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Original Article

Evaluation of the diagnostic potential and DIVA capability of recombinant LigBCon1-5 protein of *Leptospira interrogans* serovar Pomona in canine leptospirosis

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Abstract

Background: Canine leptospirosis is a serious public health concern. **Aims:** This study aims to investigate the feasibility of conserved first to fifth domains of recombinant *Leptospira* immunoglobulin like protein B antigen (rLigBCon1-5) as a serodiagnostic marker for detecting canine leptospirosis. **Methods:** A total of 340 unvaccinated canine serum samples were screened using microscopic agglutination test (MAT) and rLigBCon1-5 based immunoglobulin G (IgG) indirect-enzyme-linked immunosorbent assay (I-ELISA). Further, 60 vaccinated canine sera were screened using MAT and rLigBCon1-5 based latex agglutination test (LAT). **Results:** Microscopic agglutination test results revealed seropositivity of 28.6%. The relative sensitivity, specificity, and accuracy of IgG I-ELISA in comparison to MAT were 100%, 96.0%, and 97.2%, respectively. Out of 60 vaccinated sera, 46 sera reacted with MAT alone, and eight sera reacted by both tests, while six sera were non-reactive with both tests. Anti-LigB antibodies were detected in eight canine sera by rLigBCon1-5 based LAT. In five LAT reactive sera, agglutinins of locally circulating *Leptospira* serovars Grippotyphosa (n=4) and Australis (n=1) were detected. In three LAT reactive sera, agglutinins against Icterohaemorrhagiae (n=3) produced due to natural infection were present. **Conclusion:** Immunoglobulin G based indirect ELISA assay (IgG I-ELISA) can be employed as an alternative test instead of MAT. rLigBCon1-5 based LAT detected anti-LigB antibodies in eight vaccinated sera where the vaccine failure occurred partially or totally due to the limited efficacy spectrum of Nobivac® RL and cold chain breakage. This vaccine could not provide cross-protection against locally circulating *Leptospira* serovars. The recombinant LigBCon1-5 antigen based LAT possesses capability of differentiating infected from vaccinated individuals (DIVA capability) when employed as a pen-side test for detecting canine leptospirosis.

Key words: DIVA based test, ELISA, Latex agglutination test, Canine leptospirosis, Spatial variation

Introduction

Leptospirosis is a spirochetal zoonotic disease of ubiquitous distribution, with a much greater incidence in the tropics and possesses a broad host range affecting over 150 mammalian species (Levett, 2001; Doosti *et al*., 2012). Canine leptospirosis, referred to as Stuttgart's disease, affects canines worldwide and it is responsible for hemorrhagic diathesis, disseminated intravascular coagulopathy, uveitis, pulmonary hemorrhage as well as acute hepatitis and renal failure (Greene *et al*., 2006; Claus *et al*., 2008; van de Maele *et al*., 2008). Dogs are the maintenance hosts for serovar Canicola (André-Fontaine, 2016). In Northern Iran, unvaccinated guard dogs have been responsible for the spread of leptospirosis to cattle since a high prevalence of serogroup Canicola has been recorded in cattle having close contact with guard dogs (Abdollahpour *et al*., 2009). The age and sex of dogs seem to be potential risk factors since middle-aged male dogs, four to seven years

old, are most commonly infected (Stokes and Forrester, 2004). Even though breed of dogs does not comprise a potential risk factor, herding and hunting dogs appear to be at higher risk owing to their regular contact with wildlife (Stokes and Forrester, 2004).

The commercial vaccines available against canine leptospirosis in India are either bivalent vaccine containing antigen from *Leptospira interrogans* (*sensu lato*) serogroups Icterohaemorrhagiae and Canicola or tetravalent vaccine containing antigen from *Leptospira interrogans* (*sensu lato*) serogroups Icterohaemorrhagiae, Grippotyphosa, Pomona, and Canicola. However, seroepidemiological studies conducted in Kerala revealed Autumnalis as the predominant serovar followed by Australis (Ambily *et al*., 2013). In Tamil Nadu, serovars Autumnalis and Australis featured among the top three serovars circulating in dogs along with Grippotyphosa (Sathiyamoorthy *et al*., 2017). In Maharashtra, Pyrogenes was the predominant serovar (Patil *et al*., 2014). Hence, ample opportunity exists for developing effective autogenous *Leptospira* vaccines specific for individual agro-climatic zones of India with the inclusion of these reported serovars. In order to achieve this objective, knowledge regarding the predilection of various leptospiral serovars for different agro-climatic zones of India (spatial variation) is required (Sabarinath *et al*., 2018). Thus, the microscopic agglutination test (MAT) results of canine sera obtained from six Indian states, representing five major agro-climatic zones of India, will give impetus for developing successful national vaccination programs for controlling canine leptospirosis by shedding light on spatial variation with regard to leptospiral serovars.

