IJMPES International Journal of
Medical Parasitology & Epidemiology Sciences

http://ijmpes.com
doi 10.34172/ijmpes.4180
Vol. 5, No. 4, 2024, 106-112
eISSN 2766-6492

Original Article

Screening of Antibodies Against Leptospiral Hardjo Among Bovine Species of Nawalpur, Tanahun and Gorkha Districts of Nepal

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Abstract

Introduction: Leptospirosis, a bacterial zoonosis, can affect livestock species with considerable losses especially cattle and buffalo. *Leptospira interrogans* serovar hardjo is known to be associated with reproductive disorders in bovines. Information on the seroprevalence of antibodies against this serogroup in this territory is important in managing risks and instituting control measures for the area, such as Nepal since these diseases could be overlooked due to the absence of some surveillance practices. The aim of the study was to investigate the seroprevalence of *Leptospira interrogans* serovar hardjo antibodies among cattle and buffalo from Nawalpur, Tanahun and Gorkha districts of Nepal in order to evaluate the extent of the disease and its effect on cattle.

Methods: A total of blood samples were collected aseptically using purposive sampling from cattle and buffalo in the study area 174. These samples were analyzed serologically using PrioCHECK®L.hardjo Ab ELISA kit for antibodies to *Leptospira interrogans* serovar hardjo.

Results: The serological analysis indicated that the seroprevalence was 1.149% which points out the existence of a natural infection in cattle and buffalo reared without any immunization against leptospirosis. Geographical factors, especially region, combined with other factors like low immunogenicity of their vaccines offered to the animals, might explain the low level seroprevalence of the disease.

Conclusion: This current study has highlighted the natural occurrence of the leptospires in the hardjo serovar in cattle and buffalo within the study region. The above studies bring out the necessity for active programs for control and preventative measures to restraints leptospirosis disease among the cattle as the disease prevalence designates low seroprevalence rate.

Keywords: Zoonotic, Anestrous, ELISA, Seroprevalence, L. hardjo

Received: July 13, 2024, **Accepted:** November 25, 2024, **ePublished:** December 22, 2024

Introduction

Leptospirosis is a major zoonotic bacterial disease with global distribution caused by one or more than one serovars of about 260 serovars belonging to 23 serogroups or serotypes of pathogenic species *Leptospira interrogans* (1). All the serovars are circulating in a wide range of animal reservoir hosts including rats, other rodents, livestock and domestic pets. *Leptospira* infection in bovine sources maintenance of bacteria in the host, leading to a carrier state. The occurrence of cattle leptospirosis was first recognized in 1935 by Michin and Azinow. The Bernkopf isolated and recognized, *Leptospira* as the causative agent of disease in Palestine (2). *Leptospira interrogans*, a major zoonotic disease can constitute the major pathogenic leptospiral species that is responsible for human infection (3). People can get infection after direct contact with infected animals or indirect contact with the contaminated environments by their urinal discharges (4).

The occupational risk of exposures is mainly to the veterinarians, farmers, slaughterhouse workers, hunters, animal shelter workers, and agricultural worker and people closely connected with animals (5). *Leptospira interrogans* can readily able to penetrate abraded skin and mucous membrane barriers to establish a systemic infection via hematogenous dissemination and subsequently colonizes multiple organs, particularly the kidneys and liver and show pathogenesis on that part. While other wild rodents serve as natural reservoirs, humans and a few other domesticated animals are accidental hosts in the transmission cycle of leptospirosis (6). Globally a greater number of serovars are recognized but only a limited number are usually endemic to a particular region (7).

Leptospira is bacterial disease associated with infertility, early embryonic death and agalactia/oligolactia /mastitis. Leptospiral serovar hardjo, Pomona and Grippotyphosa are implicated in bovine abortion that causes heavy economic losses of dairy farmers (8). Among many leptospiral serovar, hardjo serovar is considered the most frequent and important serovar in bovine species (9). Examination of serological data of notified human cases

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from 1999 to 2017 presented a decline in leptospirosis cases with serovars hardjo (0.81–0.44 per 100 000) and Pomona (0.43–0.24 per 100 000) but a rise in cases with serovars Ballum (0.23–0.38 per 100 000) and Tarassovi (0.11–0.15 per 100 000) (10).

