Sero-prevalence and risk factors of leptospirosis in commercial cattle herds of Rupandehi district, Nepal

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Abstract

Nepal has an agrarian-based economy where the livestock sector contributes about 12 percent to the national GDP. Rupandehi district is one of the largest dairy pocket areas in the nation, but the disease, such as leptospirosis, posed a negative impact on their production and productivity. Despite the economic importance of cattle leptospirosis, the disease is concerned for the people's occupational risk and food safety. Although leptospirosis is a priority zoonosis in Nepal, the effective implementation of the control program lacks both in animal and human health. A cross-sectional study was conducted to estimate the prevalence and identify associated risk factors of cattle leptospirosis from March 2019 to April 2020. Altogether, 206 cattle herds located in all the sixteen local levels of the district were visited. The owners were interviewed to record the cattle management system's information and their knowledge of the zoonotic diseases. A total of 383 cattle serum samples were collected, and an indirect antibody ELISA was performed to estimate the infection status of leptospirosis in the cattle herds. All the data were analyzed by Open epi and R software for the descriptive and analytical study. Bivariate and multivariable logistic regression was applied to assess the potential herd level and animal level risk factors. Out of seven potential herd-level risk factors, the purchased cattle herds (OR: 7.2, 95% CI: 1.24-136.5, $p=0.025$) and cattle with herd size >10 (OR: 14.92, 95% CI: 2.61-283.38, $p=0.025$) were identified as significant risk factors for leptospirosis. At the animal level, the cattle taken for grazing in pastureland accessed by the community dog was a significant risk factor (OR: 4.16, 95% CI: 1.13- 14.06, $p=0.034$). Surprisingly, none of the farmers had heard about leptospirosis before. The outcome of this research could be an epidemiological insight in control of priority zoonosis to protect the livestock economy and reduce their negative impact on public health.

Background

Leptospirosis is a bacterial zoonosis, caused by the infection of leptospira species, which has a worldwide importance [1]. The natural carrier of this bacteria are wild and domestic animals, rodents, and dogs [2] . Among the various serovars, *Leptospira hardjo* is common in cattle [3-4], and they are the maintenance host for this serovar [4]. In cattle, leptospirosis causes reduced conception rates and reproductive problems like abortions, stillbirths, and decreased milk production [2,5].

People get infection after direct contact with infected animals or indirect contact with the contaminated environments by their urinals [6]. The occupational risk of exposures is mainly among the veterinarians, farmers, slaughterhouse workers, hunters, animal shelter workers, and agricultural workers[3]. World Health Organization (WHO) has considered leptospirosis a neglected and re-emerging zoonotic disease for humans and animals [7,8].

Leptospirosis is one of the six priority zoonoses listed for Nepal [9]; however, it's adequate surveillance and reporting is still lacking in Nepal [10,11]. With the first report of human leptospirosis in 1981, numerous leptospirosis cases were reported among human and animal populations [12-15]. Most of these studies supported that the source of infection in humans is due to animal contact [13,14]. However, the actual socio-dynamic transmission of leptospirosis between the animals and the disease's economic burden in both animals and humans was not well known.

Nepal's one-third of the economy is based on agriculture, and the livestock sector singly contributes around 12% to the national GDP [15]. Cattle rearing is a traditional practice of Nepal [16], and the cattle ranks first among (7.2 million heads) all large ruminants in the country [17]. Moreover, the dairy industry has undergone rapid development since the last decades [18], but

reproductive diseases such as leptospirosis in cattle have not been well assessed yet. The economic impact of the cattle production and its negative consequences on the livelihood of the poor and the marginal farmers is humongous [19].

There are only a few research articles and documentation about animal leptospirosis in cattle. According to research conducted in the Bhaktapur district of Nepal, the seroprevalence of *Leptospira interrogans* serovar hardjo was estimated at 5.11% (9/176) [20]. Another study over the various ecozones conducted in large ruminants of Nepal, the seroprevalence of *Leptospira* hardjo was 3.75% (12/320) during the pre-monsoon period and 6.88% (22/320) in post-monsoon period [21]. Similarly, a research conducted in infertile cattle and buffalo of western hills of Nepal showed the seropositivity of 11% (12/114) in cattle and 5.5% (5/86) in buffaloes [22].

