Development of an In-house Indirect ELISA Test as a Diagnostic Tool for Bovine Leptospirosis

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Abstract

Background: Leptospirosis is one of the most common zoonotic diseases, which is caused by a Spiral shaped bacterium called *Leptospira*. The recommended diagnosis method is to perform microscopic agglutination test (MAT) which is both hazardous, due to using live bacteria, and time-consuming. As a result, many attempts have been recently made to develop other serological methods, such as ELISA.

Objective: This study aimed to develop an indirect ELISA method using leptospiral whole antigens for diagnosing pathogenic *Leptospira*.

Materials and Methods: In this study, four pathogenic serovars of *Leptospira* were used and cultured in selective culture medium. The cultured bacteria were sonicated and the extracted antigens were used as captured antigen in ELISA method. A total of 74 samples from bovine suspected to leptospirosis and 43 samples from healthy animals were examined by MAT method.

Results: According to the study results, 42 samples (56.7%) out of 74 suspected ones were found positive while 32 ones (43.2%) were determined negative by MAT analysis. All of the 43 negative control samples were found negative after performing MAT. The sensitivity and specificity of ELISA, compared to those of MAT, were measured as 87.5% and 84.2%, respectively.

Conclusion: Taking into account the high sensitivity and appropriate specificity of the developed indirect ELISA method, it was recommended that ELISA be employed as an accurate method for early and rapid diagnosis of bovine leptospirosis.

Background

Leptospirosis is a zoonotic disease caused by spirochete *Leptospira*. Due to its rapid spread among humans and animals, this infection has been considered as a global public health issue.[1] The leptospirosis is endemic mainly in tropical as well as subtropical climates with high humidity, and it could be isolated from different kinds of wild and domestic animals reservoirs.[2,3] The infection is usually transmitted to people or animals through direct or indirect contact with water, soil, or food contaminated by urine from the infected animals.[4,5] The leptospirosis which is considered as occupational disease and mainly *found* in warmer seasons is under control in Iran.[6] Bovine leptospirosis causes significant economic losses and can lead to abortion, infertility, stillbirth, and reduced milk production.[7] The clinical signs of leptospirosis are nonspecific, making it difficult to diagnose the disease through a clinical approach.[8] Thus, laboratory tests play an important role in diagnosing and controlling the disease.[9] Traditionally, isolation and culture of *Leptospira* is golden test for diagnosing leptospirosis. Determination of specific antibody by microscopic agglutination test (*MAT*) has been considered as a reference test for laboratory diagnosis.[8,10] However, it faces some disadvantages, including safety concerns associated with laboratory maintenance of bacterial live cultures which requires technical expertise.[11] Therefore, several methods such as indirect immunofluorescent, ELISA, and *nucleic acid* amplification (NAA) *test* have been developed recently.[12] Evaluation of specific antibody levels by indirect ELISA has been considered as a rapid screen test for both human and animal leptospirosis.[8,13] This method has many advantages over MAT method with reasonable sensitivity and specificity.[10] In most cases, the ELISA method is implemented based on *Leptospira* whole antigens; but due to the recent development of *Leptospira*

recombinant proteins, they have also been used as capture antigens to design ELISA system for improving specificity and reproducibility.[14]

This study aimed to develop and evaluate an indirect ELISA test based on *Leptospira* whole-cell lysates as capture antigen. The data obtained from this study may have proven useful in developing an easy and inexpensive alternative serologic diagnosis test for bovine leptospirosis.

Materials and Methods
Bacterial Culture and Antigen Preparation
Four different *Leptospira* serovars consisting *of L. icterohaemorrhagiae, L. Canicola, L. grippotyphosa and L. serjoe hardjo* were obtained from *Leptospira* reference laboratory (RVSRI, Karaj, Iran), cultured on *Ellinghausen–McCullough–Johnson–Harris* (*EMJH*) modified medium (Difco, USA), and enriched by adding 10% *rabbit serum. The cultures were incubated at 37°*C under aerobic condition. After 7-10 days, the bacterial growth was examined by dark field microscope.

Equal amounts of bacterial culture were mixed and centrifuged at 3000 G for 20 minutes. The precipitate was re-suspended in distilled water and incubated for 45 minutes at 66°C in a water bath.

Alternatively, the bacterial cell lysates were prepared by sonication of bacterial culture on ice using three 10-second bursts at high intensity. The concentration of the sample protein was calculated by Lowry method.