Literature Review

Overview of Leptospira

Spirochaetes of the genus *Leptospira* are actively motile, delicate and have numerous closely wound spirals with characteristic hooked ends. Several *Leptospira* are saprophytes, while quite a few are potential pathogens of rodents, domestic animals and humans. The genus *Leptospira* consists of two important species, which are *Leptospira interrogans* and *Leptospira biflexa* (11). The genus has at present, 13 pathogenic species and six non-pathogenic (Table 1). It is caused by the bacteria named *Leptospira*, a type with 71 species (12) and over 300 serovars (13) worldwide. They are spiral bacteria (5-20 μm × 0.1 μm) with many closely set coils. Their ends are hooked and look like umbrella handles. They are actively motile by rotatory movements. They cannot be seen under light microscope due to its thinness, best observed by dark field microscopy, phase contrast and electron microscope. They stain poorly with aniline dyes; it may be stained with Giemsa stain or silver impregnation techniques (11). The microorganism survives in the environment if mean temperature remains at about 22 ˚C year around and the fluctuations are not more than 5 ˚C. Thus, leptospirosis is an extremely important disease in tropical and subtropical climates (7).

Epidemiology

It is seen that the actual incidence of leptospirosis in the Asia Pacific region is not well- documented, similar to the condition in many regions worldwide. The prevalence of leptospiral serovars are varies from country to country and depends upon weather condition, rainfall, humidity, presence of carrier animals and soil components (14). In Nepal, species wise prevalence in cattle, buffalo and chauries (cross of yak and cattle found in High Mountain) was 6% (10/160), 0.64% (1/156) and 25% (1/4), respectively in pre-monsoon and 4.51% (7/155), 0.69% (1/145) and 70% (14/20), respectively in post-monsoon samples (15). The incidence was highest in chauries followed by cattle and buffaloes; post- monsoon sera demonstrated higher prevalence than the pre-monsoon samples & furthermore, seasonality had shown clear effect on higher incidence of leptospirosis as evidenced by the detection of leptospiral antibodies in post-monsoon sera (15). The disease is seasonal, with high incidence occurring during the summer or fall in temperate regions, where temperature is the limiting factor in persistence of leptospires, and during rainy seasons in warm-climate regions, where rapid dryness would otherwise prevent survival (5). According to research done by (16) exotic pure breeds are more susceptible followed by indigenous pure breeds and cross breeds with different leptospiral serovar infection.

Table 1. Pathogenesis and Non-pathogenesis leptospiras

Pathogenic	Non- Pathogenic
Leptospira alexanderi	*Leptospira biflexa*
Leptospira alstonii	*Leptospira kmetyi*
Leptospira borgpetersenii	*Leptospira meyeri*
Leptospira fainei	*Leptospira yanagawae*
Leptospira interrogans	*Leptospira wolbachii*
Leptospira inadai	*Leptospira vanthielii*
Leptospira kirschneri	
Leptospira licerasiae	
Leptospira noguchii	
Leptospira santarosai	
Leptospira terpstrae	
Leptospira weilii	
Leptospira wolffii	

Relation of Leptospira With Animals

Leptospira is a gram–negative spirochete, flexible, spiral shaped with internal flagella (17). The three species of Leptospira are *Leptospira interrogans, Leptospira biflexa and Leptospira parva*. *Leptospira interrogans* contains large number of serogroups whose strain are pathogenic and parasitic for human and animals. (18). *Leptospira biflexa* serogroups are large in number whose strains are found in fresh surface water and moist soil and rarely isolated from human and animals (19). *Leptospira parva* is biochemically between *L. interrogans* and *Leptospira biflexa*, it is non –pathogenic for hamsters and isolated from tap water (18). The second genus in the Leptospiraceae is *Leptonema* with single species *Leptonema illini*. *Leptonema* strains were isolated from urine of a healthy bull, a turtle and water. *Leptonema* possess cytoplasmic tubules absent in Leptospira, and the structure of basal complex on its flagella resembles to that of gram-positive bacteria but structures in *Leptospira* resemble that of gram-negative bacteria (18). The serovars (serotypes) of *Leptospira* are named and antigenically organized are grouped into serogroups. *Leptospira parva* contains a single serovar, *Leptonema illini* contains two serovars, *Leptospira interrogans* is known for 19 serogroups that has more than 170 serovars, *Leptospira biflexa* contain 38 serogroups with 60 serovars (18). Rats and rodents are the important reservoirs of the *Leptospira*. A wide range of mammals can actually host the *Leptospira* species (i.e., support kidney colonization) (20). *Leptospira* represents public health issue due to its involvement in human, wild and domestic animals. They may be maintenance host (asymptomatic renal carriers, that contribute to maintain

