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# Assessment of Distribution *Leptospira* Spp. In Surface Waters of Guilan Province

[1]K. Issazadeh, [2]N. Amirmozaffari, [3]S. Mehrabian and [4]S. Oryan

[1]Science and Research Branch, Islamic Azad University (IAU), Tehran, Iran
[2]Microbiology Department, Faculty of Medical, Medical science University of Iran
[3]Biology Department, Tarbiyat Moallem University of Tehran, Iran
[4]Physiology Department, Tarbiyat Moallem University of Tehran, Iran

**Abstract:** Leptospirosis is an ubiquitous zoonotic infection.It is spread worldwide. Currently the genus *Leptospira* is classified in 13 genomic species and 4 genomospecies based upon phylogenetic and phenotypic relatedness.The goal of this study was to determine the distribution of aerobic spirochetes *Leptospira* in surface waters and soils in Guilan province of Iran. A total of 222 fresh water samples were collected in the summer of 2006 for isolation of *Leptospira* spp. After filtration, the samples were inoculated into the EMJH medium.Isolated *Leptospiras* were identified by *MAT* technique.Out of 222 samples collected for isolation of *Leptospiras*, the 40 strains isolated from soils and waters in Guilan province could be divided into 4 speices and 6 serogroups.Taken together, *Leptospira* isolates indicated by seropositivity in local areas were 35% (ricefields), 35% (water channels) and 30% (rivers). It was also found that the seroprevalence for *Pomona* was higher on large-scale ricefields, but serogruop *Sarmin* was higher on large-scale rivers.With respect to chi – square analysis for evaluation relationship between studied sources and presence of *Leptospira* different areas of Guilan province with coefficient of confidence 95% were concluded that there is a significant correlation between them ($p<0.05\%$ ).

**Key words:** *Leptospira*. *MAT* test · Surface waters · Guilan province

## INTRODUCTION

*Leptospires* are gram negative tightly helically coiled spirochetes with a length of 6-20 µm and a width of 0.1-0.15 µm. Obligate aerobic Spirochetes characterized by a unique flexuous type of motility [1]. The primary maintenance for this disease is wild animals and the disease is a major cause of economic loss in the meat and dairy industry. Humans are incidental hosts in whom this disseminated disease varies in severity from subclinical to fatal.Exposure depends on chance contacts between humans and infected animals or a contaminated environment.

The degree and nature of exposure often depend on occupational (in developing countries) and/or recreational and social activities (in developed countries). Currently *Leptospiras* from urine contaminated environments such as water and soil [2]. Animal and human leptospirosis is spread worldwide, also it is of most concern in tropical regions such areas often present suitable conditions for survival and transmission of the *Leptospires*,such a regular flooding, presence of several animal species that may maintain *Leptospires*, suitable climate for survival of the bacteria in the environment and socio-economic conditions that favour transmission [3]. Leptospirosis occurs in wild and domesticated animals, both of which can be a source of human infection.

Rapid determination of the source of infection is critical in limiting determined most easily by determining the serological type (serotype ) associated with and outbreak of disease, because certain serovars often associate with specific mammalian hosts. So, it is often possible to predict potential sources of infection and thereby control the spread of the disease. However, some leptospiral serovars (e.g., serovar Pomona) appear to adapt with to several mammalian hosts and thus can complicate this analysis. To overcome existing limitation in serovar detection and identification PCR assay and molecular techniques useful method for a difficulty [4].

## MATERIALS AND METHODS

A total of 222 fresh water samples were colleted in the summer (from July to September) 2006 for isolation

**Corresponding Author:** K. Issazadeh, Science and Research Branch, Islamic Azad University (IAU) Tehran, Iran

Table 1: Anti-serum related to species and serogroups of the saprophytic Leptospiras used in this study

| Species | Serogroup |
|---|---|
| *L. biflexa* | *Patoc, Semaranga* |
| *L. meyeri* | *Ranarum, Sofia* |
| *L.wolbachii* | *Sarmin* |