Sample Collection
In this study, a total of 117 bovine serum samples (74 suspected animals and 43 healthy ones) admitted to leptospirosis reference laboratory were examined. The samples were collected from different industrial farms located in five different provinces of Iran, namely Qom, Mazandaran, Ardabil, Guilan, and Markazi, and were stored in -20°C until examination.

All the sera were tested for the presence of *leptospiral specific* antibodies by MAT using a panel of live *Leptospira* serovars such as *Grippotyphosa, Pomona, Autumnalis, Canicola, Icterohaemorrhagiae, Sejroe hardjo, and Serjoe serjoe*. The cut-off value for discrimination of positive and negative samples was set to 1/400 in MAT analysis. However, any samples with lower titer were probably associated with non-specific antibodies.

Evaluation of Antibody With Indirect ELISA
An indirect ELISA was performed for detecting anti-*Leptospira* antibody. The proper concentration of antigen, serum, and conjugated antibody were optimized with checker board analysis.

The ELISA plates (NUNC-Immuno™ 96-well MaxiSorp plate) were coated with 100 μL/well with 6 μg/mL of prepared antigen in *carbonate buffer* (pH 9.6). The plates were incubated overnight at 4°C with agitation. After blocking with 2% (w/v) gelatin, the wells were washed three times using wash buffer (0.05% Tween20, PBS pH7.4).

The samples sera were diluted 1:100 in PBS/T and the duplicates of 100 μL of each sample were applied and incubated at 37°C for 1 h. After taking additional washing steps, 100 μL/well of HRP-conjugated anti-bovine IgG (1/20000 diluted in PBS/T) was added and incubated for 1 hour at 37°C. The plates were then washed as before, and 100 μL of 3,3′,5,5′-Tetramethylbenzidine (TMB) was added as substrate. After 15 minutes incubation in room temperature, the reaction was stopped using stop solution (0.1 N sulfuric acid) and the optical density was determined at 450 nm.

Statistical Analysis
The relative sensitivity and specificity of the developed ELISA method in bovine sera were determined in comparison to those of the MAT by using the following formula:

$Sensitivity = TP/ (TP+FN) \times 100$

TP: The number of serum samples positive by MAT; FN: The number of serum samples negative by ELISA but positive by MAT.

$Specificity = TN/ (FP+TN) \times 100$

TN: The number of serum samples negative by MAT; FP: the number of serum samples positive by ELISA but negative by MAT.

Results
According to the infectivity of *Leptospira* strains, equal amounts of *Icterohaemorrhagiae, Canicola, Grippotyphosa,* and *Serjoe hardjo serovars* were prepared and used as capture antigen for implementing ELISA. To evaluate the ELISA method, its sensitivity and specificity were compared with the results from MAT.

All of the 124 negative control samples were found negative in the MAT analysis and showed no response to any of the *Leptospira* antigens. Out of 74 suspected samples, 42 (56.7%) ones reacted to at least one of the *Leptospira serovars* studied in MAT. Out of 42 positive MAT sera, 17 ones (40.5%) reacted to *Icterohaemorrhagiae*, 13 ones (30.9%) to *Serjoe hardjo*, 2 ones (4.7%) to *Grippotyphosa*, and 1 serum (2.4%) to *Canicola* serovars.

After optimization with negative samples, the ELISA cut-off was calculated as 0.38 in dilution of 1.100. The area under the ROC curve was 0.964 (95% CI = 0.932-0.987). All of the 74 suspected samples were submitted to develop in house ELISA. Out of 42 MAT positive sera, 36

Table 1. Comparison of In-house IgG ELISA Assay With Microscopic Agglutination Test

| | | MAT Results | | Total |
		Positive	Negative	
ELISA results	Positive	36	6 (2)	42
	Negative	6	26 (41)	75
	Total	42	75	117
Data analysis	Sensitivity	87.5%	-	-
	Specificity	-	84.2%	-

Note: The numbers in parentheses indicate the values related to the negative healthy animals.

samples (87.5%) reacted with the antigenic mixture used in ELISA and 6 samples (14.2%) were negative in ELISA (Table 1). Also, 6 samples (18.7%) of negative MAT sera were reactive and others (81.25%) were non-reactive in ELISA assay. The remaining negative control samples were non-reactive in ELISA assay except for 3 samples (2.4%) (Figure 1).

