Development of leptospiral outer membrane protein-based dot-ELISA assays and immunoprofiling for diagnosis of acute canine leptospirosis

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Abstract: The present study was carried out to evaluate outer membrane protein (OMP)-based relatively simple dot-ELISA assays for the diagnosis of acute canine leptospirosis and to identify the differential expression of leptospiral OMPs for identifying the markers of acute infection, if any. Among 91 canine serum samples screened, 63 samples were found to be positive by MAT. All the samples were tested by OMP-based IgM and IgG dot ELISAs. The sensitivities of IgM and IgG dot ELISA in comparison to MAT (titer: ≥1:400) were found to be 100%, whereas the specificities were found to be 89.71% and 45.31%, respectively. IgM and IgG immunoblotting of OMPs was standardized and tested with the MAT-positive samples. IgM immunoblotting of leptospiral OMP revealed proteins of 18, 21, and 32 kDa as major reactors. With IgG, proteins of 32, 41, and 66 kDa were recognized as major reactors and an 86-kDa protein was identified as a minor reactor. The 32-kDa protein was the predominant reactor in both IgM and IgG immunoblots. Thus, a combination of 18-, 21-, and 32-kDa proteins could be a good choice for the detection of IgM antibodies indicative of acute infection while that of 32-, 41-, and 66-kDa proteins could be employed for seroepidemiological studies.

Key words: Canine, leptospirosis, outer membrane proteins, diagnosis

1. Introduction

Leptospirosis is a worldwide zoonotic disease caused by spirochetes belonging to the genus *Leptospira*. The disease is gaining importance as a reemerging disease in India. It occurs in a wide range of animals and humans and can lead to multiple organ involvement and fatal complications (1). In India, the southern states and especially Kerala and Tamil Nadu are known to be endemic for leptospirosis (2–4). The disease is most often underdiagnosed due to lack of awareness of the disease, nonspecific clinical signs, and the paucity of well-equipped laboratories (5). Identification of the disease in its initial stage is necessary for initiating proper treatment. Laboratory diagnosis of leptospirosis can be made by demonstration of the organisms in blood or urine, isolation, molecular methods for antigen detection, or serological tests that detect leptospiral antibodies (6). Approximately 10^4 leptospires/mL of blood are necessary for visualization of one cell per field by DFM and thus this method proved to be insensitive (7). Isolation is a tedious process and molecular diagnostic techniques developed so far are mostly based on polymerase chain reaction (PCR) assays and in situ hybridization, requiring well-established laboratory facilities and highly skilled personnel. Thus,

serology is the most frequently used diagnostic approach for leptospirosis. The microscopic agglutination test (MAT) is the standard reference test for serological diagnosis of leptospirosis but cannot distinguish the antibodies due to acute infection from past infection or due to vaccination. Enzyme-linked immunosorbent assays (ELISAs) employing different antigens were developed and the outer membrane protein (OMP) antigens were proved to be promising in the diagnosis of leptospirosis (8,9). The results of recombinant OMP antigen-based ELISAs are significant (10). The OMP-based latex agglutination test was also found to be sensitive in diagnosing leptospirosis in the early stage (5). However, the presence of leptospiral antibodies due to either vaccination or past infection in animals in an endemic area interferes with the diagnosis of the disease (11). The reactivity of IgM antibodies to highly conserved OMPs of pathogenic leptospires has been studied by immunoblotting with sera from acute cases of canine leptospirosis (11), while the focus on the reactivity of IgG antibodies is relatively less. Hence, identification of leptospiral proteins reacting to IgM and IgG antibodies would be of great for the development of improved serodiagnostics for canine leptospirosis.

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Therefore, the present study was carried out to develop and evaluate relatively simple OMP-based dot ELISA assays for detecting acute and convalescent cases of canine leptospirosis in comparison to MAT and to identify predominant leptospiral OMPs expressed in the acute phase of the disease.

2. Materials and methods

2.1. Serum samples
A total of 91 serum samples (85 single serum samples and six paired sera samples) suspected of leptospirosis were collected from dogs presented at University Veterinary Hospitals Mannuthy and Kokkalai, Thrissur, Kerala, India, during the period from September 2010 to December 2011.