Table 2: Anti-serums related to the species and serogroups of the pathogenic *Leptospiras* used in this study

| Species | Serogroups | |
|---|---|---|
| *L. interrogans* | *Australis* | *Pomona* |
| | *Autmnalis* | *Icterohaemorragiae* |
| | *Bataviae* | |
| | *Bratislava* | |
| | *Canicola* | |
| | *Copenhageni* | |
| | *Hardjo* | |
| *L. kirschneri* | *Cynopteri* | |
| | *Grippotyphosa* | |
| *L. noguchii* | *Fortbragg* | |
| | *Panama* | |
| *L. borgpetersenii* | *Hardjo bovis* | |
| | *Ballum* | |
| | *Balanica* | |
| | *Javanica* | |
| | *Mini* | |
| | *Sejroe* | |
| *L. santarosni* | *Djasiman* | |
| | *Shermani* | |
| *L. weilii* | *Celledoni* | |
| *L. fainei* | *Fainei* | |

of *Leptospira* spp.They were collected from rivers, channels, rice fields and wet soils of different parts of Guilan province. Water samples were collected and transported in sterile 200-500-ml glass bottles. Soil from an area of 15 to 20 cm by 4 to 8 cm was taken after all loose surface material had been removed. The soil was immediately placed in a plastic bag which was then sealed. All samples were protected against sudden temperature changes during transport to the laboratory and were processed within 12h. Temperature and pH were monitored throughout the study. Water and soil temperatures were taken in the field and pH was determined upon return to the laboratory.

Water samples (100 ml) were passed through a sterile 0.22- µm pore size membrane filter and 1.0 ml was inoculated in duplicated, into the culture medium. Soil samples (l00 g) were placed in a sterile 1-liter flask with 300 ml of *Leptospire* – free distilled water and mixed by shaking. The suspension was allowed to settle for 5 to 7 min before 50 to 60 ml was centre fudged at 800 to 900 rpm in a clinical table – top centrifuge for 5 to 7 min.

The supernatants were filtered using 0.22-µm membranes and 1.0 ml was inoculated, in duplicate, into the EMJH medium. This medium was rendered semisolid by the addition of agar to a final concentration of 0.2% and 5 – fluorouracil (5-FU) was incorporated at a concentration of 100 µg /ml to help minimize contamination.

All enrichment cultures were incubated aerobically at room temperature for 30 days and examined for the presence of *Leptospires* by dark – field microscopy. If Leptospires were not detectable after 30 days of incubation, the sample was considered to be negative.The presence of the least number of four *Leptospira* in each microscopic field was evaluated as a positive result. Then, the positive samples were separated and cultured again order to raise their population to be appropriate for MAT test. The media incubated in a shaker-incubator. They were examined by dark - field microscopy and high density (= 2.10/ml) samples were selected for MAT test.Then, selected specimens with high density stored under suitable conditions in modified korthof medium containing 20% glycerol at - 20ú C. In this study two micro tubes were used at first for the MAT test.

1 ml of culture medium containing specimen added in each micro tubes. In these micro tubes saprophytic and /or pathogenic *Leptospiras* were identified. So that, saprophytic *Leptospires* can be cultured selectively in medium containing 225 µg/ml 8-azaguanine. The same medium will differentiate saprophyte, which will grow in it, from pathogens, which will not.

Then ELISA plate was used in order to determine the species and serogroups of the *Leptospiras*. Determination of species and serogroups were performed by commercial antisera. It was prepared from Indian company).

The antisera which were used in this research are shown as follows:

## RESULTS

The obtained Statistical studies showed that a total a total of 222 samples were collected from rice-fields, soils, water channels and rivers from central parts of Guilan province during July to September 2006 and 40(18. 1 %) were found to be positive.

