Utility of recombinant LipL41 based IgM and IgG ELISA in diagnosis of canine leptospirosis in an endemic area – a study from Kerala, India

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Abstract. A study was undertaken to evaluate the relevance of detecting IgM and IgG antibodies in diagnosis of canine leptospirosis in Kerala, a southern state of India, which is endemic for the disease. A total of 205 blood (35 from healthy vaccinated, 30 from healthy unvaccinated and 140 from diseased dogs) and 151 urine samples (11 from healthy vaccinated and 140 from diseased dogs) were collected from three districts of Kerala, Thrissur, Palakkad and Kozhikode with high incidence of leptospirosis. Recombinant LipL41 protein was used as antigen and IgG and IgM based ELISAs were standardized. The results were compared with the gold standard test, microscopic agglutination test (MAT). The MAT positive samples (146 samples) were divided into those having titre >1:800 and those between 1:100 and 1:400 in view that the former constituted the acute cases. It was found that IgM ELISA was more specific and sensitive in detecting acute cases (MAT >1:800) whereas IgG ELISA was less specific. In case of seroprevalence studies (MAT titre 1:100 to 1: 400), IgG ELISA was found to be more sensitive and specific than IgM ELISA. Receiver operating characteristic curves when plotted, revealed the accuracy of IgM ELISA in acute leptospirosis. Many samples were positive for both IgG and IgM antibodies. Polymerase Chain Reaction (PCR) targeting *lipl41* gene was standardized and urine and blood samples from the same dogs were tested. PCR was found to be the specific test for the early detection of leptospires in blood even before seroconversion. However, PCR analysis of the urine samples was found to be insensitive. Hence, it can be concluded that the diagnostic strategies should be modified, and a combination of serological and molecular tests is recommended in endemic areas rather than simple detection of IgM or IgG antibodies, for the early detection of acute clinical cases of leptospirosis.

INTRODUCTION

Leptospirosis is a fatal zoonosis which is emerging as a global public health problem. According to World Health Organisation (WHO), the global incidence of leptospirosis increased from approximately 500,000 cases in 1999 (WHO, 1999) to over a million cases in 2015 (Costa *et al.*, 2015; Picardeau, 2015). Kerala, a southern state of India, is endemic for the disease (WHO, 2008). As per the reports from the Directorate of health services, Thiruvananthapuram (http://dhs. kerala.gov.in), the incidence of human leptospirosis in Kerala in 2018 was 1970. The ecology of Kerala is highly favorable for the disease to stay as an endemic due to its warm and humid climate where in the

rodent population act as reservoirs, wild and domestic animals as carriers and affecting humans as incidental hosts. The infection is transmitted to human beings from a variety of mammalian hosts, especially dogs. In dogs, the clinical manifestations are erratic and indistinguishable from other febrile illnesses which make the laboratory confirmation of the disease inevitable. However, the serodiagnosis of canine leptospirosis is an enigma because the undercurrent antibodies due to past infection or vaccination complicate the confirmation of the disease. Lack of sensitivity is the major problem associated with the conventional diagnostic methods like isolation and dark field microscopy. Polymerase chain reaction (PCR) is considered as a surrogate for the isolation (Bal *et al.*, 1994; Brown *et al.*, 1995), but cannot be employed as a routine diagnostic test due to the inevitability of an established laboratory in performing the test and documenting the results. Microscopic agglutination test (MAT) is the gold standard reference serological test (Faine *et al.*, 1999) which requires a battery of reference cultures in their active growth phase as antigens thus limiting its utility as a regular diagnostic test. This led to the development of alternative serological tests and the widely accepted test is Enzyme-Linked Immunosorbent Assay (ELISA) and its simplified versions like dot ELISA (Sriram *et al.*, 2017). These tests detect Immunoglobulin M (IgM) and Immunoglobulin G (IgG) which are considered as the indicators of acute and convalescent stages of infection respectively. However, its diagnostic significance in dogs in an endemic area where regular vaccination is practiced has to be evaluated. Moreover, the sensitivity and specificity of ELISA are affected by the antigens used (Chalayon *et al.*, 2011). The whole cell preparations with widely reactive immunodominant epitopes are replaced by outer membrane protein (OMP) preparations and recombinant OMPs (Zhylkibayev *et al.*, 2018; Loong *et al.*, 2018). The present study involves the evaluation of indirect ELISA for detecting IgM and IgG using recombinant LipL41 (rLipL41) as antigen, which is conserved among pathogenic leptospires (Mariya *et al.*, 2006). The leptospires can be seen in the blood even before seroconversion. The shedding of the organisms in urine can be detected, several days post infection. This can be seen along with high antibody titre in MAT. Since isolation is highly insensitive, PCR can be employed for the detection of organisms in blood and urine (Harkin *et al.*, 2003). Hence, the present study also includes the detection of leptospires in blood and urine using *lipl41* gene specific PCR.