2.2. Microscopic agglutination test
The MAT was carried out using liquid cultures of 5–7 days old of live leptospires employing nine serovars of *Leptospira interrogans* with a density of approximately 2×10^8 leptospires per milliliter in a 96-well U-bottom microtiter plate as per the method described by the OIE (12).

2.3. Extraction of outer membrane proteins
The OMP of *L. interrogans* serovar Australis strain Ballico was extracted using sarkosyl detergent as described by Nicholson and Prescott (13). Briefly, leptospires were grown in 500 mL of *Leptospira* culture medium for about 7 to 10 days and were harvested by centrifugation at 40,000 × g for 20 min, followed by three washings with 0.01 M PBS, pH 7.2. The cells were resuspended in 10 mM HEPES buffer and disrupted by sonication at 250 V for a total of 5 min with 15 × 5 s bursts in a sonicator. The sonication was interrupted for 5 s between each burst while cooling in an ice bath. Cellular debris was removed by centrifugation at 2000 × g for 10 min at 4 °C. The supernatant was collected and centrifuged at 100,000 × g for 60 min at 4 °C. The pellet was resuspended in 2 mL of 1% sodium lauroyl sarcosinate detergent (Sigma-Aldrich), prepared in 10 mM HEPES buffer (pH 7.4), and incubated at room temperature overnight with gentle rotation. The sarkosyl insoluble (SI) fraction was sedimented by centrifugation at 100,000 × g for 60 min at 4 °C and suspended in 100 μL of 0.06 M carbonate-bicarbonate buffer, pH 9.6. The sarkosyl soluble (SS) and SI fractions were stored at –20 °C until further use. Protein estimation in extracted samples was carried out by the method of Lowry et al. (14) using a commercial protein estimation kit (Merck, Genei).

2.4. Analysis of OMPs by SDS-PAGE
The OMP profile of *L. interrogans* serovar Australis was analyzed using one-dimensional SDS-PAGE as per the method described by Laemmli (15) with 12% resolving acrylamide gel and 5% stacking gel.

2.5. Western blotting of the OMPs
The proteins fractionated in the SDS-PAGE gel were transferred onto an NCM as per the technique described by Towbin et al. (16). The sensitivity of OMPs was tested by immunoblotting with the MAT-positive sera from cases of canine leptospirosis and specificity was checked with MAT-negative sera.

2.6. Dot ELISAs
The test was performed as described by Sharma et al. (17). The concentration of leptospiral OMP was adjusted to 100 μg/mL in carbonate-bicarbonate buffer (pH 9.6). One microliter of this antigen was coated onto the center of the NCM strips. The strips were allowed to dry at room temperature. To block the unbound sites in the NCM, the strips were incubated with blocking buffer at 37 °C for 1 h. The blocked NCM strips were rinsed in PBS-T, dried, and kept in a refrigerator for further use. Later, the strips were incubated in the serum sample dilution (1:100) in blocking buffer at room temperature for 1 h and washed with PBS-T for 10 min with five to six changes of washing buffer. The NCM strips were incubated with horse radish peroxidase conjugated goat anticanine IgM and IgG for IgM and IgG dot ELISAs, respectively, at a dilution of 1:2500 in blocking buffer for 1 h at room temperature. The strips were washed four times in PBS-T and the color was developed by putting the strip in the chromogenic visualization solution at room temperature. The reaction was terminated by washing NCM strips with distilled water. The NCM strips were air-dried and the reaction was observed for the development of a brown spot.

2.7. Identification of IgM and IgG immunodominant proteins
The test was performed as described above employing goat anticanine horse radish peroxidase IgM (1:2500, in blocking buffer) and IgG conjugate (1:2500, in blocking buffer) for the detection of IgM and IgG immunodominant proteins, respectively.

2.8. Statistical analysis
Statistical analysis was done using Fisher's exact test and kappa statistics with GraphPad statistical software. The results are summarized in Tables 1–5.

