ORIGINAL ARTICLE

Veterinary Research Forum. 2023; 14 (4) 229 - 235
doi: 10.30466/vrf.2022.546475.3345
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Comparison of anti-Leptospira antibodies by microscopic agglutination test in ruminants and equines of Urmia, Iran

Aligholi Ramin[1]*, Gholamreza Abdollahpour[2], Azadeh Hosseinzadeh[1], Farid Azizzadeh[1], Pouya Ramin[3], Yousef klalili[1], Davood Sanajo[1], Sasan Iran Nezhad[1]

[1] Department of Internal Medicine and Clinical Pathology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran; [2] Department of Internal Medicine, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran; [3] Student of Dentistry, Faculty of Dentistry, Hormozgan University of Medical Sciences, Bandar Abbas, Iran.

Article Info

Article history:

Received: 09 January 2022
Accepted: 18 May 2022
Available online: 15 April 2023

Keywords:

Age
Gender
Leptospirosis
Livestock
Pomona

Abstract

Leptospirosis, a zoonotic disease, is characterized by fever, jaundice, abortion and hemoglobinuria. It is widespread and the determination of the dominant serotype in the animal species of each region accelerates the control and prevention program. 862 blood samples were prepared from ruminants and equines. Serum antibody of leptospira serovars were determined based on gender and age parameters. Sera were examined by microscopic agglutination test (MAT) with six live serotypes. The overall prevalence was 22.30%, with the highest (37.00%) and the lowest (6.60%) in Holsteins and mules, respectively. The overall male and female incidence were 12.20% and 9.86%, respectively, which was not different. The highest gender infection was in male Holsteins (19.20%) and the lowest were in male Simmentals and mules (1.72%). The highest dilution was 1:100 for *pomona* and the lowest was for *canicola*. All animals responded positively to *grippotyphosa*. The highest infection to one serovar was in Holsteins and the lowest for four serovars were in goats and Simmentals. Males less than 1.5 years old showed the highest infection. Age differences in leptospira infection except for sheep was significant. In conclusion, leptospira infection in ruminants was high compared to equines. Gender differences were not significant. The highest dilution was 1:100, with *pomona* in ruminants and *grippotyphosa* in all species. Leptospiral infection was increased with age and the differences among animals except for sheep were significant. Finally, regarding 22.30% infection rate, vaccination is necessary to Holsteins, and preventive measures for others. Health advices would be necessary for human safety.

© 2023 Urmia University. All rights reserved.

Introduction

Leptospirosis is a zoonosis among humans and animals in the tropical and subtropical areas of the world which affects domestic and wild ruminants, equines, rodent and human.[1] The prevalence of disease is common not only in tropical and temperate regions but also in relatively cold regions.[2] Pathogenic serovars of *leptospira interrogans* have been reported to cause significant economic losses in the food animal industries including severe milk loss, abortion or weak birth rate, weight loss and infertility as well as threatening to public health.[3] The disease is sometimes associated with death which increases costs of prevention, control and treatment.[4] Spirochetes are considered as the cause of disease which was first described as an infecting of several organs with severe syndromes involving jaundice and renal injuries in humans.[4] Meanwhile, the susceptible livestock can be vector, carrier or appears in the chronic form. Susceptible human and livestock are infected through the contaminated environment and water via infected mice or people.[3] Leptospirosis is also known as Weil's disease, autumn fever, swamp fever, canicola fever, rice fever, Stuttgart disease and red water disease in calf.[5] Serological investigation around Iran and other countries indicated various reports of leptospira in cattle,[6,7] buffalo,[8,9] sheep,[10,11] goats[12,13] and horses.[14,15] The definite diagnosis of disease was based on clinical signs and serological tests such as enzyme-linked immunosorbent assay (ELISA),[16] polymerase chain reaction (PCR)[17] and microscopic agglutination test (MAT)[18] in

***Correspondence:**

Aligholi Ramin. DVM, PGDVS, PhD
Department of Internal Medicine and Clinical Pathology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
E-mail: ag.ramin@urmia.ac.ir

This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 4.0 International (CC BY-NC-SA 4.0) which allows users to read, copy, distribute and make derivative works for non-commercial purposes from the material, as long as the author of the original work is cited properly.

different locations of the world in buffaloes,[9] Holsteins,[19] sheep,[20] goats,[21] and horses.[22]