Even though MAT is considered as the gold standard test for serodiagnosis of leptospirosis, the inherent pitfalls of MAT have forced disease investigators to search for alternative laboratory tests as well as field oriented tests (Budihal and Perwez, 2014). A noteworthy example for field oriented spot test is latex agglutination test (LAT), a highly economical, rapid screening pen-side test, ideally suited for large-scale screening of sera samples in endemic areas without using any sophisticated equipment (Senthilkumar *et al*., 2008). The advent of recombinant DNA technology enabled the use of recombinant outer membrane proteins (OMPs) found ubiquitously in the outer membrane of pathogenic leptospires such as LipL32 (Hartleben *et al*., 2013), Loa22 (Ye *et al*., 2014) and *Leptospira* immunoglobulin like protein B (LigB) (Deneke *et al*., 2014) to be employed successfully in molecular diagnostic assays. The recombinant OMPs can circumvent various shortcomings of whole *Leptospira* antigen-based assays such as MAT when employed in molecular diagnostic assays (Lin *et al*., 2008).

Leptospira immunoglobulin like (Lig) proteins belong to a family of bacterial immunoglobulin-like (Big) domain proteins that are ubiquitously present on the outer membrane of all pathogenic *Leptospira* species (Matsunaga *et al*., 2003). Lig proteins have been earmarked as serodiagnostic markers for acute leptospirosis and Lig protein based on immunodiagnostic assays has been an advance in addressing the under reporting of leptospirosis (Croda *et al*., 2007). A kinetic ELISA (KELA) has been developed using the recombinant antigen to the conserved region of LigA and LigB protein, which can differentiate between vaccinated and naturally infected dogs (Palaniappan *et al*., 2004). Hence, the present study focused on studying the feasibility of recombinant *Leptospira* immunoglobulin like protein B Con1-5 (rLigBCon1-5) antigen based immunoglobulin G (IgG) indirect-enzyme-linked immunosorbent assay (I-ELISA) as an alternative laboratory oriented test for MAT. Further, the study also focused on the differentiating infected from vaccinated individuals (DIVA) capability of rLigBCon1-5 based LAT to differentiate between vaccinated and naturally infected canine sera samples.

Materials and Methods

Ethical approval

The study was approved by the Institute Animal Ethics Committee (IAEC) of ICAR-Indian Veterinary Research Institute (ICAR-IVRI). The blood was drawn from either canine cephalic vein or lateral saphenous vein and the dogs were handled humanely as per the protocols laid by IAEC of ICAR-IVRI.

Serum sample collection

A total of 423 serum samples were collected from unvaccinated dogs from the six states of India, then subjected to both MAT and rLigBCon1-5 IgG I-ELISA. The details are given in Fig. 1 with regard to canine serum samples collected from six Indian states for screening by MAT and ELISA. Additionally, 60 serum samples, from canines vaccinated with the bivalent leptospiral vaccine (n=33) (Nobivac® RL, Intervet, USA) and tetravalent leptospiral vaccine (n=27) (Duramune Max® 5/4 L, Fort Dodge, Iowa, USA), were collected from Polyclinic, Indian Veterinary Research Institute. The samples were subjected to both MAT and rLigBCon1-5 antigen based LAT. Five ml of blood was drawn into serum collection tubes (BD vacutainer, USA) and the blood was allowed to clot at ambient temperature for 1 h followed by centrifugation at 2000 g for 10 min. After centrifugation, the serum samples were collected in polypropylene tubes and stored at -20°C for further laboratory analysis.

Leptospiral serovars and strains used in MAT

A battery of 16 leptospiral serovars namely, *Leptospira interrogans* serovar Australis strain Ballico, *L. interrogans* serovar Autumnalis strain Akiyami A, *L. interrogans* serovar Ballum strain S102, *L. interrogans* serovar Bataviae strain van Tienen, *L. interrogans* serovar Canicola strain Hond Utrecht IV, *L. kirschneri* serovar Cynopteri strain 3522C, *L. interrogans* serovar Djasiman strain Djasiman, *L. kirschneri* serovar Grippotyphosa strain Moskva V, *L. borgpetersenii*

Agro Climatic Zones	States	Sera samples recieved	MAT +ve sera	% Sero-positivity	Aus	Aut	Can	Cyn	Dja	Gri	Ict	Jav	Pom	Pyr	Tar
West coast Plains & Hills	Kerala	101	37	36.63%	04	05	05	06	07	24	30	15	02	14	02
	Maharashtra	35	04	11.43%	-	-	-	-	-	02	04	02	-	-	-
Southern Plateau & Hills	Karnataka	94	18	19.15%	03	-	02	-	-	15	18	04	05	10	-
Eastern Himalayan Region	Manipur	88	27	30.68%	-	01	03	-	-	19	17	06	04	11	-
Upper Gangetic Plains	Uttar Pradesh	63	24	38.10%	02	-	-	-	-	06	22	-	-	-	-
East Coast Plains & Hills	Odisha	42	11	26.19%	-	-	-	-	-	05	11	02	-	-	-
Total		423	121	28.61%	09	06	10	06	07	71	102	29	11	35	02