3. Results
Among the 91 samples tested in the present study, 63 samples were found to be positive for leptospirosis by MAT. The SDS-PAGE of the OMP-enriched SI portion revealed 8 protein bands, of which the proteins of 21, 32, and 41 kDa were found to be major and the remaining as minor (1). The SS portion of the OMPs revealed 10 protein bands of molecular weights around 20, 23, 27, 30, 32, 37, 45, 51, 61, and 84 kDa, as shown in Figure 1. The protein of 32 kDa was found to be present in both SS and SI fractions. The OMP concentration was estimated to be 0.75 mg/mL.

Table 1. Results of MAT (>1:100) and IgM dot ELISA.

	MAT-positive	MAT-negative	Total
IgM dot ELISA-positive	28	2	30
IgM dot ELISA-negative	0	61	61
Total	28	63	91

Table 2. Results of MAT (>1:400) and IgM dot ELISA.

	MAT-positive	MAT-negative	Total
IgM dot ELISA-positive	23	7	30
IgM dot ELISA-negative	0	61	61
Total	23	68	91

Table 3. Results of MAT (>1:100) and IgG dot ELISA.

	MAT-positive	MAT-negative	Total
IgG dot ELISA-positive	57	5	62
IgG dot ELISA-negative	0	29	29
Total	57	34	91

Table 4. Results of MAT (>1:400) and IgG dot ELISA.

	MAT-positive	MAT-negative	Total
IgG dot ELISA-positive	27	35	62
IgG dot ELISA-negative	0	29	29
Total	27	34	91

Table 5. Statistical analysis between different tests.

Characteristic	Tests			
	MAT (>1:100) vs. IgM dot ELISA	MAT (>1:400) vs. IgM dot ELISA	MAT (>1:100) vs. IgG dot ELISA	MAT (>1:400) vs. IgG dot ELISA
Sensitivity	100	100	100	100
Specificity	96. 83	89.71	85.29	45.31
Positive predictive value (PPV)	93.33	76.67	91.94	43.55
Negative predictive value (NPV)	100	100	100	100
Two-tailed P-value	<0.0001	<0.0001	<0.0001	<0.0001
Kappa value	0.949	0. 815	0.879	0.330
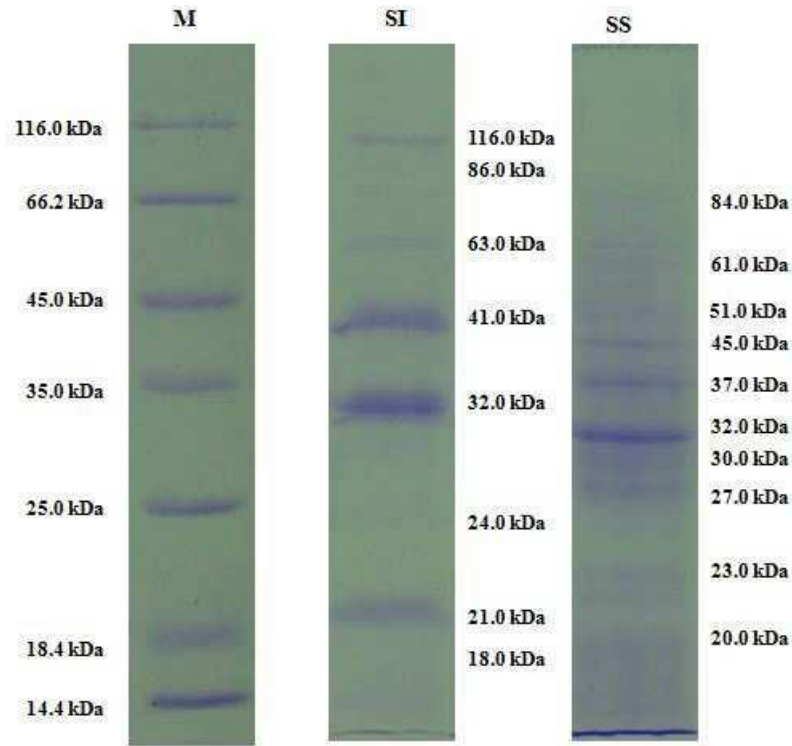
Lane M: Standard protein marker
Lane SI: Sarcosyl insoluble OMP
Lane SS: Sarcosyl soluble OMP

Figure 1. SDS-PAGE analysis of OMPs. Lane M: Standard protein marker, Lane SI: sarkosyl insoluble OMP, Lane SS: sarkosyl soluble OMP.

