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Current Status of Large Animal Leptospirosis Based on Leptospira Research Laboratory Retrospective Study in Iran

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Abstract

Background: Leptospirosis is a widespread zoonotic disease that is caused by spirochetes of the genus Leptospira. In Iran, flooding in some part of the country in 2019, caused more attention to disease distribution. Microscopic agglutination test is a gold standard for serological diagnosis of leptospirosis which is used in many reference laboratories.

Objectives: During 2010 and 2020, a total of 4900 blood samples from cows, buffaloes, horses, sheep and camels were delivered to the Leptospira Research Laboratory for serological diagnosis. These samples came from different provinces of Iran for research purposes. The aim of this study was to determine the Current status of large animal Leptospirosis based on Leptospira Research Laboratory Findings in Iran using microscopic agglutination test.

Materials and Methods: All serum samples were tested using Microscopic Agglutination Test (MAT). The MAT method was conducted as described by WHO, 2003 with some modifications. Serum samples were tested by MAT using five live *L. interrogans* serotypes, Gripptyphosa, Icterohaemorrhagiae, Hardjo, Canicola, and Pomona. Three standard controls were included in the test; one positive standard serum, one negative standard serum. Ten µL of the appropriate antigen was added to 10 µL of each diluted serum sample on a microscope slide. The microscope slide was placed in a petri-dish with moist paper to avoid evaporation, and incubated at 29°C for 90 minutes. The slide was examined under dark-field microscope (Olympus BX50), using a long working distance objective at x100 or x200 magnification. Agglutination was noted by observing clumps of leptospires. If more than 75% of the leptospira were agglutinated, the sample was considered positive at 1.100 dilution. Finally, the highest titre in which ≤75% agglutination was occurred was recorded as the final titre of antibody in the serum sample.

Results: The results of this study showed that the rate of seropositive in buffaloes 44%, cows 1.2-33.5%, horses of Guilan 31.6%, sheep 8.3-15% and in camels it was 2%. However. The highest rate of infection was in northern part of Iran, whereas the lowest rate of infection was seen in dried area. It was also noted that the rate of infection during the Covid-19 pandemic seems to be reduced in compare to the year before.

Conclusions: The results of this study showed that there is a close coloration between climate change, farm hygienic score the rate of seropositive cases. It seems that the emphasis on compliance with health protocols and disinfection by health authorities during the Covid-19 pandemic, has some effects on reducing the rate of infectious diseases as we have seen in current study. However, further epidemiological studies are needed to prove this theory.

Keywords: Leptospira, serology, MAT large animal, Iran

Introductions

Leptospirosis is a widespread zoonotic disease that is caused by spirochetes of the genus Leptospira. The first report of leptospirosis in large animal in Iran was published by Rafyi and Magami (1968) in which the prevalent serovars were Grippotyphosa, Pomona and Icterohaemorrhagia. Since then, the most prevalent *Leptospira* serovars reported in Iran include: Hardjo, Pomona, Grippotyphosa, Canicola and Icterohaemorrhagia (Maleki, 2013). Leptospirosis is considered an emerging global public health problem both in local populations and in returning travellers (Vijayachari 2008). The disease ranges from mild to lethal courses in its clinical spectrum and probably has a high proportion of sub-clinical and asymptomatic infections. Recently, WHO has established a Leptospirosis Burden Epidemiology Reference Group (LERG) (Abela-Ridder 2010) which estimated the mean global burden of endemic human leptospirosis on an incidence of 5 per 100,000 populations, recognizing this as an underestimation because it is based on severe and scarcely notified cases only and does not include epidemic leptospirosis. At the end of 1914

Inada and co-workers detected the microorganism in the liver of a guinea pig injected with the blood of a patient suffering from Weil's disease and isolated and identified '*Spirochaeta icterohcemorrhagiae'* as the causative agent of the Japanese form of Weil's disease [10]. Noguchi succeeded to isolate the organism from American wild rats, with the pathogen supposed to be identical to Japanese one (WHO 2011). A major challenge of this disease is the application of basic research to improve diagnostic methods and related vaccine development.