This study aimed to identify the herd level and animal level risk factors that could contribute to the development of leptospirosis in cattle farming. The various risk factors associated with detecting leptospirosis in bovine leptospirosis were selected based on the literature review and expert's opinion [23, 30]. The assessment of these risk factors at animal and herd levels could improve our understanding of leptospirosis, as the priority zoonosis could contribute to the development of control and contingency plan for it. It also attempts to assess farmers' knowledge regarding the leptospirosis and make them aware of being protected during their livestock handling.

Material and Methods

Study sites

This study was conducted in the Rupandehi district located in the southwestern region of Nepal (Fig 1). The district has one of the highest number of dairy cattle with around 442 cattle farms

[31], and about one hundred thousand of cattle population [17] within the total area of 530 square miles [15]. The district shares the border with neighboring nation India and has an international border quarantine where the importation of livestock exists between India and Nepal [32]. There is also a risk of disease spread due to the informal livestock trade and cattle's internal movement between other adjacent districts.

Fig 1: A map of Nepal, with the study district highlighted, including the seropositive and seronegative cattle farms.

Study design

A cross-sectional study was conducted on commercial cattle herds of Rupandehi district between March 2019 to April 2020. The semi-structured questionnaire was used to collect the information on management status, biosecurity conditions, animal movement process of each herd, and owner's knowledge of the principle zoonotic diseases such as leptospirosis, toxoplasmosis, and brucellosis. The questionnaire was initially designed in English and later translated to the local Nepali language (S1 file). A set of survey was pretested on ten farms as the pilot study. The response obtained from the owners were coded and analyzed. The response rate and clarity of the reaction were assessed to refine the survey questions. Next, blood samples were collected on the same day after interviewing the farm owners. The written consent from the owners was obtained during the questionnaire and sample collection processes.

Sampling and sample size calculation

The sampling process consisted of two stages: first, herd-level sampling was done from total 442 commercial herds located in all sixteen local levels (municipalities) in the districts. Secondly, the

propionate number of animals was selected from each herd giving a total individual sample size [33].

The sample size was determined using an open-source epidemiological software “OpenEpi” version 3.01 [34]. For selecting total herds, the expected prevalence was set as 50% with 5% desired precision at a 95% level of confidence that maximized the number of herd selection.

The formula is based on:
$$N = \frac{1.96^2 P_{exp} (1-P_{exp})}{d^2}$$

where N= sample size, P_{exp} = expected prevalence, and d = absolute precision [35].

The total number (N) of herd produced by this method is (N= 206). The same process as above, was repeated to calculate the total number of cattle to be sampled, keeping entire animal populations as 98,384 [17]. The total individual animal (n) to be sampled was (n= 383) samples. So, altogether 383 blood samples were collected from 206 selected herds located in the district.

Sample transport and laboratory testing of samples

The tubes containing blood were allowed to remain overnight at room temperature to facilitate the serum separation, and the separated serum samples were transferred to a 2 ml Eppendorf tube. The collected serum samples were stored -20° Celsius and transported to Central Veterinary Laboratory for further laboratory analysis. The indirect ELISA against leptospirosis antibody was performed using the kit brought from Prionics Lelystad B.V., the Netherlands. The process was performed according to the protocols provided within the kit (citation). The reading of the ELISA plates was performed by the ELISA reader ("Multiskan™ FC Microplate Photometer") at optical density (OD) 450nm within 15 minutes. The readings were interpreted with the software and protocol provided within the kits to determine the number of *L. hardjo* seropositive samples.

