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RESEARCH ARTICLE
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Seroepidemiology of canine leptospirosis by iELISA and MAT

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Abstract

Aim: The present study was conducted to determine the prevalence of antibodies against *leptospira* using a genus specific and serovar specific antigen by indirect ELISA (iELISA) and Microscopic Agglutination test (MAT), respectively.

Materials and Methods: 300 sera samples were collected from dogs, from Pondicherry, suspected to be suffering from Canine leptospirosis and with no history of vaccination. Using the outer membrane proteins (OMP's) of *Leptospira interrogans* serovar *canicola* as antigen the leptospiral antibodies were detected by iELISA and compared with the MAT using five commonly infecting serovars (*canicola, icterohaemorrhaegiae, grippotyphosa, pomona, australis*) as antigens. Statistical analysis of the data was carried out using chi-square test.

Result: Out of 300 sera samples, 159 (53 %) and 99 (33 %) sera samples were found to be positive for leptospiral antibodies by iELISA and MAT, respectively. The iELISA employed in this study revealed a very high sensitivity of 94.94 % in comparison with the MAT (59.11 %). However, iELISA showed less specificity (67.66 %) when compared to MAT (96.45 %). By chi-square test, a significant difference ($P<0.001$) was obtained between the results from iELISA and MAT.

Conclusion: The present study revealed that the iELISA was found to be highly sensitive, rapid and easy to perform in comparison with MAT for the detection of canine anti-leptospiral antibodies.

Key words: Canine leptospirosis, outer membrane protein (OMP), iELISA, Microscopic Agglutination test (MAT).

Introduction

Leptospirosis is a widespread zoonotic disease and is a real public health concern around the world. The disease is caused by spirochetes of the genus *Leptospira* which comprises of more than 260 serovars classified under 24 serogroups based on agglutinating antigens and is classified into multiple genomospecies based on DNA studies [1]. These highly invasive spirochaetal pathogens are capable of infecting a wide range of mammalians either by direct contact or by indirect contact with soil/water contaminated with the urine of the carrier animals.

Leptospira are obligate aerobes with an optimum growth at 28-30°C in the EMJH medium, supplemented with Tween-80 and bovine serum albumin fraction V [2]. The diagnosis of this disease is done mainly by direct microscopic examination and by isolation of these microorganisms. The microscopic examination is done using dark-field microscopy and aids in the early diagnosis of the disease. However it has two major drawbacks; i) too low concentration of leptospires (less than 104 cells / ml) which may not be detected and ii) artifacts such as fibrin and extrusions from cellular elements can be easily mistaken for Leptospira by inexperienced workers [2]. Hence microscopic examination is used as a parallel test along with the culturing of these organisms (gold standard test) which is very laborious, time consuming and does not aid in the early diagnosis of the disease [3].

Serology appears to be the most reliable tool for the diagnosis of leptospiral infections carried out with Microscopic Agglutination Test (MAT) which is recognized as the international standard technique [4]. The MAT is a specific test, which requires maintenance of live organisms that may cause problem in handling, standardization and its interpretation is relatively subjective [5]. Leptospiral outer membrane proteins (OMP) contain both transmembrane proteins, porin (OMP L1) and lipoproteins, such as LipL41 and LipL36. Most research on leptospiral antigens is being focused on Lipopolysaccharide (LPS) and their variations in the carbohydrate side chains which are responsible for the antigenic diversity among leptospiral serovars. Because of this antigenic variability, the use of leptospiral LPS has limitations for serodiagnosis. Ideally, an antigen of choice for the purpose of developing a diagnostic test which can be applied for the variety of epidemiological situations associated with human and animal leptospirosis is to be highly conserved among the diverse pathogenic leptospiral serovars. In this context, OMPs is considered to be a potential antigen as they are conserved within the various pathogenic

serovars and such proteins and their associated molecules, selectively solubilised using Triton-X 114 detergent, would be of immense diagnostic value [6].

The ELISA has been used for the serodiagnosis of a wide range of infectious diseases and also to detect leptospira – specific antibodies in humans and animals [7, 8]. This immunosorbent assay is reported to be simple, safe, specific, sensitive, easily automated and suitable for the examination of a large number of sera samples in the diagnosis of Canine leptospirosis [9].