Due to the importance of leptospirosis as a zoonotic disease, special consideration and attention should be paid in epidemiology and its control and prophylactic procedures.[23] Extensive studies in leptospirosis were based on human, animal, and environmental parameters such as multiple screening diagnostic methods using blood,[24] urines[25] and various tissue samples.[26] These have been applied to eliminate and control the leptospirosis in order to exclude human from the circulating strains. In this situation, similar activities have been carried out in different locations in Iran[6] mainly in ruminants,[27] and less in other susceptible livestock. There are various studies in animal species with different laboratory methods in different regions accompanied by effective serovars. There is no a comprehensive study in ruminants and equines to indicate the overall epidemiological aspect of leptospirosis in Urmia. Therefore, the current study not only provided a fundamental source of information to the epidemiologists, but it could also outline the strategy to deal with this disease based on the species of animals for the future. Therefore, in order to promote and benefit from such comprehensive information in leptospirosis, epidemiological features of the disease such as percentage of seropositive to leptospiral serotypes (serovars), age, sex, serum titer and serotype prevailing in ruminants and equines were evaluated and compared.

Materials and Methods

Animals and samples. Eight hundred sixty-two blood samples were prepared in Urmia, the northwest of Iran, including 203 Holsteins, 130 buffaloes, 166 sheep, 95 Simmentals, 130 goats, 80 horses and 58 mules. The overall frequency and percentage for males and females were 484 (56.20%) and 378 (43.80%), respectively. For Holsteins they were 119 and 84, for buffaloes 67 and 63, for sheep 142 and 24, for Simmentals 40 and 55, for goats 71 and 59, for horses 25 and 55 and for mules 20 and 38, respectively. Samples were prepared from animals located in the herds of different regions in Urmia suburbs. Blood was taken from purebred, crossbreed and native breeds. A 5.00 mL of blood was collected from the jugular vein into a test tube. Samples were then numbered; the gender and age information were noted and the age of animals was assessed according to their teeth conditions. The samples were refrigerated and centrifuged in 3,000 *g* for 15 min and, the sera were separated within the time. The sera were transferred to micro-tubes and frozen in a freezer (– 20.00 ˚C) to be ready for MAT. In this survey, the six common and live serovars of *leptospira pomona*, *grippotyphosa*, *harjo*, *canicola*, *icterohaemorrhagiae* and *ballum* with approved pathogenicity were used. The age of ruminants was classified into five groups of < 1.5, 1.5 - 2, 3, 4 and > 4 years old, according to the no change in deciduous teeth considered as group 1, conversion the first pair of deciduous teeth to permanent as group 2, the second pair was group 3, the third pair group 4, and the all-pairs group 5. The same category was considered for equines ages as 1 - 6, 7 - 12, 13 - 18, 19 - 22 and 25 - > 25 years old. Results were analyzed for the presence of leptospira serovars. None of the experimental animals were vaccinated against leptospirosis. This study has been received ethical review committee approval (No. 1161).

Seroprevalence examination, MAT. The MAT was performed based on the Abdollahpour *et al.* report.[6] A few hours before the MAT (Leptospira Lab Center, Faculty of Veterinary Medicine, University of Tehran), sera were taken out of the freezer and placed at laboratory temperature to gradually reach ambient temperature. The surface of the microscopic slides was divided into two rows of four (to test eight samples) and a rectangle for numbering. First, the dilution of 1:100 were performed for each of the six alive leptospira antigens. The antibody-antigen mixture was incubated at – 30.00 ˚C for 90 min and examined under a dark field microscope (BX53M; Olympus, Tokyo, Japan). At first, the samples were read as related to negative control and then positive control were examined. Samples with no agglutination, + 1, + 2, + 3, and + 4 agglutination were read. Where, 25.00% of leptospira bodies were agglutinated in each microscopic field it was assumed as + 1 (negative), where, 50.00%, 75.00% and nearly 100% were agglutinated, they were assumed as + 2 (suspicious), + 3 (positive) and + 4 (positive), respectively.

Determination of the final antibody dilution of positive samples. In order to determine the final dilution of sera that were positive in the first stage, the various dilutions of 1:100, 1:200, 1:400, 1:800 and 1: 1,600 were prepared and performed. The highest dilution in samples with + 3 or + 4 agglutinations was determined as the final antibody dilution in samples.

