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REGULAR ARTICLES

Comparative evaluation of recombinant LigB protein and heat-killed antigen-based latex agglutination test with microscopic agglutination test for diagnosis of bovine leptospirosis

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Abstract This study aimed to develop latex agglutination test (LAT) using recombinant leptospiral immunoglobulin-like protein (LigB) (rLigB) antigen and compare its diagnostic efficacy with LAT using conventional heat-killed leptospiral antigen and microscopic agglutination test (MAT) in diagnosing bovine leptospirosis. The PCR-amplified 1053-bp *ligB* gene sequences from *Leptospira borgpetersenii* Hardjo serovar were cloned in pET 32 (a) vector at *EcoRI* and *NotI* sites and expressed in BL21 *E. coli* cells as fusion protein with thioredoxin (−57 kDa) and characterized by SDS-PAGE and immunoblot. Out of 390 serum samples [cattle (n=214), buffaloes (n=176)] subjected to MAT, 115 samples showed reciprocal titre≥100 up to 1600 against one or more serovars. For recombinant LigB protein/antigen-based LAT, agglutination was observed in the positive sample, while no agglutination was observed in the negative sample. Similarly, heat-killed leptospiral antigen was prepared from and used in LAT for comparison with MAT. A two-sided contingency table was used for analysis of LAT using both the antigens separately against MAT for 390 serum samples. The sensitivity, specificity and positive and negative predictive values of recombinant LigB LAT were found to be 75.65, 91.27, 78.38 and 89.96 %, respectively, and that of heat-killed antigen-based LAT were 72.17, 89.82, 74.77 and 88.53 %, respectively, in comparison with MAT. This developed test will be an alternative/complementary to the existing battery of diagnostic assays/tests for specific detection of pathogenic *Leptospira* infection in bovine population.

Keywords *Leptospira* · LigB · Heat-killed antigen · Latex agglutination test

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Introduction

Leptospirosis is presumed to be the most widespread zoonotic disease in the world which results in high morbidity and considerable mortality in areas of high prevalence (Pappas et al. 2008) It is also an economically important bacterial infection of livestock that causes reproductive losses due to abortions, stillbirths, infertility, weak newborns, decrease in their growth rate and milk production and non-reproductive losses due to septicaemia and nephritis (Grooms 2006), caused by pathogenic spirochetes of the genus *Leptospira*. It is prevalent in most of the tropical and subtropical countries of Asia, Africa and Latin America. In India, it is mostly endemic in coastal regions but virtually common in all states of India (Srivastava 2006). The current knowledge about epidemiology and control, as well as the available diagnostic tools and the commercial vaccines, may not be adequate for the endemic regions, which leads to a frustrating scenario of endemicity and difficulties on the control of the disease (Lilenbaum and Martins 2014).

Microscopic agglutination test (MAT) is the 'gold standard' reference test for leptospirosis. Despite its widespread usage and international recognition, it has a number of limitations. These include the need to use hazardous live bacteria, expertise to test each serum sample against multiple serovars of this organism and being time consuming. MAT is inadequate for rapid case identification since it can only be performed in a few reference laboratories and requires analysis of paired sera to achieve sufficient sensitivity. Farmers living under poor socio-economic conditions and in rural areas cannot utilize the service rendered by urban-based reference laboratories. Rapid diagnostic tests are also not available for diagnosing bovine leptospirosis.

Many researchers (Smits et al. 2000, 2001; Shekatkar et al. 2010) tried using leptospiral antigens for developing rapid tests. With the introduction of recombinant DNA techniques, desired recombinant proteins have been used in place of conventional antigens. One such antigen which has been expressed only by pathogenic leptospires is leptospiral immunoglobulin-like protein B (LigB).

Hence, this study is framed to develop latex agglutination test (LAT) using recombinant LigB (rLigB) antigen and compare its diagnostic efficacy with conventional heat-killed leptospiral antigen and MAT in diagnosing bovine leptospirosis including serum samples from buffaloes.

Materials and methods

Leptospira strains

Leptospira borgpetersenii Hardjo serovar was used as a seed culture for extracting DNA and heat-extracted proteins. Ellinghausen, McCullough, Johnson and Harris (EMJH) medium was used to grow and maintain the culture. Serovars Australis, Bangkinang, Canicola, Hardjo, Hebdomadis, Icterohaemorrhagiae, Pyrogens and Tarassovi which were procured from the Indian Council of Medical Research (ICMR), Port Blair, and maintained in the *Leptospira* laboratory NIVEDI were used in MAT.

