Evaluation of real-time PCR, MAT, and recombinant LipL32-based ELISA for the diagnosis of canine leptospirosis in a disease-endemic South Indian state, Kerala

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Abstract: Canine samples (n = 425) collected from cases of suspected leptospirosis over a period of 6 years were tested using the microscopic agglutination test (MAT), recombinant LipL32-based IgM and IgG ELISA, and real-time PCR (RT PCR). A prevalence of 69.41% was recorded by MAT. Among the different tests, IgG ELISA was observed to have maximum agreement with that of MAT (k-0.810), followed by IgM ELISA (k-0.762.). RT PCR had only slight agreement with MAT (k-0.156). However, the percentage positivity of RT PCR with samples with MAT titers of 1:100 and 1:200 were 76.47% and 85.96%, respectively, indicating the reliability of RT PCR in the early phase of the disease. The values for the samples at higher MAT titers of 1:400 and 1:800 were 10.58% and 1.2%, respectively, clearly indicating the unreliability of the test for samples having high antibody titers. It was concluded that IgG ELISA could be used as the routine test for the diagnosis of canine leptospirosis in this disease-endemic region. Samples with negative results that have high symptomatic correlation with leptospirosis may be tested by PCR/RT PCR for the detection of the pathogen well ahead of antibody generation.

Key words: Microscopic agglutination test, ELISA, real-time PCR, leptospirosis, disease-endemic region

1. Introduction

Leptospirosis is reported to be the most widespread zoonosis globally (1) and is highly endemic in the South Indian state of Kerala, where this study was carried out. Diagnosis of the disease at its earliest phase can save the life of animals and can also prevent the spread of infection to contact with other animals and humans. Hence, the use of a reliable diagnostic test that can establish the disease unambiguously is of paramount importance in acute disease case management. Conventional diagnosis includes dark field microscopy (DFM), isolation of the organism, and the microscopic agglutination test (MAT). Isolation of the organism, although confirmatory, is often cumbersome and time-consuming and thus would not help in acute disease diagnosis. DFM, being a less sensitive and specific test, cannot be relied upon for confirmatory results. MAT, though considered as the gold-standard serological test, often requires paired sera examination to confirm seroconversion and hence is time-consuming

and demands technical expertise in the interpretation of the results, limiting its use as an acute disease diagnostic (2). To circumvent these disadvantages, newer diagnostics like PCR (3), real-time (RT) PCR (4–6), and recombinant protein-based ELISA were tested (7,8). However, the diagnostic suitability of these tests depends on the stage of infection and the kinetics of IgM and IgG antibodies in the infected animals as leptospires are detected 4–7 days after the infection and they are cleared following the production of antibodies, and also on the type of antibodies generated predominantly (9). Therefore, to know the suitability of the various currently available diagnostic tests for establishing the disease beyond doubt, they need to be tested simultaneously in clinical samples collected for a reasonably long time, so as to obtain samples at various stages of infection. Furthermore, the choice of different diagnostics may differ considerably when they are used in a disease-endemic locality when compared to a disease-free region. This could be attributed to the

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presence of undercurrent antibodies due to past exposure or vaccination, which could probably interfere with the building up of a detectable level of leptospires in the blood and may yield false positives in serological detection (10). There are no reports of simultaneous testing of various diagnostic tests for the diagnosis of leptospirosis in animals from a disease-endemic region, though a few reports are available on humans (11). The present study was conducted for a period of 6 years (2009–2015) in Kerala State, South India, a well-known disease-endemic region, with 425 canine clinical samples. The samples were tested using MAT and recombinant LipL32-based IgM and IgG ELISA for serological diagnosis and RT PCR for the detection of leptospiral DNA.

