Explor Anim Med Res,
Vol.12, Issue - 2, 2022, p. 167-175
DOI : 10.52635/eamr/12.2.167-175

ISSN 2277- 470X (Print), ISSN 2319-247X (Online)
Website: www.animalmedicalresearch.org

Research Article

A STUDY ON SEROPREVELENCE AND RISK FACTORS OF BOVINE LEPTOSPIROSIS IN LOWER ASSAM, INDIA

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Received 18 July 2021, revised 17 June 2022

ABSTRACT: The present study was conducted to ascertain the seroprevalence of *Leptospira* infection in cattle among certain districts of the lower Brahmaputra valley in the state of Assam from March 2017 to February 2018. Two serological tests viz. IgG ELISA and Microscopic Agglutination Test (MAT) were used to detect the leptospiral antibodies. The anti-leptospiral antibodies were detected by IgG ELISA and compared with MAT using antigens from 12 pathogenic serovars. Out of 380 sera samples, 68 (17.89 %, 95% CI: 6.89-27) and 44 (11.58%) were positive by IgG ELISA and MAT respectively. The circulating *Leptospira* serovars identified were Autumnalis (6.05%), Ballum (2.63%), Batavia (1.31%), Ichterohaemorrhagie (0.7%), Javanica (0.5%) and Sejroe (0.2%). The sensitivity and specificity of IgG ELISA in comparison to MAT were calculated and found to be 100% and 92.85% respectively with a concordance of 93.68%. An epidemiological investigation was carried out to find the association of various risk factors with *Leptospira* infection under this study in cattle using a pre-tested questionnaire. The present study will serve as baseline data for the prevention and control of *Leptospira* infection in cattle.

Key words: Leptospirosis, Bovine, IgG ELISA, MAT, Risk factors, Seroprevalence.

INTRODUCTION

Leptospirosis, a spirochaetal zoonotic disease caused by the genus *Leptospira* has now been emerged as a serious veterinary and public health problem globally. Bovine leptospirosis is a disease commonly associated with the birth of weak calves, mastitis, milk drop syndrome, blood in milk, infertility, stillbirth, and abortion (Pearson *et al*. 1980, Ellis *et al*. 1986, Srivastava 2008). Based on LPS antigen, more than 300 different serovars of pathogenic *Leptospira* (Saito *et al*. 2013) and 60 serovars of nonpathogenic *Leptospira* (Cerqueira and Picardeau, 2009, Angeliki 2010) have been documented. The disease is more predominant in tropical and subtropical countries which experience heavy rainfall, humidity, presence of marshy land and paddy cultivating areas (Favero *et al*. 2017). Most commonly, wild rodents serve as natural reservoirs of infection. The main source of infection includes contamination of soil, pasture, drinking water, and feed by the urine of infected animals or healthy carriers, aborted fetus, and uterine discharges (Adugna 2016). Generally, dairy cattle serve as a natural host of leptospiral serovars. The most commonly reported serovars responsible for the infection in man and animals are Australis, Autumnalis, Ballum, Canicola, Grippotyphosa, Hardjo, Hebdomadis, Icterohaemorrhagiae and Pomona (Saminathan *et al*. 2016). Leptospirosis often remains undiagnosed because of its subclinical infections or healthy carrier status at field condition (Iqbal *et al*. 2011). In the North Eastern Region, especially in Assam, there is still a lack of data on leptospirosis, seroprevalence, and its predominant serovars among cattle. Hence, the present investigation was conducted to detect the sero-prevalence and associated risk factors of leptospirosis in cattle among the districts of the lower Brahmaputra River valley in the state of Assam, India.

