Serologic and bacteriologic diagnosis of bovine leptospirosis in Tehran suburb dairy farms

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Summary

Leptospirosis is a worldwide zoonosis caused by *Leptospira interrogans*. This study was conducted to evaluate serologic and bacteriologic findings of leptospirosis in clinically-suspected cows. 380 sera and 33 urine samples were collected from 6 industrial dairy farms in Tehran suburb, from December 2004 to June 2005. The prevalence of disease was determined by microscopic agglutination test (MAT), enzyme-linked immunosorbent assay (ELISA), direct dark-field microscopic (DFM) examination, indirect fluorescent antibody test (IFAT), microbiologic cultural isolation technique and polymerase chain reaction (PCR). Antibodies were detected by MAT at least against one serovar of *L. interrogans* in 55 sera (14.5%) among 380 samples at a dilution of $\geq$1:100. *L. interrogans* serovar *icterohaemorrhagiae* was the most prevalent serovar. Leptospiral antibodies were detected by ELISA in 85 sera (22.4%) among 380 samples. Four (12.1%) of 33 urine samples were suspected by DFM examination and no positive sample by IFAT was observed. Leptospires could be isolated from none of the 33 samples taken from industrial farms. In this study, positive controls were detected at a dilution of $\geq$2000 leptospires per each ml of urine sample by PCR. Therefore, no DNA from serum and urine samples were collected from 6 industrial dairy farms could be detected by this method. It seems that, to increase the accuracy in the diagnosis of the disease, using a range of reliable techniques and comparing the results is important in reaching final conclusion.

Key words: Leptospirosis, Bovine, Serology, Bacteriology

Introduction

Leptospirosis is a worldwide zoonosis caused by pathogenic *Leptospira* species. The "gold standard" serodiagnostic method, the microscopic agglutination test (MAT), is particularly useful in differentiation between infective serovars (Angela *et al*., 1998). However, studies conducted by Ellis *et al*. (1982) in Northern Ireland and by Mackintosh *et al*. (1980) in England indicate that active bovine leptospiral infections often occur in the absence of detectable agglutination titers.

Enzyme-linked immunosorbent assay (ELISA) is a highly sensitive serologic technique which has been used to aid the diagnosis of many bacterial, viral and parasitic diseases (Levett, 2001). The ELISA technique has been compared with the MAT for the diagnosis of leptospirosis in human (Adler *et al*., 1980), cattle (Adler *et al*., 1982, Cousins *et al*., 1985) and sheep (Adler *et al*., 1981). Adler *et al*. (1981) indicated that there is high correlation between the ELISA and MAT results.

Efforts for early diagnosis of leptospirosis are directed towards the detection of leptospires or their DNA or antigens in blood, cerebrospinal fluid, urine and tissues. The techniques available are direct examination for leptospires, culture, detection of leptospiral antigens with antibodies and detection of leptospiral DNA with homologous nucleic acid sequence probes (Lucchesi *et al*., 2004).

However, different diagnostic techniques possess different specificity and

sensitivity. Therefore, to increase the accuracy in the diagnosis of the disease, using a range of reliable techniques and comparing the results is important in reaching the final conclusion.

In the present study, the first step was to set up some reliable diagnostic techniques. The second objective was to determine the prevalence of leptospiral infections in Tehran suburb dairy farms by MAT and ELISA (serologic techniques), direct dark-field microscopic (DFM) examination, indirect fluorescent antibody test (IFAT), cultural isolation technique and polymerase chain reaction (PCR).

Materials and Methods

Sample collection and processing

Three hundred and eighty serum samples were collected from six industrial dairy farms (by the average of 2000 cows) in Tehran suburb, from December 2004 to June 2005. Eighty-eight serum samples were obtained from farm one, 119 from farm two, 67 from farm three, 35 from farm four, 46 from farm five and 25 samples from farm six. Cows which have shown at least one of the clinical manifestations suggestive for leptospirosis, such as jaundice, haemoglubinuria, abortion or mastitis, during sample collection or some months before they were included in this study. Samples were taken aseptically using sterile 10-ml anticoagulant-free vacutainers from tail vein. Serum was separated by centrifugation of blood at 3000 × g for 10 min at room temperature. The sera were transferred into 1.5 µl sterile microtube (Eppendorf) and were kept at -20°C until used. Midstream urine samples were collected from 33 clinically-suspected cows (28 sero-positive and 5 sero-negative cows) in sterile bottles after cleaning of the vulva and perineal area and encouraging the animal to urinate by massage method. Fifty ml of the collected urine was transferred into a sterile bottle containing 0.5 ml of filtered (0.45 µm) 40% formaldehyde. It has reported that the rate of destruction of leptospires by urine is more rapid at 20°C than 4°C (Miller *et al*., 1990). So all urine samples were placed in an ice-container in the dark and transported to laboratory as soon as possible. These samples were submitted to the Leptospira Research Laboratory of Teaching and Research Hospital of the Faculty of Veterinary Medicine at the University of Tehran.