2. Materials and methods

2.1. Microscopic agglutination test
A total of 425 serum samples collected from canines were subjected to MAT. Serum samples were collected from canines irrespective of their vaccination status; however, the vaccination history was recorded for each animal. All the collected sera were subjected to MAT on the same day or the next and the rest of the serum sample was stored at –20 °C until used in another test or for a repeat testing in MAT. The test was performed following the protocol described earlier (12). A total of 10 serovars of *L. interrogans* obtained from the Regional Medical Research Centre, Port Blair, India, were employed: Australis (strain Ballico), Autumnalis (strain Rachmati), Bataviae (strain Swart), Canicola (strain Hond Utrecht IV), Grippotyphosa (strain Moskova V), Icterohaemorrhagiae (strain RGA), Javanica (strain Poi), Pomona (strain Pomona), Hardjo (strain Hardjoprajitno), and Pyrogenes (strain Salinem). Serum samples were tested at 1:100, 1:200, 1:400, and 1:800 dilutions and those serum samples that could bring about 50% clumping of the leptospires were regarded as positive and the highest dilution at which the above observation was recorded was taken as the titer value of the sample. Samples with titers below 1:100 by MAT were considered negative for leptospirosis.

2.2. Recombinant LipL32-based IgM and IgG ELISA
All the serum samples were subjected to recombinant LipL32 protein-based IgM and IgG ELISA. LipL32 was selected as the antigen of choice because it was reported that the protein was consistently expressed in all the pathogenic leptospires and it is the most immunodominant antigen recognized by the antibodies during natural infection. The LipL32 protein was expressed in *Escherichia coli* DH5α cells, purified by nickel column affinity chromatography and incorporated at 100 ng per well of the ELISA plate. The protocol of the ELISA was standardized with the following steps. The ELISA plates were coated with 100 ng of purified rLipL32 protein per well in 100 µL of coating

buffer (carbonate bicarbonate buffer at pH 9.6) and kept overnight at 4 °C, followed by washing with PBS-Tween 80 three times. Subsequently the wells were filled with blocking buffer (5% skim milk in PBS) and incubated at 37 °C for 1 h, followed by washings as above. In the next step, canine serum was diluted to 1:100 in blocking buffer and 100 µL of this was placed in the wells, incubated at 37 °C for 1 h, and washed as above. This was followed by adding 100 µL of anticanine IgM conjugate and IgG HRPO conjugated antibodies at 1:2500 dilution (Immunology Consultants Laboratory, USA) for IgM and IgG ELISA, respectively, incubated at 37 °C for 1 h and washed as mentioned above. After that, 100 µL of substrate solution was added (DAB, H_2O_2) and incubated at room temperature for 10 min and 100 µL of stopping solution (1 M H_2SO_4) was added and the OD was measured in an ELISA reader (Bio-Rad, USA) at 492 nm.

2.3. RT PCR
In order to design primers for the RT PCR assay, sequences of the *lipl32* gene of different leptospiral serovars were retrieved from GenBank (https://www.ncbi.nlm.nih.gov) and aligned using Clustal O Mega (http://www.ebi.ac.uk/Tools/msa/clustalo) and the DNASTAR Lasergene MegAlign program (DNASTAR, USA) to obtain a consensus sequence of 167 bp. From this consensus sequence, primers were designed (forward: 5'-CCG GAC GGT TTA GTC GAT GG-3' and reverse: 5'-ACG AAC TCC CAT TTC AGC GA-3') using the online Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3).

The genomic DNA was extracted from *Leptospira interrogans* serovar Autumnalis, one of the most predominant infecting serovars in Kerala. The extracted DNA was diluted in 10-fold dilutions in TE buffer (10 mM Tris and 1 Mm EDTA, pH 8.0, Sigma Aldrich, USA) so as to contain up to one genomic equivalent (GE) per 5 µL in the last dilution. All these dilutions were tested in RT PCR assay in triplicates to find out the limit of detection and efficiency of the newly designed primers.

RT PCR was performed by using 12.5 µL of Maxima SYBR Green qPCR master mix (2X) with ROX (Thermo Scientific, USA), 10 pM of forward and reverse primers, and 5 µL of the extracted DNA and the reaction volume was made up to 25 µL with molecular biology grade water. The reaction was carried out in an Applied Biosystems StepOnePlus thermal cycler using 8-well (0.2 mL) strips, sealed by adhesive seals. Each sample was taken in duplicate and a nontemplate control was set in every run to monitor any false positive results. The RT PCR assay for the *lipl32* gene was standardized with an initial denaturation of 94 °C for 4 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s, and signal captures at 95 °C for 15 s and 60 °C for 15 s. Specificity of the primers

was tested by melt curve analysis and also by testing the DNA from the nonpathogenic *Leptospira biflexa* serovar Patoc and other bacteria as templates, including *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Proteus mirabilis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Pasteurella multocida*.

