International Journal of Veterinary Sciences and Animal Husbandry

ISSN: 2456-2912
VET 2023; SP-8(5): 242-247

www.veterinarypaper.com
Received: 25-07-2023
Accepted: 30-08-2023

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Sero-prevalence and associated risk factors of *Leptospira hardjo* in dairy cattle of western dairy pocket area in Chitwan District of Nepal

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Abstract
Leptospirosis is a serious spirochete zoonotic disease of increasing worldwide prevalence and distribution. More than 300 distinct *Leptospira*l serovars recognized and these are arranged in 25 serogroups. Leptospires belonging to *Leptospira* serovar Hardjo are the most common cause of leptospirosis in cattle.
Objective: To detect the sero-prevalance and associated risk factor of *Leptospira hardjo* in dairy cattle of western dairy pocket area in Chitwan district of Nepal
Methods: Methodology was two stage sampling survey. 5 Village development committee of western Chitwan were taken. Total of 382 serum sample were collected and identified individually. Samples were tested with Priocheck *L. hardjo* antibody detection kit, ELISA. Data entry, analysis was done in Microsoft Excel, Open Epi and SPSS.
Results: Out of 382 serum sample collected and tested, 19 serum samples (4.97%) were found to be positive. Statistical analysis of the risk factors shows significant difference (p<0.05) in case of animal with history of abortion, mastitis, nervous signs, presence of rodents and dry ground surfaces whereas no significant difference (p>0.05) is found among location, age, breed, parity, body condition score, housing system, mating system, history of hemoglobinuria, and history of reproductive problems. Since, none of the sampled farms had reportedly used Leptospiral vaccine; the presence of circulating antibodies in the cattle suggested a natural exposure to *Leptospira hardjo*.

Keywords: *Leptospira hardjo*, elisa, dairy cattle, seroprevalance, serum

Introduction
Spirochete disease, leptospirosis, is public health issue (Vijayachari *et al*., 2008) [41] prevalent worldwide with its zoonotic importance (Bharti *et al*., 2003; Zavitsanou and Babatsikou, 2008) [7, 43]. The etilogical agent is spirochete bacteria *Leptospira*. Different serovars of *Leptospira* cause disease condition in affected animals (Bharti *et al*., 2003) [7]. Greater than 300 distinct serovars are classified into 25 serogroups (Picardeau, 2013) [33]. The most important causative agent in cattle is *Leptospira* serovar Hardjo (Ellis *et al*., 1978) [16].
The zoonotic disease is widely distributed to larger geographical are because of its huge mammalian hosts which are transmitted through excretion from renal tubules (Ko, *et al*. 2009) [27]. Transmission is favoured by moist environment. Primary hosts are different rodents (rats, mice) and the secondary hosts are mammals (dogs, deer, rabbits, cattle, buffaloes, sheep, goat and pigs) (Zavitsanou & Babatsikou, 2008) [43]. Transmission to human and animals occurs directly by contact with infected urine and tissues and indirectly by contact with contaminated water, food and soil (Faine, 1994) [18]. Disease in human and animals is distributed in areas contaminated with affected animal's urine with rare human-to-human transmission (Baer *et al*., 2009; WHO, 2003) [3, 42].
It is occupational disease in developing countries as well as in developed countries, such as Denmark (Holk *et al*, 2000) [21] and USA (Meites *et al*, 2004) [30] mainly affecting people who closely come in contact to infected animals like farmers, veterinary doctors and technicians, meat workers, meat analysis, workers of sewage, workers in rice field, sugar cane yielders and banana farmers (Barmettler *et al*., 2011) [6].

The spirochete survives better in warm and humid tropical climates compared to temperate areas where higher incidence occurs during summer than in fall season with higher occurrence in developing countries compared to developed countries because of greater exposure rate (Faine, *et al*., 1999) [17]. Reported case suggests dairy pocket areas in Nepal affected by infertility in crossbred and exotic cattle (Jha, 2000) [22]. *Leptospira* infected cattle are prone to decreased fertility in cattle resulting economic crisis in livestock and dairy sector (Hathaway *et al*., 1982) [20] and occupational hazard to people (Smythe *et al*., 2000) [38]. Worldwide epidemiological incidence with approximately 10 times higher incidence in tropical regions to that of temperate (Hartskeerl *et al*., 2011) [19]. One million cases of severe leptospirosis occur annually, with 58,900 deaths (Costa *et al*., 2015) [11]. In temperate areas yearly infection rate varied from 0.02 per 100,000 whereas in tropical areas 10 to 100 per 100,000 people (Pavli *et al*., 2008) [32]. Baker and Lopez (2004) [4] indicated human infections were primarily caused due to contact with cattle, and secondarily by sheep either alone or in combination with other animals.