MATERIALS AND METHODS

Samples
Blood samples were collected from 205 dogs from three different regions in the state of Kerala *viz.*, Thrissur, Palakkad and Kozhikode, with high incidence of human leptospirosis. Blood samples were collected and transferred into a heparinized vial and another one without anticoagulant for serum separation. This include 35 from healthy vaccinated, 30 from healthy unvaccinated and 140 from suspected dogs. Among the 140 samples dogs suspected for dogs, paired serum samples were collected from 12. Urine samples were collected from 151 dogs (11 from healthy vaccinated and 140 from diseased dogs). The details of samples are given in Table 1.

Microscopic agglutination test
The MAT was carried out as described by Faine *et al.* (1999) using the reference strains of *Leptospira* as antigens, *viz.*, *Leptospira interrogans* serovars Australis, Autumnalis, Canicola, Grippotyphosa, Icterohemorrhagiae, Javanica, Pomona, Pyrogenes and Hebdomadis representing nine different serogroups procured from National Leptospira Reference Centre, Regional Medical Research Centre, Port Blair, Andaman and Nicobar Islands as well as Madras Veterinary College, Chennai which were maintained in the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy. The antibody titre was the highest dilution

Table 1. Samples collected

Location	Animal details	Samples		
		Serum	Plasma	Urine
Thrissur	Vaccinated	16	16	5
	unvaccinated	14	14	
	diseased	91	91	91
		121	121	96
Palakkad	Vaccinated	9	16	4
	unvaccinated	7	14	
	diseased	25	25	25
		41	41	29
Kozhikode	Vaccinated	10	16	2
	unvaccinated	9	14	
	diseased	24	24	24
		43	43	26
TOTAL		205	205	151

of test serum showing agglutination of 50% or more leptospiral organisms. Reciprocal agglutination titres of greater than or equal to 100 were considered as positive reactions, according to Office Internationale des Epizootis (OIE) recommendations. The titres $\geq$1:800 were taken as acute cases as per previous reports (Ooteman *et al.*, 2006).

Polymerase Chain Reaction
The DNA was extracted from all the blood and urine samples as per Veloso *et al.* (2000) using proteinase K method followed by phenol chloroform extraction. Urine samples could be collected from 151 animals only. Primers specific for *lipl41* gene was used in PCR as per Senthilkumar *et al.* (2007).

Production of recombinant LipL41 antigen
The DNA was extracted from *Leptospira interrogans* serovar Canicola by proteinase K method followed by phenol chloroform as described by Veloso *et al.* (2000). The gene encoding LipL41 was amplified from the genomic DNA as per the method described by Senthilkumar *et al.* (2007). The amplified *lipl41* gene was excised using the restriction enzymes *Nco1* and *Xho1* and cloned into the pPROEXHT$_b$ expression vector (Life Technologies) digested with the

same enzymes. The ligated mixture was transformed into *Escherichia coli* DH5α cells. The positive clones were selected and the recombinant protein production was induced using 1 mM IPTG (Isopropyl-α-D-thiogalactopyranoside) for 8 h. The polyhistidine (6X-His) tagged fusion protein LipL41 was purified under denaturing conditions by Nickel chelating affinity chromatography (GeNei).