Statistical analysis. SPSS Software (version 23.0; IBM Corp., Armonk, USA) and Chi Square test were used to analyze the data.

Results

Out of 862 blood samples from ruminants and equines, 37.00% of Holsteins, 16.75% of buffaloes, 17.75% of sheep, 6.60% of Simmentals, 12.75% of goats, 9.12% of horses and 6.90% of mules showed a positive reaction to at least one leptospiral serotypes. The percentages of male and female infection to leptospiral serotypes in Holsteins were 19.20%, 16.80%, buffaloes were 26.90%, 23.80%, sheep were 15.70%, 3.60%, Simmentals were 2.10%, 11.60%, goats were 13.00%, 6.20%, horses were 1.90%, 10.40% and mules were 1.70%, 5.20%, respectively.

The overall prevalence to leptospira serovars was 22.30%. The highest infection was observed in Holsteins and the lowest was recorded in mules (Fig. 1).

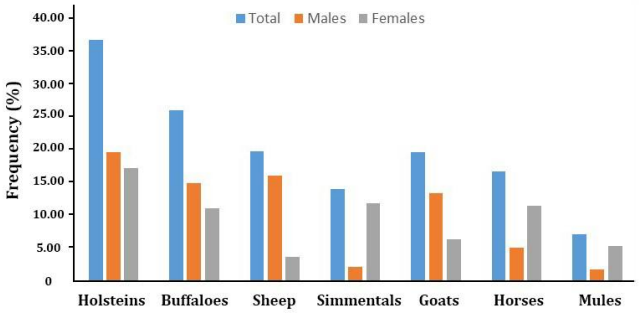

Fig. 1. Comparison of sero-positive to leptospira spp. in males and females of ruminants and equines.

Infection of males (12.20%) was significantly ($p < 0.05$) higher than females (9.86%). Holstein females showed the highest (16.75%) and ewes the lowest (3.60%) infection. The difference was significant ($p < 0.05$). Holstein males showed the highest (19.20%) and male mules the lowest (1.72%) seropositive to leptospira infection which was also different ($p < 0.05$). The comparison of male and female infection to leptospira serotypes showed significant differences only in sheep and Simmentals ($p < 0.01$). There was no gender difference in Holsteins, buffaloes, goats, horses and mules (Fig. 1).

Frequency and percentage dilution of anti-leptospira antibodies by serotype and predominant serotype showed that the highest frequency was in the dilution of 1:100 and the lowest was in 1:400 (Table 1). The headline was 1:100 for *L. pomona*, followed by *L. grippotyphosa* and *L. harjo*, and the lowest was for *L. canicola*. The lowest frequency of dilution was 1:100 and 1:200 in equines (Fig. 2). Ruminants were susceptible to *L. pomona*, *grippotyphosa* and *harjo* serovars, however, horses were not susceptible to *L. pomona*.

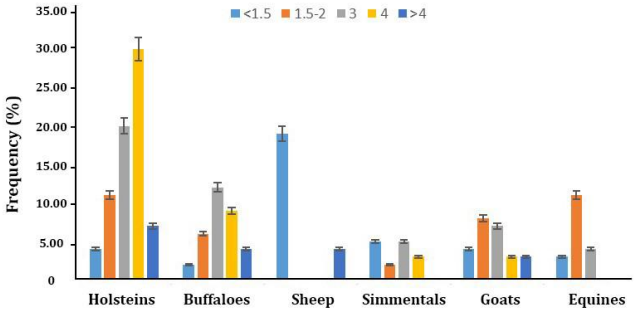

Fig. 2. Comparison of antibodies titer (1:100) against leptospira serotypes in ruminants and equines.

Table 2 shows a comparison of the frequency and percentage of multi-infection to leptospira serovars in ruminants and equines. Seropositive to leptospira in Holsteins varied from one to four serovars: The highest prevalence of one serovar (29.44%) was in Holsteins and the lowest was four serovars (0.51%) in goats and Simmentals. Equines were not affected by more than two serovars, but up to four serovars were observed in ruminants. In total, the percentage of animals with one serotype (72.59%) was over three times higher than with two serotypes (22.84%).

Age frequency of positive reaction to leptospira in animals (Fig. 2) showed that the highest and the lowest seropositive responses were in < 1.5 (3.10%) and > 4 years old (0.80%). Age comparison (Chi-square) showed that there was not age difference only in sheep, however, there was significant difference in other animals ($p < 0.01$).