2.4. DNA extraction from clinical samples

From all the animals under the study, approximately 2 mL of the blood was collected in EDTA vials and centrifuged at 2000 rpm for 2 min to settle the suspended cells. The supernatant, i.e. the plasma, was separated and taken in another sterile microcentrifuge tube and centrifuged at 12,000 rpm for 10 min at 4 °C. Then 200 µL of the plasma at the bottom along with the small pellet was retained in the tube and the rest of the supernatant was discarded. The contents in the centrifuge were mixed well by gentle vortexing and DNA was extracted using the QIAamp DNA Extraction Kit (QIAGEN, USA). Five microliters of this DNA was used as a template in RT PCR.

2.5. Statistical analysis

The percentage of agreement between different diagnostic tests and the kappa coefficient was calculated using statistical software (SPSS 21). The chi-square value, sensitivity, and specificity were calculated using the online JavaStat 2 × 2 contingency table (http://statpages.info/ctab2x2.html).

3. Results

3.1. Microscopic agglutination test

Among the 425 serum samples screened by MAT, 295 (69.41%) were found to have antibodies specific to the various serovars tested. The percentage of seroprevalence for various serovars were as follows: Australis, 69 samples (23.39%); Autumnalis, 55 samples (18.64%); Canicola, 33 samples (11.19%); Pomona, 31 samples (10.51%); Grippotyphosa, 24 samples (8.13%); Icterohaemorrhagiae, samples 30 (10.17%); Hebdomadis, 21 samples (7.12%); Pyrogenes, 16 samples (5.42%); Bataviae, 9 samples (3.05%); Javanica, 5 samples (1.69%), and Hardjo, 2 samples (0.68%).

3.2. Recombinant LipL32-based IgM and IgG ELISA

The recombinant protein was expressed in *E. coli* and purified successfully (Figure 1). The recombinant LipL32 protein was proved sensitive and specific, as it reacted with *Leptospira*-specific hyperimmune sera and failed to react with hyperimmune sera raised against various other bacterial agents. The mean cut-off OD values for recombinant LipL32 protein-based IgM and IgG ELISA were 1.82 ± 0.06 and 2.31 ± 0.15, respectively. The average OD values for IgM ELISA for the serum samples at MAT titers of 1:100, 1:200, 1:400, and 1:800 were 2.11 ± 0.13, 2.44 ± 0.11, 3.13 ± 0.14, and 3.92 ± 0.18, respectively. The

average OD values for IgG were 3.23 ± 0.19, 3.61 ± 0.21, 4.20 ± 0.31, and 5.14 ± 0.26, respectively.

3.3. RT PCR

The standard curve for the RT PCR assay standardized for the present study could be observed with a slope of 3.32, Y intercept of 32.59, and efficiency of 99.3% (Figure 2) and the amplification plot for generating the standard curve is depicted in Figure 3. The assay was found to have high specificity, as it failed to amplify any of the other bacterial agents tested, as evident from the melt curve analysis depicting a single peak, specific for the amplification of the *lipl32* gene from pathogenic *Leptospira* spp. (Figure 4). The diagnostic sensitivity of the RT PCR assay was found to be one genomic equivalent. A total of 116 samples (27.29%) were observed positive in RT PCR, with the highest percentage positivity at 1:200 MAT titer (85.96%), followed by 1:100 (76.47%), 1:400 (10.58%), and 1:800 (1.2%). It was also observed that among the MAT-negative samples (n = 130), 12.31% of the samples were found positive in RT PCR. Among these samples, 2 samples were both IgG and IgM ELISA-negative and 10 samples were negative in IgM ELISA alone.

3.4. Comparison of different tests for testing the samples and statistical analysis

The number of samples that yielded positive and negative results are depicted in Table 1. The results were compared and statistically analyzed as depicted in Table 2.