and share the infection shedding Leptospira with urine in the environment, accidental host (accidently gets contact with the Leptospira infected urine and represent the first cause of infection that could produce clinical disease). Maintenance host species; linked to a specific *Leptospira* serovar; Icterohaemorrhagiae and Ballum serogroups are associated with rodents, Pomona and Tarassovi serogroups with pigs and wild boar, Bratislava serogroup with horses and Sejroe serogroup with bovines and ovine (21). Clinical infections of *Leptospira* in animals are as: Pigs (reproductive failure, abortions, stillbirths, septicemia in piglets, renal disease in young pigs), cattle (abortion, stillbirths, agalactia, septicemic in young animals, acute hemolytic disease in calves), horse (abortions, periodic ophthalmia), dogs (acute nephritis in pups, acute renal disease in adults), sheep (acute hemolytic disease, abortions), human (influenza like illness, occasionally liver or kidney disease). *Leptospira* can infect animal of any age group including young animals (22).

Zoonotic Importance of Leptospira

Leptospira is a fatal bacterial zoonosis that affects people and animal worldwide. It is estimated that 1.03 million human cases and 58 900 deaths occurs worldwide each year (23). Leptospirosis is an extremely important disease in tropical and sub-tropical climate and disease is of seasonal importance, observed during spring and autumn, usually after heavy rainfall. The micro-organisms in the environment survives if temperature is 22 °C around and temperature is not fluctuated around 5 °C around (7). Human infection is highest in developing countries with warm, humid climate, but it is also increasingly seen in developed countries due to the travelers visiting in endemic areas. Increase participation in recreational and sport activities include contact with water (24). Leptospira are shed on body fluid (urine, vagina, placental fluid) and infection occurs when pathogen penetrate the skin through small abrasions or mucosal membrane (eye, mouth). Veterinarians, farmer, plumbers, garbage collectors get direct contact with the infected urine (25). A wide variety of peridomestic animals (rats, horses, cows, dogs, and pigs) and feral animals (bats, coyotes, sea lions, and even frogs) can transmit *Leptospira* bacteria in their kidneys and therefore apparently excrete the pathogen into the environment. The amount of pathogen that these animals shed is likely to be very significant for the formation of environmental sources and the risk of infection upon exposure to those sources. Rats shed about 5.7×10^6 *Leptospira* bacteria/ml of urine; and cows, deer, dogs, mice, and humans have been reported to shed an average of 3.7×10^4, 1.7×10^5, 1.4×10^2, 3.1×10^3, and 7.9×10^2 *Leptospira* bacteria/mL of urine, respectively (26).

Diagnostic Approach

The diagnosis of leptospirosis in animal depends on the basis of good clinical and vaccination history and the availability of diagnostic testing at a laboratory with experience in the diagnosis of leptospirosis. Similarly, the co-ordination between the diagnostic laboratory and the veterinarian is necessary to maximize the chances of making an accurate diagnosis. Diagnostic tests for leptospirosis can be divided into those designed to detect antibodies counter to the organism and those intended to detect the organism or its DNA in tissues or body fluids of animals (27). The combination of tests that comprises serological tests and detection of leptospires are both allow maximum sensitivity and specificity in establishing the diagnosis.

Serologic Tests

The microscopic agglutination test (MAT) is the most widely used method for diagnosing leptospirosis in animals. Serology is inexpensive, more sensitive, and commonly available. The MAT involves mixing appropriate dilutions of serum with live leptospires of serovars prevalent within the region of occurrence & the presence of antibodies is indicated by the agglutination of the leptospires (27). However, the test remains restricted to specialized laboratories that are capable of maintaining strains for the preparation of live antigens (28). In resource poor countries like Nepal where laboratories performing MAT or keeping cultures are rarely available, serological tests like ELISA can get well show the scenario of the disease prevalence (29).

Detection of Leptospires

Immunofluorescence (fluorescent antibody tests or FA) and polymerase-chain-reaction (PCR) assays are other techniques available for the diagnosis of leptospirosis in livestock & involves procedures to detect leptospires or leptospiral DNA in tissues or body fluids. PCR testing of urine is more reliable than testing of tissues (27). Organisms can also be cultured from infected animals but culture is expensive, takes many weeks, and is generally only available in reference laboratories. Fluid media are used for primary culture and it is seen that greater yields and faster growths are obtained in Tween (oleate)-albumin media such as EMJH (Ellinghausen, McCullough, Johnson, Harris) than media with rabbit serum (8-10% v/v) (30). The culture of these organisms takes almost 3 months or even more so the method is impractical for immediate diagnosis.