Serum samples

A total of 390 serum samples [crossbred cattle (n=214), non-descriptive river buffaloes (n=176)] were collected from randomly selected seven farms in southern India. These animals were kept for dairy purpose. None of the animals were vaccinated against leptospirosis. Among them, 14 samples had reproductive problems like abortion or retention of foetal membranes.

Hyperimmune serum in rabbits

Sera collected from four rabbits were subjected to MAT in which one rabbit (rabbit 1) turned to be positive and it was used as positive control. MAT-negative rabbits were used for raising hyperimmune serum against rLigB protein (1 mg/mL) expressed in *E. coli* (rabbit 2) and heat-killed *L. borgpetersenii* Hardjo serovar (-1×10^8 cells/mL) (rabbit 3) and as negative control (rabbit 4). Each antigen was mixed with 1 mL of Freund's complete adjuvant separately and injected into the respective rabbits. Two boosters were given every 2 weeks with Freund's incomplete adjuvant. Hyperimmune serum was tested and collected 1 week after the final booster injection (Senthilkumar et al. 2007).

Recombinant truncated LigB protein expression

The *ligB* gene is of −5.7 kb (5670 bp) in length having molecular weight of −212 kDa. The primers were designed (F-5′-GCTTCTAGCGAGAATATAATAGG-3′; R-5′-GTGTATTT GTAATACCTTTACTTC-3′) with *EcoRI* and *NotI* restriction sites along with His tag for producing recombinant protein from N-terminal *ligB* gene (883–1935 nt) sequences. PCR was carried out for amplifying partial *ligB* gene from DNA extracted from *L. borgpetersenii* Hardjo serovar culture. Further, the amplified PCR product was cloned into a pET32a vector and expressed in a BL21 strain of *E. coli* (*D3 PLyS*). The expression was induced at 30 °C using 1 mM isopropyl-β-D-thiogalactoside (IPTG). Purification of the recombinant protein was performed with a cobalt affinity column (Thermo Scientific, USA) as per the procedure described by the manufacturer. Sodium dodecyl sulphate (SDS)-PAGE was carried out as per standard procedure followed by Western blot to confirm the expressed protein by using positive standard serum (serum from which *Leptospira* has been isolated). The thioredoxin portion of the fusion protein from the pET vector was also purified using the cobalt affinity column to check for reactivity by coating on latex beads.

Preparation of heat-killed leptospiral antigen

L. borgpetersenii Hardjo was grown in EMJH medium for 10–12 days at 30 °C in a shaking incubator and used for antigen preparation. Briefly, 5 mL of sterile diluted Tween 80 (1:10) to 500-ml culture was added (inoculated 5 days back) and further incubated for another 5–6 days. Before harvesting, abundant growth and purity were checked. After ensuring the quality of the culture, leptospires were killed with formalin (0.5 % final conc.). After 1 h, killed *Leptospira* culture was heated in boiling water for 30 min, shaking every 5 min. After cooling to room temperature, the culture was centrifuged for 30 min at 10,000*g* and the supernatant was used as antigen (Vijayachari et al. 2007).

MAT

Live leptospires (representing eight serovars belonging to eight serogroups) cultured in EMJH media of 4 to 7 days old were added to 1:100 and its multiple dilutions of serum specimens in 96-well U-bottomed microtitre plates and incubated at 37 °C for 2 h; along with negative control, titres of all the eight serovars were screened for MAT. Agglutination was examined by dark-field microscopy at a magnification of ×20 and ×40. The titre was calculated as the reciprocal of the highest dilution that agglutinated at least 50 % of the cells for each serovar (Cole et al. 1973). A MAT is considered positive at titre of ≥100.