Materials and Methods

Serological methods: hygienic

A total of 4900 blood samples were collected and serum separation was performed in standard condition. All serum samples were tested using Microscopic Agglutination Test (MAT). The MAT method was conducted as described by WHO, 2003 with some modifications as follows. Centrifugation was performed on tubes containing blood for 10 minutes at 3000 rpm to separate the serum. Then the serum was transferred to the microtube using a sterile Pasteur pipette. Serum-containing tubes were kept frozen at −20 °C until conducting of the MAT, using five live *L. interrogans* serotypes, Gripptyphosa, Icterohaemorrhagiae, Hardjo, Canicola, and Pomona. All samples were transferred to the Leptospirosis research laboratory Faculty of Veterinary Medicine, University of Tehran on ice container for MAT. To perform the test, all serum samples to be tested were initially diluted 1:50 in phosphate buffer solution (PBS) in Eppendorf tubes by adding 20 µL of serum into 980 µL of PBS. Microscopic slides were divided in 8 square using diamond pen. Using a P-10 Gillman pipette, 10 µL of the appropriate antigen was added to each square on the microscope slide. Ten µL of each diluted serum sample was added beside the antigen on the slide, then the serum was mixed with the antigen. Care was taken to keep each dilution in its own square on the slide. Three standard controls were included in the test; one positive standard serum, one negative standard serum. The slide was placed in a petri-dish with moist paper to avoid evaporation, and incubated at 29°C for 90 minutes. The slide was examined under dark-field microscope (Olympus BX50), using a long working distance objective at x100 or x200 magnification. Agglutination was noted by observing clumps of leptospires. If more than 75% of the leptospira were agglutinated, the sample was considered positive at 1.100 dilution. For determination of the final titre, all positive serum samples were serially diluted in PBS in a micro-titre plate (Greiner), starting from 1.100 dilution, using 2-fold dilution (1.100, 200, up to 1600). The MAT was again performed using serial dilutions against reacting antigen. Finally, the highest titre in which ≤75% agglutination was occurred was recorded as the final titre of antibody in the serum sample.

The microscopic agglutination test (MAT) was performed using a set of hyperimmune rabbit antisera as positive controls. Cultures of 5 to 7 days were used as antigens. The slides were gently covered and placed in Petri-dish to exclude debris and prevent evaporation, and incubated at 30°C for 90 minutes. All samples were screened for agglutination at titre ≥1:100 under dark-field microscope. Agglutination of at least 75% of the leptospires was considered as positive.

Culture of Leptospires:

Mid-stream urine samples were collected from animals with suspicious sings of leptospirosis with care and hygienic conditions and two drops of the clinical samples were inoculated into semi-solid EMJH media supplemented with antimicrobial agents (5-fluorouracil, 100 μg/mL) and incubated at 30°C in an incubator at the leptospira reaesrch laboratory, University of Tehran for 2 months. The presence of leptospires was examined under dark-field microscopy using 20x and 40x magnification twice a week for 60 days. Leptospires can be distinguished by other spirochetes based on their characteristic thin helical structures with prominent hooked ends and motility.

Results

Serological test:

The general results of this study are given in table 1. The cut-off points titre in which the sample was considered as positive was 1:100. The results of this study showed that the rate of seropositive in different provinces of Iran was between 1.4 up to 44 percent. However, these results showed that the rate of seropositive in buffaloes of Khuzestan was 44%, in cows of different area it was 1.2-33%, horses of Guilan 31.6%, sheep 8.3-15% and in camels of Sistan & Baluchestan it was 2%. However. The highest rate of infection was in northern parts of Iran, whereas the lowest rate of infection was seen in dried area and during the period of least rainfall in last two years.

The highest serological titer up to 3200 was see in horses of Giulan provinces which were grazing in forest during autumn and winter time and also in buffaloes of Khuzestan with the highest titer of

800. However most of seropositive animals did not show and clinical signs of leptospirosis. The exception was seen in animals with titer of 800 and higher which showed at least one of the main clinical signs such as hemoglobinuria, jaundice, fever or ERU in horses at the time of sampling.

Isolation and identification of Leptospires:

A total of 125 urine samples from cows/calves were cultured and finally 13 test tubes culture became positive for growth of leptospira in days 18-23 after culture inoculation. Most of the positive culture were belonged to animals with clinical signs of leptospirosis and with positive titre one of the Grippotyphosa positive culture was isolated from urine sample of a cows with icterus carcase and red urine in slaughter house of Guilan province. The cross-agglutination absorption test showed that the three isolates were very close to the Leptospira interrogans Pomona and the rest of them were very close to Grippotyphosa serotype.