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MATERIALS AND METHODS

Study area and sampling design

The present investigation was conducted among the districts of the lower Brahmaputra valley of Assam, *viz.* Baksa, Barpeta, Bongaigaon, Darrang, Dhubri, Kokrajhar, Kamrup (M), Kamrup (R), Nalbari, and Udalguri, to screen the seroprevalence of *Leptospira* infection in cattle. A total of 380 representative cattle blood samples were collected randomly from smallholder, traditional and commercial dairy herd. Samples were collected from both clinically suspected and healthy animals irrespective of age, sex, breed, etc. Simultaneously, on each farm visited, a pretested questionnaire was followed for each district to collect the relevant information regarding breed, age, sex, vaccination history, husbandry practices at the farm level, and reproductive disorder like abortion, repeat breeding, infertility, and frequent fever and reduced milk yield, etc.

Collection of serum sample

Around 3-5 ml of blood sample was collected to yield serum in a sterile labeled clot activator (BD vacutainer™) aseptically from the jugular vein and the samples were transported to the laboratory by maintaining the temperature at 2- 4 degree centigrade in an airtight sample carrying box. Vacutainers were allowed to stand in an inclined position at room temperature to facilitate clotting and separation of serum. Later on, the serum sample was separated from the coagulated blood and centrifuged at 3000 rpm for 5 minutes. In certain cases where the amount of serum obtained inside the vacutainer is poor, the same was subjected for a holding period of 12-24 hours in the refrigerator (2-8^{0}C) to obtain an appreciable quantity. Further, the serum sample was collected in a sterile screw-capped cryovial (Tarsons™), labeled properly, and stored at -20ºC till further analysis.

Serological test for *Leptospira* sp.

Samples were subjected to two-step serological tests, *viz.* Enzyme-linked immunosorbent assay (ELISA) and Microscopic agglutination test (MAT) to diagnose the *Leptospira* infection.

Enzyme-linked immunosorbent assay (ELISA)

A commercial indirect ELISA kit namely, Bovine *Leptospira* IgG (Lep IgG) ELISA Kit (SiNCERE™ Cat no-E13812731, 96T/48T) was used for the detection of antibodies against leptospiral infection in serum. The tests were performed strictly as per the protocol outlined in the user's manual supplied with the kit. Optical densities (OD) were read in the microwells using a microplate reader at a wavelength of 630 nm. ELISA optical density (OD) readings were transformed to serum/positive percentage (PP) according to a specific equation cited by the manufacturer.

Microscopic agglutination test (MAT)

The following Leptospiral reference strains representing 12 different serovar groups (Australis, Autumnalis, Ballum, Canicola, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Javanica, Pomona, Pyrogenes, Sejroe and Tarasovi) received from Department of Microbiology, Bharatidasan University, Tiruchirapalli, Tamil Nadu were used in this study for the conduct of MAT. *Leptospira* antigens for MAT were prepared according to the method provided by World Organization for Animal Health (OIE 2008) on leptospirosis. These serovars were kept at 28-30°C in screw-capped test tubes in 0.1 percent semisolid EMJH agar using *Leptospira* medium base supplemented with 10% enrichment (Diffco, USA). In 10 ml of liquid EMJH medium, 0.5 ml of each representative strain from the panel of 12 serovars were inoculated. Incubation was done at 30°C for five to seven days. A loopful of culture was checked under dark field microscope to confirm the absence of contamination or clumps and the presence of viable leptospires. A 5-8 day old liquid culture of live Leptospires (12 different serovars) incubated at 29 ±1°C, containing a density of $2x10^8$ Leptospires per mL was used as live antigen (Panwala and Mulla 2015). The subculture was maintained in the *Leptospira* laboratory at DBT twinning project on "Serosurveillance of *Leptospira* infections in animals of Northeastern region of India" at the Department of Veterinary Microbiology, College of Veterinary Science, AAU, Khanapara, Guwahati, Assam, India.