Test result validation and interpretation criteria

After the measurement of optical density, the corrected OD value is calculated by subtracting the OD value of reference (controls) and test sera (samples) with the mean OD value of the blanks (ELISA buffer). Now, the percentage positivity (PP) of reference sera and the test sera were calculated as follows.

$$PP = \frac{\text{corrected OD of the test sample}}{\text{corrected OD of the positive control}} * 100$$

The result was validated if

- i) The mean OD of the blanks was < 0.150
- ii). The corrected OD of positive control was ≥ 1.000
- iii). The mean PP of negative control was < 20
- iv). The mean PP of weak positive control was between 20 to 60

For each sample, the cut off for percentage positivity for the test sera was determined as follows:

PP < 20 was recorded negative, PP between 20-45 was classified as inconclusive, and PP ≥ 45 was considered positive for *L. hardjo* antibodies.

Statistical Analysis.

First, the raw data were entered manually on MS excel® spreadsheet from the paper-based questionnaire. After cleaning the data, the excel file was converted to a csv file and analyzed by R version 3.6.1 (R core Team 2019). The descriptive data analysis was performed to calculate the proportion of the seropositive herds with at least an animal positive to *L. hardjo* antibodies in it, and the percentage of seropositive animals among all herds. The true herd-level prevalence and the animal level prevalence were calculated by the use of open-source software EpiTools by

Ausvet [36]. The estimated sensitivity (Se) and the specificity (Sp) of the indirect antibody ELISA were estimated to be 96.4% and (Sp) 96.7%), respectively [37].

The continuous variables (herd size, age category, parity number) and an ordinal variable (body condition score) were converted to binary categorical variables using the quartiles (median as cut off) to avoid the problem of linearity [38]. The association between the categorical variables were assessed by Pearson's Chi-square test. Highly related variables were assessed, and the biologically plausible variables were retained in the model for further analysis. For instance, there was a high correlation between the age category and the parity number and the cattle with common grazing ground and cattle grazing in community dog access. We believe that age could contribute to more critical information, and the community dog was a biologically the reservoir of leptospirosis, and these two variables were retained [39-43]. The remaining variables, the parity number and the common grazing ground, were ultimately dropped from the final model.

Bivariate analysis was performed to measure the association of each categorical variable with the outcome "seropositivity to leptospirosis" (Table 1 and 2). The variables with the significance level $p \leq 0.2$ following the bivariate analysis and those with the biological plausibility were manually entered in the final multivariable model [44]. A backward stepwise variable selection was used to add the variable with the lowest p-value to construct a final model with a significance level of $p \leq .05$. Any variables with p-value < 0.05 were considered statistically significant and considered the risk factors. (Table 3 and Table 4).

The multicollinearity among the independent variables was checked for variance inflation factors (VIF) by "vif" function in R, and variables with "vif" coefficient > 10 were considered high. None of the independent variables in the analyses showed multicollinearity.

The potential confounding effects of the variables were checked with the changes in the point estimates of the variables that remain in the model [43-45]. Any changes in the coefficient with >20% were included in the final model.

Alternatively, a full model was built in the first place, and the appropriate model was selected based on the automatic stepwise Akaike's Information Criteria (AIC) selection process (both backward and forward direction). The initial manually derived models were compared to the automated derived models, but all of the approaches ended up the same set of variables on the final model (Table 3 and Table 4). The fitness of the final models was assessed by Hosmer-Lemeshow goodness of fit function (hoslem.test) in R, and they were fit.

Ethical statement:

Ethical statements for animal subjects were approved by Nepal Veterinary Council (NVC), Ref. No 179 (TG)/2076.77, the official statutory body of animal health. A written consent of form from the cattle owner were obtained during the interview. The experienced and registered veterinarian handled all the animals during animals' sampling, taking account of minimal pain during the blood collection. The owners also requested to provide the test results of their cattle herds through confidential means.

Results

Descriptive study

Of the total 206 herds selected, the median herd size was 10 with a range of one to 200 cattle.