Comparing the data on the overall isolation percentage, it is apparent that a higher cell density occurred in rice field and water channels. The frequency of *Leptospiras* in rivers were 30% and all of the soil

Table 3: Summary of isolation data for the region and sources

| Sources | Rice field | | Soil | | Water channel | | River | | Total | |
|---|---|---|---|---|---|---|---|---|---|---|
| regions freq. | No.(+) | % | No.(+) | % | No.(+) | % | No.(+) | % | No.(+) | %(+) |
| Sangar | 3 | 15.7 (7.5) | 0 | 0 (0) | 10 | 52 (25) | 6 | 31.5 (15) | 19 | 47.5 |
| Khoshkebijar | 3 | 33.7 (7.5) | 0 | 0 (0) | 2 | 22.2 (5) | 4 | 44.4 (10) | 9 | 22.5 |
| Anzali | 6 | 60 (15) | 0 | 0 (0) | 2 | 20 (5) | 2 | 20 (5) | 10 | 25 |
| Rasht | 2 | 100 (5) | 0 | 0 (0) | 0 | 0 (0) | 0 | 0 (0) | 2 | 5 |
| Total | 14 | 35 | 0 | 0 | 14 | 35 | 12 | 30 | 40 | 100 |

Table 4: Pathogenic and saprophytic *Leptospira* spp. isolated from different regions of Guilan

| Bacteria | *L. interrogans* | | *L. biflexa* | | *L. kirschneri* | | *L. wolbachii* | | Total | |
|---|---|---|---|---|---|---|---|---|---|---|
| region Freq. | No.( +) | % | No.(+) | % | No.(+) | % | No.(+) | % | No.(+) | % (+) |
| Sangar | 3 | 15.7 (7.5) | 8 | 42 (20) | 0 | 0 (0) | 8 | 42.08 (20) | 19 | 47.5 |
| Khoshkehbijar | 3 | 33.3 (7.5) | 2 | 22.2 (5) | 0 | 0 (0) | 4 | 44.4 (10) | 9 | 22.5 |
| Anzali | 4 | 40 (10) | 2 | 20 (5) | 2 | 20 (5) | 2 | 20 (5) | 10 | 25 |
| Rasht | 0 | 0 (0) | 0 | 0 (0) | 0 | 0 (0) | 2 | 100 (5) | 2 | 5 |
| Total | 10 | 25 | 12 | 30 | 2 | 5 | 16 | 40 | 40 | 100 |

Table 5: Pathogenic and saprophytic *Leptospira* spp. isolated from different sources of Guilan

| Species | *L. interrogans* | | *L. biflexa* | | *L. kirschneri* | | *L. wolbachii* | | Total | |
|---|---|---|---|---|---|---|---|---|---|---|
| Freq. Region | No.(+) | % | No.(+) | % | No.(+) | % | No.(+) | % | No.(+) | %(+) |
| Rice field | 10 | 50 (25) | 2 | 10 (5) | 2 | 10 (5) | 0 | 0 (0) | 14 | 35 |
| Soil | 0 | 0 (0) | 0 | 0 (0) | 0 | 0 (0) | 0 | 0 (0) | 0 | 0 |
| Water channels | 0 | 0 (0) | 7 | 63.6 (17.5) | 0 | 0 (0) | 7 | 50 (17.5) | 14 | 35 |
| River | 0 | 0 (0) | 3 | 33.3 (7.5) | 0 | 0 (0) | 9 | 66.6 (15) | 12 | 30 |
| Total | 10 | 25 | 12 | 30 | 2 | 5 | 16 | 40 | 40 | 100 |

Table 6: Pathogenic and saprophytic serogroups isolated from different sources of Guilan

| Freq. | Pomona | | Patoc | | Ictero haemorragiae | | Semaranga | | Sarmin | | Grippotyphosa | | Total | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| sources | No.(+) | % | No.(+) | % | No.(+) | % | No.(+) | % | No.(+) | % | No.(+) | % | No.(+) | %(+) |
| Rice field | 6 | 0(0) | 0 | 0(0) | 0 | 0(0) | 0 | 0(0) | 0 | 0(0) | 0 | 0(0) | 0 | 0 |
| Soil | 6 | 42.8(15) | 1 | 7.1(2.5) | 4 | 28.5(10) | 1 | 7.1(2.5) | 0 | 0(0) | 2 | 14.2(5) | 14 | 35 |
| Water channel | 0 | 0(0) | 5 | 45.4(12.5) | 0 | 0(0) | 2 | 18.1(5) | 7 | 63.3(10) | 0 | 0(0) | 14 | 35 |
| River | 0 | 0(0) | 1 | 8.3(2.5) | 0 | 0(0) | 2 | 16.6(5) | 9 | 75(22.5) | 0 | 0(0) | 12 | 30 |
| Total | 6 | 15 | 7 | 17.5 | 4 | 10 | 5 | 12.5 | 16 | 40 | 2 | 5 | 40 | 100 |

