Short Communication

Seropositivity and Associated Risk Factors for Bovine Leptospirosis in Dairy Farms

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Abstract | Leptospirosis is one of the most economically important diseases of the cattle and buffaloes population worldwide and is caused by serovars of pathogenic *Leptospira*. A pilot study was conducted in some of the dairy farms located in Telangana and Andhra Pradesh, endemic states of India, during 2017 to investigate the seropositivity of bovine leptospirosis in dairy animals and associated risk factors at the farm level. The semi-structured questionnaire was designed as per the literature and used to collect leptospirosis associated risk factors along with serum samples from eight non-vaccinated dairy herds. The collected serum samples (n=56) were examined for detection of leptospiral antibodies using the Microscopic Agglutination Test (MAT), the gold standard serological test. The Chi-square and odds ratio analyses were employed to identify the important risk factors for leptospirosis in dairy farms. The study revealed that the seroprevalence of bovine leptospirosis at an individual animal and farm level was 39.8% and 75%, respectively, associated with age (p=0.041) and the health status of the animal (p=0.0001) as the significant risk factors. The predominantly observed reacted serovars were Icterohemorrhagiae (31.8%), Hebdomadis (27.3%), Bangkinang (22.7%), Australis (18.2%), Djasiman, Hurstbridge, Panama, and Pyrogenes (13.6%). Further, studies are required to identify the appropriate farm level/animals level risk factors associated with bovine leptospirosis using large-scale sampling with a refined survey instrument tool.

Keywords | Bovine leptospirosis, Risk factors, Microscopic Agglutination Test, PCR, Seroprevalence

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INTRODUCTION

Leptospirosis, a spirochaetal zoonosis, has emerged as a serious global veterinary and public health problem affecting the health of both animals and humans in tropical and subtropical countries (Costa et al., 2015). Leptospirosis is caused by pathogenic species of the genus *Leptospira* and results in high morbidity and mortality (Ellis, 2015). The disease is prevalent in humans and a variety of mammalian hosts such as rodents, bovine, sheep, goats, dogs,

horses, etc., including wild mammals, affecting both public health and the livestock economy. The disease has gained much importance as it is often undiagnosed (Ellis, 2015). Among animals, cattle and wild rodents are the leading hosts that excrete leptospirae in their urine and act as carrier/reservoir animals. Transmission of leptospirosis occurs mainly by exposure to water or soil contaminated by the urine of infected animals or by direct contact with infected animals materials (Ellis, 2015).

Leptospirosis has been reported as one of the major causes of reproductive failures in cattle and other ruminants, causing abortions, decreased fertility, reduced milk yield, decreased growth rate, mortality in calves, stillbirths, weak newborns (Ellis, 2015). Leptospires colonize the proximal renal tubules of various mammals and are intermittently excreted through the urine of carrier animals (Lilenbaum and Martins, 2014). The first animal isolate was reported in cattle in 1928 from USSR, and the serovar was named Grippotyphosa. Since then, the disease was reported globally, through infection by a wide variety of serovars and with varied clinical outcomes. In India, leptospirosis is endemic for human and animal populations (Balamurugan et al., 2018) and is a global hotspot (Costa et al., 2015; Torgerson et al., 2015). The prevalence of leptospirosis with various *Leptospira* serovars in farm animals from enzootic coastal states of India, especially the coastal belt from Odisha, Maharashtra, Kerala, Tamil Nadu, and Gujarat states, and Andaman islands, ranged from 14.55% to 54.14% (Alamuri et al., 2020) were reported as an emerging and important urban zoonosis (Hotez, 2017). Dairy farmers need to know farm practices to instigate effective disease control measures. Assessing the prevalence of leptospirosis and associated risk factors at the farm level is essential for implementing sustainable interventions, which will help understand the host-pathogen interactions and identify the potential pathways to control the transmission of leptospirosis. Hence, in the present study, besides the prevalence level, important dairy farming practices are elicited in the existing conditions of Indian farming practices for assessing the associated risk factors for bovine leptospirosis.

MATERIAL METHODS

Study area and farms description

The study was carried out in two southern states of India endemic to bovine leptospirosis, namely Andhra Pradesh and Telangana (Alamuri et al., 2019; Prameela et al., 2013). The bovine population in these states includes crossbred (crossed with Holstein Friesian and Jersey cattle), indigenous breeds (Sahiwal and Gir cattle and Murrah buffaloes), and non-descriptive breeds of animals. The number of animals in each farm range from 1 to 100. Some farms rear exclusively cattle or buffaloes or some with mixed species and breeds. A pilot study was conducted in January-April 2017 in eight dairy farms, four each from Telangana and Andhra Pradesh states, and blood samples of seven animals from each farm were collected besides the questionnaire data.