The reactivity of IgM dot ELISA was found to be 33% (30 samples), as shown in Figure 2, and that of IgG dot ELISA was 68% (62 samples), as shown in Figure 3. Furthermore, eight samples (9%) were found to be positive only for IgM dot ELISA (Figure 2), 40 (44%) samples were found to be positive by IgG dot ELISA alone (Figure 3), and 22 (24%) samples reacted with both IgM and IgG dot ELISA.

Among the MAT-negative (titer: <1:100) samples, IgM dot ELISA recognized two samples as positive and IgG dot ELISA recognized five samples as positive. The samples positive for IgM and IgG dot ELISA were also subjected to separate immunoblots to analyze the proteins expressed or upregulated during acute infection. Western blotting using IgM-positive sera against leptospiral OMPs recognized
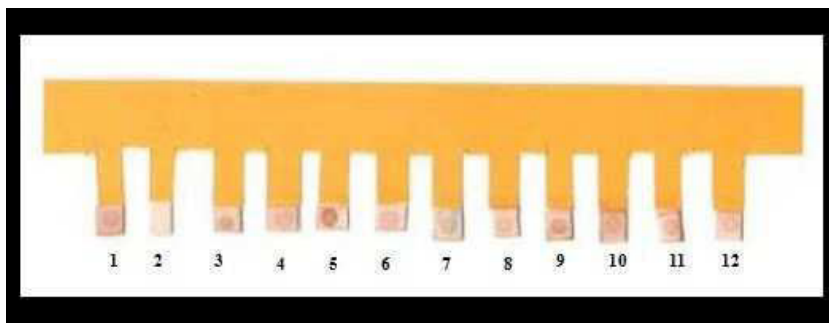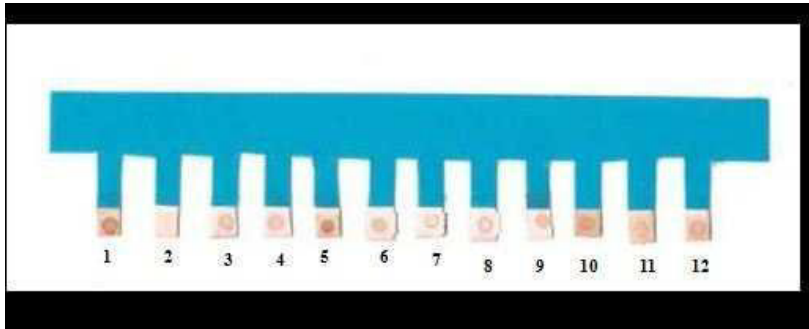

Figure 2. IgM dot ELISA. 1: Positive control, 2: negative control, 3–12: representative positive samples.

1: Positive control, 2: Negative control, 3-12: Representative positive samples

Figure 3. IgG dot ELISA. 1: Positive control, 2: negative control, 3–12: representative positive samples.

protein bands ranging from 18 kDa to 32 kDa with 18-, 21-, and 32-kDa proteins as major reactors being recognized in all acute cases (Figure 4). In IgG immunoblotting, the high-molecular-weight proteins of 32, 41, and 66 kDa were found to be the major reactors and an 86-kDa protein was a minor reactor (Figure 5).