Aus: Australis; Aut: Autumnalis; Can: Canicola; Cyn: Cynopteri; Dja: Djasiman; Gri: Grippotyphosa; Ict: Icterohaemorrhagiae; Jav: Javanica; Pom: Pomona; Pyr: Pyrogenes; Tar- Tarassovi

Cumulative figure of sera positive for various serovars (n= 288) exceed total MAT positive sera (n=121) as several sera reacted with multiple leptospiral serovars.

Fig. 1: Map of India showing locations from where unvaccinated canine serum samples were collected for MAT and recombinant *Leptospira* immunoglobulin like protein B Con1-5 antigen based ELISA. The results at 1:100 cut off value show agglutinins for various leptospiral serovars. MAT: Microscopic agglutination test

serovar Hardjo-Bovis strain JB197, *L. interrogans* serovar Hebdomadis strain Hebdomadis, *L. interrogans* serovar Icterohaemorrhagiae strain RGA, *L. borgpetersenii* serovar Javanica strain Veldrat Batavia 46, *L. noguchii* serovar Louisiana strain LSU 1945, *L. interrogans* serovar Pomona strain Pomona, *L. interrogans* serovar Pyrogenes strain Salinem and *L. borgpetersenii* serovar Tarassovi strain Perepelitsin were used for MAT.

Microscopic agglutination test

Microscopic agglutination test was employed for screening of agglutinins against various leptospiral serovars in canine sera using standard protocols (Alexander, 1986). Briefly, serum samples were diluted 1:50 in phosphate buffer saline (PBS) and a volume of leptospiral antigen, equal to the diluted serum volume, was added to each well, making the final serum dilution 1:100 in the screening test. Live leptospiral antigens (approx. 2×10^8 leptospires/ml) of 16 reference serovars, with 4-8 days old, were used in this study. The microtitre plates are incubated for 2 h at 29°C and the serum-antigen mixtures were examined using dark field microscopy. A positive outcome of MAT, exposure/seropositivity, was defined as >50% reduction in the number of free non-agglutinable leptospires in a serum sample at 1:100 dilution in comparison with the control for at least one leptospiral serovar.

Induction of expression and purification of rLigBCon1-5 antigen

Escherichia coli M15 strain harboring expression vector pQE30 (Qiagen, USA) was used for production of rLigBCon1-5 antigen. The vector contained the N-terminal conserved region of LigB gene without the signal sequence from *L. interrogans* serovar Pomona strain Pomona. This recombinant antigen is well characterized since it was used earlier in a seroprevalence study of bovine leptospirosis in India; this antigen was employed in both I-ELISA and LAT to compare with the gold standard test, MAT (Deneke *et al*., 2014). This serodiagnostic antigen, comprising D_1-D_5 Big tandem repeats, which corresponds to amino acid sequence positions 34-434 of the LigB protein (GenBank Accession No. AF534640), was modeled using homology modeling in Swiss-model server (Fig. 2A). This *E. coli* strain was grown in Luria Bertani broth (Difco, USA) till the spectrometric reading at OD_{600nm} reached 0.5-0.7. The cells were then induced with 1 mM Isopropyl ß-D-1-thiogalactopyranoside (IPTG) and allowed to grow further for 6 h at 37°C. Cells were harvested and the proteins analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using standard protocol (Laemmli, 1970). The recombinant protein was purified using Nickel nitrilotriacetic acid (Ni-NTA) agarose affinity chromatography (Qiagen, USA) as per instructions of the manufacturer. Analysis of the purified recombinant protein was done on SDS-PAGE. The purified recombinant LigBCon1-5 was checked for immunogenicity using high titred canine sera by Western blot analysis as per standard protocol (Towbin *et al*., 1979).

rLigBCon1-5 IgG I-ELISA for testing canine sera with unknown vaccination history

Sera from dogs with unknown vaccination history were analyzed by I-ELISA using standard protocol