Questionnaire survey

The semi-structured questionnaire was designed as per the available literature and used for the collection of leptospirosis risk factors along with serum samples from eight non-vaccinated herds comprising a total of 56 individual animals. A questionnaire data was also collected on the herd and individual dairy cattle through one-to-one interaction with the farmer. The questionnaire had information on the name of the farm, location, herd size, location of the farm, mixing with other animals, other animals present in the farm including rodents, breed of the animal, age, sex, breeding methods, level of hygiene, presence of water bodies, source of water, sources of fodder, the health status of the animal, calf management, dog access to the farm and type of flooring in the shed.

Samples

A five ml of blood was collected, and serum was separated and aliquoted in a cryovial, labeled and stored at -20°C at Veterinary Microbiology Laboratory, Veterinary College, Korutla, Jagityala. Sera samples were transported using a thermocol box with an ice pack to the Leptospira Research Laboratory, National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Bengaluru, for conducting serological and molecular assays/tests.

Serological/Molecular assays

All the serum samples were screened for *Leptospira*-specific antibodies by MAT using a reference panel of 18 live cultures of pathogenic *Leptospira* serovars covering 16 serogroups (Table 1). Any herd with at least one seropositive animal was categorized as a positive herd. Briefly, the antigen and antibody interaction were examined under the dark-field microscope at 200 magnifications for observing the agglutination reactions. The endpoint (titer) was taken as that dilution which gives 50% agglutination, leaving 50% of the cells free when compared with antigen control (no agglutination should be seen in the antigen control) was considered positive at ≥1:100 dilutions.

The sera which reacted with *Leptospira* serovar(s) in MAT were used for further investigations of active *Leptospira* infection by *LipL32* gene-based PCR (Stoddard et al., 2009) for confirmation. The DNA was extracted from 200µl of serum samples using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) by following the manufacturer's protocols and eluted in 50 µL volume. In the PCR, the extracted DNA (10 µL) along with designed specific primers and 2X master mix (Ampliqon, Odense, Denmark) in 25 µL

Table 1: Reference strains of *Leptospira* used in the study

Sl. No.	Species	Serovar	Serogroup	Strain
1	*L. interrogans*	Australis	australis	Ballico
2	*L. interrogans*	Bankinang	autumnalis	Bankinang 1
3	*L. interrogans*	Canicola	canicola	Hond Utrech IV
4	*L. interrogans*	Hardjo	sejroe	Hardjoprajitno
5	*L. interrogans*	Hebdomadis	hebdomadis	Hebdomadis
6	*L. interrogans*	Pyrogenes	pyrogenes	Salinem
7	*L. borgpetersenii*	Tarassovi	tarassovi	Perepelicin
8	*L. inadai*	Kaup		LT 64-68
9	*L. interrogans*	Icterohaemorrhagiae	icterohaemorrhagiae	RGA (ATCC443642)
10	*L. interrogans*	Copenhageni		M 20
11	*L. santarosai*	Shermani	shermani	1342 K
12	*L. kirschneri*	Grippotyphosa	grippotyphosa	MoskvaV
13	*L. fainei*	Hurstbridge	hurstbridge	BUT 6
14	*L. borgpetersenii*	Javanica	javanica	Poi
15	*L. noguchii*	Panama	panama	CZ 214 K
16	*L. interrogans*	Djasiman	djasiman	Djasiman
17	*L. interrogans*	Pomona	pomona	Pomona
18	*L. interrogans*	Bataviae	bataviae	Swart

Table 2: Individual animal level risk factors associated with bovine leptospirosis

Variable		Positive	Negative	Chi-square analysis	p-value	odds
Species	Cattle	19	25	0.655	0.418	2.28
	Buffaloes (Ref)	3	9			
Breed	Indigenous	19	24	1.08	0.29	2.63
	Crossbred (Ref)	3	10			
Age	>2year	14	11	4.15	0.04*	3.11
	<2 year (Ref)	9	22			
Sex	Male	4	0	2.92	0.08	#
	Female (Ref)	22	30			
Health status	Reproductive disorder	15	5	14.389	0.0001**	12.4
	Apparently healthy (Ref)	7	29			