Materials and Methods

Survey Design

Population design and study sample: Animals in this region are not identified individually and are owned by many different owners. So, all the animals in the village are taken as a large herd owned by many different owners. The animals are in close contact and are under similar management practices. So, simple random sampling from such population is difficult and no sampling frame is available. So, a sampling design used by Vázquez-Barquero *et al*, (1986) [40] called as Two Stage Sampling Survey Design was used. Firstly, from 10 different VDCs of western part of Chitwan district, 5 VDCs (pocket area for milk production as given by District Livestock Service Office, DLSO Chitwan) namely Divyanagar, Gunjanagar, Mangalpur, Geetanagar, Sharadanagar were chosen. This division was applied for the design of the survey at first stage where sampling frame is available and required sample size was calculated on a Pocket area basis. The pocket areas are displayed in Figure 1 developed using Arc Map 10.3.

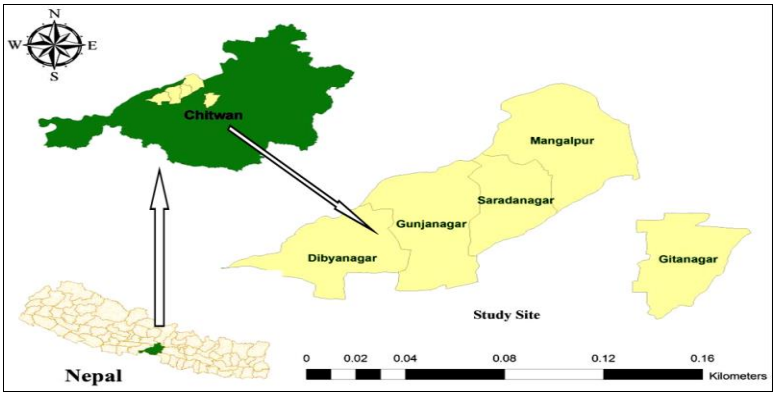

Fig 1: Map of the study site

Sample size and selection of sample

Reliable livestock population data of the above VDCs are known, probability proportional to size (PPS) design was used at the first stage. At the second stage, a fixed number of animals were chosen from selected VDC using simple random sampling. The sample size was calculated as 328 from the Epi Tools epidemiological calculators (Sergeant, 2017) [36] which is an online software. It uses the Daniel's formula (Daniel, 1999) [45] for sample size calculation.

$N = Z^2p(1-p)/e^2$ (for large population)

Where, N = no of sample

Z = 1.96 (from Z table at 95% confidence interval)

P = 0.5 (no previous prevalence data in Chitwan so we take 0.5)

E = 0.05 (precision level)

Using this formula sample size is calculated as 385. We have population size as 2206 so, adjusted sample size (n') is calculated as 328 using the formula n' = (N x n)/(N+n).
During sampling and questionnaire survey, there is high chance of occurrence of error or in some samples some data might be missing. To overcome this 10% more of calculated sample size has been suggested to be collected. Thus, the required sample size is 361. Fixed number of animals was selected at second stage of sampling from each VDC with the total sample size of 382. Table 2 demonstrates the selected VDCs with number of samples collected.

Table 1: Selected VDCs and No. of calculated Sample

S.N.	Selected VDCs	Total cattle	Number of samples
1	Gunjanagar	110	76
2	Divyanagar	100	75
3	Sharadanagar	450	77
4	Mangalpur	504	77
5	Geetanagar	1042	77
	Total	2206	382

(***Source*:** DLSO, 2071/72: Chitwan)

Rapport building: The farmers in the study area was informed about the research program and its objectives, expected outcomes and its impact or usefulness on them in the day before sample collection. They were informed and were expected for their kind support and cooperation.

Nature and sources of data

Primary sources of data: For the collection of primary data, semi structured questionnaire was used. In order to collect the most reliable useful information, primary data was collected by using household survey. The blood samples were also examined and the result obtained was used as primary source of data.

Secondary source of data: The secondary source of the data was collected from DLSO office of Chitwan and relevant literatures.

Collection of data: The conduction of questionnaire survey about information regarding reproductive problems, age, breed, parity, presence of clinical signs and managemental factors was done by farmer's interview.

Laboratory: In all, 382 blood samples were collected from 5 VDCs (Gunjanagar-76, Divyanagar-75, Sharadanagar-77, Mangalpur-77 and Geetanagar-77) which was selected on random basis. Blood samples were collected in sterile 10 ml Vaccuitainer and centrifuged. Thus, separated serum was removed and stored at -20 °C until testing. Laboratory work was performed at Central Veterinary Laboratory, Tripureshor, Kathmandu.