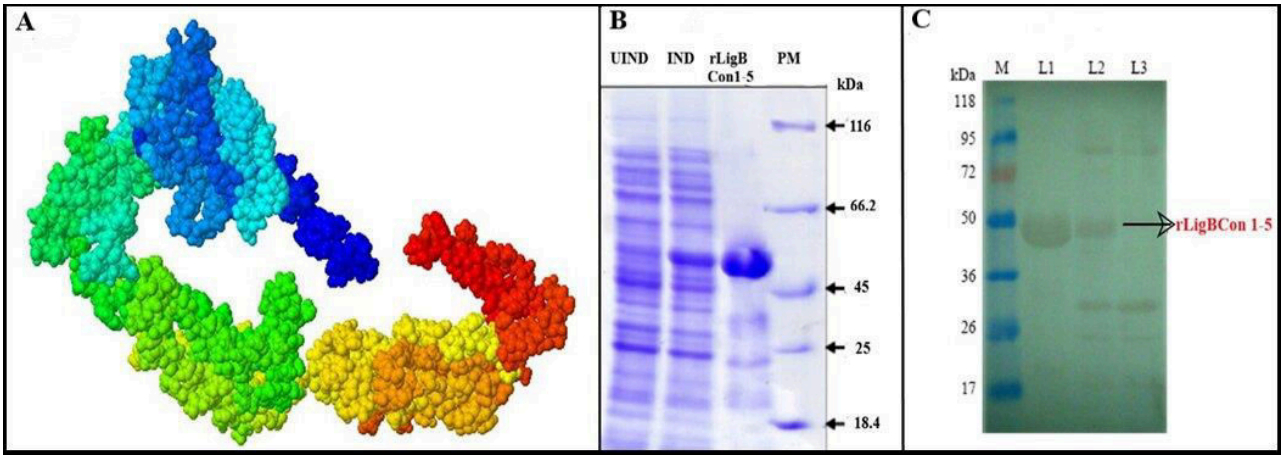

Fig. 2: Structure prediction, expression, and immuno-reactivity of recombinant *Leptospira* immunoglobulin like protein B Con1-5 antigen. (**A**) Homology modelling of rLigBCon1-5 protein in Swiss-model server. Amino acids 34-434 of rLigBCon1-5 protein of *L. interrogans* serovar Pomona strain Pomona corresponding to D_1-D_5 Big tandem repeats. The color scheme from N terminal to C terminal runs from blue to red, (**B**) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showing the elution fractions of rLigBCon1-5 protein after affinity chromatography. UIND: Uninduced by IPTG, IND: Induced by IPTG, rLigBCon1-5: Recombinant *Leptospira* immunoglobulin like protein B Con1-5 antigen, and PM: Protein marker, and (**C**) Western blot analysis showing immunoreactivity of rLigBCon1-5 protein against high titred canine sera. M: Pre-stained protein marker, L1: Lane containing purified rLigBCon1-5 antigen, L2: Lane containing induced *Escherichia coli* M15 strain harboring expression vector pQE30, and L3: Lane containing uninduced *Escherichia coli* M15 strain harboring expression vector pQE30

(Engvall and Perlmann, 1971). rLigBCon1-5 was used as diagnostic antigen. Various concentrations of rLigBCon1-5 antigen (31.25 ng to 500 ng in carbonate bicarbonate buffer (pH = 9.6)) were added to each well of immunoassay plates (Maxisorp®, Nunc, Denmark) in duplicates. A constant 1:100 dilution of known positive and negative serum samples were also considered. Negative control sera for ELISA standardization was obtained from apparently healthy dogs. The sera collected from Polyclinic, IVRI were found to be sero-negative for leptospiral antibodies using MAT at a 1:50 dilution. The checkerboard titration was performed using various dilutions of rabbit anti-canine IgG horseradish peroxidase (HRPO) conjugate such as 1:2500, 1:5000, 1:10000, and 20,000 in order to find the optimum dilution of HRPO conjugated secondary antibody to be employed in ELISA to screen canine serum samples. The ELISA wells were filled with substrate solution (Ortho-phenylenediamine and H_2O_2 (Sigma-Aldrich, USA)). Color development was stopped by adding 2 M H_2SO_4, after 10 min of the plates incubation in dark. Absorbance was recorded at 492 nm wavelength in an ELISA reader (BioRad 680 micro plate reader, USA). The mean OD_{492nm} value of the known negative sera was calculated, and double of this value was taken as the cut-off value as described previously (de Savigny and Voller, 1980).

Statistical analysis

The relative sensitivity, specificity, and accuracy (in percentage) of rLigBCon1-5 based I-ELISA in comparison with MAT for the serodiagnosis of canine leptospirosis was evaluated as described below:

$$\text{Sensitivity} = a/(a + c) \times 100$$

Where,

a: The number of serum samples positive by both MAT and rLigBCon1-5 based I-ELISA
c: The number of serum samples positive by MAT but negative by rLigBCon1-5 based I-ELISA

$$\text{Specificity} = d/(b + d) \times 100$$

Where,
d: The number of serum samples negative by both MAT and rLigBCon1-5 based I-ELISA
b: The number of serum samples negative by MAT but positive by rLigBCon1-5 based I-ELISA

$$\text{Accuracy} = a + d/(a + b + c + d) \times 100$$

An intuitive method for calculating predictive values (in percent) for positive and negative test results was done as per standard method (Jacobson, 1998).