Table 7: Pathogenic and saprophytic serogroups isolated from different regions of Guilan

| Sources Freq. | Pomona | | Patoc | | Ictero haemorragiae | | Semaranga | | Sarmin | | Grippotyphosa | | Total | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No.(+) | % | No.(+) | % | No.(+) | % | No.(+) | % | No.(+) | % | No.(+) | % | No.(+) | % (+) |
| Sangar | 2 | 10.5(5) | 5 | 26.3(12.5) | 1 | 5.2(2.5) | 3 | 15.7(7.5) | 8 | 42.1(20) | 0 | 0(0) | 19 | 47.5 |
| Khoshkehbijar | 2 | 22.2(5) | 1 | 11.1(2.5) | 1 | 11.1(2.5) | 1 | 11.1(2.5) | 4 | 44.4(10) | 0 | 0(0) | 9 | 22.5 |
| Anzali | 2 | 20(5) | 1 | 10(2.5) | 2 | 20(5) | 1 | 10(2.5) | 2 | 20(5) | 2 | 20(5) | 10 | 25 |
| Rasht | 0 | 0(0) | 0 | 0(0) | 0 | 0(0) | 0 | 0(0) | 2 | 100(5) | 0 | 0(0) | 2 | 5 |
| Total | 6 | 15 | 7 | 17.5 | 4 | 10 | 5 | 12.5 | 16 | 40 | 2 | 5 | 40 | 100 |

samples taken were negative. This distribution may be correlated with the moisture content of the soil sample. Water and soil samples were collected from four different regions that the most number of positive cases was found to sangar region, i.e. 6 cases (47.5%) an the minim of number was related to Rasht region, i.e.2 cases (5%) (Table 3.)

According to the chi-square analysis for evaluation relationship between investigated sources (ricefields, rivers, water channels and soil ) and the total number of *Leptospira' s* distribution in the central part of Guilan province and with the coefficient of confidence 95% were concluded that a significant difference (correlation) between them. (Chi square = 11.962, df = 3, sig.level = 0.008). Generally, Four species of *Leptospiras* were isolated as follows: *L.interrogans*, *L.kirschneri, L.wolbachii and L. biflexa.*The most important subspecies of pathogenic and saprophytic *Leptospires* isolated from different samples, were *Pomona, patoc, Icterohaemorrhagiae, semaranga, sarmin* and *Grippotyphosa* (Table 6.)

## DISCUSSION

We attempted in this study to examine the distribution of *leptospiras* in waters and soils within a given area, by an enrichment culture method using a standard volume of sample material. It is clear from the results presented that the density of *leptospires* from one body of water to the other is quite variable. The frequency of isolation for the ricefields and water channels are equal (35%) followed by the rivers (30 %) and finally the soil (0%). A higher percent of positive samples were in sangar region.It seems that in this region the carriers of *leptospiras* are more than the other parts, i.e. much more percentage of domestic animals such as horse, cow and sheep which traverse to the farms in these parts, are the carriers of *leptospiras.*Leptospirosis is now identified as one of the emerging infectious disease, exemplified by recent large outbreaks in Nicaragua [5-9] Brazil, India [10] southeast Asia, the United States [11,12] and most recently in several countries as a result of the Ecochallenge sabah 2000 competition in Malaysia [13-15].

Because the extended of the forest around these parts, it is possible that wild animals which are the reservoir of *leptospira* enter the farms and led to the spread of this bacterium. A remarkable point is that even positive samples found in the rivers are related to the sampling parts of rivers where water is almost stagnant or flows very slowly and there is the possibility that the wild and domestic animals have passed their urine there.