Statistical analysis: Data entry, management and analysis was done using program Microsoft Office Excel 2007. The association between different risk factors of disease such as location, age, breed, parity, history of reproductive problems, history of clinical signs like swollen lymph nodes, posterior paresis and managemental factors like multiple use of hypodermic needles and RP gloves, disinfection after dehorning, types of housing and presence of hematophagus insects were compared and analyzed statistically by a Chi-square (χ^2) analysis and fisher exact test using computer software Open Epi (Open Source Epidemiologic Statistics for Public Health - Version 3.01), Dean, Sullivan & Soe, (2013) [13] with significance level defined at the $p<0.05$. Odd Ratio for breeds, age group, parity considered and different risk factors were calculated using Open Epi (Open Source Epidemiologic Statistics for Public Health) (Version 3.01, Dean *et al*., 2013) [13].

Serological Test: Serological test was done using Indirect ELISA which was carried out as per the guidelines provided in PrioCHECK®*L. hardjo* Antibody Test Kit (Prionics, Netherland).

Result

Seroprevalance of *Leptospira hardjo*: Out of 382 serum sample collected and tested, 19 serum samples (4.97%) were found to be positive whereas remaining 363 serum samples (95.03%) were found to be negative.

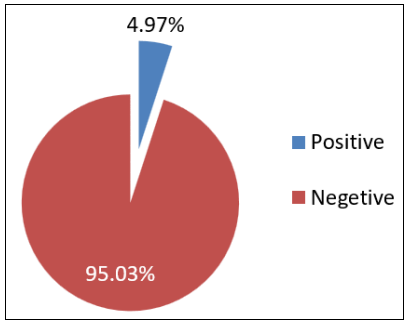

Fig 2: Seroprevalance of *Leptospira hardjo*

VDC wise prevalence of *Leptospira hardjo:* Out of 76 total sample of Mangalpur VDC, 8 samples (10.53%) were positive whereas in Divyanagar, Gunjanagar and Geetanagar 3 sample were positive out of 72, 74 and 74 sample respectively. There was no significant difference in prevalence rate among these VDCs ($p>0.05$), suggesting that cattle in all those VDCs are equally at risk to *L. hardjo*.

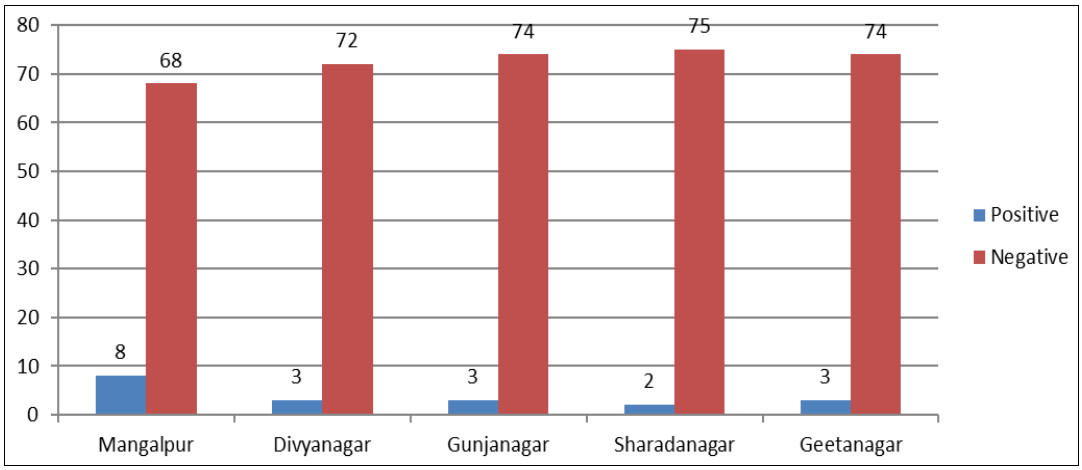

Fig 3: VDC wise prevalence of *Leptospira hardjo*

Different Parameter Wise Prevalence of *Leptospira hardjo*

Table 2: Different parameter wise prevalence of *L. hardjo*

S. No.	Parameter		Total	Positive	Prevalence %	Odd Ratio	P-value
1.	Breed	Jersey cross	219	15	6.85%	2.923(0.9516-8.978)	Chi (0.051) Non-significant
		Holstein Cross	163	4	2.45%		
2.	Age	< 5 years	297	14	4.71	0.7915 (0.2768-2.264)	Chi (0.207) Non-Significant
		> 5 years	85	5	5.88%		
3.	Parity	Up to 2nd Parity	242	9	3.72%	N/A	Chi (0.257) Non-significant
		3-5 Parity	132	9	6.82%		
		> 5th Parity	8	1	12.50%		
4.	BCS	< 3	234	14	7.22%	1.82(0.6417-5.162)	Chi (0.2548) Non-significant
		> = 3	148	5	2.66%		

All the parameters (breed, age, parity and body condition score) are not-significant (p>0.05) as shown in table 2. This results shows that breeds, different age group, different parity and variation in body condition score does not affect the chance of infection and all are at equal risk.