*p-value at 5 % significance ** p-value at 1% significance

because one cell frequency value is '0', it is not possible to calculate the odds

Table 3: Farm level risk factors associated with bovine leptospirosis

Variable	Total no. of farms positive for variables	No. of Farms positive to leptospirosis
Size of farm		
<50 animals on the farm	5	3
>50 animals on the farm	3	3
Location of farm		
Isolated farm	6	5
Farm located nearer to water bodies	2	1
Calves management		
Separate housing for calves	2	2

Calves mixing with adults	6	4
Presence of other Species		
Sheep	2	2
Goat	3	3
Dog	8	2
Rodents	5	5
Water system		
Borewell water used in the farm	5	5
Tap water used in the farm	2	0
Stagnated water used in the farm	0	0
Feeding system		
Farms with rodent access to feed	5	5
Farms with dog asses to pasture lands	7	7
Farmer's own land fodder	5	5
Drylands fodder	2	2
Disease history of the farm		
Farms with abortion history	2	2
Farms with reproductive disorders	4	4
Farms with mastitis cases	2	2
Artificial insemination	5	3
Natural service	3	3
Good level of sanitation	5	5
Poor level of sanitation	3	1

volume reaction were used for amplification with the described PCR cycling conditions, and the resulting amplicons were resolved in agarose gel electrophoresis.

Statistical analysis

Data generated from laboratory investigations and questionnaire surveys were coded and recorded using Microsoft® Excel 2016. The Chi-square test and odds ratio was calculated using the IBM SPSS Statistics for Windows, version 22 (IBM Corp., Armonk, N.Y., USA) to determine the associated risk factors (sex, age, breed, parity, and health status of the animals). Differences among groups of each factor were considered significant at $p<0.05$ for all parameters tested.

RESULTS

In this study, among 56 animals tested, 22 animals had antibodies against at least one serogroup of *Leptospira* (MAT Titre ≥ 1:100), generating an individual animal seropositivity of 39.8% (22/56) with the farm level seropositivity of 75 % (6/8). Further, an individual and farm level seropositivity of 66.7% and 100 % in Andhra Pradesh and 17.85% and 50% in Telangana were observed, respectively. Further, the serovars that predominantly reacted with the serum samples were Icterohemorrhagiae (31.8%), Hebdomadis (27.3%), and Bangkinang (22.7%). The overall predominant *Leptospira* serogroup specific antibodies that determined by the frequency distribution of the employed serovars as follows, Icterohemorrhagiae (59.1%), Hebdomadis (27.3%), Bangkinang (22.7%), Australis (18.2%), Djasiman (13.6%), Hurstbridge (13.6%), Panama (13.6%), and Pyrogenes (13.6%). On Chi-square analysis, it was found that out of five variables, the age of the animal (p=0.041) and health status (p=0.0001) were associated with the seropositivity of leptospirosis (Table 2). The odds ratio results revealed that the animals with >2 year age groups were having 3.11 times higher chances than an animal with < 2 year age groups of being associated with leptospirosis. Similarly, bovines with reproductive problems had 12.4 times more chances to be positive than those with apparently healthy bovines (Table 2). The farm level determinants included were farm size, mixing with other animals, presence of other animals in the farm, breed, age, sex, method of breeding, level of hygiene, purchase of new cattle, migration of animals, rearing system, presence of water bodies, animal crossing water body while moving out, source of water for drinking and washing the animals, sources of fodder and dog access to the farm (Table 3). However, since the samples size was limited, multivariable models could not be fitted. Further, among 22 MAT reacted samples tested in PCR, none of the samples yielded amplicons of 285 bp specific for the pathogenic *Leptospira LipL32* gene.