Interpretation of test result

The *Leptospira* cultures without auto-agglutination were used as antigens in MAT. MAT was carried out at 2-fold dilution starting from 1:20 and the positive samples were titrated up to end titers. Titres of 1:40 or above to any of the serovars were considered evidence of leptospiral infection. A titer of 1:40 was used as the cut-off because it is the closest dilution to the usual cut-off of 1:50 used in other reported sero-epidemiological surveys (Everard *et al.* 1985). The serum-antigen mixtures were examined under a dark field microscope for visible agglutination. For observation, one drop of mixture is transferred with a platinum loop or pipette from a well to a microscopic slide and examined under 20X objective without a cover slip. The titer was considered to be

positive when the dilution gave 50% agglutination, leaving 50% of the cells free. Comparisons were made with a control suspension of leptospires diluted in PBS (pH 7.2) without serum to rule out the possibilities of auto-agglutination.

Risk factors associated with *Leptospira* infection in cattle

To find out the risk factors associated with *Leptospira* infection in cattle, relevant data like age, sex, breed, herd size, location of the herd, feeding type, source of water, grazing, exposure to other animals, rodent infestation, etc were recorded. History of different diseases such as abortion, reduced milk yield, retention of placenta, repeat breeding, infertility, and other health-related problem were also recorded using a pretested questionnaire format at the time of sampling.

Geographic information system (GIS)

The locations of sample collection within different districts of Assam with their coordinates were recorded using a GPS navigation device. The longitude and latitude of respective locations were incorporated into a Microsoft Excel sheet and subsequently plotted in a map with the help of computerized software (Arc Map® GIS version 10.2) to study the spatial distribution of the *Leptospira* infection among the districts of Assam (Fig. 1). Thematic mapping / Density mapping prepared by GIS software based on input data shows the district wise spatial distribution of the infection.

Statistical analysis

Data were analyzed using the statistical software R (R Core Team 2018). The association between the seroprevalence of *Leptospira* infection and various risk factors were considered by the Chi-square test of independence (Mangiafico 2015). The association was considered significant if $p<0.05$. Seroprevalence of leptospirosis was described with a 95% confidence level.

RESULTS AND DISCUSSION

In the present study, the overall seroprevalence of leptospirosis in cattle among the districts of the lower Brahmaputra valley of Assam was found to be 17.89% with (95% CI: 6.89, 27) by IgG ELISA and 11.58% by MAT. Based on IgG ELISA highest prevalence of *Leptospira* infection was recorded in Kamrup (M) with a prevalence of 24.28% while no samples were found to be positive from the Nalbari district. The details of the *Leptospira* serovars detected through MAT and their prevalence are presented in Table 1. Concerning districts, variation in prevalence might be due to the type of