Indirect enzyme-linked immunosorbent assay
Optimum concentrations of the recombinant LipL41, IgG and IgM Horse Radish Peroxidase (HRPO) conjugates (Immunology Consultancy Limited, USA) and serum were determined by checkerboard analysis and the test was performed in 96 well microtitre plates. In order to check the specificity of rLipL41 in ELISA, hyper immune serum was raised against rLipL41 in New Zealand white rabbits and used as primary antibody for the detection of bacterial antigens *viz.*, *E. coli*, *Staphylococcus aureus* and *Salmonella* coated in ELISA plates.

Comparison of MAT and ELISA
The relative sensitivity, specificity and accuracy of both the IgM and IgG ELISAs were evaluated in comparison to the MAT

as described in Veterinary Epidemiology by Thrusfield (2005). In order to evaluate the efficacy of the tests in detecting acute cases, sensitivity, specificity and accuracy were calculated separately for the samples having MAT titre $\geq$1:800. Receiver operating characteristic (ROC) curves were plotted separately and analyzed.

RESULTS

Microscopic agglutination test
Among the 205 canine serum samples, 146 (71.12%) were found to be positive by MAT. This included 12 out of 35 healthy vaccinated animals (34.28%) and eight (26.67%) out of 30 healthy unvaccinated animals with titre of $\geq$1:100. Among the 205 dogs in the study, paired serum samples were collected from 12 dogs suspected of leptospirosis and four were negative while eight had shown titre of 1:100 by analysis of their first serum samples. After analysis of the second serum sample of these 12 dogs, eight of them were having titre above 1:400 and four having 1:200.

Polymerase Chain Reaction
In the present study, among the 205 blood samples tested, 56 (27.32%) were found to be positive. An amplicon of 1077 bp was obtained in all the positive samples. Among these, 53 (36.30%) were MAT positive and three (5.08%) were MAT negative. In addition, PCR analysis of the paired samples of 12 dogs showed that four samples exhibited positive results even before confirmation of the disease by MAT, when the second serum sample could be analyzed. The healthy animals, both vaccinated and unvaccinated, were found

to be negative. Among the 151 urine samples collected, 21 (13.09%) were found to be positive by PCR and all these were from clinically ill dogs.

Indirect enzyme-linked immunosorbent assay
Indirect ELISA was standardized by checker board method to detect anti-leptospiral antibodies using rLipL41 protein as the antigen. The rabbit anti-canine IgG HRPO conjugate in the dilution of 1:10,000 was found to be optimum. The optimum antigen concentration for rLipL41 was calculated as 100 ng / well. A 1:100 dilution of the test serum was optimum. In IgM ELISA, 50ng/ well rLipL41 was optimum and the rabbit anti-canine IgM HRPO conjugate in the dilution of 1:10,000 was found to be optimum. The ELISA test conducted using other bacterial antigens and anti rLipL41 serum was found to be negative.

The results are presented in Table 2. The MAT positive samples were divided into those with titre $\geq$1:800 and those with titre between 1:100 and 1:400 to differentiate acute and past infections respectively. The sensitivity, specificity, positive predictive value and negative predictive value and accuracy of IgG and IgM ELISA in com-parison with the gold standard test MAT were calculated (Table 3). The ROC curves revealed the high accuracy of IgM ELISA having area under the curve (AUC) 0.811 (Fig. 1), compared with IgG having AUC 0.675 (Fig. 2) in acute cases whereas IgM ELISA showed a lesser accuracy with AUC 0.745 (Fig. 3) compared with IgG ELISA (AUC 0.969) (Fig. 4), when analyzed using serum samples with MAT titres between 1:100 to 1:400.

