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Short Communication

Evaluation and comparison of native and recombinant LipL21 protein-based ELISAs for diagnosis of bovine leptospirosis

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A 21-kDa leptospiral lipoprotein (LipL21) was evaluated for its diagnostic potential to detect bovine leptospirosis by ELISA. Both native LipL21 (nLipL21) and recombinant LipL21 (rLipL21) proteins were tested and compared regarding diagnostic efficiency, and no statistically significant difference was observed. The sensitivity of rLipL21 ELISA for 62 microscopic agglutination test (MAT) positive sera was 100% and the specificity with 378 MAT negative sera was 97.09%. Thus, rLipL21 protein-based ELISA could be used as an alternative to MAT for the diagnosis of bovine leptospirosis.

Keywords: ELISA, leptospirosis, microscopic agglutination test, nLipL21, rLipL21

Leptospirosis is an acute febrile zoonosis with a global distribution, widely recognized as an emergent or re-emergent disease [7]. The non-specific symptoms of the disease, fastidious nature of the organism, and complexities associated with the standard serological test-microscopic agglutination test (MAT) often make diagnosis difficult [9]. Recombinant protein-based ELISAs have been utilized as an alternative form of diagnostics, with high sensitivity and specificity [6]. Recombinant proteins that have been well characterized for their diagnostic potential include leptospiral lipoprotein (LipL) 32 [1], LipL41 etc [8]. Recently, a 21-kDa LipL21 was reported to be the second most abundant outer membrane protein in the *Leptospira interrogans* serovar Lai [2]. Though, this protein has been evaluated as a vaccine candidate [3]. Its diagnostic potential has not been explored. Previous analysis of the amino acid sequence of LipL21 protein in serovar Lai revealed a fatty acid incorporation site [3] which will be absent in the recombinant LipL21. The present study evaluated and compared the potential of both native and recombinant LipL21 as antigens in ELISA for the serodiagnosis of leptospirosis in bovines to ascertain whether the presence of fatty acid influence the diagnostic potential of the protein significantly.

Native LipL21 (nLipL21) was isolated in pure form following the protoco [10]. To achieve production of recombinant LipL21 (rLipL21), the gene coding for the 21-kDa protein *lipL*21 was amplified using specific primers, excluding the 54 bp long signal sequences from the 5′ end of the *lipL*21 gene. The amplified product was then cloned into pPRO.EX.HTc expression vector (Life Technologies, USA) and transformed into competent *Escherichia coli* M15 cells. The transformed cells were plated on Luria- Bertani (LB) ampicillin (100 μg/mL) agar plates (Sigma, USA) and confirmed by colony PCR. To achieve expression of the cloned gene, the transformed cells were subcultured onto LB broth and induced with 1 mM concentration of isopropyl-beta-D-thiogalactopyranoside in log growth phase and then further incubated for 37°C for 7～8 h. After the bacterial cells were pelleted by centrifugation at 8,000 × g for 10 min, 7 mL of lysis buffer (100 mM NaH_2PO_4, 10 mM Tris, 8 M urea, pH 8.0) was added to 0.5 g (wet wt.) of the bacterial pellet, which was kept at room temperature for 1 h with intermittent vortexing. Cell debris was removed by centrifugation at 8,000 × g for 15 min. The supernatant was incubated with 700 μL of Ni-NTA resin (Qiagen, USA) for 1 h in the presence of 20 mM imidazole to induce binding of polyhistidine-tagged recombinant protein to the affinity matrix. Then, the Ni-NTA resin cell lysate mixture was packed into the column, and the unbound proteins were allowed to pass through the column and discarded. The column was washed thoroughly with

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15 mL of wash buffer (100 mM NaH_2PO_4, 10 mM Tris, 8 M urea, pH 6.3), and the fusion protein was eluted with 3 mL of elution buffer (100 mM NaH_2PO_4, 10 mM Tris, 8 M urea, pH 4.5). The eluted rLipL21 protein was then dialyzed against phosphate- buffered saline (pH 7.4) overnight at 4°C, after which the concentration was estimated to be 3 mg/mL.

The rLipL21 was analyzed by Western blotting using the hyperimmune serum raised against nLipL21 following standard protocol [12]. A strong reaction was observed (Fig. 1, lane 1～3), proving the identity and immunogenicity of rLipL21. The immunoreactivity levels of rLipL21 and nLipL21 to the sera of bovines affected with leptospirosis were also tested (Fig. 1, lane 4～6), and reactivity was observed. Further, the specificity of rLipL21 was tested by further Western blot analysis employing different bacterial organisms *viz*., *Brucella abortus*, *Campylobacter jejuni*, *Mycoplasma bovis*, *Pasteurella multocida*, and *Salmonella typhimurium*, using the hyperimmune sera raised against rLipL21, and no cross-reactivity was observed (Fig. 1, lane 7～12).