Materials and Methods

The purposive sampling technique was applied to collect the serum samples from dairy cattle and buffalo in different districts of Nepal in July - August of 2021 AD. Serum samples from female dairy cattle and buffaloes of more than two years of age having history of reproductive problems along with other cases like abortion, repeat

breeding, anestrus, hematuria, hemolactia were taken into consideration. Trained veterinary doctors followed ethical guidelines while drawing blood samples from the animals. Our study involved only the collection of blood samples from the jugular vein of bovine species, a routine procedure that does not cause undue distress or harm to the animals. We adhered to established protocols for handling and sampling, ensuring minimal discomfort to the animals involved. Number of samples collected from different districts for different propose is given (Figure 1).

The test was carried out using the ELISA kits for the detection of antibodies directed against *Leptospira interrogans* serovar hardjo in serum of cattle and buffalo. The manual provided in the ELISA test kits (PrioCHECK®L.hardzo Ab ELISA) were followed in the lab of National Cattle Research Program, Rampur. The reading of the ELISA plates was performed by the ELISA reader (Multiskan™ FC Microplate Photometer) at optical density (OD) 450 nm within 15 minutes. The readings were interpreted with the software and protocol provided within the kits to determine the number of *Leptospira* hardjo seropositive samples.

Test Result Validation and Interpretation Criteria

After the measurement of OD of the wells at 450 nm within 15 minutes of stopping the color development, we calculate the mean OD_{450} value of the blanks (well A1 and B1). Then we calculate the corrected OD_{450} value of all samples by subtracting the mean OD450 of the blanks. Now calculate the percentage positivity (PP) of the reference samples 2,3 and test the samples according to the formula given in procedure.

$$\text{Percentage Positivity}(\text{PP}) = \frac{\text{Corrected OD450 test sample}}{\text{Corrected OD450 Reference Serum1}} \times 100$$

Validation Criteria

The result was validated if

- The mean OD of the blanks was < 0.150
- The corrected OD of positive control was ≥ 1.000
- The mean PP of negative control was < 20
- The mean PP of weak positive control was between 20 to 60

Interpretation of Percentage Positivity

- If PP ≤ 20 %, It is negative for *Leptospira* hardjo specific antibodies
- If PP = 20% to 45%, It is inclusive (antibodies may be present)
- If PP ≥ 45%, Positive for *Leptospira* hardjo specific antibodies

Statistical Analysis

Data was entered in MS Excel 2019 and data analysis was done using OpenEpi Open-Source Epidemiological Statistics for Public Health (Version 3.01).

Results and Discussion

Out of 174 samples tested in laboratory, 8 sample has changed the color i.e., it may contain the antibodies against the *Leptospira* hardjo (Figure 2). But when PP was calculated according the formula given in the ELISA test procedure, only 2 samples were seropositive for the presence of *Leptospira* hardjo specific antibodies (Figure 3). Thus, the seroprevalence was found to be 1.1494%. The prevalence of leptospirosis studied by

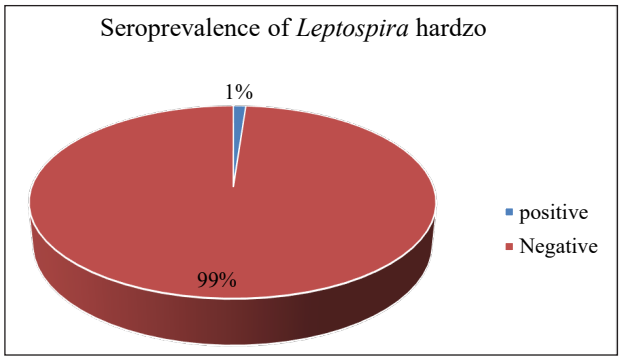

Figure 2. Seroprevalence of Leptospira Hardjo Determined by Using PrioCHECK®L.hardzo Ab ELISA

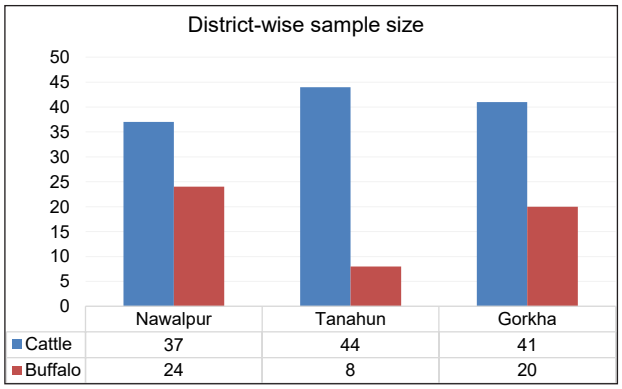

Figure 1. Serum Samples Collected and Tested for Leptospirosis From Different Districts