Table 2. Results of MAT, IgM ELISA, IgG ELISA and PCR

MAT + (n= 146)		Only IgM +	Only IgG +	Both IgM and IgG +	Blood PCR	Urine PCR
$\geq$1:800	61	2	11	48	51	3
1:100 – 1:400	85	0	58	27	2	18
MAT -ve	59	1	2	1	3	0

Table 3. Efficacy of the ELISAs and PCR in comparison with MAT

	MAT titre ≥1:800 positive			MAT titre 1:100–1:400 positive		
	IgG ELISA	IgM ELISA	Blood PCR	IgG ELISA	IgM ELISA	Blood PCR
Sensitivity (%)	98.63	81.97	82.76	98.63	52.74	2.35
Specificity (%)	38.35	81.29	97.96	95.08	96.72	94.92
Accuracy (%)	67.5	81.1	93.66	96.9	74.7	40.28
Positive predictive value (%)	40.13	63.29	94.12	97.96	97.47	40.00
Negative predictive value (%)	96.67	91.97	93.51	96.67	46.09	40.29
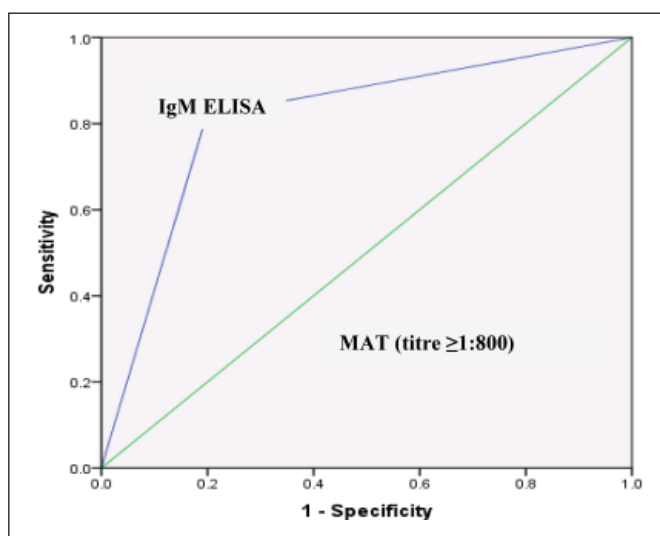
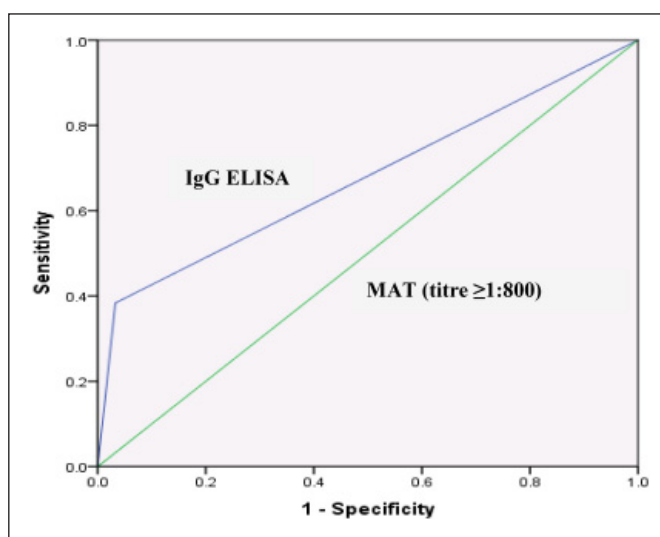Figure 1. MAT (≥1:800) Vs IgM ELISA
Area under the curve 0.811