Microscopic agglutination test (MAT)

MAT was performed mainly as described by Turner (1968) with some modifications in Leptospira Research Laboratory as follows: a 7–10-day-old culture of *Leptospira interrogans* in liquid medium was used as antigen. The density of leptospires was assessed using a counting chamber (Petroff-Hausser USA) and adjusted to 2×10^8 leptospires/ml. Six reference strains of *L. interrogans* which were used as antigen includes: *hardjo*, *pomona*, *icterohaemorrhagiae*, *grippotyphosa*, *canicola* and *ballum*. All serum samples were serially diluted in phosphate buffer solution (PBS) in a microtiter plate (Greiner), starting from 1:50 dilution, using 2-fold dilution (1:100, 200, 400, 800 and 1600). Then, 10 µL of serum dilution was added to 10 µl of appropriate antigen on a microscopic slide and was placed in a plate with moist paper to avoid evaporation, and incubated at 30°C for 90 min. Finally, the slide was examined under dark-field microscope (Olympus BX50). One antigen control and two (positive and negative) standard serum controls were used each time. Titres of $\geq$1:100 were considered positive. The end-point titre was determined as the highest serum dilution showing agglutination of at least 50% of the leptospires.

Enzyme-linked immunosorbent assay (ELISA)

This assay was performed as described by Cousins *et al*. (1985) and Surujballi and Mallory (2004) with some modifications in Leptospira Research Laboratory in the following manner. The wells of ELISA flat-bottomed microtiter plates (Grainer) were coated by sonicated bacterial cell derived from six standard serovars of *L. interrogans* and incubated overnight at 4°C then washed three times with PBS containing 0.05% tween 20 (PBST). The density of leptospires was assessed using a counting chamber (Petroff-Hausser USA) and adjusted to 5×10^7 leptospires/ml. Before coating, antigen

suspension was sonicated at 20 kHz for 20 sec (2 periods). Then, 200 µL of 1% bovine serum albumin (BSA) was added to each well, incubated for one hr at 25°C and washed three times in PBST. Serum samples were diluted in PBST (1:400) and 100 µl volumes were dispensed into each well, incubated at 37°C for one hr (all samples and controls were tested in duplicate) and then washed three times in PBST. Peroxidase conjugated rabbit anti-bovine IgG was diluted (1:7000) in PBST and 100 µl volumes were dispensed into each well, incubated at 37°C for one hr, and then washed four times in PBST. One-hundred µl of OPD substrate solution was dispensed into each well, incubated in the dark at 25°C for 30 min. The reaction was stopped by addition of 50 µl of 8 N H_2SO_4 and the optical density (OD) produced in each well was measured at 492 nm using a Titertek Multiskan (Anthus labtec instrument) and recorded using a computer. Four quality controls—three known positive control sera (high, medium and low positive) and one negative serum—were included on each plate. If any control had more than 10% variation from its target OD, the test was repeated for all sera on that plate and if the variation of duplicates of a sample exceeded 15% of coefficient of variation (CV), the test was repeated for that serum. The OD accepted for each sample was determined by averaging the results obtained from the duplicate samples. The standard error (SE) for each of assays was calculated from the mean difference between duplicate OD's. Cut-off point was determined as described by Cousins *et al*. (1985) as follows: the mean OD's of 40 negative sera were 0.254 and standard deviation (SD) was 0.0318. The mean plus 3 SD was 0.349 and this OD value was used to signify the lowest level at which specific IgG was considered to be present.

Direct dark-field microscopic (DFM) examination

This test was carried out as described by Abdollahpour (1995a) as follows: one ml of each formalinised and filtered (0.22 µm Millipore filter) urine sample was centrifuged at 12000 × g for 20 min at 4°C. Supernatant was removed and the pellet was resuspended in 100 µl of remained urine. Twenty µl of this suspension was placed on a clean microscopic slide and 20 field were examined for each urine sample with dark-field microscope (Olympus BX50), using a dry condenser.

