Prospective evaluation of the diagnostic potential of LipL32 based latex agglutination test for Bovine leptospirosis

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Abstract. The Livestock Sector of India plays an important role in livelihood security and socioeconomic development of rural households. Leptospirosis is an important zoonotic disease responsible for septicaemia, interstitial nephritis, jaundice, abortion, reproductive problem in most of the animal species. Reproductive disturbances in bovine population is most often restricted to investigation of brucellosis, however apart from brucellosis, there are many undiagnosed diseases like leptospirosis that takes a toll in the reproductive anomalies of cattle and buffalo. Hence, the present study was elucidated to screen the seroprevalence of Leptospira in cattle and buffalo in various hamlets of North India using a user friendly screening test i.e. LipL32 latex agglutination test. The overall seropositivity was found to be 26.01% (230/884) in case of bovine in this study and the LipL32 LAT showed a profound sensitivity and specificity with level of 94.97% and 99.53% respectively.

INTRODUCTION

The Livestock Sector of India plays an important role in livelihood security and socioeconomic development of rural households. Despite its numero-uno status in bovine population and milk production, India is facing challenges in terms of milk productivity and compromised revenue generation (DADF, 2016). Infectious diseases such as brucellosis, leptospirosis are a bane for dairy industry and are responsible for lion share of reproductive failure in bovines. Leptospirosis is an important zoonotic disease responsible for septicaemia, interstitial nephritis, jaundice, abortion, and reproductive problem in most of the animal species (Adler & Moctezuma, 2010). Disease in bovines is usually caused by *Leptospiraborgpeterseni* serovar Hardjo for which they are carrier animals (Dhaliwal *et al.*, 1996). However, the severe form of the disease occurs when bovines are infected with non-host adapted serovars (Dhaliwal *et al.*, 1996). In India many a time's reproductive anomalies in bovine rested on serological or molecular diagnosis of brucellosis. However apart from brucellosis, there are several infectious agents e.g. leptospirosis, campylobacteriosis, trichomoniasis, candidasis, aspergillosisetc involved in reproductive disturbances in bovine population (Ellis, 1994). But these diseases are under diagnosed or under reported. Reports suggesting co-infection involving brucellosis and leptospirosis are very limited with respect to Indian sub-continent.

Outer membrane proteins with their lion share in the bacterial outer membrane (OM) play a significant role in adhesion, immunity and pathogenicity. Transmembrane (TM) proteins, lipoproteins and peripheral proteinsare the three classes of leptospiral outer membrane proteins (OMPs) identified so far (Haake *et al.*, 2000; Nally *et al.*, 2005; Cullen *et al.*, 2005). Among them, several OMPs have been identified and characterized for their usage in diagnostics or as vaccine candidates (Haake & Matsunga, 2002; Veerapandian & Natarajaseenivasan, 2015). LipL32, the most abundant antigen which has been extensively studied for the past two decades (Veerapandian & Natarajaseenivasan, 2015). The usefulness of highly conserved lipoprotein of pathogenic leptospires, LipL32 has already been investigated in human and animal sera (Bomfim *et al.*, 2005; Dey *et al.*, 2004).

The aim of this study was to determine the sero-prevalence of anti leptospiral antibodies by using LipL32 LAT in bovine population in certain hamlets of Northern India to assess the status of the disease and to assist in decision making regarding control to be adopted so that, the goal of augmenting milk productivity of bovines can be achieved in near future.

MATERIALS AND METHODS

Bovine serum samples were submitted to *Leptospira* Laboratory, Veterinary Bacteriology & Mycology, Indian Veterinary Research Institute from different dairy farms located in Madhya Pradesh, Uttar Pradesh, Haryana and Uttaranchal state of Northern India with previous history of abortion, repeat breeding, haemorrhagic mastitis etc. Most of the animals were kept under small holder unit under semi intensive rearing practices and breeds of cattle include crossbreed Holstein Friesian, Jersey, Red Sindhi and Sahiwal etc. and Murrah breeds of buffalo.

Blood samples were collected from 884 (819 Cattle + 65 Buffalo) animals by jugular venipuncture in vacutainer tubes. Blood samples were chilled and transported to the laboratories where they were centrifuged at 1500g for 10min. Separated serum was stored in 1.5 ml eppendorff tubes and kept at -20°C for further evaluation.