4. Discussion

Early and accurate diagnosis of leptospirosis has paramount importance in acute disease case management. The most commonly employed diagnostic methods include serological tests like MAT and ELISA and molecular tests like PCR or RT PCR. When employing serological tests for testing the disease in a disease-endemic region, it has to be kept in mind that preexisting antibodies in the animals by virtue of prior infection or vaccination must be excluded from giving false positive results. This could be ensured by observing the fourfold titer in the paired sera sample, which may not be of much diagnostic value in acute cases. An alternative could be observing a high single titer of 1:400 or above (13). However, all cases of leptospirosis may not necessarily have this high titer value. It is already known that the antibodies responsible for MAT results may be formed in 5–7 days of time and samples collected prior to 5 days may not have any detectable antibodies. Furthermore, there are reports of long-term persistence of IgM and IgG antibodies in infected humans, which may give false positive serological reactivity. Hence, these factors must be considered while devising a diagnostic strategy for a disease-endemic region. The present study was carried out for a period of 6 years using sera collected from dogs in a disease-endemic South Indian state, Kerala,
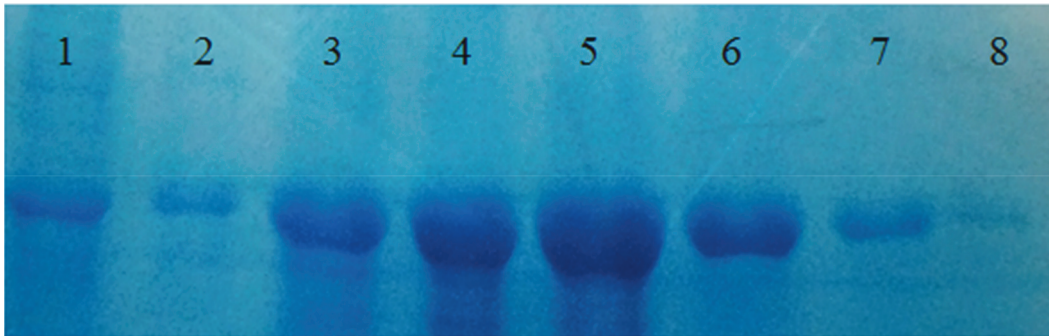
Figure 1. Purification of recombinant LipL32 protein of *Leptospira*. Lane 1- induced, Lane 2- uninduced, Lanes 3–8- purified protein at 2, 3, 4, 5, 6, and 7 h of IPTG induction.
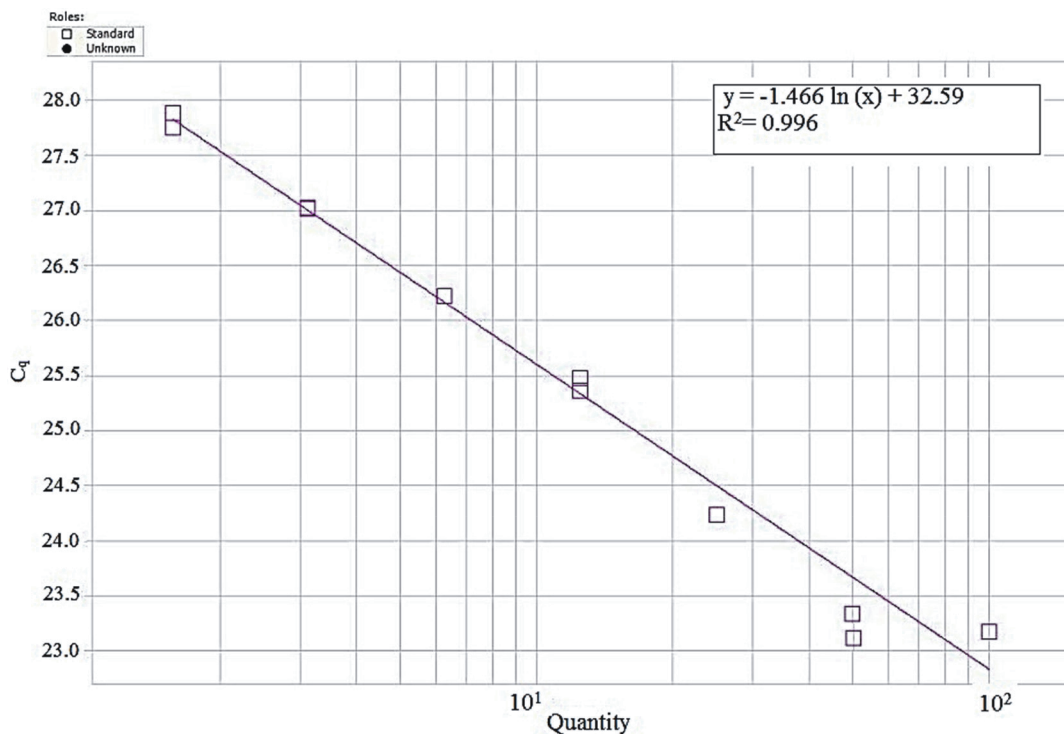