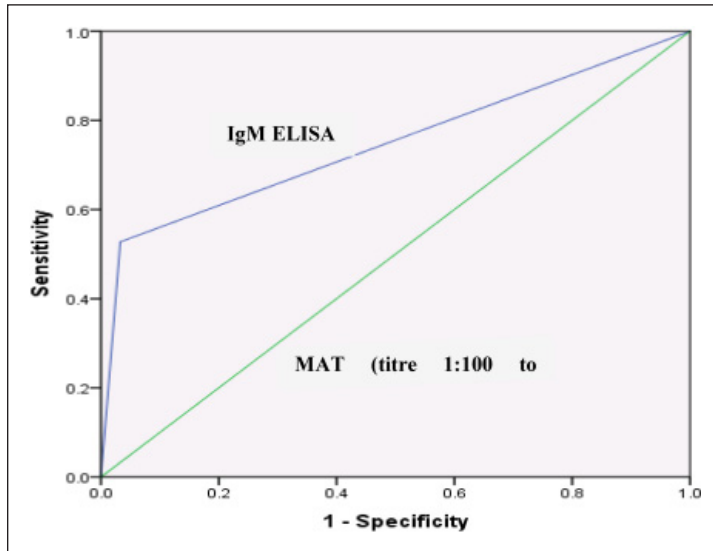Figure 2. MAT (≥1:800) Vs IgG ELISA
Area under the curve 0.675

Figure 3. MAT (titre 1:100 to 1:400) Vs IgM ELISA
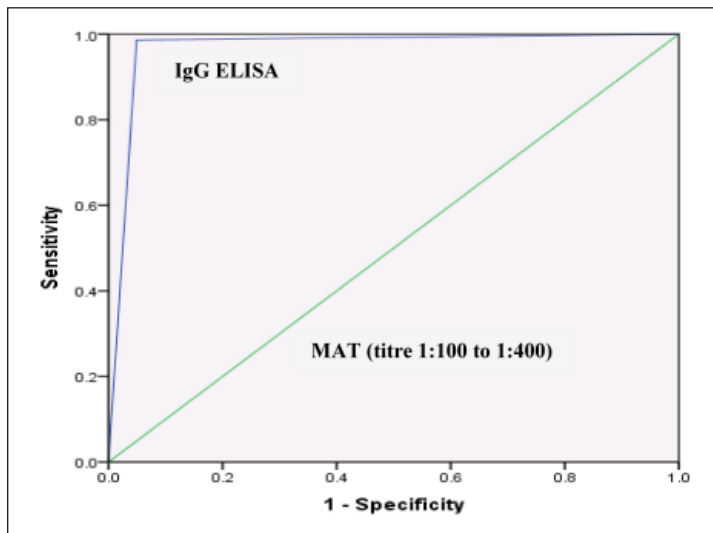Area under the curve 0.747

Figure 4. MAT (titre 1:100 to 1:400) Vs IgG ELISA
Area under the curve 0.962

DISCUSSION

The present study evaluates the relevance of detecting IgG and IgM antibodies in diagnosis of leptospirosis in an endemic area. The tests employed were IgG ELISA, IgM ELISA, MAT and PCR.

The recombinant LipL41 was used as the antigen in ELISAs and the gene *lipl41*

specific primers were used in PCR of both urine and blood samples, as the gene was reported to be conserved among pathogenic leptospires only (Natarajaseenivasan *et al.*, 2004).

Leptospiraemia is detectable within five days after infection and remains for 10 days (WHO, 2008). This indicates that PCR can detect circulating leptospires before the

antibodies appear in the blood which is in accordance with the findings of Ooteman *et al.* (2006). A positive PCR result on whole blood in the presence of a negative urine result can occur during the first two weeks of infection. The presence of amplification inhibitors in clinical samples like urine can cause false negative results, particularly in animal specimens (OIE, 2008). In case of asymptomatic chronic carriers, there will be shedding of *Leptospira* in the urine intermittently for weeks to months (McDonough, 2001). Thus a negative whole blood result in the presence of a positive urine PCR result in a dog with clinical signs of infection suggests that the dog was likely infected at least two weeks prior to sample collection (Levett, 2001). In the present study, none of the MAT negative samples were positive for urine PCR. Due to the collection of single urine sample from each animal, the PCR results of urine might have underestimated the infection status in the present study which is in accordance with Cetinkaya *et al.* (2000).

