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Original Article

Detection of *Leptospires* serogroups, Which Are Common Causes of Human Acute Leptospirosis in Guilan, Northern Iran

*HR Honarmand, *SS Eshraghi*

Dept of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

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Abstract

Background: This study is performed to reveal most common species and subspecies of leptospires that are main causes of human leptospirosis in Guilan, Northern Province of Iran.

Methods: We performed IgM-ELISA and MAT on 282 blood samples from patients who attended to 3 hospitals in the flat area of Guilan Province with clinical symptoms consisted with leptospirosis. All specimens with titers ≥ 160 against at least one pathogenic strain in MAT and with titers ≥160 in IgM-ELISA were regarded confirmed positive cases indicative acute disease. For any confirmed positive cases, we determined the strains, which had the highest titer to determine the frequency of most common serovars and serogroups.

Results: Seventy of 282 sera had titers ≥160 against at least one pathogenic strain in MAT and titers ≥ 160 in IgM-ELISA. We determined frequency of common causative serogroups which had highest titers in 70 positive cases and only cases which had high titers in MAT and in IgM-ELISA were selected which is a reliable criterion to detect acute disease and to determine causative serogroup.

Conclusion: Nine serogroups including sejroe, grippotyphosa, mini, ictero haemorrhagiae, celledoni, autumnalis, cynopteri, pomona, and javanica were more responsible of acute leptospirosis in Guilan.

Keywords: *Serogroups, Leptospirosis, IgM-ELISA, Microscopic agglutination test*

Introduction

Leptospirosis is the most important zoonosis that is particularly prevalent in humid tropical and subtropical regions with a low social-economic status (1-4). "The risk of leptospirosis may be related to the occupations closely associated with water or sewage such as fish workers, miners and sewage workers. Farmers and domestic animal keeper may also become infected. The infected host animals play as a carrier for long time and excrete bacteria in their urine". (1, 5). Excreted *Leptospires* can live in environmental waters and moist soil for a long time especially in warm temperature and can penetrate the body of another host (animal or human) through skin abrasion and continue its epidemiological cycle (6-10). Determination of common and endemic serovars in any area is very important and represents the main step for epidemiological studies (11-14). "As the clinical symptoms and signs of leptospirosis are often nonspecific, the disease is easily mistaken for other major infectious diseases" (15-17). "Clinical presentations of leptospirosis may vary, and different types of disease may be observed, from relatively mild influenza-like symptoms to severe diseased with renal failure, liver impairment, and haemorrhage (Weil's syndrome)" (18-21). "Because of the wide variety of symptoms, leptospirosis is easily confused with many other fibril illnesses including haemorrhagic fevers, e.g., dengue fever" (22-24).

Guilan Province is located in north of Iran, near Caspian Sea with all condition facilitations the prevalence of leptospirosis, but there is not enough information about epidemiology of this disease in the area. Detection of common and endemic se-

***Corresponding to author:** Tel: + 9821 88973660, Fax: + 9821 88954913, E-mail: eshraghs@tums.ac.ir

rovars or serogroups is a basic and important step to determine epidemiological features of leptospirosis in any area (25-29). In the present study, we tried to find which serogroups of *Leptospires* are common causes of human leptospirosis in this endemic area by performing Microscopic Agglutination Test (MAT) on blood samples of patients who were suspected for bearing leptospirosis.

Materials and Methods

Blood samples were collected from patients hospitalized in three big general hospitals of the area (Imam Khomeini in Somesara, Razi in Rasht, and 22 Aban in Lahijan). The included patients were suspected to bear leptospirosis according to their clinical symptoms and diagnosis of physicians. All patients who had some common symptoms of leptospirosis according to WHO guidelines (30) such as: fever, severe headache, conjunctiva suffusion icterus, myalgia, arthralgia, general malaise, stiff neck, anorexia, nausea, and vomiting and also had history of exposure to wild or domestic animals, environmental water and rice farming were selected for the study. Ten ml vein blood was taken from any patient and was centrifuged to separate serum. All serum samples were stored in-20° C for examination.

We tested all specimens with a semi-quantitative ELISA method according to the manual of a reference laboratory (KIT Biomedical Research, Amsterdam) to determine positive samples and to decrease number of MAT examination for saving cost and time (31). By using ELISA method we can titrate and detect specific IgM and IgG antibodies against leptospira surface antigens. We used plates coated with antigens of wijenberg strain (Copenhageni serovar, Icterohaemorrhagia, serogroup), dilution buffer for making serial dilution of serums from 1:10 to 1:10240. Peroxides antihuman conjugate IgM and IgG[1], and 5- amino –2 salycylid pH= 5.8- 6 with 0.05% H_20_2 as substrate. Plates were washed by automatic washing machine with wash buffer. Automatic ELISA reader were used for reading ODs at 492 nm was used to measure cut off number and titers according to the manual (31).

MAT was performed according to the same manual by using a microbial panel which include 25 pathogenic and 3 nonpathogenic strains (Table l) and by allocating 3 plates for any specimen (one plate for each microbial panel). After adding 50ul sterile PBS with pH: 7.2 to all wells and adding extra 40 ml of the same PBS to all wells of column 2, we add 10 µl homogenized serum specimen to the same wells, mix well, and then make serial dilution from 1:20 to 1:20480 for all specimens by using multi-pipette. Then, 50 µl of a certain s well grown strain culture was added to all wells of each row and incubated in 30° C for 2 h. Dark field microscope was used for reading the results. Reading was done from first well of first row of first plate (panel I) and continue from left to right to find a well with half crowded of bacteria comparing to the first well which has original bacterial culture and consider its dilution as titer of that serum to that strain. All sera with titers ≥ 160 against at least one pathogenic serovar and with the same titer in IgM-ELISA were considered as confirmed positive cases.