4. Discussion
Canine leptospirosis is an important zoonotic disease of global distribution. A wide variety of serovars have been found to cause leptospirosis in dogs in different regions. Although vaccines are available, the lack of cross-protection among serovars as well as the emergence of
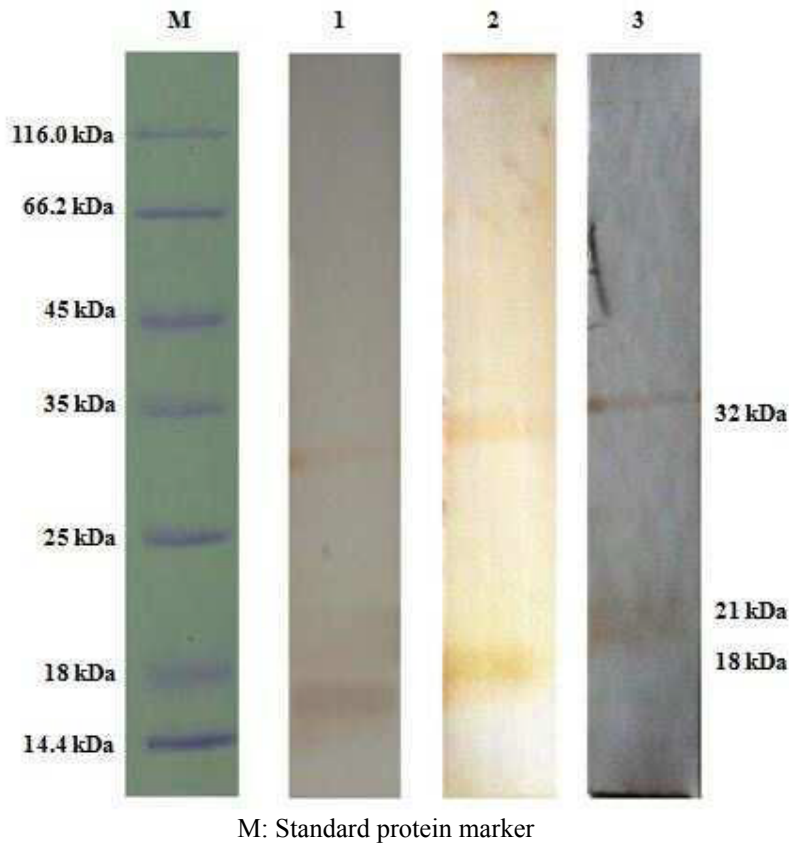
M: Standard protein marker

1, 2, 3: Representative positive samples

Figure 4. IgM immunoblotting of leptospiral OMPs. M: Standard protein marker, 1–3: representative positive samples.
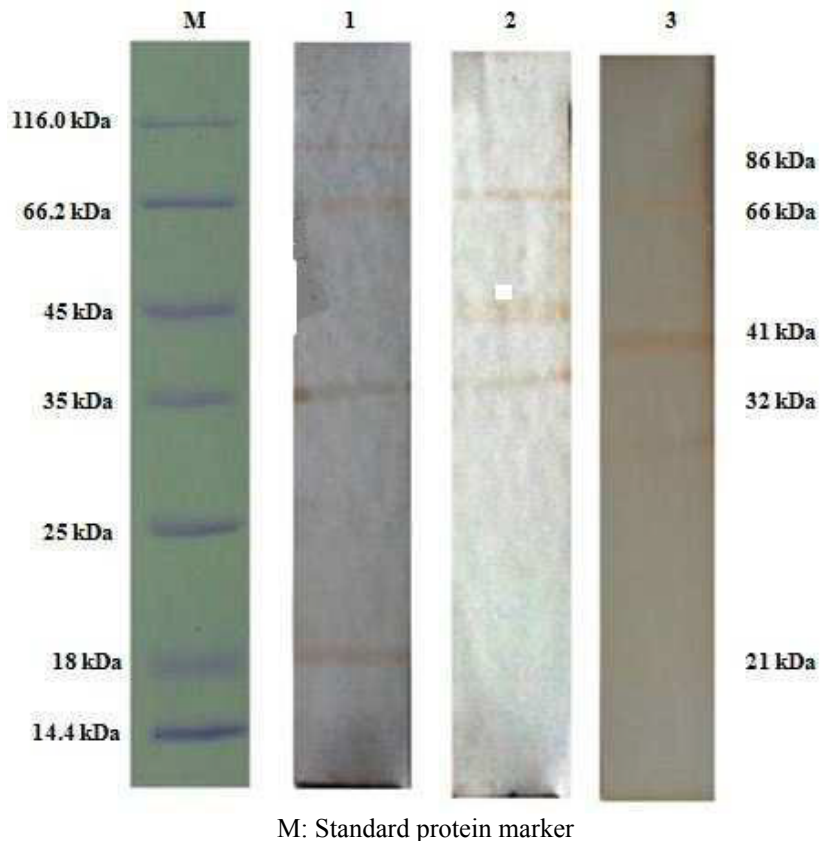
M: Standard protein marker
1, 2, 3: Representative positive samples

