Short Paper

Seroprevalence of leptospirosis among suspected cattle in eastern part of India: a comparative study between rLipL32ELISA and MAT

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Summary

Leptospirosis in cattle is a worldwide problem associated with abortion, agalactia, still birth and infertility resulting in major economic losses to dairy industry. There is lack of data regarding seroprevalence of bovine leptospirosis in eastern India. So, with the aim to investigate the prevalence of the disease in Odisha and West Bengal state of eastern India, a total of 350 cattle serum samples were collected with distinct geographical attributes having history of infertility, abortion, and haemogalactia. Serum samples were tested by microscopic agglutination test (MAT) using a battery of twelve *Leptospira* serovars as live antigen to detect the serovars present in the studied area. Further a recombinant protein (LipL32) based ELISA was carried out for comparative study. Overall seropositivity using MAT and rLipL32ELISA were found to be 50.85% and 56%, respectively. The sensitivity and specificity of rLipL32ELISA relative to MAT was calculated and found to be 100% and 89.53%, respectively. In the current study among the serovars, *icterohaemorrhagiae* was the most predominant serovar reported in this study. So to conclude, this study warrants further investigations in this area to establish the risk factors involved in disease transmission cycle.

Key words: Cattle, Leptospirosis, MAT, rELISA, India

Introduction

Leptospirosis in cattle is a worldwide problem associated with abortion, agalactia, still birth and infertility resulting in major economic losses to dairy industry (Faine *et al*., 1999; Lilenbaum and Souza, 2003). The organism persists in the kidneys and genitals of animals without showing clinical signs of disease (Ellis *et al*., 1986). Carrier cows often excrete leptospires in their urine and such animals are important sources of infection for other cows, for dairy farm workers and for other people (Waitkins, 1986). The disease is widely prevalent in different parts of India (Pirmanayangam *et al*., 2001; Pushpa and Kumari, 2005; Mariya *et al*., 2007; Balakrishnan *et al*., 2011a). It has recently been recognized as a reemerging infectious disease among animals and humans and also has the potential to become even more prevalent with anticipated global warming (Kamath and Joshi, 2003; Yang, 2007). With sparse reports from the eastern part of India regarding the study of the prevalence of the disease, it was decided to include two eastern states like Odisha and West Bengal in this study.

Gold standard test for *Leptospira* detection is microscopic agglutination test (MAT) (OIE, 2008). No single serological test is free of shortcomings. To overcome various short comings with MAT and other culture isolation methods, researchers have long been searching for a rapid, sensitive test for genus specific detection of antibodies and devised methodologies to characterize novel antigens present in the proteome of outer membrane of pathogenic *Leptospira* and make user friendly tests to study the seroprevalence rate of the disease. LipL32, LipL21, LipL41 are important leptospiral lipoproteins expressed only in pathogenic *Leptospira* spp. (Shang *et al*., 1996). Recombinant LipL32 (rLipL32) is a major outer membrane protein which is the recent target antigen for carrying out immuno diagnosis (Flannery *et al*., 2001; Dey *et al*., 2004; Bomfim *et al*., 2005). So, the present study was designed to determine the seropositivity of leptospirosis in cattle by using rLipL32ELISA and also to find the correlation of this test with gold standard MAT.

Materials and Methods

Animals and sampling

Cattle serum samples (n=350; 200 from Odisha and 150 from West Bengal) having distinct geographical conditions like coastal belt, plane area and hilly area with high relative humidity were collected during the period between September 2011 and February 2012. Of the 350 sera, approximately 10% of each herd in different village settings spanning over 20 different districts of both Odisha and West Bengal states was collected. Simultaneously, a questionnaire was made for each village to collect information regarding breed, age, sex of animals and management, sanitary practices at farm, etc.

The serum samples were collected from animals with history of infertility, abortion and haemogalactia, etc. No animal had history of earlier vaccination. Sera were kept in sterile micro centrifuge tubes and stored at -20°C until further use.

MAT and ELISA

The collected serum samples were tested by MAT (OIE, 2008) for detecting antibodies using 12 leptospiral serovars viz. *australis*, *autumnalis*, *ballum*, *canicola*, *grippotyphosa*,*hardjo*,*hebdomadis*, *icterohaemorrhagiae*, *pomona*, *pyrogenes*, *javanica* and *tarrasovi*. A 7–8-day-old culture grown in Ellinghausen McCullough Johnson Harris (EMJH) medium containing approximately 2 × 10^8 live *Leptospira*/ml were used as antigen in the study. These serovars used as antigens were maintained in Veterinary Bacteriology and Mycology Division, Indian Veterinary Research Institute, India. Sera showing titer more than or equal to 1:100 were considered to be positive.