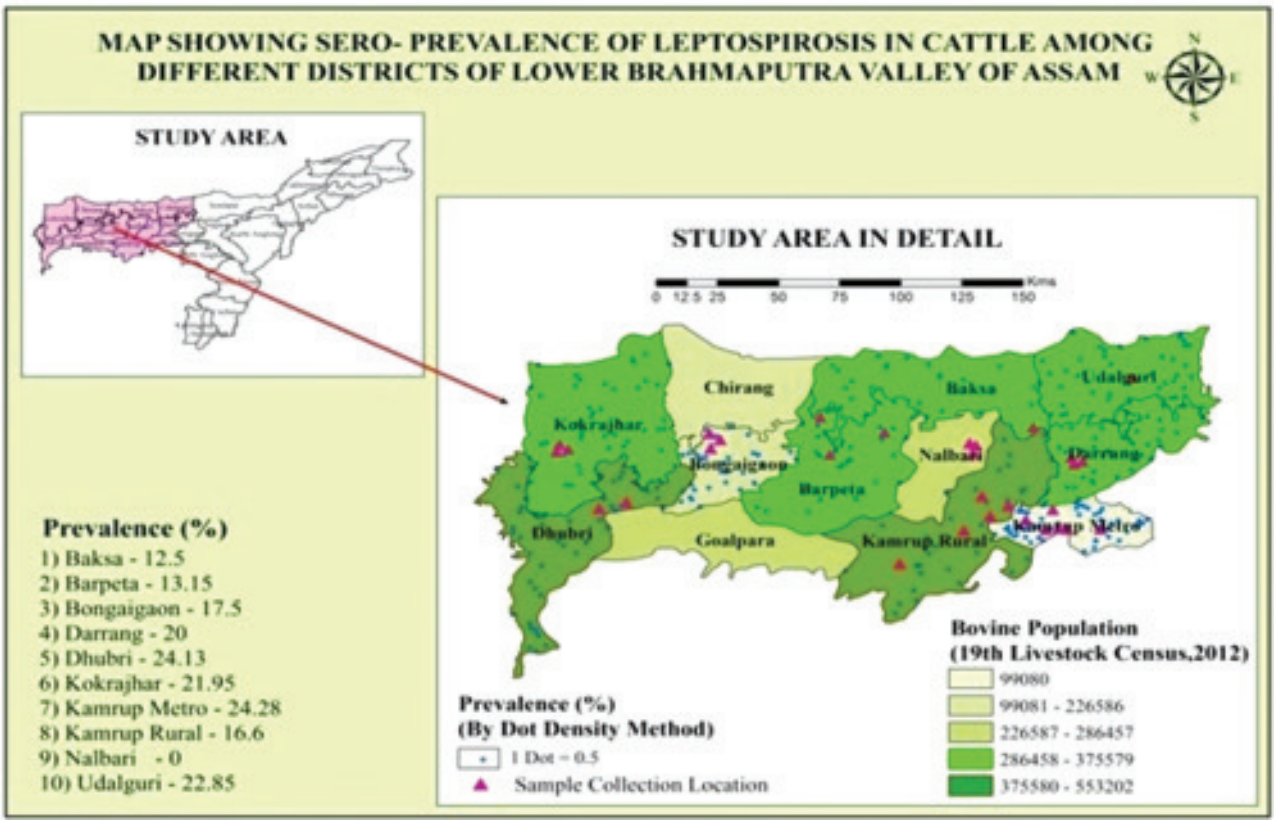

Fig. 1. District wise spatial distribution of *Leptospira* infection.

breeding, management, ecology of the disease, and poor animal husbandry practices. The urban areas from where sampling was done were generally more polluted which may favor the growth and multiplication of *Leptospira* organisms. Similar findings on a district-wise variation in seroprevalence of *Leptospira* infection in cattle were also reported earlier (Pandian *et al*. 2015, Patel *et al*. 2017). Pandian *et al*. (2015) reported a total 9.11% prevalence rate from randomly selected cattle reared in nine districts of Bihar and the highest herd prevalence rate of 34.92% was observed in Patna district which might be due to sampling from organized farms. On the other hand, the highest prevalence in the Valsad district of Gujarat was attributed to its location (temperate zone) and comparatively higher rainfall as compared to another district of South Gujarat (Patel *et al*. 2017). But, Milton *et al*. (2019) also reported 8.33% seroprevalence in cattle from the high rainfall area of Meghalaya, India.

The most predominant serovars found circulating within the cattle population of the districts under the study was Autumnalis (6.05%), followed by Ballum (2.63%), Batavia (1.31%), Ichterohaemorrhagie (0.7%), Javanica (0.5%) and Sejroe (0.2%). However, no antibody was detected against serovars, *viz.* Australis, Canicola, Grippotyphosa, Hebdomedis, Pomona and Pyrogenes (Table 1). Though, there was no previous report on seroprevalence of bovine leptospirosis from Assam except that of Khan *et al*. (2012) who reported Icterohaemorrhagiae, Australis, Grippotyphosa, and Pomona in a human serum sample from Dibrugarh, Assam. The present finding is in accordance with Mitra *et al*. (2015), who reported the most prevalent serovars were Automnalis, 58/108 (53.70%) from South Andaman Islands, India. Distribution of serovar-specific anti-leptospiral antibodies against Autumnalis, Ballum, Bataviae, Ichterohaemorrhagie, Javanica, and Sejroe was recorded at dilution ratios of 1:40, 1:80, and 1:160 respectively (Table 1). Serum samples showing the agglutination phenomenon above the baseline dilution as stated above were considered serovar-specific anti-leptospiral antibodies, details of which have been shown in Fig. 2.