Hence, this study was planned on to standardize the indirect ELISA and compare it with internationally recognized standard i.e., MAT test, for its specificity and sensitivity.

Materials and Methods

Bacterial cultures: Eight standard serovars of Leptospira viz; *Leptospira interrogans* serovars *canicola*, *icterohaemorrhagiae*, *pomona*, *grippotyphosa*, *javanica*, *australis*, *autumnalis*, *hebdomadis* were obtained from the National Leptospira Laboratory, Portblair, Andaman and Nicobar Islands and are maintained at the Department of Microbiology, RAGACOVAS, Pondicherry.

Triton X-114 extraction: *L. interrogans* outer membrane protein was extracted as per the method described by Haake [6]. Briefly, leptospires were washed in phosphate-saline–5 mM $MgCl_2$ and then extracted in the presence of 1% protein-grade Triton X-114 –150 mM NaCl–10 mM Tris (pH 8)–1mM EDTA at 4°C. The insoluble material was removed by centrifugation at 17,000 g for 10 min. After centrifugation, 20 mM $CaCl_2$ was added to the supernatant. Phase separation was performed by heating the supernatant to 37°C followed by centrifugation at 1000 g for 10min. The detergent and aqueous phases were then separated and precipitated with acetone.

Serum samples: 300 sera samples were collected from dogs suspected to be suffering from Canine leptospirosis and those with no history of vaccination from Pondicherry for the detection of leptospiral antibodies by MAT and iELISA techniques.

Anti sera: Hyperimmune serum was raised in dogs against *leptospira interrogans* serovar *canicola* by repeated injections of formalin-killed antigen. Serum dilution ranging from 1:10 to 1:100 dilution of hyper immune sera raised against *Leptospira interrogans* serovar *canicola* was used.

Optimization and dilution of OMP antigen: An end point titration was carried out to determine the optimum single working dilution of *Leptospira interrogans* serovar *canicola* OMP antigen to be used to coat the ELISA plates. It was performed by keeping the serum dilution constant 1:100 against serial two fold dilutions of *Leptospira canicola* antigen. A serial two fold dilutions of *Leptospira interrogans* serovar *canicola* OMP antigen was prepared in carbonate - bicarbonate buffer (pH-9.6) to provide dilutions ranging from 3.66 µg/well to 25ng/well across the columns.

Indirect ELISA: Antigen in carbonate - bicarbonate buffer was coated on to each well by incubating at 4° C overnight. On the next day, the plates were washed five times with PBS with 0.05% Tween 20. 300 micro litre of 5% skim milk powder was added to block the uncoated sites and incubated at 37° C for one hour. The plates were washed as above and 100 microlitre of 1:50 diluted test serum samples was added to individual wells in duplicates followed by incubation at 37°C for one hour. Then the plates were washed and 100 microlitre of 1:1000 diluted rabbit anti dog HRPO conjugate was added to all the wells and again incubated at 37°C for one hour. Then the plates were final washed and 100 microlitre of freshly prepared chromogen - substrate solution containing OPD and urea H_2O_2 as substrate was added to each well and the plate was kept at room temperature (25°C) for 10 min at dark. The enzyme substrate reaction was stopped by adding 50 µl of 1M H_2SO_4. The optical density of each of the samples was recorded at 492 nm by ELISA reader (Biorad).

Microscopic agglutination test (MAT): To assess the antibody titres of canine sera samples against Leptospira, MAT [10] was performed in microtitre plates using 5 Leptospiral interrogans serovars viz., canicola, icterohaemorrhagiae, pomona, grippotyphosa and australis. A serum dilution of 1:50 was first used to screen the samples for leptospirosis. The positive samples were identified and titrated using serial two fold dilutions from 1:50 to 1:3200 in the following manner. 100 micro litre of PBS was added to wells 2 to 8 in 96 well flat bottomed microtitre plate. 100micro litre of pre-diluted serum (1:25) was added to wells 1 and 2 including positive controls corresponding to those serovars. Doubling dilution of sera was made from well 2 to 8. 100micro litre of antigen (5-7 day old live culture) was added to wells 1 to 8 so that the final dilution was 1:50 to 1:6400. The mixture was gently tapped to mix the contents and incubated at 37°C for 2 to 4 hours. A microscopic slide with a drop of the mixture was observed under dark field microscope for agglutination. The titre was the highest dilution at which 50% agglutination was observed (Figure-1).