Recombinant LipL32 antigen was availed from Veterinary Bacteriology and Mycology Division, Indian Veterinary Research Institute, India. Indirect recombinant ELISA was done as per the method adopted by Bomfim *et al*. (2005) with slight modifications. The optimum concentration of the antigen for coating the plates as well as serum was attained by checker board method. The cut off value for positive sample was taken as average of ten known negative samples OD ± 2 SD.

Statistical analysis

The relative sensitivity, specificity and concordance value of the recombinant LipL32 antigen based ELISA for the detection of anti-leptospiral antibodies in cattle sera were compared with MAT as per the statistical method described by Thursfield (1995).

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

where,
a: The number of serum samples positive by both ELISA and MAT
c: The number of serum samples positive by MAT but negative by ELISA

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

where,
d: The number of serum samples negative by both ELISA and MAT
b: The number of serum samples negative by MAT but positive by ELISA

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

Results

In this study, overall seroprevalence of leptospirosis in cattle was found to be 50.85% and 56% by MAT and rLipL32ELISA, respectively. In addition to this, Odisha (55.5%) had more seroprevalence than its counterpart West Bengal (44.66%) (Table 1).

In age wise seroprevalence, antibodies were detected more in older age (>5 years) cattle and least in animals of <6-month-old (Table 2).

In sexwise comparison study, out of 350 sera samples examined 119 were from male and 231 from female. Seropositivity for *Leptospira* was found to be frequent in female 120 (51.94%) and 132 (57.14%) than male 58 (48.73%) and 64 (53.78%) by both MAT and rLipL32ELISA, respectively.

Seropositivity among five breeds showed higher prevalence in nondescript (56.04%) and crossbred cattle (54.59%) than native breeds (Table 3).

In this study, most of the sera showed multiple serovar positive. *icterohaemorrhagiae* was recorded as

Table 1: State wise seroprevalence of bovine leptospirosis

State	No. of samples	MAT (%)	rLipL32ELISA (%)
Odisha	200	111 (55.5)	121 (60.5)
West Bengal	150	67 (44.66)	75 (50)
Total	350	178 (50.85)	196 (56)

Table 2: Age wise seroprevalence of leptospirosis in cattle

Age (yrs)	No. of samples	MAT (%)	rLipL32ELISA (%)
0.6-1.0	87	32 (36.78)	36 (41.37)
1.5-2.0	38	15 (39.47)	18 (47.37)
2.5-3.0	52	30 (57.69)	33 (63.46)
3.5-4.0	47	26 (55.31)	26 (55.31)
4.5-5	40	21 (52.5)	24 (60)
>5	86	54 (62.79)	57 (66.27)
Total	350	178 (50.85)	196 (56)

Table 3: Breed wise seroprevelance of leptospirosis in cattle

Breed	No. of samples	MAT (%)	rLipL32ELISA (%)
Cross Breed Jersey	185	101 (54.59)	108 (58.37)
Sahiwal	27	7 (25.92)	11 (40.74)
Gir	23	4 (17.34)	4 (17.34)
Holstein Friesian Cross	24	15 (62.5)	18 (75)
Non-descript	91	51 (56.04)	55 (60.34)
Total	350	178 (50.85)	196 (56)

Table 4: Seropositivity of different serovars

Serovar reported	No. of samples (Odisha)	No. of samples (West Bengal)	No. of samples and % seropositivity (overall)
icterohaemorrhagiae	69 (62.16%)	52 (77.61%)	121 (67.98%)
hebdomadis	48 (43.24%)	11 (16.41%)	59 (33.14%)
grippotyphosa	20 (18.01%)	32 (47.76%)	52 (29.21%)
hardjo	36 (32.43%)	10 (14.92%)	46 (25.84%)
australis	24 (21.62%)	0	24 (13.48%)
pomona	4 (3.06%)	2 (2.98%)	6 (3.37%)
autumnalis	0	2 (2.98%)	2 (1.12%)
pyrogens	0	1 (1.49%)	1 (0.56%)

Table 5: Comparative evaluation of rLipL32ELISA in comparison to MAT

Test	Microscopic agglutination test		Total
	Positive	Negative	
rLipL32ELISA			
Positive	178 (a)	18 (b)	196
Negative	00 (c)	154 (d)	154
Total	178	172	350

a: No. of sera positive by both the tests, b: No. of sera negative by MAT only, c: No. of sera positive by MAT only, and d: No. of sera negative by both tests. Specificity = 89.53%, sensitivity = 100%, concordance = 94.86%, and k = 0.99

the predominant serovars in West Bengal as well as in Odisha. The frequency distribution of serovars is illustrated in Table 4.

Sensitivity and specificity of rLipL32ELISA in comparison to gold standard MAT was 100% and 89.53%, respectively with *k*-value 0.99 (Table 5).