Table 1. Percentages of antibodies dilution against leptospira serotypes in ruminants and equines.

Animals	*L. pomona*		*L. grippotyphosa*		*L. canicola*		*L. icterohaemorrhagiae*		*L. harjo*	
	1:100	1:200	1:100	1:200	1:100	1:200	1:100	1:200	1:100	1:200
Holsteins	21.90 ^{a}	28.60 ^{a}	17.60 ^{a}	13.20 ^{ab}	-	-	-	-	1.10 ^{a}	17.60 ^{a}
Buffaloes	13.10 ^{a}	3.10 ^{b}	4.60 ^{ab}	2.30 ^{ab}	-	-	0.76 ^{a}	0.79 ^{a}	1.50 ^{a}	3.10 ^{a}
Sheep	19.10 ^{a}	2.40 ^{c}	40.50 ^{b}	21.40 ^{a}	-	-	-	-	2.38 ^{a}	-
Simmentals	0.71 ^{b}	3.60 ^{a}	1.42 ^{b}	0.71 ^{b}	0.71	0.71 ^{a}	0.71 ^{a}	1.42 ^{a}	2.84 ^{a}	2.84 ^{a}
Goats	30.90 ^{a}	19.10 ^{a}	38.10 ^{b}	7.17 ^{ab}	-	2.38 ^{a}	22.20 ^{a}	2.38 ^{a}	-	4.80 ^{a}
Horses	-	-	16.70 ^{b}	11.10 ^{ab}	-	11.10 ^{a}	-	-	27.80 ^{a}	11.10 ^{a}
Mules	24.10	17.90	22.90	12.30	0.41	1.60	2.45	1.60	5.30	11.40

^{abc} Different letters in each column indicate significant difference at $p < 0.05$.

Table 2. Comparison of frequency and (%) of co-infection with leptospira serotypes in ruminants and equines.

Animals	1 serotype	2 serotype	3 serotype	4 serotype	Total
Holsteins	58 (29.44)	12 (6.09)	3 (1.52)	-	73(37.00%)
Buffaloes	27 (13.71)	6 (3.05)	-	-	33(16.75%)
Sheep	22 (11.17)	10 (5.08)	3 (1.52)	-	35(17.75%)
Simmentals	8 (4.06)	3 (1.52)	1 (0.51)	1 (0.51)	13(6.60%)
Goats	11 (5.58)	13 (6.60)	-	1 (0.51)	25(12.72%)
Horses	17 (8.63)	1 (0.51)	-	-	18(9.12%)
Total	143 (72.59)	45 (22.84)	7 (3.55)	2 (1.02)	197(100%)

Discussion

The presence of leptospira serovars in the sera of ruminants and equines indicates the attendance of leptospirosis in Urmia livestock. By determining the rate and type of infection, the strategy for prognosis, favorable diagnosis, control and treatment would be appropriated.[28] Although all isolated serotypes of leptospira are related to *interrogans*,[29] these serotypes are often specific to areas with high precipitation and swamps such as West Azerbaijan and Urmia, which is considered as an epidemiological feature of the disease. The disease has been reported in human, equines, ruminants and carnivores in different parts of Iran with different serological infection.[8,11,27]

The prevalence of cattle leptospirosis has been reported up to 53.80% in Ahvaz,[30] which was greater than the findings of this study (37.00%) and for sheep it was 18.40% in Tabriz, which was slightly more than that of our study (17.75%).[31] Infection of 16.75% of buffaloes was less than 35.30% in East Azerbaijan[8] and it seems that the climatic conditions of Khuzestan and East Azerbaijan are more favorable for leptospirosis than West Azerbaijan in survival and transmission of bacterial agents. The 12.72% prevalence of goats in our study was slightly more than 11.00% reported by others in Khuzestan.[32] The prevalence of 9.12% in horses and 6.90% in mules in this study was still less than 20.00% and 9.10% mentioned in Lorestan and central regions of Iran.[14,33] Despite the equines of this study, inhabitants in Turkey, Iraq and Azerbaijan borders exposing to the heavy traffic of imports and exports, this infection seems not much serious among Urmia equines.

