovar in the cattle. The higher prevalence of *grippotyphosa* found in this study could be explained by the fact that the cattle had close contact with the reservoirs of this serovar. In addition, the longer immune response induced by this serovar and the higher frequency of new infections with this serovar may account for the observed results, as suggested by Guitián et al. (25).

Several factors such as herd size, co-grazing with infected cattle, access to contaminated water sources, use of infected bulls, inadequate husbandry practices, and replacement with animals from other farms have been

found to be associated with leptospiral infections in cattle (25,26). In this study, the seroprevalence of leptospirosis in different locations varied significantly ($P < 0.05$). Higher seroprevalence rates were detected in the town of Kırıkhan and in the village of Aşağıoba, where most of the factors mentioned above, such as co-grazing, larger herd size, and contaminated water sources, were possible. In other places, low or zero positivity rates were detected because animal husbandry is carried out to meet family requirements only and mostly involved fewer than 10 animals (average 1-3 cattle) per family. In these places, contact with other cattle, co-grazing, or access to contaminated water sources were not observed.

The results indicated that neither age nor sex had a significant effect on the frequency of leptospirosis ($P > 0.05$), and were in agreement with those of previous studies (10,27).

When ELISA results were compared with those of MAT, the specificity and sensitivity of ELISA were 94.2% and 82.2%, respectively. These values were lower than reported by Bercovich et al. (15), but were in agreement with those given by Woodward et al. (28). The observed differences in the ELISA results may be due to the different antigen preparation methods used in these studies.

Our results show that leptospirosis in cattle has a low prevalence, at least in Hatay region, when compared with previously conducted studies in various places in Turkey. However, further studies should be performed to understand the epidemiology of leptospirosis in farm animals and its association with human leptospirosis. Furthermore, some drawbacks of the serologic tests could be overcome using molecular techniques such as PCR.

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