The collected serum samples were tested by MAT for detecting anti leptospiral antibodies (OIE, 2012). A 5–7 day old culture of 12 leptospiralserovars viz. Australis, Autumnalis, Ballum, Canicola, Grippotyphosa, Hardjo, Hebdomadis, Icterohaemorrhagiae, Pomona, Pyrogenes, Javanica and Tarrasovi grown in Ellinghausen Mccullough Johnson Harris (EMJH) medium containing approximately 2×10^8 live Leptospira/ml were used as antigen in the study. Sera showing titre more than or equal to 1:100 were considered to be positive. Seroreactivity was recorded with titre ranging from 1: 100 to 1: 400.

DH5α cells harbouring LipL32 gene in pPROHTb plasmid available with GEB Laboratory of Indian Veterinary Research Institute was used for expression of recombinant LipL32 protein (rLipL32). The expression of rLipL32 (Fig. 1) was achieved by inducing DH5α using 1mM IPTG during early log phase of growth. The log phase of growth was determined by a spectro-photometric reading of 0.5-0.7. After keeping the induced bacterial culture overnight, the bacterial pellet obtained by centrifugation was lysed using lysis buffer (pH 8.0) & then again centrifuged twice. The supernatant obtained was passed through a Nickel-Nitrilotriacetic acid affinity chromatography column. The column was later washed with Wash buffer (pH 6.3) to remove any unbound protein in the column. His instead of 6x tagged LipL32 protein remain in the column which is eluted on addition of Elution buffer pH 4.5. The eluted protein was then dialysed with 1X PBS to remove urea. After dialysis, LipL32 protein was then buffered with glycine buffered saline (pH 8.2) & this protein was then used to coat latex beads buffered with the same reagent. Purified recombinant protein was coated with latex bead as per the method described earlier (Senthilkumar *et al.*, 2010; Dey *et al.*, 2007). Test was performed by adding 25µl of suspected serum samples and equal volume of sensitized latex bead on a clean glass slide. Reagents are gently mixed by rotating the glass slide in
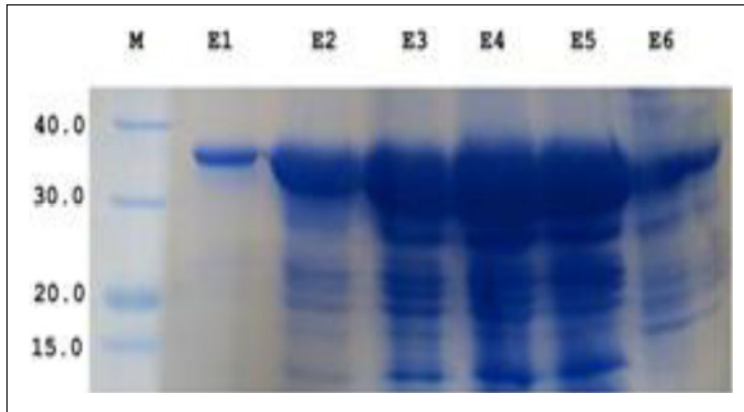
Figure 1. SDS-PAGE showing purified recombinant LipL32 protein in various elusion fraction.
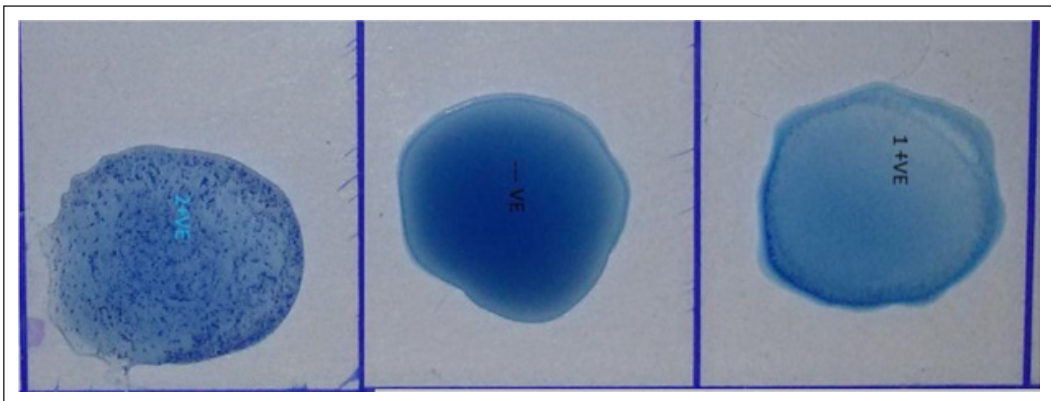