LAT

LAT was performed as per the method of Smits et al. (2000) with some modifications. Coloured latex beads (0.8-μ diameter, Sigma USA) were washed in 0.025-M 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer, pH 6.0, twice by centrifugation at 8000 rpm for 2 min each time. Ten per cent of this was coated with equal volume of antigen (25 μg/dL) using 0.025-M MES buffer, pH 6.0, and kept at 37 °C for 6 h with constant shaking. The sensitized beads were centrifuged at 8000 rpm for 3 min, and the pellet was re-suspended as 1 % suspension in MES buffer containing 5 mg/mL of bovine serum albumin (BSA). The latex beads were left overnight at 37 °C with constant shaking. They were centrifuged as before and re-suspended in MES buffer containing 0.5 mg/mL of BSA and 0.1 % sodium azide as 0.25 % suspension. This was kept at 4 °C until use. Similarly, the latex beads are sensitized with heat-killed antigen from leptospires. Control latex (non-coated and coated with only thioredoxin fusion protein) was prepared to screen for non-specific reactions.

On a glass slide, 20 μL of latex beads was taken and 20 μL of serum was added near the latex. Serum was mixed thoroughly with the latex beads and spread uniformly in a circular fashion. The slide was rotated slowly and gently. The results were recorded in 3 min. In stronger reactions, fine granular clumps tend to settle at the edge of the circle. Agglutination that occurs beyond 3 min may be due to evaporation of reagents and should not be considered as positive. Standardization of LAT was done with positive and negative serum of rabbit.

Statistical analysis

The sensitivity of a diagnostic method is the proportion of true positives that are detected by the test. The specificity of the method is the proportion of true negatives that are detected. Positive predictive value is the proportion of positives that are true positives, and negative predictive value is the proportion of negatives that are true negatives.

$$\text{Sensitivity} = a/(a+c);\ \text{specificity} = d/(b+d)$$

$$\text{Positive predictive value} = a/(a+b);\ \text{negative predictive value} = d/(c+d)$$

where a = the no. of true positives, b = the no. of false positives, c = the no. of false negatives and d = the no. of true negatives.

Statistical analyses of the tests were carried out through software Win Episcope 2.0 (Thrusfield et al. 2001).

Kappa statistic has been performed for rLigB-based LAT and heat-killed leptospiral-protein-based LAT. Kappa values have been estimated at 95 % confidence interval using the Win Episcope 2.0 software and the results are compared.

Results

Out of 390 serum samples subjected to MAT, 115 samples [cattle (n=108), buffaloes (n=7)] showed reciprocal titre ≥100 up to 1600 against one or more serovars (71, 15, 16, 10, 2 and 1 samples against 1, 2, 3, 4, 5 and 6 serovars, respectively). The rest of the samples (n=275) had no significant titre.

PCR amplicon of size 1053 bp was obtained from DNA extracted from *L. borgpetersenii* Hardjo serovar. On further cloning in pET 32 (a) vector and expression in BL21 cells, recombinant protein along with fusion protein (−57 kDa) was expressed, which was characterized by SDS-PAGE (Fig. 1) and Western blot. Heat-killed leptospiral antigen was also extracted following the protocol. Both rLigB and heat-killed leptospiral antigens were further used in LAT (Fig. 2), and agglutination was observed in the positive sample and no agglutination was observed in the negative sample. Similarly, no agglutination was observed with non-coated and thioredoxin-fusion-protein-coated latex beads.

A 2×2 contingency table was presented for LAT using both the antigens separately against MAT for 390 serum samples (Table 1). The sensitivity and specificity of rLigB LAT against MAT were 75.65 % (67.808–83.496; confidence interval (CI) at 95 %) and 91.27 % (87.937–94.609; CI at 95 %), respectively, and positive and negative predictive values were 78.38 % (69.351–85.398; CI at 95 %) and 89.96 % (85.674–93.115; CI at 95 %) with the kappa value of 0.676. The sensitivity and specificity of heat-killed Ag LAT were 72.17 % (63.983–80.365; CI at 95 %) and 89.82 % (87.937–94.609; CI at 95 %), respectively, and positive and negative predictive values were 74.77 % (65.480–82.322; CI at 95 %) and 88.53 % (84.056–91.909; CI at 95 %) with the kappa value of 0.626.