In comparison to MAT, an investigation into the efficacy of both IgG ELISA and MAT for detecting leptospiral antibodies in cattle indicated that IgG ELISA was more sensitive and straightforward to execute for the detection of anti-leptospiral antibodies in cattle. The

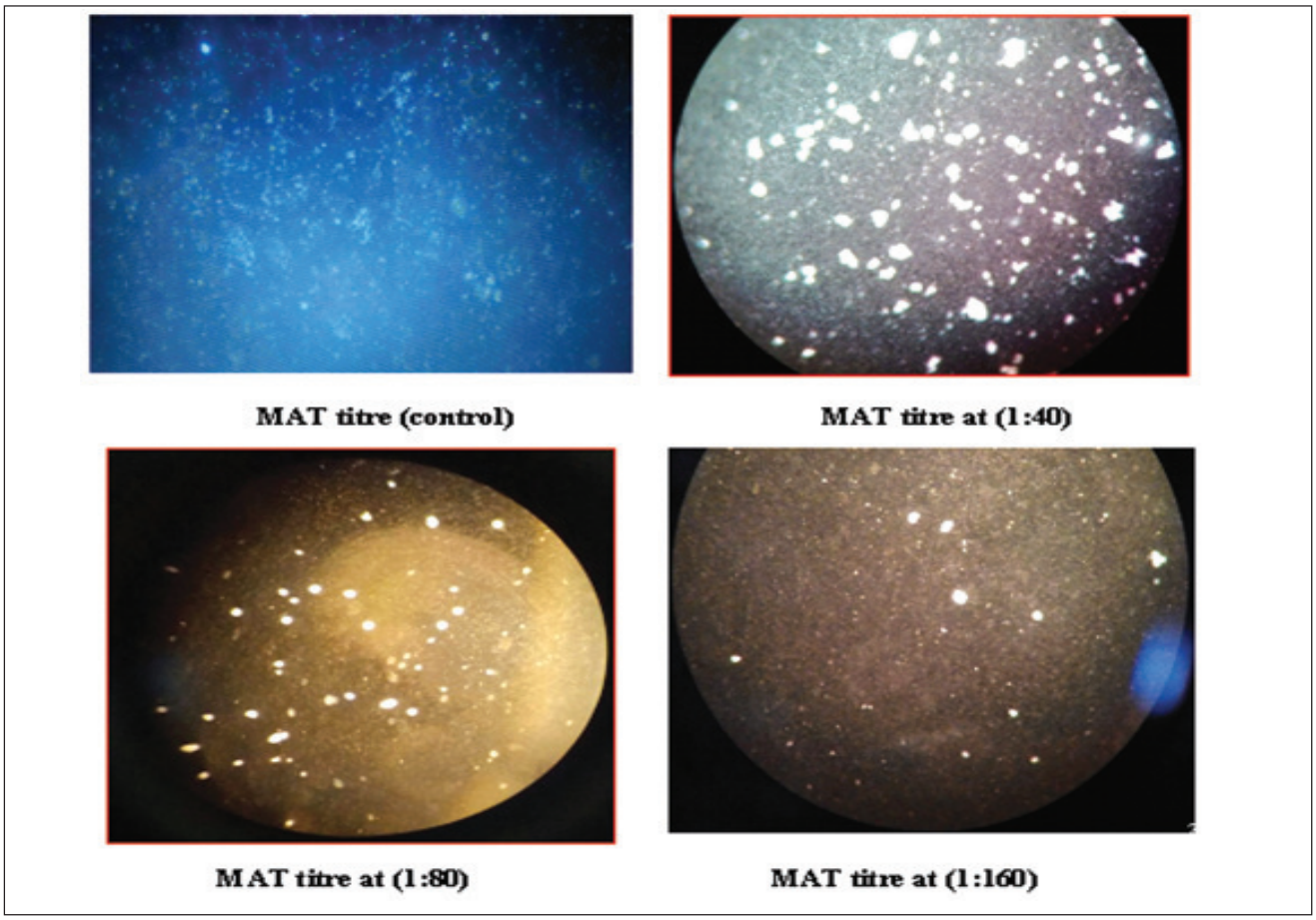

Fig. 2. Showing agglutination at different MAT titre of the positive samples.