$$y = -1.466 \ln(x) + 32.59$$
$$R^2 = 0.996$$

Figure 2. Plotting the standard curve for efficiency calculation of the primers used in RT PCR assay.

where regular vaccination is also practiced for dogs owned as pets. The samples were taken from dogs that presented with probable symptoms of leptospirosis.

The serum samples collected were subjected to MAT in 1:100 to 1:800 dilutions and rLipL32-based IgM and IgG ELISA. The blood samples collected from the animals were processed to extract the DNA and were subjected to RT PCR for the direct detection of the pathogen. MAT was performed with a battery of cultures including the most frequently circulating serovars in South India. ELISA tests were carried out using the recombinant LipL32 antigen expressed after ensuring its sensitivity and specificity in detecting leptospiral antibodies. The LipL32 antigen was

reported as one of the most promising genus-specific diagnostics for canine leptospirosis in earlier works and hence was incorporated as the antigen in IgM and IgG ELISA in the present study. The primers for the RT PCR were designed and the protocol was standardized to have a detection sensitivity of one genomic equivalent per reaction. The serum samples (n = 425) were tested by MAT to observe a seroprevalence of 69.41%. Furthermore, MAT-positive serum samples were grouped in different MAT titer groups of 51 (1:100), 57 (1:200), 104 (1:400), and 83 (1:800). Later, IgM and IgG ELISA and RT PCR were performed for the collected samples. When the reliability of the different tests was analyzed against
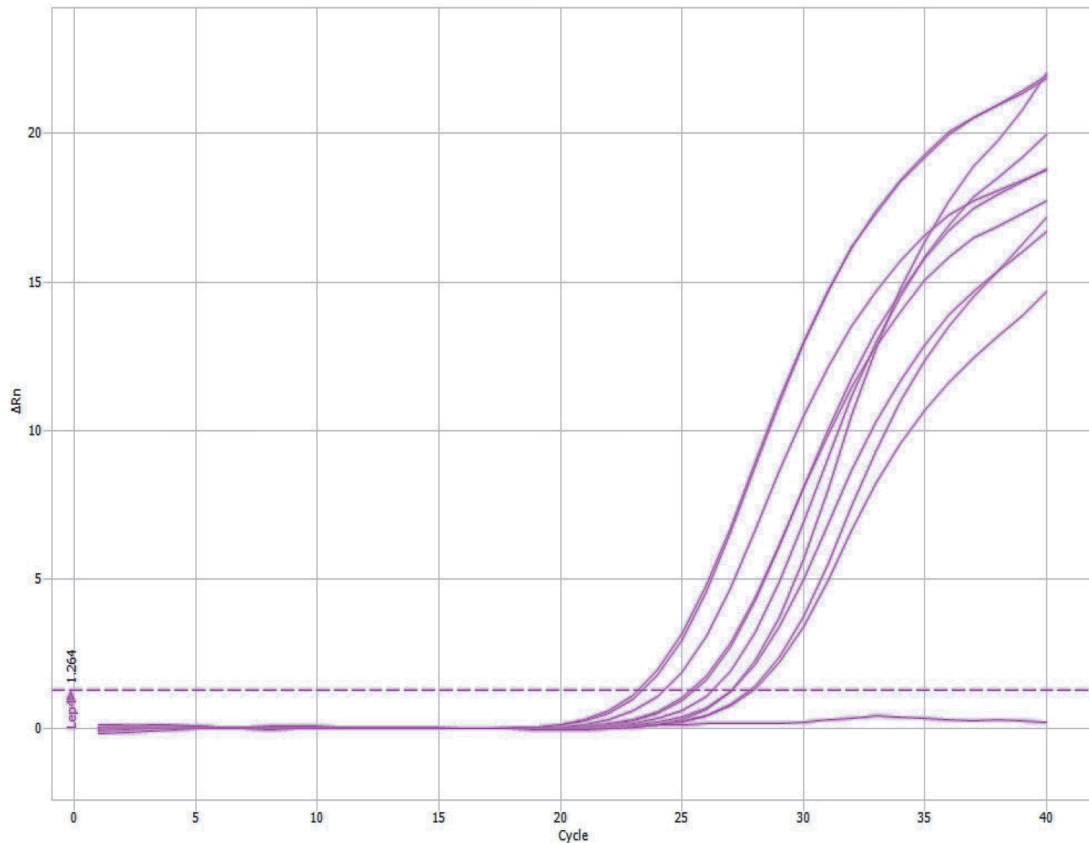
Figure 3. Amplification plot depicting the Ct values for the tenfold dilutions of the leptospiral DNA in RT PCR.