Discussion

Leptospirosis is a disease which remains undiagnosed in most of the cases in bovine causing severe reproductive and productive anomalies leading to significant economic loss to dairy industry. Serological tests like microscopic agglutination test (MAT), latex agglutination test (LAT) and enzyme linked immuno-sorbent assay (ELISA), specially Dot-ELISA and Indirect-ELISA are frequently used in detection of the disease. Though MAT is the gold standard test for leptospirosis diagnosis (OIE, 2008), it has certain limitations, it is a cumbersome and time taking procedure, it poses risks to laboratory personnel and is ineffectual to differentiate between acute and chronic infection. In recent years, recombinant based ELISA has become the preferred test compared to whole cell based ELISA as a serodiagnostic tool of leptospirosis due to better sensitivity and specificity (Dey *et al*., 2004; Bomfim *et al*., 2005).

Overall seropositivity (Table 1) recorded in this study by MAT corroborates the findings of Natarajaseenivasan *et al*. (2002), Segura-Correa *et al*. (2003), Shafighi *et al*. (2010), and Balakrishnan *et al*. (2011a). However, much lower prevalence rate (1.59%) was also reported earlier by Rifatbegović and Maksimović (2011) which may be due to variation in sample size and randomization during sample collection. In state wise prevalence study, current result was not in agreement with the earlier finding reported by Mandal *et al*. (2008) in West Bengal state, but is in congruence with the study conducted by Biswal *et al*. (2000) in Odisha. Moreover, higher level of seroprevalence in Odisha (Table 1) might be associated with the factor that the serum samples under this study were mainly from coastal belt, hot and humid climatic regions and from animals with history of reproductive disorders and mastitis.

The present findings with respect to age wise prevalence (Table 2) are in accord with the earlier study of Salas (1986) and Prescott *et al*. (1988) who observed more seropositivity in older cattle. Similarly, on sex wise comparison it also supports the findings of earlier studies of Sharma *et al*. (2003), and Aslantas and Ozdemir (2005), however, contrary to the findings of Miller *et al*. (1991), Thiyageeswaran (2007), and Balakrishnan *et al*. (2011b). The age and sex distribution of cattle in this study indicates that seropositivity against *Leptospira* in male group was frequent in young and adult animals; however, there was the preponderance in females.

The seroprevalence in breed of cattle in this study is in concurrence with earlier studies like Varma *et al*. (2001), and Nagarajan (2005) who reported more seropositivity among cross breed cattle. It may be due to the low acclimatization factor of cross bred cattle in these environmental conditions and comparatively lower resistance to diseases.

icterohaemorrhagiae was recorded as the predominant serovar in West Bengal as well as in Odisha, that corroborates the findings of earlier studies in

India by Sawhney and Saxena (1967), and Srivastava and Kumar (2003) and in Trinidad by Suepaul *et al*. (2011). But the result is in partial variance with earlier findings in West Bengal and Odisha as *hardjo* was recorded as the predominant serovar (Biswal *et al*., 2000; Mandal *et al*., 2008). The evolvement of new serovar in contrast to earlier studies in these states may be due to environment and pathogen interaction during the considerable period of time between the present and earlier studies. In comparison, the specificity and sensitivity of rLipL32ELISA relative to MAT was calculated and was found to be 89.53% and 100%, respectively (Table 5). This finding is in agreement with Bomfim *et al*. (2005), and Srivastava *et al*. (2006). There is a possibility of false negative result in MAT as the sera under test may have antibody against one serovar for which the corresponding antigen (live culture of the corresponding serovar) is not included in the panel of test antigens. Under these circumstances, the serum samples that were found to be MAT negative may be positive in rLipL32ELISA. In earlier studies, many researchers reported higher sensitivity of ELISA than MAT (Thierman and Garet, 1983; Trueba *et al*., 1990; Surujuballi *et al*., 1995). Thus, in the perspective of the present study it may be explained that because of this advantageous property rLipL32ELISA could detect *Leptospira* specific non agglutinating antibodies in the 18 sera samples that were detected negative by MAT (Table 5). Concordance is greatly affected by sensitivity and specificity of the tests under consideration (Thursfield, 1995). In this study, concordance between tests was calculated to determine the agreement between the tests to understand whether this combination of serological tests can be applied for diagnosing the disease correctly. Strengthening the findings of relative sensitivity and specificity, concordance percentage between MAT and rLipL32ELISA was calculated to be 94.86%. For further evaluation of the tests kappa statistics has been applied. By applying kappa statistics, it was found that there was perfect agreement between two tests (k=0.99). This result showed that rLipL32ELISA was able to detect all the samples that were found positive by MAT.

In conclusion, *icterohaemorrhagiae* is the predominant serovar in case of cattle in two eastern India states. It warrants further studies in these states to establish the risk factors involved in disease transmission using randomized samples. This study's results also suggested that indirect ELISA using rLipL32 as antigen may be of higher sensitivity as well as specificity than MAT for detection of leptospiral antibodies in cattle. However, further studies with a wide range of samples from several other animal species with distinct temporo-spatial distribution may be helpful to validate the significance of this test in diagnosis of leptospirosis.

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