Comparison of the results of our study with the findings in Holsteins in Brazil (6.44%)[1] and India (48.50%),[34] showed lower and higher occurrences in Urmia Holsteins, respectively. The rate of 8.43% and 15.70% leptospirosis in buffaloes of Thailand[7] and India[35] were lower than those in Urmia buffaloes. The occurrence of sheep leptospirosis in Brazil[20] and India[10] were 15.00% and 22.20%, respectively, which were lower and higher than sheep in Urmia. Goat leptospirosis in India[36] and Italy[37] were reported 26.60% and 47.00%, respectively. In Italy the rate was higher than in goats of this study. Equine leptospirosis in South Africa[38] and the Colorado, USA [39] were reported 20.00 - 39.00% and 77.00%, respectively, that were quite higher than Urmia horses. Thus, Holsteins showed the higher rate of infection to leptospirosis and rest of the animals showed relatively the lower infection rate in comparison with the other findings. One explanation for the relatively lower partial level of leptospira infection compared to the other reports, despite the lack of local vaccination program, could be the observance of hygienic standards due to continuous and annual disinfection program of the health department of the Veterinary Organization, private sector veterinarians and the careful attention of farmers to the fight against rats in their livestock farms and fundamental control of rodents as the main factor in the disease transmission.

The overall infection to leptospirosis in Urmia animals (22.30%) was not high in comparison with the other areas of Iran[31,33] and other countries[38,39] except for Holsteins which showed the higher rate. It means that the sheep, goats, Simmentals, equines and mules of this study are less sensitive to leptospira serovars than Holsteins, therefore, the mentioned rate of infection is not serious and with improving hygiene standard the level of infection level will also decreases and will partially close to the normal level and not be an important problem for public health and human too, but for Holsteins the vaccination together with the improved hygiene procedures will be necessary.[28]

Understanding the role of gender in leptospira infection can help find a way to set up control and prevention strategies. From the epidemiological point of view, the higher and non-significant leptospiral infection in males of this study might not be basically important as much as females due to the males' short economic lifetime. In this regard the epidemiology of the disease including its expansion, development and transfer in males will be less than in the females which have longer lifespan, thus, in males the disease not only does not spread in environment, animals and human (zoonosis), but also will be automatically limited. This result was consistent with the findings of Balamurugan *et al*. that mentioned seropositivity to leptospira in males was significantly higher than in females.[34] In general, the role of gender in leptospiral infection in animals is controversial and different results have been recorded. Among females of this study, Holsteins have shown the highest infection rate (37.00%) which should be considered serious. Lack of significant differences between genders in leptospira infection is reported by authors for animals,[30] however, in human, males are significantly more infected than females.[40,41] In contrary, Johnson *et al*.[42] showed that there was not significant gender differences in human.

The lowest dilution of anti-leptospira antibodies was 1:100 which has been reported in most regional[8] and global studies.[30] It is quite comprehensive and not considered as an acute clinical infection, however, the observation of 1:400 in one goat and Simmentals shows a serious form of leptospirosis which was not observed in other species. The observation of serum dilution up to 1:800 and 1:3,200 were reported by others[30,34] but not in this study. Equines of this study revealed the lowest frequency of dilutions, thus, the prevalence of seropositivity to leptospira in equines was very low compared to ruminants. Among the serotypes, *L. pomona* was the predominant not only in this study but also in many parts of Iran and the world,[4,15,37,43] and the other serovars were mentioned too.[27] In the last decades the predominant serovar in Iran was *L. grippotyphosa*, which

has now been replaced by *L. pomona*. Differences in predominant serovars in various regions could be due to the number of samples, season of sampling and reservoirs in the regions.[6] In overall, the predominant serovar in Iran is variable but for Urmia is *L. pomona*.

In this study, the infection of animals varied from one to four serovars but they were often infected with one serovar (16.60%) which was consistent with the results of others.[8] The local and global results, especially in ruminants, show that in each region, a specific antigen with a high frequency is usually predominant and the rest of the serovars are at a lower level. Haji Hajikolaei *et al*. recorded the multi-serovar infection up to five serovars in one animal which was not observed in global studies.[27] Simultaneous response to *L. grippotyphosa*/ *L. pomona*, *L. grippotyphosa*/ *L. canicola* and *L. pomona*/ *L. canicola* with different dilutions in this study could be evidence of concomitant serotypes. Therefore, considering the highest seropositive to leptospira in Holsteins (37.00%) as well as the presence of three serovars (1.52%), Holsteins play a more important role in the epidemiology and survival of leptospira than other ruminants and equines, thus, Holsteins should be considered in disease control and prevention strategies.[44]