$$\text{Positive predictive value (PPV)} = a/(a + b) \times 100$$

$$\text{Negative predictive value (NPV)} = d/(c + d) \times 100$$

The evaluation of rLigBCon1-5 based I-ELISA for detection of anti-*Leptospira* antibodies in canines as compared with MAT was determined using Chi-square test and Kappa statistics.

rLigBCon1-5 based LAT for testing vaccinated canine sera

Latex beads were sensitized with rLigBCon1-5 antigen as described by Deneke *et al*. (2014) with slight modifications. A 2.5% suspension of latex particles (1.0 µm diameter, Polybead® Polystyrene Blue and Red Dyed Microsphere, Polysciences, USA) was washed thrice with glycine buffered saline (Glycine 0.1 M, NaCl 0.17 M; pH = 8.2). Finally, the latex beads were made into a 2% suspension with glycine buffered saline which was later mixed with an equal volume of rLigBCon1-5 antigen (1 mg/ml) diluted in the same buffer. The

mixture was incubated at 37°C for 6 h in a shaking platform to ensure constant mixing. The sensitized latex beads were further blocked with bovine serum albumin (Difco, USA) (5 mg/ml) and incubated overnight. Latex beads were centrifuged and the pellet was finally resuspended in glycine buffered saline as a 2% suspension containing 0.02% sodium azide. The sensitized latex beads were stored at 4°C until use. LAT was performed on glass slides by mixing an equal volume of serum (20 µL) and sensitized beads (20 µL). The result was read within 2 min. The agglutinated samples were considered positive. A score of 3^+, 2^+, and 1^+ was designated to sera which showed agglutination within 30 s, 30 s to 1 min, and 2 min, respectively as previously described (Smits *et al*., 2000). Samples were considered negative without any agglutination.

Results

MAT results revealed a seropositivity of 28.6% (121/423) for canine leptospirosis with maximum agglutinins detected for serovar Icterohaemorrhagiae (n=102) followed by Grippotyphosa (n=71), Pyrogenes (n=35), Javanica (n=29), Pomona (n=11), Canicola (n=10), Australis (n=09), Djasiman (n=07), Autumnalis (n=06), Cynopteri (n=06), and Tarassovi (n=02) (Fig. 1). The cumulative figure of sera positive for various serovars (n=288) exceeds total MAT positive sera (n=121) as several sera reacted with multiple leptospiral serovars. MAT titre ranged from 1:100 to 1:3200. Antibodies to serovars Ballum, Bataviae, Hardjoprajitno, Hebdomadis, and Louisiana were absent in all canine sera against which MAT was performed. Out of the six Indian states, Uttar Pradesh recorded the highest seropositivity (38.1%) followed by Kerala (36.6%), Manipur (30.7%), Odisha (26.2%), Karnataka (19.2%), and Maharashtra (11.4%) as depicted in Fig. 1.

In this study, a high level expression of rLigBCon1-5 protein was observed after induction of broth culture with IPTG. Incubating the broth culture overnight yielded approximately 20 mg/L of protein (Fig. 2B). Western blot analysis showed that rLigBCon1-5 protein was highly immunogenic against anti-leptospiral antibodies from canines (Fig. 2C).

The checkerboard titration revealed a maximum positive/negative (P/N) ratio when an antigen concentration of 62.5 ng/well was employed against a constant 1:100 dilution of known positive and negative serum samples. The optimum dilution of rabbit anti-canine IgG HRPO conjugate was found to be 1:5,000. The mean optical density (OD) value of the control negative serum samples was 0.151. Therefore, any sample showing twice the mean OD value of the control negative sera or more (≥0.302) was considered positive for canine leptospirosis.

Of 423 serum samples tested, 121 (28.6%) and 133 (31.4%) serum samples were found positive by MAT and IgG I-ELISA, respectively. The sensitivity, specificity, and accuracy of rLigBCon1-5 based IgG I-ELISA relative to MAT are 100.0%, 96.0%, and 97.2%, respectively. The kappa value of 0.93 suggests high agreement between rLigBCon1-5 based IgG I-ELISA and MAT in detecting leptospiral antibodies (Table 1).