MAT, irrespective of the titer values, it was observed that IgG ELISA showed almost perfect agreement (kappa value: 0.810 and percentage agreement: 92.47) and IgM ELISA had substantial agreement (kappa value: 0.762 and percentage agreement: 89.88) with MAT. This is in agreement with a previous report, which recorded that during human leptospirosis in a disease-endemic region, IgG antibodies were generated markedly. However, with the observed substantial kappa value of IgM ELISA versus MAT, our study disagrees with that report as there is little or no detectable IgM response elicited towards high-molecular-weight antigens like LipL32 during either acute or convalescent stages. This might be attributed to a different antibody kinetics in dogs dwelling in a disease-endemic region.

When RT PCR was tested for its efficiency, the kappa value and percentage agreement between RT PCR and MAT for the whole set of the analyzed serum samples were 0.156 and 50.33, respectively, indicating its unreliability for the routine diagnosis of leptospirosis. It is evident from Table 1 that with a higher MAT titer or indirectly more time elapsed after the clinical onset of the disease, the probability of getting positive values in ELISA increases

and significantly diminishes the probability of direct antigen detection using RT PCR. It was observed that the samples with low MAT titer values of 1:100 and 1:200 yielded higher percentage positive values of 76.47% and 85.96%, respectively, in RT PCR. In addition, the values with respect to higher MAT titers of 1:400 and 1:800 were 10.57% and 1.22%, respectively. It is also noteworthy to observe that 12.31% of samples regarded as MAT-negative were found positive in RT PCR, signifying its role in the detection of very early disease, where serological tests may yield negative or ambiguous results. Furthermore, 12 samples that were IgM ELISA-negative yielded positive results in RT PCR, while only two samples that were IgG ELISA-negative were found positive in RT PCR. When ELISA was performed on MAT-negative, PCR-positive samples, it was found that the mean IgM titer was 1.92 ± 0.09, and for IgG ELISA it was 2.64 ± 0.12. Thus, this study could demonstrate IgG ELISA as having more reliable diagnostic acceptability than IgM ELISA, though IgM antibodies are often considered as indicators of acute infections. Thus, though a previous report (14) observed IgM ELISA as an efficient test for the diagnosis of acute leptospirosis, our study recommends the use of IgG ELISA.