Investigation of age status in seropositive reaction to leptospira in animals showed that animals less than 1.5 years of age (3.10%) had the highest sensitivity compared to older ages, however, this result was certainly affected by the high number of male sheep infections (19 positive sheep) while in female Holsteins and buffaloes the infection was increased by increase in age. This was variable in goats, Simmentals and equines. Thus, except for sheep, the age difference was significant ($p < 0.01$) in female ruminants and the highest prevalence was up to 4 years of age. The results of this study were consistent with the findings of Tolouei and Haji Hajikolaei *et al*. who reported significant age sensitivity above two years of age in animals.[8,30] Balamurugan *et al*. reported an increasing trend in leptospira infection with age in cattle.[34] Patel *et al*. recorded that the highest seroprevalence of leptospira in buffaloes over 4 years of age was 20.00% while between 1 - 4 years of age, it was only 4.60%.[43] Agrawal *et al*. reported that the frequency of leptospirosis was increased with the age of the animals.[45]

The results of studies in the last decades on leptospiral infection in the world show that despite the improvements in health conditions and limited vaccinations in susceptible animals, not in Iran, the seroprevalence of the disease is still growing. Since leptospira serovars are associated with different symptoms, therefore, the disease has multiple clinical signs, and serum tests, especially MAT, will be necessary to confirm the diagnosis.[19] This is an excellent method with good performance where the live leptospira serovars exist in the laboratory. The gold standard for the diagnosis of leptospirosis is the MAT method, however, it also has limitations.[18] According to World Health Organization (WHO) standard, to definitively determine the disease, it is necessary to prepare two samples with two to three weeks' interval to determine a doubling of the dilution or to obtain a serum dilution of 1:400 and above in the first sample. Meanwhile, the MAT method is more sensitive to the IgM (indicating acute disease) detection than IgG, so it is less sensitive in the diagnosis of vectors, carriers and chronic forms of the disease. Accordingly, one of the reasons for the low sero-prevalence results of leptospira in any study may be related to the stage of vector, carrier or chronic form of disease, therefore, the MAT method is not able to detect them. Thus, two or three diagnostic methods are recommended to be used simultaneously with MAT to achieve an accurate result.[30] In the absence of live leptospira serovars, alternative methods are PCR and ELISA with accurate and rapid diagnosis in the first 15 days of infection. In order to diagnose and screen for leptospirosis, the combination of PCR and MAT is recommended.[12]

In conclusion, the seropositivity to leptospira was 22.30% and in ruminants it was more than equines. There was no gender difference although males were infected more than females. The highest dilution was 1:100, which *L. pomona* was common in ruminants and *L. grippotyphosa* in all species. Multi-infection to various serovars in ruminants was greater than in equines. Leptospirosis was increased with age in Holsteins and buffaloes and the age difference except for sheep was significant. Finally, based on 22.30% infection, vaccination against leptospirosis in Holstein is necessary and for other animals the prevention procedures and hygienic recommendations will be necessary to protect human from leptospirosis.

Acknowledgments

This study was funded by the Vice Chancellor for Research, Urmia University, Urmia, Iran. We would like to thank the University staff and those who participated in this study.

Conflict of interest

The authors declare that there is no conflict of interest.

References

1. Fávero JF, de Araújo HL, Lilenbaum W, et al. Bovine leptospirosis: Prevalence, associated risk factors for infection and their cause-effect relation. Microb Pathog 2017; 107: 149-154.
2. Mgode GF, Mhamphi GG, Massawe AW, et al. Leptospira seropositivity in humans, livestock and wild animals in a semi-arid area of Tanzania. Pathogens 2021; 10(6): 696. doi: 10.3390/pathogens10060696.