Indirect fluorescent antibody test (IFAT)

This method was performed mainly as described by Miller *et al*. (1989) with some modifications in Leptospira Research Laboratory in the following manner. Antisera against six reference leptospira serovars were prepared in rabbit as described by Ellis *et al*. (1982). One ml of each urine sample was centrifuged at 12000 × g for 20 min at 4°C. Supernatant was removed and the pellet was washed with 0.01 M PBS (pH = 7.4) and resuspended in reminded solution. Ten µl of this solution was placed on a clean microscope slide. After drying at 37°C, the slide was flamed lightly, fixed in acetone for 10 min and allowed to dry. Slides were covered with 20 µl of rabbit antiserum diluted in 0.01 M PBS (1:800) and were held in a moist chamber at 37°C for 40 min. Following a brief rinse in distilled water, slides were placed in PBS for 40 min and rinsed in distilled water for one min. Slides were covered with conjugate diluted 1:400 in 0.01 M PBS, then placed in a moist chamber at 37°C for 40 min. After a brief rinse in distilled water, slides were placed in PBS for 40 min and rinsed in distilled water for one min. Cover slips were applied over a drop of mounting fluid and slides were examined under a fluorescence microscope (Olympus BX50) at magnification of ×400. Seven-day-old cultures of six standard serovars of *L. interrogans* were serially diluted in negative urine sample, using 10-fold dilution, to be used as positive controls. Two positive controls were used.

Isolation and culture of leptospires

This method was performed mainly as described in World Health Organization (WHO) leptospirosis guideline (2003) by Abdollahpour (1995b). About five µl of fresh midstream urine sample was inoculated into Ellinghausen-McCulough modified by Johnson-Harris (EMJH) semi-

solid medium (ESR2), immediately after sample collection. All cultured media were incubated at 30°C for at least five weeks and examined at one-week intervals for leptospiral growth, using dark-field microscopy.

Polymerase chain reaction (PCR)

This method was carried out as described by Gerritsen *et al*. (1991) and Lucchesi *et al*. (2004) with some modifications as follows: *L. interrogans* serovar *hardjo* used in this study was provided by WHO/FAO Leptospira Reference Laboratory, Brisbane. The PCR was performed with the primers previously described by Woodward and Redstone (1994). The sequences of these were Lep13: 5'CTCGGATCCTTAGATATGCTGCAGAAGCT TG3' and Lep14: 5'AAAAGATCTTATGATTATGAATCACAACCTG3' and expected size of the amplified product was 852 bp. PCR amplifications were performed in 25 µl reaction volumes, containing 10 mM Tris-HCl (pH = 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 µM for each of dATP, dCTP, dGTP and dTTP, 50 pM of each primer and 1.1 U of Taq DNA polymerase (Roche). DNA fragments were visualized by placing the gel on an ultraviolet transilluminator (Bioimaging system UVP).

Results

Microscopic agglutination test (MAT)

Antibodies were detected at least against one serovar of *L. interrogans* in 55 (14.5%) of 380 samples at a dilution of ≥1:100. Positive titres against more than one serovar were detected in 27 sera of the positive samples (Table 1).

Table 1: Frequency (%) and number of positive serum samples by MAT at a dilution of 1:100 in terms of number of serovars among 380 samples

Number of serovars	Number of positive sera	Frequency (%)
One serovar	28	7.4
Two serovars	23	6.1
Three serovars	3	0.8
Four serovars	1	0.3
Total	55	14.5

From total of six farms, the highest prevalence of positive sera by MAT was found in farm three (43.3%), followed by farm one (20.5%), farm four (8.6%), farm five (4.4%) and farm two (2.5%), while no positive serum was seen in farm six.

Positive titres were recorded against serovar *icterohaemorrhagiae* (37 samples), *hardjo* (31 samples), *grippotyphosa* (8 samples), *pomona* (7 samples), *canicola* (3 samples) and *ballum* (1 sample) (Table 2). Table 3 presents the number and frequency of each serovar in different farms.

Enzyme-linked immunosorbent assay (ELISA)

Leptospiral antibodies were detected in 85 sera (22.4%) of 380 samples. The highest prevalence of positive sera by ELISA was found in farm one (45.5%), followed by farm three (43.3%), farm six (32%), farm four (8.6%), farm five (4.4%) and farm two (2.5%).