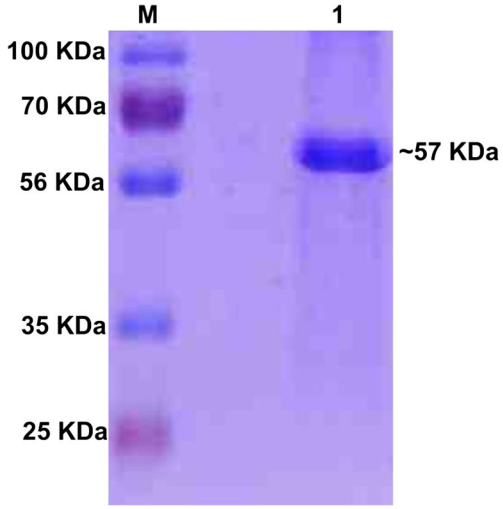

Fig. 1 SDS-PAGE profile of expressed rLigB protein. *Lane M* marker; *lane 1* rLigB elute after cobalt affinity column chromatography

Serum from rabbit 1 (MAT positive) showed reactivity against MAT, rLigB LAT and heat-killed antigen LAT, whereas that of rabbit 2 (hyperimmunized against rLigB) showed reactivity only against rLigB. Rabbit 3 (hyperimmunized against heat-killed antigen) has shown serum reactivity against MAT and heat-killed antigen LAT, and rabbit 4 (control) has not shown any serum reactivity against the above tests.

Out of 14 cattle having reproductive problems like abortion and retention of foetal membranes, eight animals were showing significant MAT titre (against serovars Autumnalis, Pyrogenes, Australis, Canicola, Tarrassovi, Sejroe, Hebdomadis and Icterohaemorrhagiae); nine animals were showing positive agglutination based on LigB and heat-killed Ag LAT. All the animals were negative for brucellosis by a Rose Bengal plate test (RBPT) (unpublished data).

Table 1 Two-sided contingency table for the presence of leptospiral antibodies by LAT (rLigB, heat-killed antigen) against MAT

	LAT	MAT		Total
		Positive	Negative	
Positive	rLigB LAT	87	24	111
	Heat-killed antigen LAT	83	28	
Negative	rLigB LAT	28	251	279
	Heat-killed antigen LAT	32	247	
	Total	115	275	390

Discussion

Leptospirosis is ubiquitous throughout the world. But, it is documented in places only where there are experts in leptospirosis and astute and aware medical and veterinary practitioners and epidemiologists, and adequate specialized laboratory facilities for accurate diagnosis are available (Faine et al. 2000). As leptospirosis is frequently underdiagnosed due to the difficulty of performing both culture and the reference serological test, MAT, it is required to develop alternative tests. Further, these alternative tests, if they can be performed under field conditions without the requirement of sophisticated equipment and skilled manpower, can then support early diagnosis, early treatment and further prevention of leptospirosis. One such test is LAT, and to avoid handling pathogenic cultures, recombinant-antigen-based tests are preferred over conventional antigen tests.

In the present study, the conserved region of the LigB protein (which is produced further based on recombinant DNA technology) has been selected as the suitable diagnostic candidate antigen, since this region is present in almost all

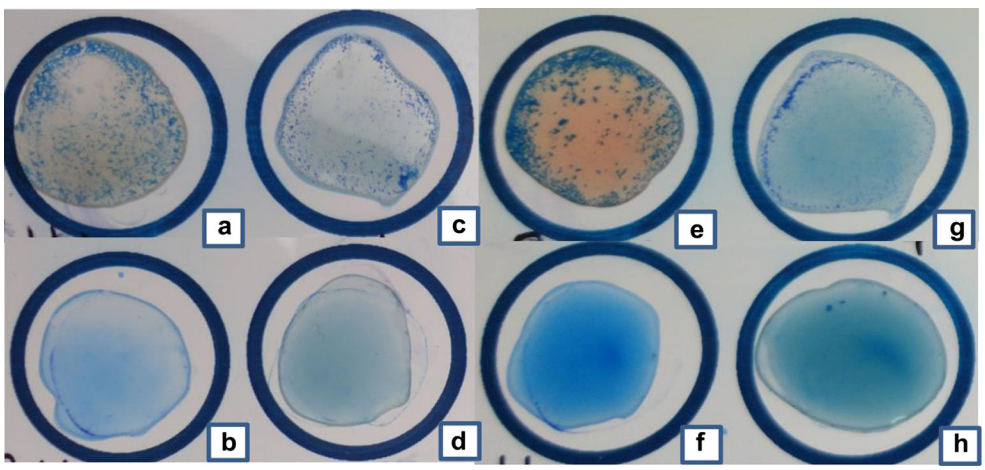

Fig. 2 Latex agglutination test for diagnosis of leptospirosis from serum of animals. **a** Rabbit positive serum and **b** rabbit negative serum with rLigB-coated latex beads, respectively. **c** Cattle positive serum and **d** cattle negative serum with rLigB-coated latex beads, respectively. **e** Rabbit positive serum and **f** rabbit negative serum with heat-killed antigen-coated latex beads, respectively. **g** Cattle positive serum and **h** cattle negative serum with heat-killed antigen-coated latex beads, respectively