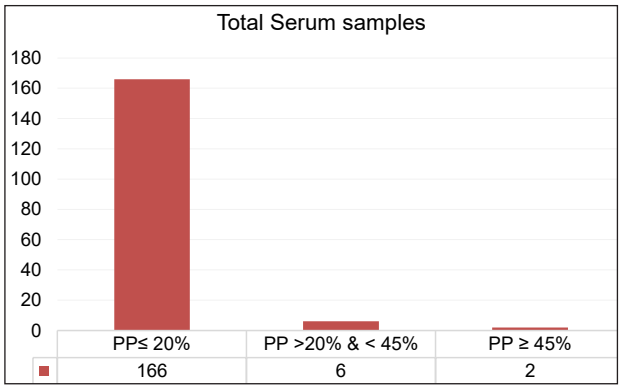

Figure 3. Percentage Positivity Calculation Using Formula Given in Procedure

other authors in Nepal was slightly higher than the result obtained from my studies. The Sero-prevalence and risk factors of leptospirosis in commercial cattle herds of Rupandehi district, Nepal was 3.814% reported by (15); 11.42% in unvaccinated dog done in Kathmandu valley (31); 10.5% in cattle and buffalo reported by (29). The Seroprevalence of *Leptospira* hardjo in cattle of Gujarat, India was 5.77% (8) and 5.11% Bhaktapur district of Nepal as reported by (32). There was no any practice of vaccination of cattle and buffalo against leptospirosis disease in the study area of my research. It can be seen that the vigorous use of antibiotic in the cattle in any case of any clinical abnormality in them can also be the contributing factor in lowering the antibody detection by ELISA as (6) in his study has explain that a significant number of cattle infected with hardjo but not detectable serologically respond well to antibiotic therapy.

Age Wise Prevalence of Leptospira hardjo

When the data analysis is done using OpenEpi Open-Source Epidemiological Statistics for Public Health (Version 3.01), *P* value is found to be 0.5511($P>0.05$). So, there is no significant difference between age and prevalence (Figure 4). The present study indicates prevalence percentage higher in age group more than 3 years; statistically it is not significant which indicates that both age groups have equal probability of getting leptospirosis. Several studies indicate older aged animals are more prone to get exposed to leptospirosis than young ones (33-37) which is in accordance with the result of present study.

Species Wise Prevalence of Leptospira Hardjo

There is no significant difference, i.e., p value is found to be 0.1620 ($P>0.05$) between species and prevalence. The present study indicates prevalence rate can be similar in both cattle and buffalo; statistically it is not significant which indicates that both species have equal probability of getting leptospirosis (Figure 5). According to research done by Balakrishnan et al (16) exotic pure breeds are more susceptible followed by indigenous pure breeds and cross breeds with different leptospiral serovar infection.

District Wise Prevalence of Leptospira Hardjo

The *P* value calculated according to OpenEpi Open-Source Epidemiological Statistics for Public Health (Version 3.01) is found to be 0.5735 ($p>0.05$) i.e., there is no significant difference between district and prevalence. Through the research it is shown that Gorkha and Tanahun have each 1-1 prevalence of leptospirosis but wasn't found in Nawalpur district (Figure 6). Statistically it is not significant which indicates that all districts have equal probability of getting leptospirosis.

Conclusion

In conclusion, this study conducted in Nawalpur, Tanahun, and Gorkha districts of Nepal provides valuable insights into the seroprevalence of Leptospira interrogans serovar hardjo antibodies in cattle and buffalo populations. The observed seroprevalence of 1.149% indicates the presence of natural infection in the absence of vaccination against leptospirosis in the study area. Factors such as low antibody titers of *Leptospira* hardjo and geographical influences likely contribute to the observed seroprevalence. These findings underscore the necessity of ongoing surveillance