Positive whole blood and urine PCR results were observed in six samples. It can occur within the first week of infection during an overlapping blood and tissue phase of infection or the animal may be a convalescent carrier currently infected with another serovar. Positive urine samples are considered a source of infection for other animals and humans. A dog with negative whole blood and urine PCR results is likely not infected if the samples were collected prior to antibiotic therapy. However, whole blood PCR results are only positive early in infection and urinary shedding can be intermittent. Therefore, if leptospirosis is still suspected, MAT testing is the suitable method to be adopted, thus limiting the utility of PCR as a single diagnostic test in an endemic area. The gold standard test, MAT was proved to be having restrictions like maintenance of reference strains, hazards to personnels and necessity of collection of paired serum samples. Thus the antibody detection was mostly based on ELISA which was made more specific by the use of recombinant outer membrane proteins.

The present study reveals that IgG ELISA is sensitive in seroprevalence studies (MAT titre 1:100 to 1:400) as well as for detecting acute cases (MAT titre $\geq$1:800). The test could not achieve 100% sensitivity since two MAT positive samples were found to be positive to IgM antibodies and negative to IgG, indicating current infection without any previous exposure which can happen, although rare in an endemic area. IgM usually appear in blood within three to ten days after the onset of symptoms but delayed appearance for as long as three to four weeks after initial infection has been reported (Faine *et al.*, 1999). Two healthy unvaccinated animals which were MAT negative gave a positive reaction in IgG ELISA. This may be due to a low titre of antibodies in the animals which could not be detected by MAT, whereas ELISA being more sensitive, detected them. Several reports state that ELISA is more sensitive than MAT (Ribotta *et al.*, 2000; Agunloye *et al.*, 2001) whereas, in some studies, sensitivity of ELISA is reported from 77.8% to 100%, which is mostly related to the time of blood sampling (Levett and Branch, 2002; Vitale *et al.*, 2004).

The specificity of IgG ELISA was found to be unsatisfactory in acute cases (MAT titre $\geq$1:800), whereas IgM ELISA was found to be specific. However, when a MAT titre of $\geq$1:100 was taken as positive, the sensitivity of IgM ELISA was not satisfactory. This can be explained by the fact that agglutinating antibodies detected in MAT can be both IgG and IgM (Adler *et al.*, 1980). Chronic carriers and vaccinated animals in endemic areas harbour certain level of IgG anti-bodies, which were detected in MAT and IgG ELISA, which is of least diagnostic value in endemic areas. These were not detected in IgM ELISA. In acute cases (MAT titre of $\geq$1:800), IgM antibodies appeared and thus IgM ELISA gave a satisfactory sensitivity of 81.97%. The studies on dynamics of IgM and IgG antibodies in leptospiral infection in human beings (Chernukha *et al.*, 1976) and dogs (Hartman *et al.*, 1984) had been conducted earlier. There are reports that IgM antibodies are better indicators of acute cases of leptospirosis (Rosa *et al.*, 2017) and

IgM ELISA can be employed as a better diagnostic tool with higher specificity compared with IgG ELISA. The results of the present study are in agreement with the above statement. The analysis of the ROC curves revealed the higher accuracy of IgM in detecting acute cases. However, the higher sensitivity and accuracy of IgG ELISA in seroprevalence studies cannot be overlooked. In an endemic area, there are chances of overlapping of IgG and IgM antibodies and the latter may last for several months in the serum of the infected animal, challenging its diagnostic efficacy. Moreover, it was revealed from the study that molecular tests like PCR could detect leptospiral DNA in blood even before seroconversion.

CONCLUSION

The present study reveals that both IgG and IgM ELISAs are sensitive in detecting acute cases of canine leptospirosis. However, IgM ELISA was found to be more specific compared with the former. The blood PCR was observed to be sensitive as specific early diagnostic tool even before sero-conversion. On the other hand, urine PCR was found to be highly insensitive, but specific in diagnosing leptospirosis. Hence, the diagnostic strategies should be modified, and a combination of serological and molecular tests is recommended in endemic areas rather than simple detection of IgM or IgG antibodies, for the early diagnosis of clinical cases of leptospirosis.

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