pathogenic *Leptospira*, whereas it is absent in non-pathogenic *Leptospira* (Palaniappan et al. 2004). In addition, the LigB protein is expressed only in vivo and not in vitro culture of leptospires. Also, the conserved region of leptospiral immunoglobulin-like A and B proteins as DNA vaccine elicited a prophylactic immune response against leptospirosis in hamsters (Forster et al. 2013).

On expression of cloned partial *ligB* gene in the pET 32 (a) vector in BL21 cells and upon comparison of protein profiles of recombinant clones with that of control (vector pET 32 (a) and BL21 cells), after 4 h of induction, MW size of 57-kDa expressed protein was observed in case of recombinant clone, which is co-relating well with the predicted size of 36 kDa with the fusion protein thioredoxin (18 kDa) in the vector. The intensity of bands increased gradually up to 8 h post-induction. In un-induced and control cultures, such specific bands were not observed even after prolonged incubation. The optimum time of harvest was 6 h post-induction (PI), and not much difference was observed in the rate of expression at either 6 or 7 h PI, but thereafter, there was a reduction in expression. This reduction could be attributed to autolysis of bacterial cells.

The purified rLigB and heat-killed antigen-based LAT were standardized initially using hyperimmune sera raised in rabbits followed by positive and negative sera of rabbit and subsequently standardized in positive and negative cattle sera.

So far, LAT has been developed using conventional antigens and recombinant antigens and tried in human as well as animal serum samples, in case of human samples LAT against conventional antigens (on comparison with MAT). Smits et al. (2000) reported 82.3 % sensitivity and 94.6 % specificity (using heat-stable, broadly reactive antigen prepared from the pathogenic strain Lely 607); Shekatkar et al. (2010) reported 90.62 % sensitivity and 91.96 % specificity (using five different prevalent leptospiral strains). In dridot LAT (Smits et al. 2001), the sensitivity of the assay varied with the stage of the disease and was 72.3 % for samples collected during the first 10 days of the illness and 88.2 % for samples collected at a later stage. The specificity was 93.9 and 89.8 %, respectively. Senthilkumar et al. (2008a) used a recombinant rLipL41 antigen for latex agglutination, and the overall sensitivity and specificity of this test were found to be 89.7 and 90.45 %, respectively. Anti-LigB assay had a sensitivity of 81 % for sera from patients with less than 7 days of illness. The specificity of the assay was 93 to 100 and 90 to 97 % among sera from healthy individuals and patients with diseases, respectively (Croda et al. 2007). Chalayon et al. (2011) concluded that rLipL32 and rLoa22 as antigen gave high accuracy when rLipL21, rLipL32, rLipL41 and rLoa22 antigens were used. A commercial LAT for human leptospirosis showed 90 % sensitivity and 10 % specificity, which are unacceptable for diagnosis of leptospirosis (Effler et al. 2002).

In case of animal sera, using rLipL41 protein, the sensitivity and specificity of IgG-ELISA and LAT were 86.84 and 93.16 % and 95.42 and 98.33 %, respectively, for bovine serum samples (Senthilkumar et al. 2010). In the rOmpL1-based LAT, the sensitivity and specificity were observed as 93.05 and 91.95 %, respectively, in the diagnosis of canine leptospirosis (Senthilkumar et al. 2008b).