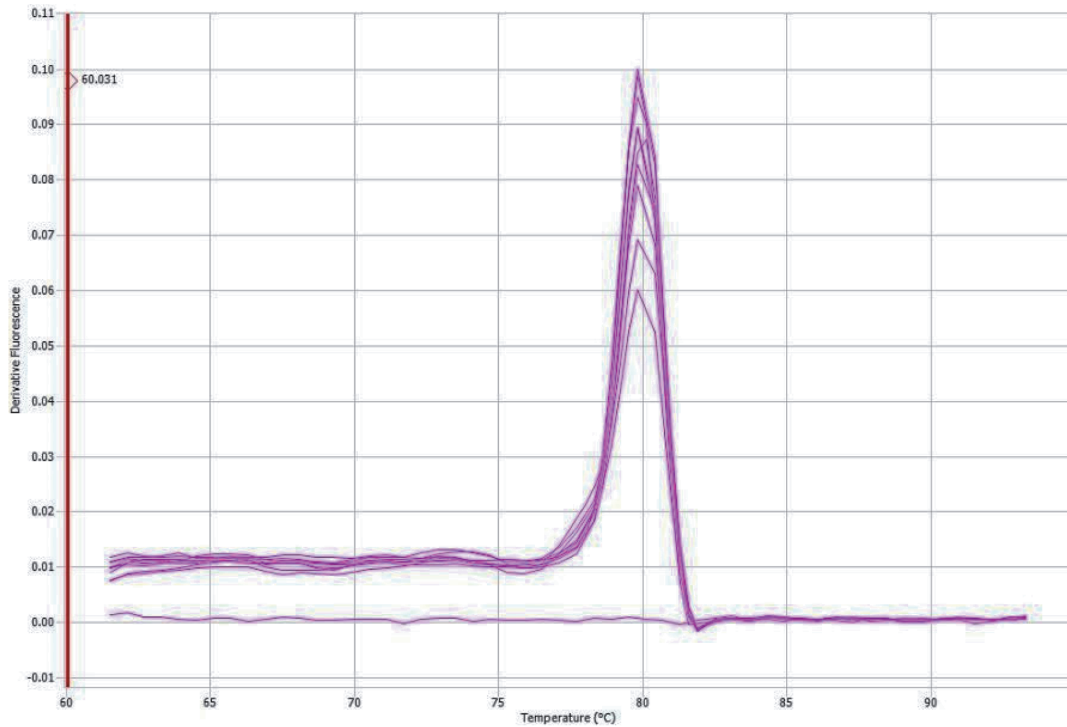
Figure 4. Melt curve analysis of *lipl32* amplicons indicating single peak and specificity of PCR reactions.

Table 1. Results of IgM and IgG ELISA and RT PCR as compared to MAT.

MAT	IgM ELISA		IgG ELISA		RT PCR	
	+	-	+	-	+	-
1:100 (51)	36	15	51	0	39	12
1:200 (57)	51	6	57	0	49	8
1:400 (104)	104	0	104	0	11	93
1:800 (83)	82	1	83	0	1	82
Negative (130)	21	109	32	0	16	114

Table 2. Comparison of different diagnostic tests (MAT, IgM ELISA, IgG ELISA, and RT PCR).

Tests in comparison	Percentage of agreement	χ^{2*}			Kappa*	Sensitivity**	Specificity**	PPV**	NPV**
		PU	Y	MH					
MAT vs. IgM ELISA	89.88	246.940	243.371	246.359	0.762	92.5	83.8	92.9	83.2
MAT vs. IgG ELISA	92.47	289.032	284.799	288.352	0.810	100	75.4	90.2	100
MAT vs. RT PCR	50.35	21.197	20.123	21.147	0.156	33.9	87.7	86.2	36.9
IgM ELISA vs. RT PCR	49.88	40.932	39.295	40.836	0.187	34.9	98.0	98.3	31.1
IgG ELISA vs. RT PCR	52.24	30.794	29.497	30.722	0.188	35.3	90.8	89.7	90.8

PU: Pearson uncorrected, Y: Yates corrected, MH: Mantel–Haenszel, PPV: positive predictive value, NPV: negative predictive value.
*$P < 0.0001$, **Confidence interval 95%–100%.

This predominance of IgG antibodies rather than IgM could be attributed to the disease endemicity, where many of the animals could have a presensitization of *Leptospira* either through a prior clinical/subclinical infection or due to vaccination, and the subsequent exposure would have resulted in an anamnestic immunological surge predominated by IgG antibodies. A similar observation may not be expected while examining the serum samples from a region where the disease occurrence is sporadic. Thus, we could conclude that for the screening of sera for leptospirosis from a disease-endemic region like Kerala, it would be most appropriate to employ IgG ELISA for the unambiguous identification of the positive cases, followed by RT PCR/conventional PCR for the IgG ELISA-negative samples.