DISCUSSION

Leptospirosis is one of the neglected tropical diseases affecting humans and animals and is endemic in India's coastal belt (Alamuri et al., 2019). Variation in rainfall, humidity, and climate, coastal regions are linked to a high prevalence of bovine leptospirosis in Andhra Pradesh. The study results, were in agreement with the findings of Alamuri et al. (2019), who observed 68.08% of seropositivity in Andhra Pradesh, whereas Prameela et al. (2013) observed 44.7% of seropositivity with Pomona, Autamnalis, and Hardjo as predominant serovars. However, Balamurugan et al. (2016a) reported the seroprevalence ranging from 45 to 75% in other enzootic states of India. In this pilot study, the seroprevalence in the farms' animals of Telangana state was 17.8 % which is concordant with the earlier studies where the seroprevalence was 16.3% (Prameela et al., 2013). The seropositivity of bovine leptospirosis reported in the states/UTs adjoining to the Telangana and Andhra Pradesh was 37% in Odisha (Balamurugan et al., 2017), 41% in Maharashtra (Balamurugan et al., 2016b), 87% in South India (Natarajaseenivasan et al., 2011), 44% in Tamil Nadu and Karnataka (Saranya et al., 2021; Balamurugan et al., 2018). The divergence might have arisen due to variations in the sample size, geography, animal management, husbandry practices, location of farms, and the number of serovars included in the MAT. Moreover, the leptospiral infection in bovine usually remains sub-clinical even though serologically found positive. The predominantly reacted pathogenic serovars representing serogroup-specific antibodies prevalent in the present study were Icterohemorrhagiae, Hebdomadis, Bangkinang, Australis, Djasmin, Hurstbridge, Panama, etc. The prevalence pattern of predominant serovars was similar to the earlier studies reported by Alamuri et al. (2019) and Prameela et al. (2013), except for Djasmin and Hurstbridge serovars which were observed along with other pathogenic serovars. Even though Hardjo is considered common in cattle, other serovars were also noticeable in many parts of India and the world (Balamurugan et al., 2016a; Patel et al., 2017). The present study findings are in congruence with the results of a previous study by Sritharan (2012), where the serovars observed were Pomona, Tarassovi, Hebdomadis, Lai, Australis, Ballum.

The present study demonstrated age and health status of the animal were risk factors significantly associated with the occurrence of leptospirosis. Our finding in respect to the age-wise prevalence was in agreement with earlier studies and Leahy et al. (2021), who reported more seropositivity in older cattle. However, Dogonyaro et al. (2020) found no significant association among the different age groups with bovine leptospirosis in South Africa. Further, the results were also in concordance with studies of Behera et al. (2014), who observed that anti-leptospiral antibodies were detected more in older aged (>5years) cattle and least in animals of < 6 months old. This could be due to the duration of exposure and persistence of antibodies against *Leptospira* pathogens in aged animals.

In the current study, the health status of the animal was also identified as one of the significant risk factors associated with leptospirosis, which is concurrent with that of Prameela et al. (2013), who reported that the history of abortion at the animal level is associated with the occurrence of bovine leptospirosis. It is also in agreement with the findings of Fávero et al. (2017), where the cows which were seropositive to *Leptospira* spp. had eight times more chances of developing reproductive disorders. Ismail et al. (2019) reported that animals with a previous history of abortions were found significantly positive with *Leptospira* Hardjo serovar. It is important to reinforce that *Leptospira* infection has a chronic presentation in bovines and can cause severe reproductive problems such as abortion, stillbirths, and low fertility (Ellis, 2015). Bovines are considered maintenance hosts for the serovars Hardjoprajitno and Hardjobovis, which are transmitted directly among bovines and are associated with reproductive failure. Therefore, by initiating the appropriate sanitary practices, we can reduce the occurrence of *Leptospira* infection. Many researchers have applied multivariate analysis for the identification of important farm-level risk factors (Desa et al., 2021). The same could not be applied in the present study, as the sample size is insufficient for generalizing the result. However, larger and appropriate samples and sampling sizes will provide better results to identify appropriate risk factors in bovines, which helps design effective program planning in preventing and controlling leptospirosis, especially in bovines.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

NOVELTY STATEMENT

Assessed the farm level associated risk factors for leptospirosis occurrence in dairy animals.

AUTHORS CONTRIBUTIONS

SM, VB, and VMB: Designed and conceptualized the work with overall monitoring, analyzed and interpreted the data. RD, SI, and RPT provided guidance and support. SM wrote the original draft and VB and VMB edited the manuscript. SM conducted the survey. SM and KVK carried out laboratory experiments. GG carried out the statistical analysis of risk factors. BRS: Provided guidance and support to carry out the research work at ICAR-NIVEDI. All authors read and approved the final manuscript.

Compliance With Ethical Standards Statement Of Animal Rights

The manuscript does not contain animal experimental trials. No ethical clearance is required for collecting small volumes of blood samples required for seroepidemiological studies, as per CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines. Moreover, the serum samples were collected by well-trained veterinarians concerning animal welfare regulations for the diagnosis of disease along with questionnaire data after obtaining the oral concern from the farm/animal owners.

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