3. Ellis WA. Animal leptospirosis. Curr Top Microbiol Immunol 2015; 387: 99-137.
4. McBride AJ, Athanazio DA, Reis MG, et al. Leptospirosis. Curr Opin Infect Dis 2005; 18(5): 376-386.
5. Faine S, Adler B, Bolin C, et al. Leptospira and leptospirosis. 2nd ed. Melbourne, Australia: Medisci Press 1999; 249-273.
6. Abdollahpour G, Shafighi ST, Sattari Tabrizi S. Serodiagnosis of leptospirosis in cattle in North of Iran, Gilan. Int J Vet Res 2009; 3(1): 7-10.
7. Chadsuthi S, Chalvet-Monfray K, Wiratsudakul A, et al. A remotely sensed flooding indicator associated with cattle and buffalo leptospirosis cases in Thailand 2011-2013. BMC Infect Dis 2018; 18(1):602. doi:10.1186/s12879-018-3537-3.
8. Tolouei M. Prevalence of serum antibodies against six leptospira serovars in buffaloes in Tabriz, Northwestern Iran. J Buffalo Sci 2014; 3(3): 76-81.
9. Ijaz A, Ghaffar A, Ali A, et al. Seroprevalence of leptospirosis and its association with reproductive and productive parameters from buffalo population of Rajanpur and Muzaffargarh districts of Pakistan. The J Anim Plant Sci 2020: 30(1): 1-7.
10. Vihol Priti D, Patel Jignesh M, Patel Jatin H, et al. Serological and clinicopathological studies on leptospirosis among sheep. J Anim Res 2016; 6(4): 571-577.
11. Zakeri S, Khorami N, Ganji ZF, et al. Leptospira wolffii, a potential new pathogenic Leptospira species detected in human, sheep and dog. Infect Gent Evol 2010; 10(2): 273-277.
12. Lilenbaum W, Varges R, Ristow P, et al. Identification of *Leptospira* spp. carriers among seroreactive goats and sheep by polymerase chain reaction. Res Vet Sci 2009; 87(1): 16-19.
13. Hassanpour A, Asgarloo S, Imandar M, et al. Seroepidemiologic study of goats leptospirosis in Khoy-Iran. J Anim Vet Adv 2012; 11(2): 229-233.
14. Khalili M, Sakhaee E, Bagheri Amiri F, et al. Serological evidence of leptospirosis in Iran; A systematic review and meta-analysis**.** Microb Pathog 2020; 138, 103833. doi: 10.1016/j.micpath.2019.103833.
15. Dewes C, Fortes TP, Batista Machado G, et al. Prevalence and risk factors associated with equine leptospirosis in an endemic urban area in Southern Brazil. Braz J Dev 2020; 6(8). 58380-58390.
16. Ye C, Yan W, McDonough PL, et al. Serodiagnosis of equine leptospirosis by enzyme-linked immuno-sorbent assay using four recombinant protein markers. Clin Vaccine Immunol 2014; 12(4): 478-483.
17. Mullan S, Panwala TH, Polymerase chain reaction: an important tool for early diagnosis of leptospirosis cases. J Clin Diagn Res 2016; 10(12): DC08-DC11.
18. Shivakumar S, Krishnakumar B, Diagnosis of leptospirosis - - role of MAT. J Assoc Physicians India. 2006; 54. 338-339.
19. Rajeev S, Berghaus RD, Overton MW, et al. Comparison of fluorescent antibody and microscopic agglutination testing for Leptospira in pregnant and non-pregnant cows. J Vet Diagn Invest 2010; 22(1): 51-54.
20. da Silva JD, Viana MP, Lima Pereira Calado LG, et al. Cross-sectional survey for sheep leptospirosis in the northeast region of Brazil. Prev Vet Med 2021; 197 105525. doi: 10.1016/j.prevetmed.2021.105525.
21. Viana MP, da Silva JD, César Lima AM, et al. Epidemiological and geospatial characterization of goat leptospirosis in Northeast region of Brazil. Small Rumin Res 2022; 206, 106589. doi: 10.1016/j.small rumres.2021.106589.
22. Wood PL, Steinman M, Erol E, et al. Lipidomic analysis of immune activation in equine leptospirosis and Leptospira-vaccinated horses. PLoS One 2018; 13(2): e0193424. doi: 10.1371/journal.pone.0193424.
23. Allan KJ, Biggs HM, Halliday JEB, et al. Epidemiology of leptospirosis in Africa: a systematic review of a neglected zoonosis and a paradigm for 'One Health' in Africa. PLoS Negl Trop Dis 2015; 9(9): e0003899. doi: 10.1371/journal.pntd.0003899.
24. Shrestha R, McKenzie JS, Gautam M, et al. Determinants of clinical leptospirosis in Nepal. Zoonoses Public Health 2018; 65(8): 972-983.
25. Hamond C, Pestana CP, Medeiros MA, et al. Genotyping of Leptospira directly in urine samples of cattle demonstrates a diversity of species and strains in Brazil. Epidemiol Infect 2016; 144(1): 72-75.
26. De Olivera D, Figueira CP, Zhan L, et al. Leptospira in breast tissue and milk of urban Norway rats (*Rattus norvegicus*) Epidemiol Infect 2016; 144(11): 2420-2429.
27. Haji Hajikolaei MR, Ghorbanpour M., Gharibi D, et al. Serologic study on leptospiral infection in sheep in Ahvaz, southwestern Iran. Iran J Vet Res 2007; 8(4): 333-336.
28. Martins G, Lilenbaum W. Control of bovine leptospirosis: Aspects for consideration in a tropical environment. Res Vet Sci 2017; 112: 156-160.
29. Casanovas-Massana A, Pedra GG, Wunder EA, et al. Quantification of Leptospira interrogans survival in soil and water microcosms. Appl Environ Microbiol 2018; 84(13): e00507-18. doi: 10.1128/AEM.00507-18.
30. Haji Hajikolaei MR, Ghorbanpour NM, Abdolahpour GR, Serological study of leptospirosis in cattle in Ahwaz. J Vet Res 2005; 60(1): 7-14.
31. Tooloei M, Abdollapour GR, Karimi H, et al. Prevalence of serum antibodies against six Leptospira serovars in sheep in Tabriz, Northwestern Iran. J Anim Vet Adv 2008; 7(4): 450-455.
32. Rezaei S, Haji Hajikolaei MR, Ghadrdan Mashhadi AR, et al. Comparison of Leptospira interrogans infection in the goats and sheep. Iran J Vet Med 2016; 10(2): 113-119.