The median age of the cattle in the herd was five years old. Of the total 206 herd, the

composition of home bred and purchased animals was 60.68% (125/206) and 39.32% (81/206). The majority, 93.69% (193/206), of the herds, were based on stall-feeding system, and only 6.31% (13/206) of them were taken for occasional grazing. About eighty-five percentage (175/206) of the farms applied an artificial insemination process for breeding their animals, and the remaining 15.04% (31/206) of the herds used to apply the natural mating system. Interestingly, 22% (67/300) of the farmers used to work both in the rice field and the cattle rearing. However, none of the farmers had heard about the leptospirosis before, and none had any information to prevent transmission of that diseases from their livestock.

Herd level seroprevalence and potential risk factors

Of the total 206 herds tested against leptospirosis, 10 (4.85%, 95%CI: 1.92- 7.79) herds had at least one seropositive animal. The potential herd level variables were classified broadly into two categories: i) Herd characteristics and Herd management system [24-25, 29, 39, 43, 46].

Animal level seroprevalence and potential risk factors

Of the 383 blood samples collected, 16 samples produced hemolysis, and they were discarded from testing. Of the total 367 serum samples, 14 (3.81 %, 95%CI: 2.29, 6.3) were tested positive to *L. hardjo*. The potential animal level risk factors selected were: i) animal characteristics such as sex, age, breed, body condition score, parity number, pregnancy status, stage of lactation, tick infestation ii) animal movement patterns such as taking animals in the grazing field and pasture access by the community dogs [24,29,43,47].

Univariate regression analysis for herd and animal level risk factors

There were 11 herd-level risk factors included in the univariate analysis, as depicted in Table 1.

The purchased cattle herds had higher odds of detecting *Leptospira hardjo* (OR: 6.21, 95% CI: 1.13-115.6, $p=0.09$) compared to the home-bred herds, but the finding was only borderline significant. The cattle herds with median herd size of >10 were 12 times more likely to be seropositive compared to those with a herd size of ≤ 10 (OR: 12.00, 95% CI: 2.19-223.44, $p=0.02$). Among the potential herd-level risk factors, the variables with the cut-off values of $p \leq 0.20$ were qualified to enter in the final multivariable model. Such variables were cattle herd origin, herd size, herd structure, herd fed with soil contaminated feed, presence of rodents on farms, and the herd whose owners worked in the rice field with bare feet (Table 1).

Table 1: Herd level factors potentially associated with seropositive to leptospira by univariate logistic regression analysis

Determinants	Factor level	Total number of herds (n=206)	Positive farms	Negative farms	OR	95% CI	P- value
Herd Characteristics							
Cattle origin	Purchased	125	9	116	6.21	(1.13-115.6)	0.09
	Home breed	81	1	80			
Herd Size (median=10)	>10	93	9	84	12.00	(2.19-223.44)	0.02*
	<=10	113	1	112			
Management system							
Herd structure	Head to head	71	6	65	3.29	(0.84-16.02)	0.10 ^a
	Tail to tail	25	1	24	1.49	(0.07-12.19)	0.74
	Single	110	3	107	Ref		
Bedding type	Mat	22	1	21	1.81	(0.08,16.1)	0.62
	None	144	3	141	0.58	(0.1,3.2)	0.51
	Saw dust	96	4	92	0.92	(0.16-5.2)	0.93
	Straw	105	6	99	Ref		
Source of water	Tap	116	6	110	1.17	(0.32-4.71)	0.81
	Well	90	4	86	Ref		
Feeding system	Occasional grazing	13	1	12	1.704	(0.09-10.25)	0.63
	Stall feeding	193	9	184	Ref		
Breeding type	AI	175	9	166	1.70	(0.09-10.25)	0.65

	Natural Breeding	31	1	30	Ref		
Cat feces seen around farms	Yes	72	5	67	1.94	(0.52-7.20)	0.31
	No	134	5	129	Ref		
Feed contamination with the soil	Yes	127	8	119	2.59	(0.63-17.45)	0.20 ^a
	No	79	2	77	Ref		
Rodents seen around farms	Yes	139	9	130	4.57	(0.83-85.16)	0.09
	No	67	1	66	Ref		
Owners worked on rice field with bare feet	Yes	148	9	139	3.691	(0.67,68.84)	0.15 ^a
	No	58	1	57	Ref		

a: these non-significant variables were entered in the final model with cut off criteria of $p \leq 0.2$, including the significant variables.