comparative study showed a sensitivity and specificity of IgG ELISA to be 100% and 92.85% respectively, in comparison to the gold standard (MAT) with a concordance of 93.68%, which also corroborated the earlier report (Behera *et al*. 2014). Higher sensitivity and specificity of IgG ELISA than MAT have also been documented in various studies (O' Keefe 2002, Pandian *et al*. 2015). Thus, from the perspective of the present study, it may be stated that IgG ELISA could detect *Leptospira* genus-specific non-agglutinating antibodies, which were found negative by MAT. Concordance is greatly affected by the sensitivity and specificity of the tests under consideration (Thrusfield 1995). In this study, concordance between tests was calculated to determine the agreement between the tests as it is a powerful epidemiological tool to understand whether the combination of serological tests can be applied to diagnosing the disease accurately. Strengthening the findings of relative sensitivity and specificity, the concordance percentage between IgG ELISA and MAT was calculated to be 93.68%. This result showed that IgG ELISA was able to detect all the samples that were found positive by MAT.

Seroprevalence of *Leptospira* infection in relation to breed, sex, age, and health status of cattle based on ELISA

Out of 380 samples, crossbred cattle were found to be more susceptible (20.29%), in comparison to indigenous cattle (15.16%) as shown in Table 2. The higher seropositivity in crossbreed cattle as recorded might be due to poor disease resistance, and more susceptibility to any incoming pathogens. Lower seropositivity in indigenous cattle might be due to their being better adaptive, tolerant, and resistant in natural climatic conditions. According to Varma *et al*. (2001) and Nagarajan (2005), cross-breed cattle had higher seropositivity, which could be owing to the low acclimation factor of cross-bred cattle in these environmental conditions and their significantly inferior disease resistance. Exotic pure breeds, followed by crossbreed and indigenous cattle, are more susceptible (Balakrishnan *et al*. 2011, Pandian *et al*. 2015).

Table 1. MAT titres against the predominant *Leptospira* serovars.

Sl. No.	Serovar	MAT titre					Total
		1:20	1:40	1:80	1:160	1:320	
1	Australis	-	-	-	-	-	-
2	Autumnalis	-	14	6	3	-	23
3	Ballum	-	6	2	2	-	10
4	Bataviae	-	3	2		-	5
5	Canicola	-	-	-	-	-	-
6	Grippotyphosa	-	-	-	-	-	-
7	Hebdomadis	-	-	-	-	-	-
8	Pomona	-	-	-	-	-	-
9	Pyrogens	-	-	-	-	-	-
10	Sejroe	-	1	-	-	-	1
11	Ichtero haemorrhagie	-	3	-	-	-	3
12	Javanica	-	1	1	-	-	2
	Total	-	28	11	5	-	44

Sex-wise prevalence of *Leptospira* infection was found to be higher in females (20.58%) in comparison to male animals (6.75%) as depicted in Table 2. Gender-associated stress factors such as lactation, pregnancy, and parturition in the female population may increase the risk of infection (Sharma *et al*. 2003, Agrawal *et al*. 2005). The lower prevalence in male cattle might be attributed to the smaller size of males in a herd. However, there were reports which depicted either more seropositivity in males (Miller *et al*. 1991, Mandal *et al*. 2008, Balakrishnan *et al*. 2011) or no sex bias (Ramin and Azizzadeh 2013). Animals of the age group belonging 5 years and above were found to be more susceptible (23.72%), followed by animals of the age group of 3 to 5 years (20.66%) and lowest in the age group between 1 to 3 years (8.03%) (Table 2). The higher rate of seroprevalence recorded in the age group of above 5 years might be due to cattle belonging to the older age group making them more prone to *Leptospiral* infection. Salas (1986) and Prescott *et al*. (1988), who found higher seropositivity in older cattle, agree with the findings of this study. The lower seroprevalence of *Leptospira* infection recorded in young animals (1-3 years) of the age group might be attributed to the infrequent repeated exposure to infection (Balakrishnan *et al*. 2011, Patel *et al*. 2014, Pandian *et al*. 2015). The seroprevalence during the current investigation which recorded more (21.12%) in cattle with clinically ill/with a history of abortion, repeat breeding, reduced milk yield, and mastitis than in apparently healthy animals (15.96%) (Table 2). Similar kind of findings were also reported earlier by (Momtaz and Moshkelani 2012, and Patel *et al*. 2014).