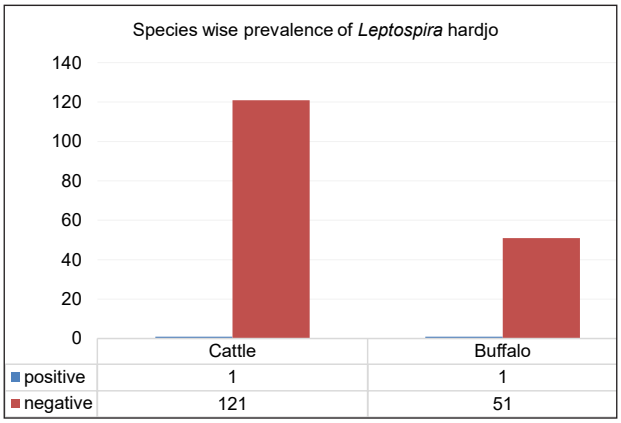

Figure 5. Species wise prevalence of *Leptospira* Hardjo

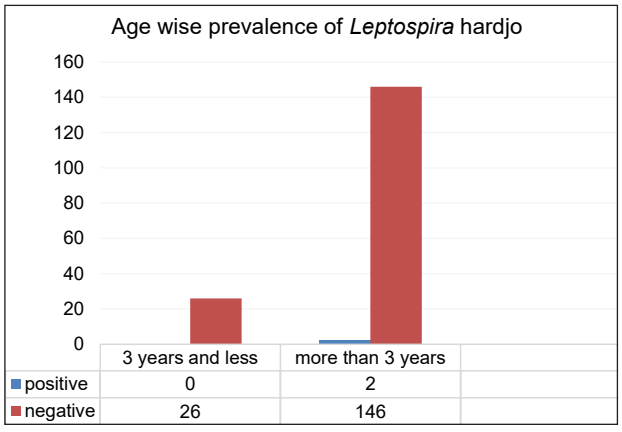

Figure 4. Age Wise Prevalence of *Leptospira* Hardjo

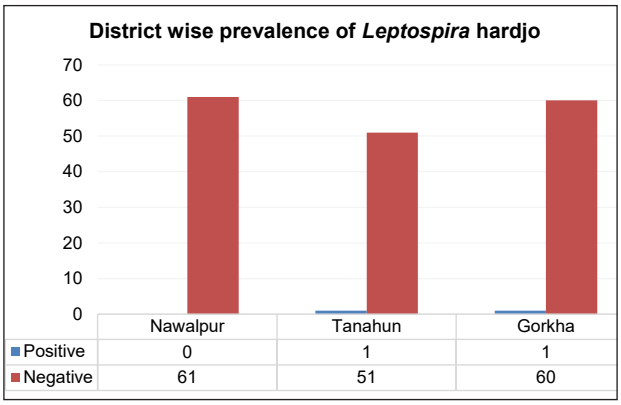

Figure 6. District Wise Prevalence of Leptospira Hardjo

and management strategies to effectively mitigate the impact of leptospirosis on bovine populations in the studied districts. Continued research and monitoring efforts are crucial to better understand the dynamics of leptospirosis transmission and to implement appropriate control measures. Vaccination against leptospirosis, along with improved husbandry practices and environmental management, are recommended to reduce the prevalence of the disease and minimize its impact on bovine health and productivity. Overall, this research emphasizes the importance of proactive measures to safeguard the health and welfare of cattle and buffalo populations, as well as to protect public health by reducing the risk of zoonotic transmission of leptospirosis.

Acknowledgments

I would like to acknowledge Dr. Chet Raj Pathak, Asst. Prof. Dr. Shambhu Shah, Prof. Dr. Krishna Kaphle, Professor Dr. H. B. Rana, Asst. Prof. Dr. Shrijan Bastola, Asst. Prof. Dr. Ganga Prasad Yadav, Dr. Pratik Kiju, Dr. Santosh Panta, Dr. Jivan Kharel, Dr. Manoj Adhikari, Dr. Sudhakar Gupta, Dr. Chiran Krishna Tiwari, and all my classmates.

Authors' Contribution

Conceptualization: Chet Raj Pathak, Rashok Khanal.
Data curation: Chet Raj Pathak.
Formal analysis: Rashok Khanal.
Funding acquisition: Rashok Khanal.
Investigation: Chet Raj Pathak, Rashok Khanal.
Methodology: Rashok Khanal.
Project administration: Chet Raj Pathak, Rashok Khanal.
Resources: Chet Raj Pathak.
Software: Chet Raj Pathak.
Supervision: Chet Raj Pathak.
Validation: Chet Raj Pathak, Rashok Khanal.
Visualization: Rashok Khanal.
Writing–original draft: Rashok Khanal.
Writing–review & editing: Chet Raj Pathak, Rashok Khanal.

Competing Interests

The authors declare that there is no conflict of interest.

Ethical Approval

Not applicable.

Funding

It is self-funded by Rashok Khanal.