With respect to Lig antigen for the diagnosis of leptospirosis, Palaniappan et al. (2004) reported that the overall sensitivity of recombinant Lig antigens rCon, rVarA and rVarB was 76, 41 and 35 %, respectively, to MAT-positive canine sera (MAT >1600) in a kinetic ELISA. In rLigB-based LAT in bovine samples, it showed sensitivity and specificity of 93.68 and 92.31 %, respectively (Deneke et al. 2014). The sensitivity and specificity of ELISA were 80.0 and 87.2 %, respectively, compared to those of MAT utilizing a recombinant LigA fragment comprising the 4th to 7.5th repeat domain (LigACon4-7.5) as a diagnostic antigen for equine leptospirosis (Saleem et al. 2013), whereas Ye et al. (2014) reported sensitivity and specificity of 82.39 and 86.15 %, respectively, based on rLigACon4-8 ELISA. In the case of rLig-based ELISA for diagnosis of canine leptospirosis, the sensitivity and specificity of 84.5 and 76.9 %, respectively, were reported (La-Ard et al. 2011). Thome et al. (2014) observed sensitivity ranging from 89 to 95 % and 70 % specificity in rLigB, rLigA and rLipL32-based multi-antigen Print Immunoassay for diagnosis of canine leptospirosis.

Thus, a very wide variation has been observed in the sensitivity and specificity in the tests to diagnose leptospirosis with respect not only to the nature of the antigen, test format and species intended but also to the period of exposure and population under study (random vs diseased population). Ramadass et al. (1999) observed a non-significant reduction in sensitivity of LAT against ELISA in human and animal sera. Hull-Jackson et al. (2006) also observed variation in the sensitivity and specificity and recommended to evaluate assays for use with different populations. In the present study also, we have obtained less sensitivity and specificity to that of the study conducted by Deneke et al. (2014), which may be due to the test population disease status and geographical location. In their study, they have targeted animals having reproductive problems and the study population belongs to northern and eastern India, whereas in the present study, diseased animals were not targeted and study population belongs to southern India. In this study also, sensitivity increased to 87.5 % in suspected population (animals with abortion and other reproductive problems) although the specificity suffered, which may be due to the fact that all the sera interpreted as MAT negative might not actually be negative since only eight *Leptospira* serovars were used as antigens for antibody detection. Less number of buffalo serum samples was positive for leptospiral antibodies may be due to well maintenance of farms as well as animals were not allowed for wallowing. It

is also pertinent to mention that culture plus MAT is an imperfect gold standard against which to compare alterative tests for the diagnosis of leptospirosis (Limmathurotsakul et al. 2012).

Based on kappa value analysis, good agreement (>0.6) exists between rLigB-based LAT and heat-killed leptospiral-protein-based LAT with MAT. Hence, it can be used at field level where there is no facility for MAT and other diagnostic facilities available for screening of leptospirosis. In addition, it can complement already existing tests. Positive and negative predictive values may vary over the prevalence rate.

As such, there is no much difference between rLigB and heat-killed antigen-based LAT in terms of sensitivity and specificity, but when the objective is differentiating vaccinated and infected animals, rLigB-based tests may be preferred even over the MAT. This is evident from non-reactivity of serum (obtained from rabbit injected with heat-killed leptospiral antigen) against rLigB antigen, whereas reactivity was observed with heat-killed antigen and MAT.

In our study involving abortion samples, isolation of leptospires from mother and aborted foetuses has not been attempted due to strategic difficulties, and hence, gold standard diagnosis could not be established. However, from the associated tests such as MAT (which can be considered as a serological gold standard test till now) and LAT developed under this study, they suggest a strong relationship between the presence of leptospiral antibodies and abortions. Further detailed study is needed with more number of clinical samples to ascertain the percentage of leptospiral contribution in causing reproductive problems especially abortion and stillbirth in bovines. However, an active surveillance approach should also include asymptomatic animals because they may shed the bacterium to the environment and consequently play an important role in the epidemiology of leptospirosis (Hamond et al. 2015). Tropical regions have many particularities such as climate or topography, management factors and husbandry practices that affect the occurrence of the infection, and its routes and disease severity (Martins and Lilenbaum 2013).

In conclusion, when routine vaccination to effectively curb bovine leptospirosis is practiced, rLigB-based LAT tests can be of paramount importance compared even to MAT, with widespread field applicability. Further, it can be used as an initial screening test which can be complemented by other tests. Also, this study appears to be the first to use serum samples from buffaloes along with that of cattle for evaluating LAT using rLigB.

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Statement of animal rights The use of rabbits in this research has been approved by the Institutional Animal Ethics Committee.?

Conflict of interest The authors declare that they have no conflict of interests.

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