Prevalence in relation to Clinical Signs

Table 3: Clinical Signs wise prevalence of *L. hardjo*

S. No.	Clinical Signs	Total Sample	Positive	Prevalence	Odd ratio	P –Value
1.	History of Reproductive Problem	89	7	7.87%	1.999 (0.7623-5.242)	Fisher Exact(0.2521) Non-significant
	No reproductive Problem	293	12	4.10%		
2.	History of Abortion	25	5	20.00%	6.125 (2.006-18.7)	Fisher Exact (0.009901) Significant
	No abortion	357	14	3.92%		
3.	History of Mastitis	69	12	17.39%	9.203 (3.475-24.38)	Fisher exact (0.00001784) Significant
	No mastitis	313	7	2.24%		
4.	Yellow/orange milk clot	30	5	16.67%	N/A	Chi (0.000004424) Significant
	Red milk clot	12	2	16.67%		
	Normal milk with clot	27	5	18.52%		
	No milk clot	306	7	2.24%		
5.	History of Hemoglobinuria	14	2	14.29%	3.441 (0.7131-6.61)	Fisher Exact (0.2986) Non-significant
	No hemoglobinuria	368	17	4.62%		
7.	History of Nervous Sign	6	3	50%	22.5 (4.207-120.3)	Fisher Exact (0.003818) Significant
	No nervous sign	376	16	4.26%		

According to the clinical signs, result shows history of abortion, history of mastitis, clotted milk and history of nervous signs are statistically highly significant (p<0.01). The animal with these clinical signs are at greater risk to infection. However, History of reproductive problems and history of hemoglobinuria are statistically non-significant (p>0.05) and signifies that animal with or without those clinical signs are equally at risk of infection.

Prevalence in relation to management system

Table 3: Prevalence in relation to management practices

S. No.	Management System	Total	Positive	Prevalence	Odd Ratio	p-value
1.	Intensive housing	338	15	4.44%	2.153 (0.6813-6.806)	Fisher Exact (0.3266) Non-significant
	Semi-intensive	44	4	9.09%		
2.	Presence of Rodents	194	14	7.22%	2.847 (1.005-8.067)	Chi (0.04055) Significant
	Absence of rodents	188	5	2.66%		
3.	Artificial Insemination	261	13	4.98%	1.005 (0.3725-2.71)	Chi (0.9926) Non-significant
	Natural Mating	121	6	4.96%		
4.	Wet Ground Surface	107	0	0%	N/A	Chi (0.005284) Significant
	Dry Ground Surface	275	19	6.91%		

In case of management system, presence of rodents and dry ground surface are significant (p<0.05) and implies as a risk factor to *L. hardjo* infection in cattle whereas housing system and mating system are non-significant (p>0.05).

Discussion

Above result represents the prevalence of *Leptospira hardjo* in the dairy cattle of the western dairy pocket area of Chitwan district of Nepal to be 4.97%. The current result is in congruence with the study by Çetinkaya, Ertaş, Öngör & Muz, (2000) [8] where 4.02% of total sample were positive by PCR. In a study by Microscopic Agglutination test (MAT), 11% prevalence of *L. hardjo* in cows and 5.5% in buffaloes with problems of infertility (Joshi and Joshi, 2000) [24], 9.3% sample were positive for *L. hardjo* of the 118 repeat breeder and aborted cows (Jha VC, 2002) [23], Serological survey throughout Nepal shows 17% prevalence rate of *L. hardjo* in different species of livestock (Dyson *et al*., 2000) [14] and similarly in the previous study done by Joshi, Joshi and Shrestha, (2001) [25] in the high hills of Nepal was found 8.5% in cattle which are higher than in our study. However, much lower prevalence rate of 1.59% was reported by Rifatbegovic and Maksimovic, 2011 [34]. Higher to our study, Vakili, Hassanpour & Khakpour, (2013) [39] found 7.14% seroprevalance, Ajaj and Farwachi, (2013) [2] in Nineveh Province, Iraq found 6.3%, Ebrahimi, Nasr and Kojouri, (2004) [15] in Shahrekord district, central Iran found 17.33% for *L. hardjo* in cattle. Current result is not in agreement with the study done by Sharma *et al*., (2003) [37] in Andhra Pradesh, India, where 39% seropositivity in cattle was observed.