In conclusion, this study recommends the use IgG ELISA as the best serological test for the diagnosis of canine leptospirosis in a disease-endemic region. However, samples that yield negative or doubtful results with very a narrow margin with the cut-off in IgG ELISA should only be tested in molecular tests like PCR or RT PCR, thus avoiding unnecessary processing and testing of the all the samples in costly and labor-intensive molecular tests.

References

1. Adler B, Moctezuma A. *Leptospira* and leptospirosis. Vet Microbiol 2010; 140: 287-296.

2. Branger C, Blanchard B, Fillonneau C, Suard I, Aviat F, Chevallier B, André-Fontaine G. Polymerase chain reaction assay specific for pathogenic *Leptospira* based on the gene hap1 encoding the hemolysis-associated protein-1. FEMS Microbiol Lett 2005; 243: 437-445.

3. Joseph S, Thomas N, Thangapandian E, Singh VP, Verma R, Srivastava SK. Evaluation and comparison of native and recombinant LipL21 protein-based ELISAs for diagnosis of bovine leptospirosis. J Vet Sci 2012; 13: 99-101.

4. Bourhahy P, Bremont S, Zinini F, Giry C, Picardeau M. Comparison of real- time PCR assays for the detection of pathogenic *Leptospira* spp. in blood and identification of variations in target sequences. J Clin Microbiol 2011; 49: 2154-2160.

5. Waggoner JJ, Balassiano I, Abeynayke J, Sahoo MK, Mohamed-Hadley A, Liu Y, Vital-Brazil MJ, Pinsky AB. Sensitive real-time PCR detection of pathogenic *Leptospira* spp and a comparison of nucleic acid amplification methods for the diagnosis of leptospirosis. PLoS One 2014; 9: e 112356.

6. Denipitiya DTH, Chandrasekharan NV, Abeyewickreme W, Hartkeerl CM, Hartskeerl RA, Jiffrey AM, Hapugoda MD. Application of a real time polymerase chain reaction (PCR) assay for the early diagnosis of human leptospirosis in Sri Lanka. Biologicals 2016; 44: 497-502.

7. Dey S, Mohan CM, Kumar TM, Ramadass P, Nainar AM, Nachimuthu K. Recombinant LipL32 antigen-based single serum dilution ELISA for detection of canine leptospirosis. Vet Microbiol 2004; 103: 99-106.

8. Deneke Y, Sabarinath T, Gogia N, Lasiamthara J, Viswas KN, Chaudhuri P. Evaluation of recombinant LigB antigen-based indirect ELISA and latex agglutination test for the serodiagnosis of bovine leptospirosis in India. Mol Cell Probe 2014; 28: 141-146.

9. Budhihal VS, Perwez K. Leptospirosis diagnosis: competancy of various laboratory tests. J Clin Diagnostic Res 2014; 8: 199-202.

10. Guerreiro H, Croda J, Flannery B, Mazel M, Matsunaga J, Reis MG, Levett PN, Ko AI, Haake DA. Leptospiral proteins recognized during the humoral immune response to leptospirosis in human. Infect Immun 2001; 69: 4958-4968.

11. Ooteman MC, Vago AR, Koury MC. Evaluation of MAT, IgM ELISA and PCR methods for diagnosis of human leptospirosis. J Microbiol 2006; 65: 247-257.

12. Goris MG, Hartskeerl RA. Leptospirosis serodiagnosis by the microscopic agglutination test. Curr Protoc Microbiol 2014; 6: 32-35.

13. Rajapakse S, Weeratunga P, Niloofa R, Fernando N, de Silva LN, Rodrigo C, Maduranga S, Nandasir N, Premawansa S, Karunanayake L et al. A diagnostic scoring model for leptospirosis in resource limited settings. PLoS Negl Trop Dis 2016; 10: e0004513.

14. Niloofa R, Fernando N, de Silva NL, Karunanayake L, Wickramasinghe H, Dikmadugoda N, Premawansa G, Wickramasinghe R, de Silva JH, Premawansa S et al. Diagnosis of leptospirosis: comparison between microscopic agglutination test, IgM-ELISA and IgM rapid immunochromatography test. PLoS One 2015; 10: e0129236.