Figure 2. Seroreactive of bovine sera with LipL32 latex agglutination test.

horizontal and vertical direction. In positive test, agglutination was observed by formation of fine granular particle settled at the edge of reagent mixture, where as if homogenous suspension was obtained, then test was declared negative (Fig. 2).

Statistical analysis

The relative sensitivity, specificity and concordance value of the recombinant LipL32 antigen based ELISA for the detection of anti-leptospiral antibodies in bovine sera were compared with MAT as per the statistical method described by Senthil Kumar *et al.* (2010).

% Sensitivity = a/(a+c) x100, where "a" is the number of serum samples positive by both ELISA and MAT, "c" is the number of serum samples positive by MAT but negative by ELISA.

% Specificity = d/(b+d) x100 where "d" is the number of serum samples negative by both ELISA and MAT, "b" is the number of serum samples negative by MAT but positive by ELISA.

Concordance = (a+d)/ (a+b+c+d) x 100

RESULTS

Out of 884 bovine sera sample screened by in house latex agglutination test using recombinant LipL32 protein, the overall seropositivity was found to be 26.01% (230/884). Of the different farms screened,
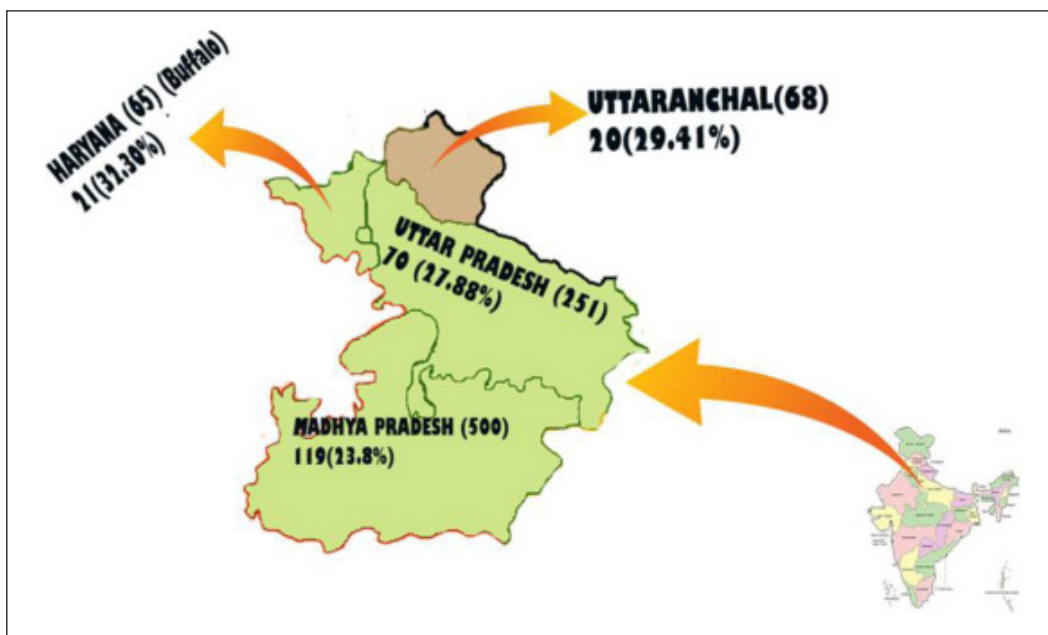
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Figure 3. Percentage seropositive of bovine sera for leptospirosis with LipL32 latex agglutination test.