33. Maleki S, Zakian A, Abdollahpour GR. Seroprevalence of Leptospira interrogans infection in equids of Lorestan province: Investigation the role of probable risk factors. Iran Vet J 2021; 16(4):108-118.
34. Balamurugan V, Thirumalesh SR, Sridevi R, et al. Seroprevalence of bovine Leptospirosis in Odisha, India. World J Vet Sci 2013; 1: 1-7.
35. Patel JM, Vihol PD, Dabas VS, et al. Seroepidemiological study of leptospirosis in buffaloes of south Gujarat, India. Buffalo Bull 2016; 35(3). 383-388.
36. Chandan L, Vinod KK, Vimal RR, et al. Trend in the seroprevalence of Leptospirosis among cattle and goat populations of South Andaman. Indian J Vet Res 2017; 26(1): 37-40.
37. Tagliabue S, Figarolli BM, D'Incau M, et al. Serological surveillance of Leptospirosis in Italy: two-year national data (2010–2011). Vet Ital 2016; 52(2): 129-138.
38. Simbizi V, Saulez MN, Potts A, et al. A study of leptospirosis in South African horses and associated risk factors, Prev Vet Med 2016; 134: 6-15.
39. Fagre AC, Mayo CE, Pabilonia KL, et al. Seroprevalence of Leptospira spp. in Colorado equids and association with clinical disease. J Vet Diagn Invest 2020; 32(5): 718-721.
40. Jansen A, Stark K, Schneider T, et al. Sex differences in clinical leptospirosis in Germany: 1997-2005. Clin Infect Dis 2007; 44(9): e69-e72.
41. Goris MGA, Boer KR, Duarte TAT, et al. Human leptospirosis trends, the Netherlands, 1925-2008. Emerg Infect Dis 2013; 19(3): 371-378.
42. Johnson MAS, Smith H, Joseph P, et al. Environmental exposure and leptospirosis, Peru. Emerg Infect Dis. 2004; 10(6): 1016-1022.
43. Patel JM, Vihol PD, Raval JK, et al. Seroprevalence of leptospirosis in clinically ailing bovine. J Anim Res 2015; 5(1): 31-35.
44. Shafighi T, Abdollahpour G, Zahraei Salehi T, et al. Serological and bacteriological study of leptospirosis in slaughtered cattle in north of Iran (Rasht). Afr J Microbiol Res 2010; 4(20): 2118-2121.
45. Agrawal R, Kumar M, Kumar M, et al. Epidemiological pattern of leptospirosis in livestock of Uttaranchal state. Indian J Comp Microbiol Immunol Infect Dis 2005; 26(2): 109-113.