The results of 60 vaccinated canine sera screened using both MAT and rLigBCon1-5 based LAT are depicted in Table 2. rLigBCon1-5 based LAT was sero-negative for MAT^{+ve} (n=46) vaccinated sera since this test gave a homogeneous suspension with these canine sera (Fig. 3A). A total of six sera did not react by both MAT and rLigBCon1-5 based LAT which indicated vaccine failure (Fig. 3Bb). Latex agglutination test showed DIVA capability since it showed clear-cut agglutination with naturally infected canine sera (n=08) which can be visualized easily (Figs. 3Ba and c). A positive correlation exists between MAT titre and LAT score. Canine sera (n=04) with MAT titres ≥ 1:1600 showed LAT score of 3^{+ve} that means the agglutination occurred within 30 s and the intensity of the agglutination was high. The agglutinins formed a halo at the periphery while the center was virtually empty (Fig. 3Ba). Serum samples (n=02) with MAT titres ≥ 1:400 gave LAT score of 2^{+ve} that means the agglutination occurred between 30 s-1 min with a moderate intensity of agglutination. Two borderline sera with MAT titre of ≥ 1:200 gave LAT score of 1^{+ve} and agglutination occurred within 2 min with low intensity of agglutination (halo at periphery not prominent) (Fig. 3Bc).

Table 1: Relative sensitivity, specificity, and accuracy values of recombinant LigBCon1-5 IgG based I-ELISA for detection of anti-leptospiral antibodies in canine sera as compared to MAT

		MAT				Low 95% C.I.	High 95% C.I.
		Positive	Negative	Kappa	0.93	0.89	0.96
rLigBCon1-5 based I-ELISA	Positive	121 (a)	12 (b)				
	Negative	0 (c)	290 (d)	Sensitivity	100.0%	96.2%	100.0%
				Specificity	96.0%	92.9%	97.8%
				Accuracy	97.2%	95.1%	98.5%
				† PPV	90.9%	84.4%	95.0%
				‡ NPV	100.0%	98.3%	100.0%

rLigBCon1-5: Recombinant *Leptospira* immunoglobulin like protein B Con1-5 antigen, IgG: Immunoglobulin G, I-ELISA: Indirect ELISA, MAT: Microscopic agglutination test, C.I.: Confidence interval, a: Represents both MAT^{+ve} and rLigBCon1-5 based $I\text{-}ELISA^{+ve}$, b: Represents MAT^{-ve}/rLigBCon1-5 based $I\text{-}ELISA^{+ve}$, c: Represents MAT^{+ve} and rLigBCon1-5 based $I\text{-}ELISA^{-ve}$, d: Represents MAT^{-ve}/rLigBCon1-5 based $I\text{-}ELISA^{-ve}$, † PPV: Positive predictive value, and ‡ NPV: Negative predictive value

Table 2: Results from 60 vaccinated canine sera screened using both MAT and rLigBCon1-5 antigen based LAT

Number of dogs vaccinated using tetravalent Duramune Max® 5/4 L	Number of dogs vaccinated using bivalent Nobivac® RL	Number of vaccinated canine sera screened by both MAT and rLigBCon1-5 based LAT	Number of vaccinated canine sera showing sero-positivity by MAT	Number of vaccinated canine sera showing sero-positivity by both MAT and rLigBCon1-5 based LAT	Number of vaccinated canine sera showing sero-positivity by MAT and sero-negativity by rLigBCon1-5 based LAT	Number of vaccinated canine sera showing sero-negativity by both MAT and rLigBCon1-5 based LAT	Agglutinins against leptospiral serovars in sera of dogs vaccinated using tetravalent Duramune Max® 5/4 L				Agglutinins against leptospiral serovars in sera of dogs vaccinated using bivalent Nobivac® RL			
							Can	Gri	Ict	Pom	Can	Ict	Gri	Aus
27	33	60	54	8	46	6	27	27	27	27	30[a]	33[b]	4[c]	1[d]

MAT: Microscopic agglutination test, rLigBCon1-5: Recombinant *Leptospira* immunoglobulin like protein B Con1-5 antigen, LAT: Latex agglutination test, Can: Canicola, Gri: Grippotyphosa, Ict: Icterohaemorrhagiae, Pom: Pomona, Aus: Australis, a and b: Canine sera showing agglutinins against serovar Canicola (n=30) and Icterohaemorrhagiae (n=33) suggestive of vaccination failure followed by natural infection with serovar Icterohaemorrhagiae in three rLigBCon1-5 based LAT^{+ve} dogs, c and d: Five rLigBCon1-5 based LAT^{+ve} canine sera from Nobivac® RL vaccinated dogs showing agglutinins against serovar Grippotyphosa and Australis (not included in vaccine) besides Canicola and Icterohaemorrhagiae suggestive of antibody generation by both vaccination and natural infection