*P value <0.05 are the significant variables.

Ten potential animal level risk factors were selected and classified under broad category: animal characteristics and animal movement pattern, as shown in Table 2. In the univariate analysis, the non-lactating cattle were four times (OR: 4.09, 95% CI: 1.27-12.73, $p=0.019$), more likely to be seropositive with leptospirosis compared to lactating cattle. The cattle taken for common grazing in the fields were four times more likely to be seropositive to leptospira (OR: 4.11, 95% CI: 1.22,12.51, $p=0.015$) compared to animals that were stall-fed on farms. The animals grazed on the field with the community dog access had significantly higher odds of leptospira seropositivity (OR: 5.21, 95% CI: 1.53,16.012, $p=0.005$). However, none of the other animal level risk factors were associated with the seropositive to leptospirosis among cattle.

The variables “parity number” and “common grazing ground” were dropped because they were highly associated with “age category” and “pasture grazing with community dog access,” respectively. The set of variables such as the breed of cattle, animals' parities, history of repeat breeding, stage of lactations, and grazing in pasture with the community dog access were selected for multivariable analysis.

Table 2: Animal level factors potentially associated with seropositive to leptospira by univariate logistic regression analysis

[illegible]

Common grazing field with other domestic animals	Yes	47	5	42	4.11	(1.22,12.51)	0.015* ^b
	No	320	9	311			
Grazing in pasture with the community dog access	yes	39	5	34	5.21	(1.53,16.012)	0.005*
	No	328	9	319			

a: this variable is highly correlated with age category and finally dropped from the model.

b: variable is highly correlated with community dog access and finally dropped from the model.

*P value <0.05 is statistically significant

Multivariable logistic regression Analysis for herd and animal level risk factors

Multivariable logistic regression analyses were separately conducted for a group of the herd-level and the animal-level risk factors.

Seven variables from the set of herd-level and four variables from the animal level risk factors were qualified to enter the final multivariable models based on cut off criteria of p-value ≤ 0.2 .

In the final herd level multivariable model, the two herd-level risk factors: cattle origin and herd size were associated with seropositivity to leptospirosis (Table 3). The purchased cattle herds had significantly higher odds of detecting leptospirosis (OR: 7.2, 95% CI: 1.24-136.5, p=0.025) compared to home-bred cattle herds. Cattle herds with herd size >10 animals were about fifteen times more likely to seropositive for leptospirosis (OR: 14.92, 95% CI: 2.61-283.38, p=0.025) than herd size of ≤ 10 animals.

In the final animal level multivariable model (Table 3), the animals taken for grazing in the pastureland with access to community dogs had significantly higher odds of seropositive to leptospirosis (OR: 4.16, 95% CI: 1.13- 14.06, p=0.034). The cattle herd owners who

worked in the rice fields showed an increased likelihood of leptospira seropositivity in their cattle herds (borderline significance; OR: 5.67, 95% CI: 0.96-108.75, $p=0.057$). Non-lactating animals that was a risk factor in the univariable analysis was not a risk for leptospira seropositivity in the multivariable model (OR: 4.09, 95% CI: 0.84-9.59, $p=0.089$).

Table 1: Herd level and animals risk factors associated with leptospirosis infection on commercial dairy farms by multivariable logistic regression analysis.