References

1. Ko AI, Goarant C, Picardeau M. *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. Nat Rev Microbiol. 2009;7(10):736-47. doi: 10.1038/nrmicro2208.
2. Ellis WA. Animal leptospirosis. Curr Top Microbiol Immunol. 2015;387:99-137. doi: 10.1007/978-3-662-45059-8_6.
3. Hashemi SA, Arzamani K, Abdollahpour G, Beheshti N, Alavinia M, Azimian A, et al. Seroprevalence of *Leptospira* infection in occupational risk groups in North Khorasan province, Iran. Heliyon. 2021;7(1):e05983. doi: 10.1016/j.heliyon.2021.e05983.
4. Haake DA, Levett PN. Leptospirosis in humans. Curr Top Microbiol Immunol. 2015;387:65-97. doi: 10.1007/978-3-662-45059-8_5.
5. Levett PN. Leptospirosis. Clin Microbiol Rev. 2001;14(2):296-326. doi: 10.1128/cmr.14.2.296-326.2001.
6. Adler B, de la Peña Moctezuma A. *Leptospira* and leptospirosis. Vet Microbiol. 2010;140(3-4):287-96. doi: 10.1016/j.vetmic.2009.03.012.
7. Burriel AR. Leptospirosis: an important zoonotic disease. In: Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. Spain: FORMATEX; 2010. p. 687-93.
8. Prajapati A, Kushwaha A, Chayanika D, Subhashree N, Varsha P, Marcia A, et al. A review on bovine leptospirosis with special reference to seroprevalence in India. Int J Curr Microbiol Appl Sci. 2018;7(4):1813-20. doi: 10.20546/ijcmas.2018.704.205.
9. Radostits OM, Gay CC, Hinchcliff KW, Constable PD. Veterinary Medicine: A Textbook of the Diseases of Cattle, Horses, Sheep, Pigs and Goats. Edinburgh, UK: Saunders Ltd; 2007.
10. Nisa S, Wilkinson DA, Angelin-Bonnet O, Paine S, Cullen K, Wight J, et al. Diverse epidemiology of *Leptospira* serovars notified in New Zealand, 1999-2017. Pathogens. 2020;9(10):841. doi: 10.3390/pathogens9100841.
11. Garry FB. Miscellaneous infectious diseases. In: Rebhun's Diseases of Dairy Cattle. Elsevier Health Sciences; 2008. p. 606-39. doi: 10.1016/b978-141603137-6.50018-1.
12. Korba AA, Lounici H, Kainiu M, Vincent AT, Mariet JF, Veyrier FJ, et al. *Leptospira ainlahdjerensis* sp. nov., *Leptospira ainazelensis* sp. nov., *Leptospira abararensis* sp. nov. and *Leptospira chreensis* sp. nov., four new species isolated from water sources in Algeria. Int J Syst Evol Microbiol. 2021;71(12):005148. doi: 10.1099/ijsem.0.005148.
13. Picardeau M. Virulence of the zoonotic agent of leptospirosis: still terra incognita? Nat Rev Microbiol. 2017;15(5):297-307. doi: 10.1038/nrmicro.2017.5.
14. Balamurugan V, Alamuri A, Kumar KV, Varghese B, Govindaraj G, Hemadri D, et al. Prevalence of anti-leptospiral antibodies and frequency distribution of *Leptospira* serovars in small ruminants in enzootic South Peninsular India. Vet World. 2021;14(8):2023-30. doi: 10.14202/vetworld.2021.2023-2030.
15. Gompo TR, Jyoti S, Pandit S, Sapkota RC, Pandey A. Sero-prevalence and risk factors of leptospirosis in commercial cattle herds of Rupandehi district, Nepal. bioRxiv [Preprint]. July 29, 2020. Available from: https://www.biorxiv.org/content/10.1101/2020.07.29.226464v1.
16. Balakrishnan G, Roy P, Govindarajan R, Ramaswamy V, Murali Manohar B. Seroepidemiological studies on leptospirosis among bovines in an organized farm. Int J Agro Vet Med Sci. 2011;5(6):511-9. doi: 10.5455/ijavms.20110802091129.
17. Johnson RC. *Leptospira*. In: Baron S, ed. Medical Microbiology. 4th ed. Galveston, TX: University of Texas Medical Branch at Galveston; 1996. Available from: https://www.ncbi.nlm.nih.gov/books/NBK8451/.
18. Yasuda PH, Steigerwalt AG, Sulzer KR, Kaufmann AF, Rogers F, Brenner DJ. Deoxyribonucleic acid relatedness between serogroups and serovars in the family *Leptospiraceae* with proposals for seven new *Leptospira* species. Int J Syst Bacteriol. 1987;37(4):407-15. doi: 10.1099/00207713-37-4-407.
19. Adler B, Faine S. The genus *Leptospira*. In: Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E, eds. The Prokaryotes. New York, NY: Springer; 2006. p. 294-317. doi: 10.1007/0-387-30747-8_10.
20. Cordonin C, Turpin M, Bringart M, Bascands JL, Flores O, Dellagi K, et al. Pathogenic *Leptospira* and their animal reservoirs: testing host specificity through experimental infection. Sci Rep. 2020;10(1):7239. doi: 10.1038/s41598-