Table 1. Evaluation of diagnostic values of rLipL32 based LAT in comparison to MAT

	MAT +ve	MAT –ve	Total
rLipL32 LAT +ve	227 (a)	03 (b)	230
rLipL32 LAT -ve	12 (c)	642 (d)	654
Total	239	645	884

Sensitivity = 227/239×100=94.97%; Specificity = 642/645×100=99.53%; Concordance = 869/884×100=98.30%.

seropositivity was found in all farms with Mathura recorded highest seropositivity (37.81%). Seropositivity in different farms is depicted in Fig. 3.

Among the serovars reported in the present study, Icterohaemorrhagiae was the predominant serovar showing sero-positivity with 180 sera (21.97%), followed by Grippotyphosa 83(10.13%), Autumnalis 24(2.93%), Hardjo 7(0.85%), Australis 7(0.85%), Hebdomadis 7(0.85%), Javanica 4(0.48%) and Canicola and Pomona, each with 3(0.42%) seropositivity. Sensitivity, specificity and concordance value of Lipl32 LAT for serodignosis of bovine leptospirosis has been depicted in Table 1.

DISCUSSION

India has an agrarian economy with live-stock as an integral part of agricultural community for their livelihood security and sustainability. Leptospirosis is an important livestock disease incurring production and reproduction losses in animals and significant public health importance causing morbidity and mortality in occupationally exposed groups.

India enjoys a tropical climate with high humidity and significant rain fall, which is conducive for *Leptospira* to survive in the soil and water of the country. Reactivity to leptosirosis is slightly higher because of

grazing habit of animals in rice fields, swampy and water logged area and wallowing habits of buffaloes in mud water. Husbandry practices, herd management, rodent abundance in surrounding farm area may attribute the prevalence of leptospiral seropositivity among animals. The laboratory diagnosis of leptospirosis is mainly based on serological methods, since culture is both less sensitive and time consuming (Dey *et al.*, 2007). The microscopic agglutination test (MAT), the reference method, is serogroup specific but 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 accuracy (OIE, 2012).

LipL32 is a major leptospiral outer membrane protein expressed ubiquitously in all pathogenic *Leptospira* species and is absent in saprophytic leptospires. It is the most prominent protein in SDS-PAGE protein profile (Dey *et al.*, 2007) and is also the most frequently recognized antigen in immunoblots with patient sera. Disease diagnostics need to be frugal and cost effective in order to gain popularity in developing South East Asian nations. So that they can percolate to grass root level to benefit destitute, marginalised livestock farmers. Recombinant LipL32-antigen based Latex agglutination test with proven sensitivity and specificity can be produced at low cost and hence is an important candidate as a *Leptospira* spot test for implementation in developing countries such as India (Behera *et al.*, 2014). Another advantage of LipL32 based LAT is that it is easy to perform and interpret, which makes it a suitable spot test to be performed by a semi-skilled animal health worker in peripheral animal health settings. LAT using recombinant LipL32 protein has been elucidated earlier with good sensitivity and specificity in bovine, canine (Senthilkumar *et al.*, 2010; Dey *et al.*, 2007) and porcine leptospirosis (Behera *et al.*, 2014). Reports in Northern India demonstrated that seroreactivity in cattle ranging from 10.1% to 15.8% (Srivastava *et al.*, 2006) and in buffaloes it ranges from 5.8% to 26.66%

(Verma *et al.*, 2001; Srivastava *et al.*, 2006; Agarwal *et al.*, 2005). 25.5% seroprevalence reported in cattle and 32.30% seropositivity in buffaloes are comparable with earlier study in India by Verma *et al.* (2001). Reacting serovars of the present study viz. Icterohaemorrhagiae, Grippotyphosa, Autumnalis, Hardjo, Australis, Hebdomadis, Javanica Canicola and Pomona was quite similar to earlier finding in same area (Sawhney & Saxena, 1967; Srivastava & Kumar, 2003) indicating endemicity in distribution of these serovars and absence of *Leptospira* vaccination practices for large ruminants in this region in particular and India in general.

CONCLUSION

Leptospirosis is a disease caused by multiple serovars, which has immense potential to impact adversely the health and productivity of livestock and pose serious public health consequences. So, more attention and intensive diagnosis and control programme need to be instituted for effective management of the disease to ensure increasing productivity of animals which will boost the burgeoning livestock sector of India and protection of animal and public health.

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