Figure 5. IgG immunoblotting of leptospiral OMPs. M: Standard protein marker, 1–3: representative positive samples.

new serovars is a major limitation and as a result new cases occurs regularly, especially in endemic regions like Kerala and Tamil Nadu in India. Accurate diagnosis of the disease in the initial stage is important for initiating effective therapy. MAT is the only gold-standard test for leptospirosis and the lack of suitable reference laboratories for leptospirosis warrants the development of suitable and rapid bedside diagnostics for leptospirosis.

In the present study, 63 samples were found to be MAT-positive. The SDS-PAGE of the OMP-enriched SI portion revealed 8 protein bands and the SS portion of the OMPs revealed 10 protein bands. Similar banding patterns were observed by Abhinay et al. (11), Nicholson and Prescott (13), Alves et al. (18), Cullen et al. (19), and Joseph (20), who reported 21-, 32-, and 41-kDa OMPs as major in SI fractions and a number of low-molecular-weight proteins in SS fractions. The sensitivity of OMPs was analyzed by western blotting using MAT-positive sera and a strong reaction was observed against the major OMPs, indicating the identity of the OMPs. Further, the specificity of OMPs was tested with MAT-negative sera collected from dogs with other febrile diseases and no cross-reactivity was

observed. A similar method of testing has been employed by other workers (21,22). The sensitivity and specificity of IgM dot ELISA in comparison to MAT (titer: ≥1:100) was found to be 100% and 96.83%, respectively, while that of IgG dot ELISA was 100% and 85.29%, respectively. The samples that are MAT-negative may not be true negatives since only nine *Leptospira* serovars were used in the study; this could be the reason for the low specificity of dot ELISAs in comparison to MAT (22). According to the World Health Organization (1), a MAT titer of 1:100 is positive, but in endemic areas a high initial MAT titer (≥1:400) could be considered as an acute case (23). Hence, in Kerala, which is a highly endemic area, a cut-off titer of 1:400 is more suitable for identifying acute cases by MAT. At this cut-off titer, IgM dot ELISA reacted to seven serum samples that were MAT-negative (<1:400), while, IgG dot ELISA showed reactivity to 35 MAT-negative samples. The sensitivities of IgM and IgG dot ELISA in comparison to MAT (titer: ≥1:400) were found to be 100%, whereas the specificities were found to be 89.71% and 45.31%, respectively. The low specificity of IgG dot ELISA in an endemic area might be due to the presence of background

antibodies due to past infection or vaccination and hence it could not be employed for the diagnosis of acute infections, whereas IgM dot ELISA could be the test of choice because of its high sensitivity and specificity in detecting acute cases. These results are in agreement with the findings of several other workers (17,24,25).

A strong reaction was observed in western blotting against proteins of 18, 21, and 32 kDa by IgM conjugate. The proteins of 32, 41, and 66 kDa were found to be the major reactors and the 86-kDa protein was found as a minor reactor by IgG immunoblotting. These results are in accordance with the findings of Guerreiro et al. (26) and Natarajaseenivasan et al. (27), who reported the upregulation of 32- and 41-kDa proteins in acute infection, and these could be employed for the serodiagnosis of leptospirosis. The protein of 32 kDa was found to be immunodominant as it stained more intensely in the blot and was also observed to react to both IgM and IgG antibodies. Furthermore, cloning, expression,

and functional characterization of these proteins would be helpful for the development of rapid serodiagnostic techniques, capable of differentiating acute infections from past infections or vaccinations.

In conclusion, in the present study a leptospiral OMP-based IgM dot ELISA assay was developed and found to be sensitive in comparison to MAT in detecting acute canine leptospirosis, and the IgG dot ELISA assay could be used for seroprevalence study. Furthermore, the study identified the leptospiral OMPs of 18, 21, 32, 41, and 66 kDa as immunodominant and these proteins could be used for developing better diagnostics or as vaccine candidates for controlling leptospirosis in canines.

Acknowledgment

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