Evaluation of MAT and iELISA: The relative sensitivity and specificity of the iELISA for serodiagnosis of canine leptospirosis in comparision to MAT was carried out as described by Thrusfield [11].

Statistical analysis: Relative sensitivity and specificity of iELISA for the detection of leptospiral antibodies in dog sera were determined in comparison with MAT, as described by Zweing and Robertson [12]. Comparative evaluations of both serological tests were carried out by chi-square test (SPSS 17.0 software).

Table-1. End point titration to find out the optimum conentration of antigen for iELISA

Hyperimmune serum	1:50 dilution							
Antigen concentration (µg/µl)	3.66	1.83	0.915	0.4575	0.228	0.114	0.0571	0.0285
OD values (492 nm)	0.7515	0.7942	0.8565	1.0417	1.0126	1.0062	0.9802	0.8574

Table-2. Distribution of PP values obtained from dog serum samples

Range of PP values	No. of samples	Results
0-29.01	141	Negative
29.02*-58.02 (*cut-off value)	135	Positive
58.03-87.03	21	Highly Positive
87.04-116.04	03	Highly Positive
Total	300	

Table-3. Relative sensitivity and specificity of MAT compared with iELISA for the detection of anti-leptospiral antibodies

iELISA	MAT		
	Positive	Negative	Total
Positive	94	65	159
Negative	05	136	141
Total	99	201	300

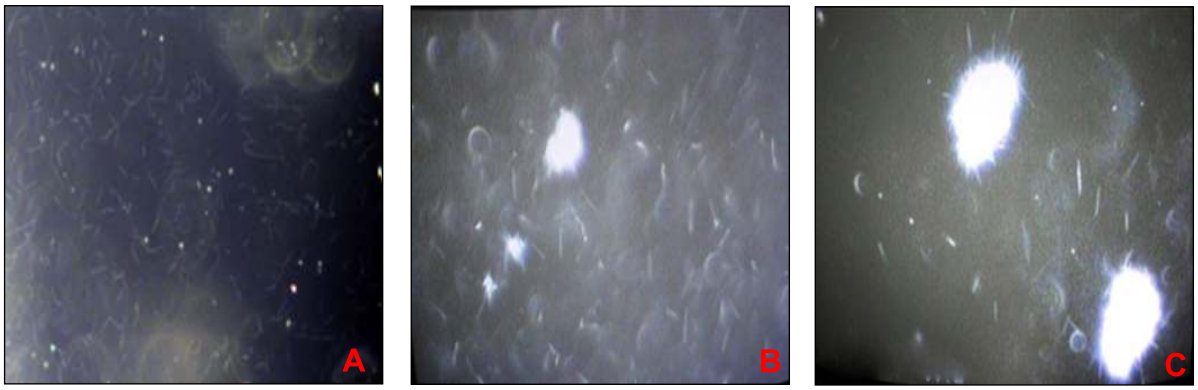

Figure-1. Photomicrograph of Microscopic Agglutination Test (MAT) using dark-field microscopy (a) Live leptospiral organism with no agglutination (Negative control), (b) 50% Agglutination, (c) 100% Agglutination.

Results and Discussion

Antigen extraction and Protein estimation: A seven day old *Leptospira interrogans* serovar *canicola* with a concentration of 4.0 x 10^7 organisms per ml was used to obtain OMPs by Triton X-114 extraction method. The concentration of OMP extracted was 3.66 µg/µl by Lowry's method. The advantages of using Triton X-114 extracted OMP antigen in iELISA is that the bulk amount of the extracted antigen can be obtained without handling the live Leptospira organisms. It is safer than MAT because it eliminates the risk of handling live leptospires. Another advantage with this antigen is that it can detect genus-specific antibodies which MAT does not.