020-64172-4.
21. Cilia G, Bertelloni F, Fratini F. *Leptospira* infections in domestic and wild animals. Pathogens. 2020;9(7):573. doi: 10.3390/pathogens9070573.
22. Lattes R. Annals New York Academy of Sciences: Discussion. Ann N Y Acad Sci. 1966;135(1):504-5. doi: 10.1111/j.1749-6632.1966.tb45498.x.
23. Guernier V, Goarant C, Benschop J, Lau CL. A systematic review of human and animal leptospirosis in the Pacific Islands reveals pathogen and reservoir diversity. PLoS Negl Trop Dis. 2018;12(5):e0006503. doi: 10.1371/journal.pntd.0006503.
24. Andersen-Ranberg EU, Pipper C, Jensen PM. Global patterns of *Leptospira* prevalence in vertebrate reservoir hosts. J Wildl Dis. 2016;52(3):468-77. doi: 10.7589/2014-10-245.
25. Bierque E, Thibeaux R, Girault D, Soupé-Gilbert ME, Goarant C. A systematic review of *Leptospira* in water and soil environments. PLoS One. 2020;15(1):e0227055. doi: 10.1371/journal.pone.0227055.
26. Barragan V, Olivas S, Keim P, Pearson T. Critical knowledge gaps in our understanding of environmental cycling and transmission of *Leptospira* spp. Appl Environ Microbiol. 2017;83(19):e01190-17. doi: 10.1128/aem.01190-17.
27. Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, et al. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis. 2003;3(12):757-71. doi: 10.1016/s1473-3099(03)00830-2.
28. Turner LH. Leptospirosis I. Trans R Soc Trop Med Hyg. 1967;61(6):842-55. doi: 10.1016/0035-9203(67)90045-4.
29. Regmi L, Pandey K, Malla M, Khanal S, Pandey BD. Sero-epidemiology study of leptospirosis in febrile patients from Terai region of Nepal. BMC Infect Dis. 2017;17(1):628. doi: 10.1186/s12879-017-2733-x.
30. Johnson RC, Harris VG. Differentiation of pathogenic and saprophytic leptospires I. Growth at low temperatures. J Bacteriol. 1967;94(1):27-31. doi: 10.1128/jb.94.1.27-31.1967.
31. Central Animal Quarantie Office, Kathmandu. Animal Quarantine Monthly Report 2018. Nepal; 2018. Available from: http://www.caqo.gov.np/uploads/files/5453250888.pdf.
32. Rawal G, Shrestha D. Sero-detection of *Leptospira* Hardjo in cattle of Bhaktapur district of Nepal. Int J Appl Sci Biotechnol. 2019;7(3):378-81. doi: 10.3126/ijasbt.v7i3.25713.
33. Ryan EG, Leonard N, O'Grady L, More SJ, Doherty ML. Seroprevalence of *Leptospira* Hardjo in the Irish suckler cattle population. Ir Vet J. 2012;65(1):8. doi: 10.1186/2046-0481-65-8.
34. Garedaghi Y. Seroprevalence of *Neospora caninum* in stray dogs of Tabriz, Iran. J Anim Vet Adv. 2012:11(6):723-6.
35. Garedaghi Y. Seroepidemiology of *Neospora* sp. in horses in East-Azerbaijan province of Iran. J Anim Vet Adv. 2012;11(4):480-2.
36. Garedaghi Y, Khakpour M. Molecular differentiation of sheep and cattle isolates of *Fasciola hepatica* using RAPD-PCR. Arch Razi Inst. 2012;67(2):109-15. doi: 10.22092/ari.2016.103894.
37. Garedaghi Y. Seroepidemiology of *Neospora caninum* in cattle in East-Azerbaijan province, North West Iran. J Anim Vet Adv. 2012;11(5):645-8.