And also considering the time spread of this disease that is in accordance with the season of rice cultivation, it is concluded that the spread of *leptospirosis* has close relationship with the rice farming in the Guilan.

In 1961, Baker showed that the contaminated waters and soils of rainforests in west region of Malaysia contained 13 serogroups and 29 serovars among 1362 isolates [16]. The isolated *leptospiras* included serogroups of *icterohaemorragiae* (55.6%), *Grippotyphosa* (4.9%), *Pomona* (3.2%) and *Ranarum* (0.1 %), but in different areas of central parts of Guilan the highest number has been belonged to *sarmin* subspecies (40%) and frequency rate of *icterohaemorrhagiae, grippotyphosa* and *Pomona* serogroups were 10%, 5% and 15% respectively. Generally, varied and different findings in various regions may reflect the differences in size, kind of samples and time of sampling.

For example, in 1961 Baker by studies carried out of the soil mainly isolated *Bataviae, Autumnalis* serovars and a few number of *Icterohaemorrhagiae* strains. Consequently, the abundance extent of different serovars of *Leptospiras* depends on place and time of sampling, pH and humidity of soils, water pH and stagnancy of waters, entryway for wild and domestic animals [16].

During 1988 to 1992, the studies carried out by Yang w. and Pang J., lic in the west and southwest of the yunan, indicated that the topic pathogenic *Leptospiras* in the water and soil is one the most important geographical and epidemiological problems of the *Leptospiras*. The total abundance extent of *Leptospiras* isolates were 3.31%, (i.e. 8 out of 242 samples). Proportion of positive samples from water and soil were 2.14% and 4.9% respectively. Obtained strains in this research belonged to 6 serogroups which included *Icterohaemorrhagiae, Pyrogenes, grippotyphosa, Sejroe a*nd *Australis* serogroups. The recovered serogroups of *Leptospiras* from soil and water were in conformity with isolated serogroups of sick people and animals in the same area with Leptospirosis[17].

By study which Dr.honarmand and etal carried out in 2004, in Guilan province totally, 289 samples of collected serum were studied so that, by using MAT test, 70 samples of serum were certainly positive in point of view of existence of *Leptospiras*. The maximum abundance extent has been related to *Sejroe* serovar. (i.e. 20 out of 282 specimens ) and the minimum extent have been related to the serovars of *Tarasovi*, *Ballum* and *Hebdomadis* with the same abundance of 1 case.The frequency extent of isolates were *Grippotyphosa,Icterohaemorrhagiae*

and *Panama* serovars have been 15.4 and 2 cases respectively [18]. These results and also the results of our experiment indicate that *Grippotyphosa, Icterohaemorrhagiae, Pomona* serovars are of much important in Guilan and can be transmitted to human by environment and cause the infection. In other study carried out by Dr. Qanaõ I and his colleagues in Guilan, in 1999 from 237 serum specimens suspected to *Leptospira* in Rasht hospitals (center of Guilan) carried out by serologic tests, specially MAT, 74 cases were reported as positive [19]. The percentage of soil humidity is an important factor for survival of pathogenic Leptospiras [20]. But in our research the number of positives samples were 0 % because of unknown factors. Studies in different countries of the world is indicated that the clinical cases of isolated *Leptospira* was equal to the numbers of *Leptospiras* in the environment, specially in waters and the same *Leptospira*s cause leptospirosis in human beings. Therefore, farmers and all of the people who are, by any means, in danger should be given necessary knowledge about the dangers of this disease so that they should use long boots and latex gloves, they never enter the farms with bare feet and never leave the animals into the farms.Medical center must put enough surveillance on controlling this disease, specially in summer.The percentage of organic matters play an important role for survival of *Leptospiras* in soil, so that the same researcher showed that the maximum positive number of soil samples related to the peat soils containing of high humidity and a lot of organic matters.21 out of 28 cases (75%) of the samples of peat soil around of spring areas are considered positive but no positive sample was found in the areas where were extremely sandy [20].