Standardization of indirect ELISA (iELISA): iELISA was standardized by optimising antigen dilution at varying dilutions of hyperimmune sera ranging from 1:10 to 1:100 and healthy serum collected from dogs before immunisation that served as a negative control and rabbit anti-dog IgG-HRPO conjugate at a constant dilution of 1:1000. The OD values were obtained with respect to varying dilutions of OMP antigen against a constant dilution of hyperimmune serum in each column (Figure-2). It was observed that the antigen concentration of 0.4575 µg/well at 1:50 of hyperimmune sera and 1:1000 dillution of rabbit anti-dog HRPO was found to be satisfactory to screen the test serum samples (Table-1). At this concentration of antigen, the OD value of strong positive at 492 nm was 1.0417. So, the antigen concentration of 0.4575 µg/well was taken as optimal antigen concentration to coat the nunc maxisorb ELISA plates for screening of test sera samples.

Determination of the cut-off value: Ribotta [13] used at a much higher concentration of antigen, 2.2 µg per well and a serum dilution of 1:200. Surujballi [14] used comparatively lower concentration of antigen, 0.1 µg per well which gave an OD of one with strong positive serum, 0.6 with moderate and 0.05 with negative serum at a dilution of 1:200. But in the present study we used a very minimal concentration of antigen and this is likely due to the quality and purity of antigen extracted. A concentration of 0.4575 µg per well of antigen, average OD values of 1.2, 0.6 and 0.18 were obtained for strong positive, moderate and healthy serum, respectively. The conversion of raw OD values into percent positivity (PP) values offers advantages, as it obviates the variations arising out of OD values of test samples in comparison with control values of each plate on a day to day basis. Thus results interpreted in the present study based on PP values offer a means to distinctly separate a population of animals which are either positive or negative. The mean OD value of control negative serum samples at 1:50 dilution was 0.180 and that of control positive serum samples was 1.500 using 0.4575 µg/well of OMP antigen. Based on these OD values, twice the mean OD values of the negative control serum was considered as the cut-off OD value to screen the test serum samples. Therefore any serum sample showing the OD of 0.36 and above was considered as positive for leptospirosis.

Calculation of the percent positivity (PP) values: The

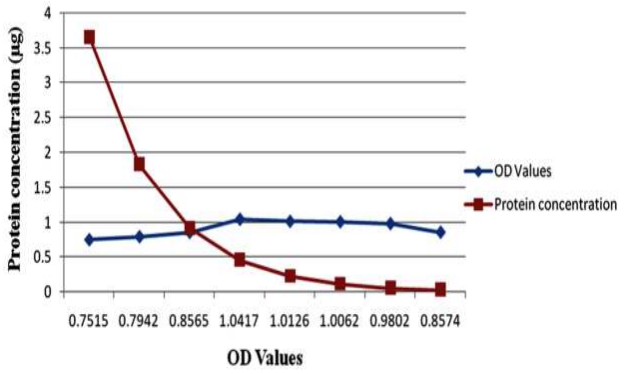

Figure-2. Determination of optimum concentration of OMP antigen for iELISA

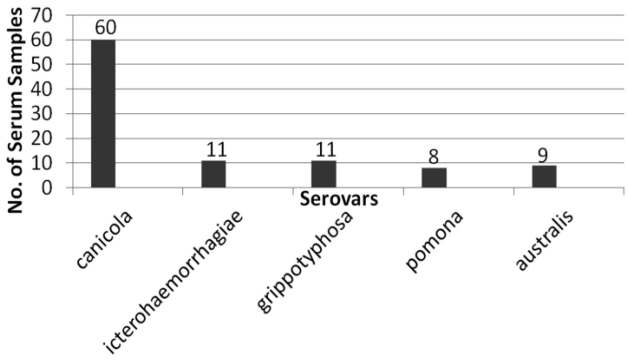

Figure-3. Comparative seroprevalence of leptospiral serovars in the dog population by Microscopic Agglutination Test (MAT).

PP values were calculated using the average OD values obtained from the negative control serum samples. Based on these OD values, the mean PP value of the negative control group was calculated as 14.51. Therefore, any serum sample showing a PP value of 29.02 (twice the mean negative PP value) or more was considered as positive for leptospirosis (Table 2). Out of these 300 sera samples, 159 (53 %) were found to be positive for leptospirosis by iELISA.