Our statistical analysis of the risk factors shows significant difference (p<0.05) in case of animal with history of abortion, mastitis, nervous signs, presence of rodents and dry ground surfaces whereas no significant difference (p>0.05) is found among age, breed, parity, body condition score, housing system, mating system, history of hemoglobinuria, and history of reproductive problems.

The seroprevalence in relation to age group and parity of cattle, history of reproductive problems (p>0.05) are different from the findings of Ngbede *et al*., (2012) [31], Kocabiyik and Cetin (2004) [28] and Balakrishnan *et al.*, (2011) [5] which may be due to variation in sample size, randomization during sample collection, geography, climate and differences in test procedure.

The seroprevalance in relation to breed, body condition score (p>0.05) is similar to findings of Ngbede *et al*., (2012) [31]. Similarly, significant difference in relation to abortion is similar to findings of Ellis *et al*., (1978) [16], Chiebao *et al*., (2013) [10], and does not correlate to the fnding of Chappel *et al*., (1989) [9] and percentage of abortion is lower i.e., 4.7% in the study of Sanhueza *et al*., (2013) [35]. Similarly, our finding is not statistically significant (p>0.05) in case of housing system does not correlate to the finding of Kingscote, (1986)

[26] who found significance in relation to intensive housing.

The seroprevalance associated with risk factor of presence of rodents is significant which gives information that rodents are important carrier. This transmission is proven by the study of Kositanont *et al*., (2003) [29]. Artificial insemination as a risk factor for *L. hardjo* is non-significant in our finding which does not co-relate to the finding of Chiebao *et al*., (2013) [10]. Our finding of dry ground surface as significant factor of *L. hardjo* to cattle does not correlate to the study of Adugna (2016) [1] which states ground surface moisture and water on bedding or soil are the important factor in tropical regions governing the persistence of the organism, it can persist as long as 183 days in water. This may be due to the prevalence of *L. hardjo* in systemic circulation or other risk factor to be the cause of transmission. Adugna (2016) [1] review gives similar finding to mastitis and abortion as a significant risk factor to *L. hardjo* as in our finding. Mastitis and yellow clot milk is significant risk factor in our finding as described by Zelski R, 2007 [44].

Summary, Conclusion and Recommendation

Summary

A total of 382 serum samples of improved dairy cattle were collected from 5 VDCs from western dairy pocket area of Chitwan district of Nepal and examined at Central Veterinary Laboratory, Tripureshwor, Kathmandu. Seroprevalance of *L. hardjo* was found to be 4.97%. Different risk factor analysis showed significant difference between wet and dry ground surface, cattle with history of previous mastitis and clot color of milk, History of abortion and presence of rodents ($p<0.05$). No significant difference ($p>0.05$) was found in case of VDCs, Breed, Parity, Age, Body Condition Score (BCS), History of Reproductive Problems, types of housing, history of hemoglobinuria and types of breeding.

Conclusion

This study shows that antibodies of *L. hardjo* are circulating in the improved cattle of western dairy pocket area of Chitwan district of Nepal, abortion, mastitis, nervous signs, presence of rodents and dry ground surfaces are potential risk factors of *L. hardjo* and also determines the need for continuous monitoring of *L hardjo* in animals and humans in that area and all over the nation. Further studies should be performed using greater sample size, greater geographical area and modern confirmatory techniques such as PCR, DNA-hybridization or loop-mediated isothermal amplification (LAMP) for detailed findings.

Recommendation

This result shows that *L. hardjo* is prevalent in the dairy cattle of the western Chitwan of Nepal. Since not a single farm where sample were taken had record of vaccination, the antibody positive case in our research study suggest a natural infection in cattle's life time. Once infected, cattle serve as source of infection to other animals and humans and spread the causative organism continuously or intermittently. It is a disease of zoonotic importance and causes serious problems in many species of animals. So, it's very important to adopt the preventive measures like vaccination and giving importance in preventing the risk factors. It is recommended to control the rodents, urine of infected animals, and contact with animal tissue those working in close proximity with animals, using gloves and proper clothes, must not use urine for drinking on religious purpose. Awareness should be improved. This result also recommends more broad study and research on this disease. Furthermore, its recommends there is chance of transmission among other animals in close proximity and thus preventive measures and treatment must be done.

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