## REFERENCES


1. Levett, P.N., 2001. Leptospirosis. Clinical Microbiology Reviews, 14: 296-326.
2. Faine, S., 1982. Guidelines for the control of leptospirosis. World Health Organization (WHO). Geneva, Switzerland. pp: 171.
3. Faine, S., B. Adler, C.A. Bolin and P. Perolat, 1999. *Leptospira* and Leotospirosis. Medical Sciences. Melbourne, Australia. pp: 272.
4. Richard, L. and A. Bolin, 1997. Differentiation of *Leptospira interrogans* isolated by IS1500 hybridization and PCR assys. Journal of Clinical Microbiology, pp: 2612-2617.
5. Brandling-Bennett, A.D. and F. Pinheiro, 1996. Infectious diseases in Latin America and the Caribbean: are they really emerging and increasing? Emerg Infect Dis., 2: 59-61.
6. Centers for Disease Control and Prevention. Outbreak of acute febrile illness and pulmonary hemorrhage-Nicaragua, 1995. Morb Mortal Wkly Rep., 1995; 44: 841-843.
7. Levett, P.N., 1999. Leptospirosis: re-emerging or re-discovered disease? J. Med. Microbiol., 48: 417.
8. Schwartz, D.A., 1997. Emerging and reemerging infections: progress and challenges in the subspecialty of infectious disease pathology. Arch Pathol Lab Med., 121: 776.
9. Trevejo, R.T., J.G. Rigau-Perez, D.A. Ashford, E.M. McClure, C. Jarquin-Gonzalez, J.J. Amador, J.O. de los Reyes, A. Gonzalez, S.R. Zaki, W.J. Shieh, R.G. McLean, R.S. Nasci, R.S. Weyant., C.A. Bolin., S.L. Bragg, B.A. Perkins, R.A. Spiegel, 1998. Epidemic leptospirosis associated with pulmonary hemorrhage-Nicaragua. J. Infect. Dis., 178: 1457-1463.
10. World Health Organization. Leptospirosis, India: report of the investigation of a post-cyclone outbreak in Orissa, November 1999. Wkly Epidemiol Rec WHO. 2000; 75: 217-223.
11. Centers for Disease Control and Prevention. Outbreak of acute febrile illness among athletes participating in triathlons-Wisconsin and Illinois, 1998. Morb Mortal Wkly Rep., 1998; 47: 585-591.
12. Centers for Disease Control and Prevention. Update: leptospirosis and unexplained acute febrile illness among athletes participating in triathlons-Illinois and Wisconsin, 1998. Morb Mortal Wkly Rep., 1998; 47: 673-676.
13. Centers for Disease Control and Prevention. Outbreak of acute febrile illness among participants in EcoChallenge Sabah 2000-Malaysia, 2000. Morb Mortal Wkly Rep., 2000; 49: 816-817.
14. Communicable Disease Surveillance Centre. Leptospirosis outbreak in Eco Challenge 2000 participants. Commun Dis Rep., 2000; 10: 341.
15. Evans, M. and G. Baranton, 2000. Leptospirosis outbreak in Eco Challenge 2000 participants, Eurosurveillance Weekly, 4: 000921.
16. Alexander, A.D. and M.F. Baker, 1975. Pathogenic Leptospiras isolated from Malaysian surface waters. Applied Microbiology, 3: 30-33.

17. Yang, W. and J. Pan, 1994. An investigation on the distribution of *Leptospira interrogans* in southwest of Yunnon province. 15(5): 289-91.
18. Honarmand, A., 2002. Identification of species and subspecies Leptospires, agents of human acute leptospirosis in Giulan province with serological and molecular methods, Doctoral thesis, Tehran University of Medical sciences.
19. Mansour Ghanaei, F. and A. Sarshad, 2005. Leptosoira in Guilan, a northern province of Iran: Assesment of the clinical presentation of 74 cases. Med. Sci. Monit., 11(5): 219-223.
20. Henry, R.A. and R.C. Jhonson, 1978. Distribution of the genus *Leptospira* in soil and water. Applied and Environmental Microbiology, 2: 492-499.
21. Sofia, B., 2002. *Leptospira* infection among pigs in southern Vietnam, Doctoral thesis, Swedish University of Agricultural Sciences.