MAT: Out of the 300 dog serum samples tested against 5 serovars of leptospira, 99 (33 %) serum samples were found to be positive for at least one serovar tested. In that 60 (60.60 %) serum samples were found to be positive for serovar *canicola*, 11 (11.11 %) serum samples for serovar *icterohaemorrhagiae*, 11 (11.11 %) serum samples for serovar *grippotyphosa*, 8 (8.08 %) serum samples for serovar *Pomona* and 9 (9.09 %) sera samples were found to be positive for serovar *australis* (Figure-3). It was observed that 40 serum samples cross reacted with various serovars, A total of 11 sera samples cross reacted with *canicola* and *icterohaemorrhagiae*; 9 sera with *canicola* and *pomona*, 8 sera with *canicola* and *australis*; 5 sera with *grippotyphosa* and *canicola*, 2 with *australis* and *pomona*, and 5 sera with *icterohaemorrhagiae* and *pomona*. These cross reacted sera were verified in different dilutions to find out the serovar.

Among many serovars of *leptospira interrogans*, we have selected only five serovars for this study to test the antibodies in the dog sera. Most clinical cases of Leptospirosis in dogs are caused by serovars *canicola*, *icterohaemorrhagiae*, *grippotyphosa* and *pomona* [15]. In addition, other serovars namely *australis*, *javanica*, *ballum*, *hardjo*, *autumnalis* and *pyrogenes* were recorded by Senthilkumar [16]. Immunity to leptospirosis is largely humoral and is relatively serovar specific [7]. MAT is the most commonly used serological diagnostic method, although it also detects antibodies produced by vaccination [17]. According to our study the frequency of leptospiral infection with *canicola* serogroup is more common in canines of Pondicherry.

Sensitivity and specificity of iELISA and MAT: The sensitivity and specificity of the iELISA in comparison with MAT was 94.94 % and 67.66 % respectively (Table-3). The sensitivity and specificity of MAT in comparison to iELISA was 59.11 % and 96.45% respectively. Surujballi [14] showed a higher sensitivity and specificity 95.3 % and 100 %, respectively. The iELISA developed by Ribotta [13], showed a higher sensitivity (100%) and higher specificity (95.6 %) than our study. The relative sensitivity of iELISA obtained in our study was 94.94 % suggesting that it was as efficient as MAT in detecting true positive animals. However, the specificity was only 67.66 % signifying the inability of the iELISA to detect all the MAT negative animals as iELISA was negative too. This could be explained on the basis that all those sera turned out to be MAT negative may not actually be negative as only 5 leptospira serovars were used as antigens for antibody detection in our MAT technique. However, the findings supported the prevailing view that the iELISA is more sensitive than MAT [18].

Statistical analysis: Chi-square analysis of the data revealed that there is a significant difference between iELISA and MAT employed in this study, where the chi-square value is 104.4 with a Probability value of 0.0001 i.e., <0.01. So, it is highly significant at 1 % level.

Conclusion

The present study revealed that the iELISA was found to be highly sensitive, rapid and easy to perform in comparison with MAT for the detection of canine anti-leptospiral antibodies. The immunosorbent assay employed a non-hazardous, highly purified, reproducible antigenic preparation which can be prepared in large quantities and stored for long periods. Moreover, they do not require the maintenance of a constant supply of live leptospiral cultures of different serovars as in the case of MAT. Therefore iELISA can be recommended as a valuable test in routine diagnostic laboratories. The most prevalent serovar was found to be *Leptospira interrogans* serovar *canicola* by serovar specific MAT test. Many of the samples subjected in the present study were from animals either convalescing or suffering with subclinical leptospirosis. Data showed that 53% of these animals were at least one time or

other exposed to *Leptospira* resulting in development of antibodies detectable by iELISA.

Authors' contributions

RSK and RMP were involved in the design of this research work. The research was done by RSK. RMP has monitored all the activities being a supervisor. VMVS, SVP, HKM, PXA and JT have assisted this research work. VMVS and RSK drafted and revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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