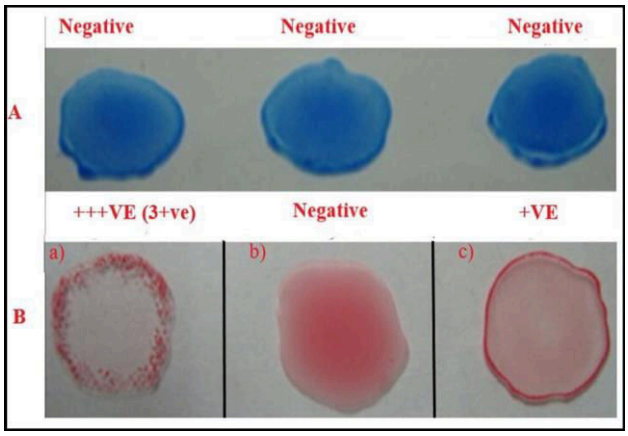

Fig. 3: Latex agglutination test for screening of vaccinated canine sera performed using sensitized latex beads coated with recombinant LigBCon1-5 (rLigBCon1-5) antigen. (**A**) Complete protection by vaccine (LAT^{-ve}, MAT^{+ve}) (MAT with agglutinins against vaccine serovars) in 46 vaccinated canine sera (**B**) Latex agglutination test in association with MAT detecting partial and complete vaccination failure in separate instances in vaccinated canine serum samples. (a) and (c) Partial protection by vaccine (LAT^{+ve}, MAT^{+ve}) (MAT with agglutinins of serovars both included and not included in vaccine) in eight vaccinated canine sera (b) Complete vaccine failure (LAT^{-ve}, MAT^{-ve}) (MAT without agglutinins against vaccine serovars) in six vaccinated canine sera

Discussion

Leptospirosis is an important re-emerging disease worldwide and requires early confirmatory diagnosis since antibiotic treatment is most effective at the early stage of the disease (Al-orry *et al*., 2016). Analysis of leptospiral antigens targeted by the humoral immune response revealed that antibodies were generated against LigB protein during infection with pathogenic *Leptospira* spp. (Matsunaga *et al*., 2003; Croda *et al*., 2007). rLigBCon1-5 protein based ELISA/LAT as an alternative laboratory test in remote locations, where MAT is not readily accessible, would permit the intervention strategies based on early case detection and the subsequent initial treatment. This strategy would prevent disease progression and the severe outcomes associated with leptospirosis (Croda *et al*., 2007).

rLigBCon1-5 based IgG I-ELISA gave results in concordance to MAT in terms of sensitivity, specificity, and accuracy. In this study, all 121 MAT positive sera were positive by rLigBCon1-5 based IgG I-ELISA that indicates its high sensitivity in comparison with MAT. However, 12 serum samples showing negative results by MAT, tested positive by IgG based I-ELISA. The probable reason for the slightly lower specificity might be that all the sera construed as MAT negative might not actually be negative, since the battery of leptospiral antigens used for MAT was only sixteen *Leptospira* serovars. More than 250 pathogenic serovars belong to 24 serogroups reported within the species *L. interrogans* (Xu *et al*., 2017). Therefore, it makes practical sense to maintain in a *Leptospira* laboratory only the most prevalent serovars in an endemic region. When a dog is infected with a newly emerging serovar or a relatively rare serovar having low prevalence rate, MAT invariably gives a false negative test result due to incomplete panel of leptospiral serovars available for performing MAT (Musso and Scola, 2013).

MAT results of the present study indicate the predilection for various leptospiral serovars to thrive in specific agro-climatic zones of India. Even though the landlocked state of Uttar Pradesh recorded the highest seropositivity (38.10%), there was a lack of serovar diversity in this state as well as in states such as Maharashtra and Odisha. However, the serovar epidemiology in Kerala (11 serovars) and Karnataka (7 serovars) indicates that these states are bastions for leptospiral serovars. Both these states fall in the perhumid region (windward side) of Western Ghats and receive the bulk of the South-West monsoon. This is the first instance in India, where serovars Djasiman and Cynopteri have been reported in dogs from Kerala. The northeastern state of Manipur also showed serovar diversity (7 serovars). This propensity of leptospiral serovars to thrive in different ecological zones was also observed by Lau *et al*. (2012) and Sabarinath *et al*. (2018) in American Samoa and India, respectively. This

seroepidemiological study revealed that the agglutinins against serovars Pyrogenes (n=35) and Javanica (n=29) were significantly higher than agglutinins against serovars Pomona (n=11) and Canicola (n=10) whose bacterins are present in tetravalent leptospiral vaccines. Hence, according to our research and previous seroepidemiological studies in India (Ambily *et al*., 2013; Patil *et al*., 2014; Sathiyamoorthy *et al*., 2017), we believe that commercial vaccines should include newly emerging leptospiral serovars such as Autumnalis, Australis, Pyrogenes, and Javanica along with established leptospiral serovars such as Icterohaemorrhagiae, Canicola, Grippotyphosa, and Pomona in order to prevent leptospirosis in vaccinated dogs.