Table 4 indicates number of positive and

Table 2: Number and frequency (%) of positive serum samples according to serovar, at various dilutions

Serovar	Dilutions											
	1:100		1:200		1:400		1:800		1:1600		Total	
	N	F (%)	N	F (%)	N	F (%)	N	F (%)	N	F (%)	N	F (%)
G	7	8.1	1	1.2	0	0	0	0	0	0	8	9.2
P	4	4.6	2	2.3	1	1.2	0	0	0	0	7	8.1
I	17	19.5	9	10.3	9	10.3	1	1.2	1	1.2	37	42.5
C	2	2.3	1	1.2	0	0	0	0	0	0	3	3.5
B	1	1.2	0	0	0	0	0	0	0	0	1	1.2
H	19	21.8	10	11.5	1	1.2	1	1.2	0	0	31	35.6
Total	50	57.5	23	26.4	11	12.6	2	2.3	1	1.2	87	100

G: *grippotyphosa*, P: *pomona*, I: *icterohaemorrhagiae*, C: *canicola*, B: *ballum*, H: *hardjo*, N: number and F: frequency

Table 3: Number and frequency (%) of each serovar, in different farms

Farms	Serovars													
	G		P		I		C		B		H		Total	
	N	F (%)	N	F (%)	N	F (%)	N	F (%)	N	F (%)	N	F (%)	N	F (%)
Farm 1	1	1.2	2	2.3	9	10.3	2	2.3	0	0	14	16.1	28	32.2
Farm 2	3	3.5	0	0	0	0	0	0	0	0	0	0	3	3.5
Farm 3	3	3.5	1	1.2	28	32.2	1	1.2	1	1.2	17	19.5	51	58.6
Farm 4	1	1.2	2	2.3	0	0	0	0	0	0	0	0	3	3.5
Farm 5	0	0	2	2.3	0	0	0	0	0	0	0	0	2	2.3
Farm 6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	8	9.2	7	8.1	37	42.5	3	3.5	1	1.2	31	35.6	87	100

G: *grippotyphosa*, P: *pomona*, I: *icterohaemorrhagiae*, C: *canicola*, B: *ballum*, H: *hardjo*, N: number and F: frequency

negative sera tested by MAT and ELISA in Tehran suburb dairy farms.

Table 4: Number of positive and negative serum samples by MAT and ELISA

	ELISA positive	ELISA negative	Total
MAT positive	47	8	55
MAT negative	38	287	325
Total	85	295	380

Kappa statistic for determination of overall agreement between MAT and ELISA results was 0.568 indicating moderate agreement between the two tests.

Direct dark-field microscopic (DFM) examination

Four (12%) of 33 urine samples were suspected by DFM examination. Negative sera were seen in three of them, both by MAT and ELISA. In the other one, positive serum was seen by ELISA and positive titer was detected at a dilution of 1:100 against serovar *icterohaemorrhagiae* by MAT.

Indirect fluorescent antibody test (IFAT)

Leptospires were detected at a dilution of ≥1:1000 in positive controls, however in the present study, of 33 urine samples taken from dairy farms, no sample found positive by IFAT.

Isolation and culture of leptospires

All of the 33 samples taken from industrial farms were culture-negative; no leptospires could be isolated from them. But leptospires were isolated from urine samples taken from two clinically-affected calves; the results of serologic tests for them have been negative by ELISA but positive by MAT at a dilution of ≥1:100 with more than one serovar. These two urine samples were taken from traditional farm in Tehran suburb and found negative by DFM examination.

Polymerase chain reaction (PCR)

The present method only detected and amplified DNA from positive controls (≥2000 leptospires per ml of urine sample). Therefore, in this study, no DNA from serum and urine samples which were collected from six industrial dairy farms, could be detected by PCR.

Discussion

The earliest study (1967) on the seroprevalence of leptospirosis (*L. interrogans*) in Iran indicated a positive rate of 31% in cattle and 17% in sheep (Maghami, 1967). Another study showed that the seroprevalence rate against leptospiral antigen was 24.6% in Tehran suburb dairy farms (Maghami, 1980). Results of the similar studies on seroprevalence of leptospirosis in other regions of Iran reported values between 3 and 30.7% in Tehran suburb (Moharrami *et al*., 1992), 24.24% in Mashhad suburb (Talebkhan *et al*., 1996), 32% in Shiraz suburb (Firouzi and Vandyousefi, 2000), 46.8% in Karadj suburb (Goli, 2002), 22% in Guilan province (Asadpour, 2002) and 53.73% in Ahvaz suburb (Hajikolaei *et al*., 2005).