Determinants	Response level	Adjusted OR	95%CI	p value
i. Herd level risk factors (n=206)				
Cattle origin	Purchased Homebred	7.2	(1.24,136.5)	0.025*
Herd size	>10 <=10	14.92	(2.61, 283.38)	0.001*
Owners working bare foot on rice fields	Yes No	5.67	(0.96, 108.75)	0.057
Rodents seen around kitchen and farms	Yes No	3.93	(1.53-75.72)	0.149
ii. Animal Level risk factors (n = 367)				
Grazing in pasture with the community dog access	Yes No	4.16	(1.13- 14.06)	0.034*
Production status of cattle	Lactating Non-lactating	4.09	(0.84-9.59)	0.089

CI: confidential intervals;

OR: odds ratios;

*P-value <0.05

Discussion

We estimated that the herd level and animal level seroprevalence of leptospirosis as 4.85% (10/206) and 3.81 % (14/367), respectively, among the cattle herds in Rupandehi district of Nepal. There were some previous regional studies in Nepal, that estimated 3.75 % (12/320) to 11% (12/114) of the leptospirosis in cattle population[21,22]. Nevertheless, there might be either overestimation or underestimation of the leptospirosis prevalence due to differences in epidemiological design, sample size, and geographical diversity. Probably, this is the first risk factor study of leptospirosis among cattle in Nepal to our knowledge.

The purchased herds were more likely to detect leptospirosis than the homebred herds in our study. It is epidemiologically justifiable that the herds that moved during selling are more likely to contract the disease [12, 48-49].

Also, the Rupandehi district of Nepal is located in the trade transit with India and neighboring provinces, so the cattle herd, when moved from one destination to another, have higher exposures compared to the confined herds.

Our findings show that the cattle herds of >10 animals in the herd was a risk factor. The bigger herd size is the risk factor for leptospirosis, and many of the studies have already proved that [25,29]. The high stocking density in the bigger herd size farms had a higher risk of getting the diseases due to frequent selling or purchasing of animals, and the contact between infectious and susceptible animals takes place more often [50].

The cattle herds owned by the people who worked in rice fields with bare feet were at borderline risk than herds whose owners had another occupation ($p<0.05$). It was because most of the cattle

owners in Nepal were the farmers, and they were more likely to work in the rice field to get straw for the cattle. Moreover, the possible environmental contamination might be a source of infection in the herds. The above finding was similar to the study by Alavi et al. [51] in southwestern Iran, and Sejvar et al. [52] in Malaysia. The owners who used to work in the rice fields posed risks to their cattle and themselves unless they apply the personal protective measures.

Though rodents are the carriers for leptospirosis [2], the association was not statistically significant in the multivariable analysis. However, rodents should be controlled in the farms because they could contribute to transmission of leptospira organisms.

Our findings showed that the cattle taken in the pasture with community dog access had significantly higher odds of leptospirosis. In Nepal, there are a significant number of community dogs in the cities and village areas. These dogs frequently move in search of food and defecate openly in the pasture that leads to the contamination of grazing ground. The above statement is supported by the findings in Brazil by Fávero et al. [43], who also demonstrated that the dog in the pasture was associated with detecting leptospira antibodies in cattle. This study findings might advocate the local governments to formulate plans in managing the population of community dogs around the livestock farming area.

However, our study also had some limitations. Some cattle farm owners do not remain on the farms, and the information was obtained from the less educated farmworkers, and their level of understanding may not represent the idea of the all cattle owners in general. There could be potential recall bias in obtaining the animals' information as most of them had not maintained the excellent record-keeping on the farms.

Conclusions

This research estimated the burden and identified the risk factors of cattle leptospirosis in the commercial cattle herds that provided an epidemiological insight into animal leptospirosis in Nepal. Though leptospirosis has been documented as priority zoonoses by the government, the effective implementation of the surveillance program still lacks in the nation. The outcome of this research might be useful for effective program planning in the prevention and control of leptospirosis both in animal and human health. Ultimately, the livestock production and productivity should be enhanced, food safety for the public must be assured, and farmworkers' occupational safety should be ensured. We suggest further nationwide research and application of control measures through one health approach to reduce the incidence of zoonotic diseases like leptospirosis in Nepal.

Supporting information

S1: Questionnaire used for the study

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Authors contribution

TRG: Conceived the study design, conducted the research, performed lab, analyzed data and wrote the paper, revised draft. SJ: assisted in data and sample collection, lab testing and literature review. SP and AP: Assisted in data and sample collection. RC: assisted in lab testing.

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Figure 1