The development of diagnostic assays based on recombinant LigB (rLigB) antigen, expressed only during natural infection, would be a valuable tool to identify animals that contract leptospirosis despite vaccination (Palaniappan *et al*., 2004). Further, the vaccinated sera showed high absorbance values in ELISA with whole-cell proteins of leptospires, but failed to show reactivity in Kinetic ELISA (KELA) and Western blot analysis by rLigB antigen, confirming a lack of antibodies to rLigB antigen in the vaccinated sera (Palaniappan *et al*., 2004). Hence, in the present study rLigBCon1-5 antigen was used to coat latex beads to determine vaccination failure since reactivity of vaccinated sera in rLigBCon1-5 based LAT would imply that the dog has contracted leptospirosis despite vaccination. Moreover, vaccination against canine leptospirosis is effective just in avoiding the clinical manifestations and it offers no protection against acquiring infection (Feigin *et al*., 1973).

The reason for eight serum samples reacting to both MAT and rLigBCon1-5 based LAT is a combination of vaccination failure as well as the presence of newly emerging serovars in non-vaccinated regions. Three Nobivac® RL vaccinated dog sera which reacted to both MAT and rLigBCon1-5 based LAT contained only agglutinins against serovar Icterohaemorrhagiae but not serovar Canicola. This suggests vaccination failure. The agglutinins formed against serovar Icterohaemorrhagiae in MAT might be due to natural infection rather than vaccination due to reactivity of all three sera with rLigBCon1-5 based LAT. However, the agglutinins formed in MAT against serovars Grippotyphosa (n=4) and Australis (n=1) along with Canicola (n=5) and Icterohaemorrhagiae (n=5) in five Nobivac® RL vaccinated dog sera, which reacted to the both tests, might be due to antibody generation by both vaccination and natural infection. Vaccination with Nobivac® RL generated agglutinins which will protect dogs against infection by serovars belonging to serogroups Icterohaemorrhagiae and Canicola but failed to confer cross protection against natural infection with serovars belonging to unrelated serogroups (Grippotyphosa and Australis). This main drawback of leptospirosis bacterins of limited efficacy spectrum (protection directed only against the vaccine serovars or closely related serovars within the serogroup) cannot be solved by increasing the amount of protective antigen (Levett, 2001; Adler, 2015). Moreover, the widely cross-agglutinating lipopolysaccharides (LPS) epitopes of saprophytic *L. biflexa*, which are cross-reactive in MAT, are unable to afford any cross-protection when exposed to infectious field strains (Srikram *et al*., 2011; Adler, 2015). Hence, it is of paramount importance to conduct regular epidemiological surveillance in an agro-climatic zone/geographical region to unravel temporal variation due to the emergence of new serovars which can become causative agent for canine leptospirosis despite vaccination (Higino *et al*., 2010). This tailoring of vaccines to accommodate emerging serovars can be done and inclusion of these new serovars helps in the preparation of effective vaccines (Klaasen and Adler, 2015).

The reason for six vaccinated canine sera not showing reactivity by both MAT and rLigBCon1-5 based LAT might be due to vaccination failure. The major reason for vaccination failure in India is due to a lack of awareness of field veterinarians and pet owners regarding “Window of susceptibility” (immunity gap). Window of susceptibility is a time period during which there is insufficient maternal immunity to provide full protection against natural infection, but still sufficient maternal immunity to block the ability of pup to mount a protective active immune response. Therefore, if a pup receives a vaccination for leptospirosis before the maternal leptospiral antibodies diminish, the maternal antibodies will block the protective effect of the vaccine challenge and prevent the pup from mounting an active immune response against leptospirosis. This is the reason why veterinarians usually recommend an additional booster vaccination, given after the puppy series of vaccinations has been completed, usually at about 16 weeks of age or older, which is the time period when the MDA have tapered to negligible levels (Friedrich and Truyen, 2000). Another reason could be post-manufacture factors such as cold chain breakage of *Leptospira* vaccine during transit and storage (Day *et al*., 2016). The cold chain breakage of *Leptospira* vaccine, maintained optimally between 2-8°C, can have adverse effects on vaccine potency when the vaccine is exposed to inappropriate temperatures mainly due to frequent electricity cuts in India affecting refrigeration temperatures.

In conclusion, we described the seroprevalence of canine leptospirosis in India with special emphasis on spatial variation. There is a variation of leptospiral serovar distribution in different agro-climatic zones of India. The microscopic agglutination test results will help in tailoring of existing commercial vaccines to accommodate emerging leptospiral serovars whose inclusion will help in the preparation of effective vaccines. Our observation with rLigBCon1-5 based IgG I-ELISA suggest the potential of this test to be employed as an alternative laboratory test instead of MAT in tertiary levels of the animal health care system. Further, our findings with regard to vaccinated canine sera

employing rLigBCon1-5 based LAT suggest that this penside assay has DIVA capability and can pinpoint vaccine failure as well as a natural infection due to emerging serovars not included in commercial vaccines. However, the preliminary study conducted with limited numbers of vaccinated canine sera needs further analysis which requires more field positive serum samples with known histories of vaccination and experimental infection.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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