The seroprevalence against six reference strains of *L. interrogans—hardjo*, *pomona*, *icterohaemorrhagiae*, *grippotyphosa*, *cani-*

cola and *ballum*—in Tehran suburb dairy farms by MAT in the present study (14.5%) is lower than that in previous studies. One of the main reasons for this is that our serum samples were taken from full industrial and advanced dairy farms which managed under veterinary specialist surveillance. Besides, in previous studies, serum samples have been randomly collected, but samples of the present study have been taken from clinically-suspected cows.

Results of previous studies on the prevalence of each serovar in Iran have shown that *L. hardjo* was the most (67.7%) and *L. icterohaemorrhagiae* the least (0.8%) prevalent serovars in Tehran suburb (Moharrami *et al*., 1992); that *L. icterohaemorrhagiae* was the most and *L. pomona* the least prevalent serovars in Mashhad suburb (Talebkhan *et al*., 1996); that *L. grippotyphosa* was the most prevalent serovar in Urmia (Jaafari *et al*., 1996); that *L. canicola* was the most (39.9%) and *L. hardjo* the least (4.7%) prevalent serovars in Karadj suburb (Goli, 2002); that *L. grippotyphosa* was the most prevalent serovar in Guilan province (Asadpour, 2002); that *L. canicola* was the most and *L. grippotyphosa* the least prevalent serovars in Shiraz suburb (Firouzi and Vandyousefi, 2000); that *L. canicola* was the most prevalent serovar in tribal area of west central Iran (Abdollahpour, 2003) and that *L. grippotyphosa* was the most and *L. ballum* the least prevalent serovars in Ahvaz (Hajikolaei *et al*., 2005).

Durham and Paine (1997) believed that there is a significant difference between the prevalence rates of *L. hardjo* and *L. pomona* in industrial and traditional herds. They have reported a seroprevalence of 7.27% against *L. hardjo* and a rate of 16.13% against *L. pomona* in industrial dairy farms, while in traditional dairy farms, the seroprevalence were 16.13% against *L. hardjo* and 8.1% against *L. pomona*. Hajikolaei *et al*. (2005) believe that there is a significant difference in leptospiral prevalence between industrial and traditional dairy farms, too.

In the present study, the most prevalent (*L. icterohaemorrhagiae*) and the least prevalent (*L. ballum*) serovars were different from those of previous studies. It may be depend on two factors: there is a significant difference between prevalence of serovars in industrial and traditional dairy farms, on the other hand, the type and prevalence of serovars change over the time in one area and from region to region.

The MAT has some disadvantages which indicate the need for an alternative test for routine diagnosis of leptospirosis. One major problem with the MAT is its use of live organisms as antigens. This requires the continuous culture and handling of these hazardous bacteria in laboratories; the subjective assessment of results can also make quality assurance of the MAT difficult. Another problem associated with the MAT is that it only detects agglutinating antibodies and non-agglutinating antibodies may go undetected. As a consequence of these problems associated with the MAT, some reliable bacteriologic and serological techniques for the diagnosis of leptospiral infections were used.

The ELISA technique is now widely accepted as a routine serological test for detecting antibodies against a wide range of microbial antigens. The ELISA has some advantages over the MAT. It is relatively sensitive and specific and semi-automated. It uses killed antigens, and the results can be read objectively (Surujballi and Mallory, 2004). The correlation between the ELISA and MAT was lower in the present study than was described by Cousins *et al*. (1985) and Adler *et al*. (1981).

In the present study, examination of urine sediment using DFM had intermediate sensitivity for detection of leptospires but results are subjective. The IFA had lesser sensitivity than DFM examination. Success of DFM examination and IFA technique depends on the presence of a relatively large number of leptospire and this may not always be the case—as was shown in this study—which leptospires were isolated from urine samples taken from two clinically-affected calves in which leptospires could not be demonstrated by IFA and DFM examinations. Although the IFA did not appear to be as sensitive as culture technique in this study, it offers some advantages over serologic and cultural methods. Interpretation of MAT titre is difficult, and in some confirmed cases of leptospirosis, titres have been detected neither by MAT nor by

ELISA. Demonstration of leptospires by IFA provides definitive evidence of infection. This test can be conducted within few hours, whereas culture techniques require 6–8 weeks. Culture method is time-consuming, difficult and expensive, therefore, will not be a practical technique for the routine diagnosis.

Thus, on the basis of the results presented here, we conclude that each diagnostic procedure has advantages and disadvantages as a routine diagnostic test. Therefore, comparing the results of different methods is important in reaching the final conclusion.

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