According to our results, sensitivity and specificity of the developed ELISA, compared to those of the MAT, were found to be 87.5% and 84.2%, respectively.

Discussion

Leptospirosis has been recognized as a worldwide common zoonotic disease occurring in hot and humid climate.[13] Human is an accidental host for *Leptospira* and, therefore, the infections are not the major factor in causing this disease.[15,16] Temperature and humidity are two important factors contributing to the growth and survival of these bacteria.[17,18] Therefore, there are more chances for the bacteria to survive, reproduce, and spread to other animals in warm, humid, and tropical climates with high rainfall.[19,20]

Early and accurate diagnosis of the disease is one of the most important measures to take in order to prevent, control, and treat the disease properly.[21-23] Since the humoral immune responses are usually detectable in one to two weeks after the infection, the implementation of serological methods can be postponed until this period ends.[24] Given the above discussion, this study aimed to develop and evaluate a homemade ELISA method using Iranian isolated strains in order for establishing an accurate diagnosis of *Leptospira*. Serological methods are often implemented by conducting the MAT analysis, but other serological methods such as ELISA have also been utilized recently. MAT test is time-consuming and requires considerable skills and experiences, while ELISA test is available and reproducible, and during which accuracy is very important.[25,26]

Recently, many attempts have been made to provide an ELISA method with acceptable accuracy and sensitivity for diagnosing human and animal leptospirosis. Some of

these studies have employed *Leptospira* killed whole cells as capture antigen, but they have also been considered as suitable candidates for developing ELISA tests due to the availability of recombinant antigens.[27-30]

Surujballi and Mallory developed a sensitive and specific ELISA for detection of bovine antibodies to multiple pathogenic *Leptospira* serovars which were routinely monitored in Canada. The sensitivity and specificity of this method were approximately 94% and 95%, respectively.[31]

Mulla described IgM ELISA as a rapid test which produced results quite similar to those from MAT when used for diagnosing human leptospirosis. The sensitivity and specificity of ELISA were 88% and 90.90%, respectively.[32] In another study, the ELISA was compared with the MAT analysis regarding the diagnosis of bovine leptospirosis. In the given study, ELISA showed 100% sensitivity compared to MAT and, in conclusion, ELISA appeared to be a better alternative to MAT for diagnosing bovine leptospirosis.[33] Comparing MAT and ELISA in terms of the potential for detecting leptospiral antibodies in cattle, Sakhaee et al found a remarkable correlation between these methods, so that 2.25% of MAT positive sera were determined negative by the ELISA, and 8.45% of the MAT negative sera were determined positive by the ELISA.[34]

Similarly, the results from our study showed that the developed ELISA method, compared to MAT, had appropriate consistency, sensitivity (87.5%), and specificity (84.2%). However, we believed that using the local strains of *Leptospira* spp. as antigens could increase the sensitivity of the serodiagnosis of bovine leptospirosis. Therefore, it was strongly recommended that the ELISA be adopted as a suitable test for screening suspected cases of bovine leptospirosis.

Conclusion

Overall, the results show that the ELISA method developed in this study, has acceptable sensitivity and specificity and
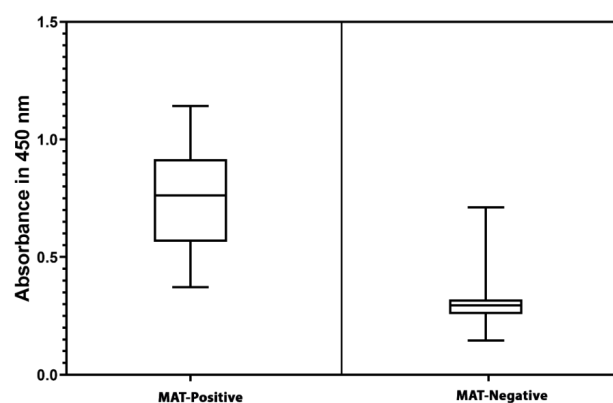

Figure 1. Evaluation of the Developed ELISA for Detection of *Leptospira* Antibodies in Positive and Negative MAT Sera.

could be used as a screening method for rapid diagnosis of bovine leptospirosis.

Authors' Contributions

MT and PK designed the study and analyzed the results. NB Performed sampling and experiments and wrote the manuscript. ME and SS performed.

Ethical Approval

The authors of this study have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

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