Seroprevalence of *Leptospira* infection in relation to herd size, location of the herd, and types of husbandry practices of cattle based on ELISA

In consideration of herd size, *Leptospira* infection was recorded higher in larger herds (21.29%) followed by

medium herds (19.42%) and small herds (11.76%) (Table 3). The highest prevalence of *Leptospira* infection was recorded in larger herds where above 30 animals were kept and lowest in smaller herds where below 10 animals were reared. Infection by *Leptospira* spp. is more likely in larger herds with high animal density (Oliveira *et al*. 2013). In Irish suckler herds, breeding herd size was revealed to be a statistically significant risk factor for leptospirosis. After controlling for region, the probabilities of a herd being positive for leptospiral infection were 5.47 times greater (p = 0.032) in herds with 14 to 23 breeding animals than in herds with less than 13 breeding animals (Ryan *et al*. 2012).

The prevalence of *Leptospira* infection was found to be higher in the urban area (20.64%) in compare to rural (16%) (Table 3). Due to the disposal of animals with inadequate milk output, the dairy farms in this region have a high animal turnover rate. When undertaken without sufficient biosecurity controls, the rapid rate of animal turnover could allow the entry of diseases like leptospirosis. Based on husbandry practices (Table-3), the prevalence was found to be more where there is poor hygienic condition prevailing in that farm (23.12%), followed by moderate (17.03%) than good (9.41%) which has shown some similarities with the previous findings when these practices are performed in an uncontrolled or unhygienic manner, it can facilitate the transmission of diseases. Hashimoto *et al*. (2015) reported that in the high density of animals in calving pens, urine accumulates and increased contact with contaminated products of birth, abortion, and reproductive failures associated with animal handling; these possible exposures may predispose animals to *Leptospira* spp. infection.

Seroprevalence of *leptospira* infection in relation to month-wise sample collection based on ELISA

The prevalence of *Leptospira* infection was found to be highest during monsoon season, *i.e.,* June-September (26.08%), followed by pre-monsoon, *i.e*., March-May (17.07%), and then post-monsoon, *i.e.,* October-

Table 2. Seroprevalence of *Leptospira* infection in relation to breed, sex, age and health status of cattle based on IgG ELISA.

Sl. No.	Breed/genetic group	No of sample tested	No of Positive	Percent positive (%) *
1	Indigenous	178	27	15.16
2	Cross-breed	202	41	20.29
	Total	380	68	17.89
Sl. No.	**Sex**	**No of sample tested**	**No of Positive**	**Percent positive (%) ****
1	Male	74	5	6.75
2	Female	306	63	20.58
	Total	380	68	17.89
Sl. No.	**Age Group (Years)**	**No of sample tested**	**No of Positive**	**Percent positive (%) *****
1	Young (1-3)	112	9	8.03
2	Middle age (>3-5)	150	31	20.66
3	Adult (Above 5)	118	28	23.72
	Total	380	68	17.89
Sl. No.	**Particulars**	**Total no of animal**	**Positive**	**Percent positive (%) ******
1	Clinical history of the animals/clinically ill	142	30	21.12
2	Apparently healthy animals	238	38	15.96
	Total	380	68	17.89

*No significant association ($p>0.05$), $\chi^2=1.36$; **Significant association ($p<0.05$), $\chi^2=6.8$; ***Significant association ($p<0.05$), $\chi^2=10.92$; ****No significant association ($p>0.05$), $\chi^2=1.28$.