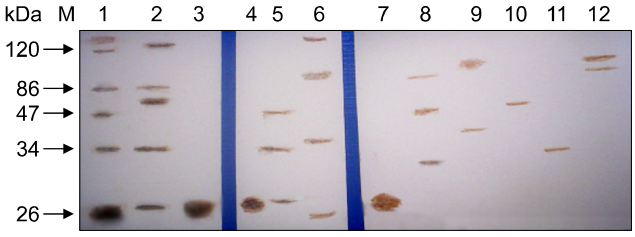

Fig. 1. Western blot analysis. Lane 1-3: checking the identity and immunogenicity of rLipL21 using nLipL21 hyperimmune serum, Lane 1: induced *Escherichia* (*E.*) *coli* cell lysate, Lane 2: non-induced *E. coli* cell lysate, Lane 3: purified rLipL21, Lanes 4-6: analysis of the immunoreactivity levels of rLipL21 and nLipL21 to MAT positive field cattle sera, Lane 4: purified rLipL21 protein, Lane 5: sarcosyl-soluble portion of OMP, Lane 6: sarcosyl-insoluble portion of OMP, Lanes 7-12: checking the cross-reactivity of rLipL21 hyperimmune sera to various bacteria, Lane 7: purified rLipL21, Lane 8: *Brucella abortus* culture lysate, Lane 9: *Campylobacter jejuni* culture lysate, Lane 10: *Mycoplasma bovis* culture lysate, Lane 11: *Pasteurella multocida* culture lysate, Lane 12: *Salmonella typhimurium* culture lysate.

A total of 440 sera samples collected from cattle suspected to have leptospirosis from different states of India were screened by employing MAT according to standard procedures [5]. The sera samples were collected from unvaccinated cattle between 2～4 weeks after showing symptoms of blood in milk as well as reproductive problems such as repeat breading, high number of services per conception, early embryonic loss, and irregular estrus cycles. Nine different leptospiral serovars *viz*., *Leptospira* (*L.*) *interrogans* serovars Canicola, Hardjo, Pomona, Pyrogenes, and Icterohaemorrhagiae along with *L. borgpetersenii* serovars Tarassovi, Javanica, Sejroe, and Ballum, were used to screen the sera samples using MAT. Sixty-two samples (14.1%) with a titer of 1 : 100 or above were designated as positive for the presence of leptospiral antibodies [13]. In addition to the 440 sera samples tested, negative control sera (n = 45) obtained from healthy calves (n = 20) and cattle with diseases other than leptospirosis (n = 25), which were found to be sero-negative for leptospiral antibodies using MAT at a 1 : 50 dilution, were used in this study for the standardization of ELISA.

ELISAs were standardized for both rLipL21 and nLipL21 according to standard procedures [4], and the optimum

Table 1. Comparison of rLipL21 ELISA with nLipL21 ELISA

	Average optical density	
	Positive samples (n = 46)	Negative samples (n = 20)
rLipL21 ELISA	0.44 ± 0.03	0.093 ± 0.009
nLipL21 ELISA	0.463 ± 0.09	0.102 ± 0.089

Table 2. Evaluation of rLipL21 ELISA for detection of anti-leptospiral antibodies in bovine sera as compared to MAT

		MAT				χ^2	*p*-value	C.I.
		Positive	Negative	Total	Uncorrected	362.824	0.00000	
					Mantel-Haenzel	355.842	0.00000	
					Yates Corrected	361.999	0.00000	
rLipL21 ELISA	Positive	62	11	73	Kappa	0.904	0.00000	
	Negative	0	367	367	Error Kappa	0.028	0.00000	
	Total	62	378	440	Sensitivity	100%		95～100%
					Specificity	97.1%		99%
					Accuracy	97.5%		99%
					PPV	84.9%		99%
					PNV	100%		95～100%

antigen concentrations were 75 ng/well and 100 ng/well, respectively. The cut-off values for interpretation of the ELISAs using the negative sera samples (n = 45) were determined as previously described [1] to be 0.158 and 0.188 for rLipL21 and nLipL21, respectively.

Comparison of nLipL21 and rLipL21-based ELISAs was performed employing 20 MAT negative control sera and 46 MAT positive sera (MAT titer $\geq$ 1 : 800). The ELISAs were carried out by coating the two sets of plates simultaneously with two different antigens, *i.e.*, one set of plates coated with rLipL21 antigen and the other set with nLipL21 antigen. The sera were used at 1 : 100 dilutions in the same order in the two sets of plates using the same reagents. The OD values obtained for the positive and negative samples at 492 nm were analysed using "Z" test and "t" test. All samples gave similar results in both ELISAs, and the only difference observed was that the nLipL21 plate showed a slightly higher OD value than the rLipL21 plate (Table 1). Upon comparison, the OD values for the positive and negative samples were not statistically significant ($p \leq 0.05$).

Out of 440 bovine sera examined, 73 (16.6%) showed OD values higher than the cut-off value and thus were considered to be positive for leptospirosis using rLipL21 ELISA. The sensitivity, specificity, and accuracy of rLipL21 ELISA when compared to MAT were calculated as previously reported [11] to be 100, 97.1, and 97.5%, respectively, at 95% confidence intervals (Table 2).

The present study compared the diagnostic efficiency of rLipL21 employed in ELISA with that of nLipL21. We observed that rLipL21 was equally good as nLipL21 in detecting leptospiral antibodies. Further, in comparison with the volume of work associated with the extraction of nLipL21, rLipL21 could be considered as more efficient due to the ease with which it can be produced in bulk amounts. Regarding its diagnostic efficacy compared to MAT, rLipL21 demonstrated 100% sensitivity, 97.1% specificity, and 97.5% accuracy. The slightly low specificity value could be due to the fact that all the sera interpreted as MAT negative might not actually be negative, since only nine *Leptospira* serovars were used as antigens for antibody detection [8]. The analyzed results were considered statistically significant ($p \leq 0.05$), and the obtained kappa value of 0.9 suggests perfect agreement between rLipL21 ELISA and MAT in detecting bovine leptospiral antibodies. Thus, it can be concluded that rLipL21 is appropriate for large-scale confirmative screening of bovine leptospirosis using ELISA, and its diagnostic efficacy is independent of fatty acid incorporation.

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