Table 1: MAT results of 4900 serum samples (Cows, Buffalos, Horse, Sheep and camels) collected from 15 provinces of Iran during 2010-2020

Province	Samples size	Positive no.	% Average
Guilan	320	70	21.9
Mazandaran	120	28	23.3
Khuzestan	982	257	26.2
East Azarbayjan	301	50	16.6
Tehran	152	24	15.8
West Azarbayjan	255	57	22.3
Khorasan	200	17	8.5
Kerman	280	35	12.5
Lorestan	425	39	9.2
Alborz	434	37	8.5
Ardabil	295	21	7.1
Golestan	250	18	7.2
Sistan & Baluchestan	300	15	5
Kurdestan	120	4	3.3
Markazi	466	7	1.4

Discussion

This is the first study reporting ten years of retrospective study of leptospirosis according to the serum or urine samples referred to the Leptospira Research Laboratory, faculty of Veterinary Medicine, University of Tehran. During this period (2010-2020) a total of 4900 blood samples and 125 urine samples were collected and referred to the Lab for diagnostic purpose. In the present report, only the results of large animal samples including cow, buffaloes, horse, sheep and camel are being used. The results of this study as shown in table 1 indicated that Leptospirosis in large animals is seen in all provinces of Iran with different infection rate. According to the present study the rate of seropositivity in different parts of Iran was between 1.4 up to 44 percent. The highest rate of infection is seen in northern provinces of Iran, whereas the lowest rate of infection is seen in central and south-east of Iran. It also showed that during last two years in which we had the least rainfall in fifty years, the rate of infection become very low. For example, the study of bovine leptospirosis in Markazi province (central Iran) showed 1.4% of 466 cows became seropositive. However, It seems that during last two years the emphasis on compliance with health protocols and disinfection by health authorities to control the Covid-19 pandemic, has some effects on reducing the rate of infectious diseases in domestic animals as we have seen in current study. It can be the results of more understandings of farmers of the role of practicing health protocols in the prevention of infectious diseases .However, further epidemiological studies are needed to prove this theory.

The results of the present study also showed

that the rate of seropositive in buffaloes of Khuzestan was as high as 44% and in horses of Guilan 31.6% which had more chance of attracting leptospira interrogans from wild animals, rodents and stagnant water in this area. However, the rate of infection in cows of different provinces was 1.2-33% which also shows the role environmental conditions on the spread of disease in each area. The same situation can be seen in sheep which the infection rate was 8.3 to 15%. Camels seems to be more resistance to leptospira infection as it shows that only 2% of them were seropositive in Sistan & Baluchestan province in south-east of Iran.

This study also is the first report of detection and characterization of leptospiral isolate from cows in Iran using serological and molecular techniques. In this study, a pathogenic species of *L. Grippotyphosa*, was isolated from urine of a cow which was suspected to leptospirosis. This finding was in line with previous studies that also isolated L. Grippotyphosa, from Mus msuculus in south-west of Iran (Maghami et als, 1997). The results of this study also showed that the chance of isolation of leptospira from clinical samples will be increased when clinically suspected animals are used for sampling.

A serological study in cows of Guilan province of Iran in 2004 showed that 53 (25.8%) samples had a positive reaction against one or more serovars of leptospira, in which the most prevalent *Leptospira* serovar was Canicola with 24 (11.7%) samples, and the least prevalent *Leptospira* serovars was Icterohaemorrhagiae with 1 (0.5%) sample (Abdollapour et al., 2009). However, one year later Shafighi et al. in 2010 reported that the most prevalent serovar in slaughtered cattle in Guilan was Pomona. Moreover, in Tehran suburbs, Pomona was found to be the predominant serovar (Abdollahpour et al., 2012).

A study in Iran also showed that bacteriological culture of 118 urine samples in isolation of 11 *Leptospira* organisms using a semisolid home-made enriched leptospira medium. All these isolates belonged to one feedlot farm in Tehran suburbs which many of the calves exhibited clinical symptoms of leptospirosis including anorexia, haemoglobinuria, jaundice and fever (Maleki 2013).

In conclusion the results of this study showed that there is a close coloration between climate change, farm hygienic score on the rate of seropositive cases in large animals. It seems that the emphasis on compliance with health protocols and disinfection by health authorities during the Covid-19 pandemic, has some effects on reducing the rate of infectious diseases as we have seen in current study. However, further epidemiological studies are needed to prove this theory.

The results of the present study also showed that there is a close genetic relatedness of these isolates to Grippotyphosa and Pomona serovars, although, there is a need for more molecular study of each isolate for identifying their differences with standard isolates. The are some more question about the pathogenicity of this isolate in other animal species and in human.

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