Table 3. Seroprevalence of *Leptospira* infection in relation to herd size, location of the herd and types of husbandry practices of cattle based on ELISA.

Sl. No.	Herd size	No of sample tested	Positive	Percent positive (%)*
1	Small(<10 animals)	153	18	11.76
2	Medium (11-30)	139	27	19.42
3	Large (above30)	108	23	21.29
	Total	380	68	17.89
Sl. No.	**Location of herd**	**No of sample tested**	**Positive**	**Percent positive (%)****
1	Urban	155	32	20.64
2	Rural	225	36	16.00
	Total	380	68	17.89
Sl. No.	**Types of husbandry practices**	**No of sample tested**	**Positive**	**Percent positive (%)*****
1	Good hygiene	85	8	9.41
2	Moderate hygiene	135	23	17.03
3	Poor hygiene	160	37	23.12
	Total	380	68	17.89

*No Significant association ($p>0.05$), $\chi^2=4.96$.; ** No significant association ($p>0.05$), $\chi^2=1.05$; *** Significant association ($p<0.05$), $\chi^2=7.20$*

Table 4. Seroprevalence of *leptospira* infection in relation to month-wise sample collection based on ELISA.

Season	Month	No. of Sample	Positive	Percent positive
Pre- monsoon	Mar-May	123	21	17.07
Monsoon	June –Sep	115	30	26.08
Post- monsoon 16.66	Oct-Nov		66	11
Winter	Dec-Feb	76	6	7.89
Total		380	68	17.89

Significant association ($p<0.05$), $\chi^2=10.58$

November (16.66 %) as depicted in Table 4. The lowest prevalence (7.89%) was recorded from December to February (winter season). During the monsoon (*i.e*., June to September) flooding has been a major problem in some parts of the lower Brahmaputra valley of Assam due to heavy rainfall which might be a cause of a higher prevalence of leptospirosis. During this season, the animals may get infected due to repeated exposure to contaminated water. Pawar *et al*. (2018) found a consistent seasonality pattern in the number of leptospirosis cases reported monthly from January 2011 to December 2015. The highest number of cases occurred around August each year, which could be due to heavy rainfall and relative humidity that leads to an increase in the number of cases due to prolonged contact of at-risk people with water bodies. Patel *et al*. (2017), reported the rainy season has the highest frequency of *Leptospira* infection.

CONCLUSION

In the present study, the most predominant serovars circulating within the cattle population among the districts were found to be Autumnalis, followed by Ballum, Bataviae, Ichterohaemorrhagie, Javanica, and Sejroe. The present study will serve as baseline data for the prevention and control of *Leptospira* infection in cattle. A paucity of information exists on transmission, host range, and other factors that enhance the disease's emergence. The findings of this study also revealed that IgG ELISA might have a higher sensitivity and specificity for detecting leptospiral antibodies in cattle than MAT. However, more comprehensive studies including detailed epidemiology of leptospirosis, culture, and molecular detection of the organism are required for precise and confirmative diagnosis. Therefore, a wide range of samples from other animal species with distinct temporal-spatial distribution may be helpful to validate the significance of the tests in diagnosing leptospirosis.

ACKNOWLEDGMENT

The authors express their sincere thanks to the DBT twinning project on "Serosurveillance of

Leptospira infections in animals of Northeastern region of India" to conduct the present investigation. The authors are also highly thankful to Assam Agricultural University, Jorhat, India for providing the necessary funding for this project.

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***Cite this article as:** Kader NA, Hussain P, Bora DP, Hazarika RA, Das S, Tamuly S, Sonowal S, Abedin SN, Arif SA, Hazarika P (2022) A study on seroprevelence and risk factors of bovine leptospirosis in lower Assam, India. Explor Anim Med Res 12 (2): 167-175. DOI: 10.52635/eamr/ 12.2.167-175.