Results

One hundred and forty two sera were obtained between 1-5 d and 140 sera were obtained in days >5 after onset of disease (totally mean date were 6.36). All 282 collected specimens were examined by semi-quantitative ELISA for screening and finding positive serum samples. Leptospira strains which were used for MAT assay are demonstrated in Table 1. We performed MAT only for IgM positive specimens to avoid paradox interpretations in determining recent causative serogroup. Fifty-nine of 282 sera had titers ≥ 320 and 11 sera had titers ≥ 160 (totally 70 cases) in MAT (Table 2).

Eighty-nine sera had titers ≥ 160 in IgM-ELISA and 110 sera had titers ≥ 160 in IgG-ELISA. Seventy sera had titers ≥ 160 in both MAT and

1 - Bio Rod Company, France.

IgM-ELISA, which were regards as confirmed positive cases. Nineteen sera had titers ≥ 160 in IgG-ELISA, but < 160 in MAT. They might were infected with strains which were not included in the microbial panel which we were used. For each confirmed positive case, we considered the serovars whose titer was very high in MAT, as the cause of the disease and by this way we determined the common serovars and serogroups which may be regarded as most common causes of the disease. Table 3 and 4 indicated the sero-groups serovars, which were common causes of acute leptospirosis in Guilan Province, respectively.

Table 1: Leptospira strains which were used for MAT assay

Panel	Strain No	Serogroup	Serovar	Strain
IA	1	Australis	Bratislava	Gez bratislava
IB	2	Ballum	Ballum	MUS 127
IC	3	Canicola	Canicola	Hond utrecht IV
ID	4	Grippotyphosa	Grippotyphosa	Duyster
IE	5	Grippotyphosa	Grippotyphosa	Mandemakers
IF	6	Hebdomadis	Hebdomadis	Hebdomadis
IG	7	Icterohaemorrhagiae	Icterohaemorrhagiae	Kantorowic
IH	8	Icterohaemorrhagiae	Copenhageni	Wijnberg
IIA	9	Javanica	Poi	Poi
IIB	10	Pomona	Pomona	Pomona
IIC	11	Pomona	Proechimys	1161U
IID	12	Sejroe	Hardjo	Hardjoprajinto
IIE	13	Sejroe	Hardjo type bovis	Lely 607
IIF	14	Sejroe	Saxdoebing	Mus 24
IIG	15	Sejroe	Sejroe	M84
IIH	16	Semaranga	Patoc	Patoc I
III1	17	Andaman	Andaman	Ch11
III2	18	Australis	Australis	Ballico
III3	19	Autumnalis	Rachmati	Rachmat
III4	20	Bataviae	Bataviae	Swart
III5	21	Celledoni	Celledoni	Ccelledoni
III6	22	Cynopteri	Cynopteri	3522C
III7	23	Mini	Mini	Sari
III8	24	Panama	Panama	CZ214K
III9	25	Pyrogenes	Pyrogenes	Salinem
III10	26	Semaranga	Semaranga	Veldrat sem 173
III11	27	Shermani	Shermani	1342k
III12	28	Tarassovi	Tarassovi	Perepelicin

Table 2: Results of MAT titers of 282 sera

Serogroup	Serovar	Strain	No titers		
			<160	>160<640	≥640
Australis	Bratislava	Jez Bratislava	277	5	0
Ballum	Ballum	Mus 127	258	22	2
Canicola	Canicola	Hond Utrecht IV	260	19	3
Grippotyphosa	Grippotyphosa	Duyster	214	51	17
Grippotyphosa	Grippotyphosa	Mandemaker	217	47	18
Hebdomadis	Hebdomadis	Hebdomadis	269	12	11
Icterohaemorrhagiae	Icterohaemorrhagiae	Kantorowic	236	39	7
Icterohaemorrhagiae	Copenhageni	Wijnberg	242	33	7
Javanica	Poi	Poi	250	28	4
Pomona	Pomona	Pomona	249	26	7
Pomona	Proechimys	1161 u	230	40	12
Sejroe	Hardjo	Hardjoprajinto	280	2	0
Sejroe	Hardjo type bovis	Lely 607	257	21	4
Sejroe	Saxkoebing	Mus 24	212	53	17
Sejroe	Sejroe	M 84	181	82	19
Semaranga	Patoc	Patoc I	125	106	51
Andaman	Andaman	Ch 11	171	81	30
Australis	Australis	Ballico	272	10	0
Autumnalis	Rachmati	Rachmat	227	48	7
Bataviae	Bataviae	Swart	262	20	0
Celledoni	Celledoni	Celledoni	220	55	7
Cynopteri	Cynopteri	3522 C	231	48	3
Mini	Mini	Sari	207	65	10
Panama	Panama	CZ 214 K	246	33	3
Pyrogenes	Pyrogenes	Salinem	244	37	1
Semaranga	Semaranga	Veldrat Sem 173	250	26	6
Shermani	Shermani	1342 K	238	39	5
Tarassovi	Tarassovi	Perepelicin	276	6	0

Table 3: Serogroups which were common causes of acute leptospirosis in Guilan Province in 2003

Frequency	Serogroup	Order
27	Sejroe(4)*	1
15	Grippotyphosa(2)*	2
10	Icterohaemorrhagiae, Mini	3
6	Celledoni	4
5	Automnalis	5
4	Cynopteri	6
3	Pomona, Javanica,	7
2	Canicola	8
1	Ballum,Panama,Shermani,Tarasovi,Hebdomadis	*9*

*: number of serovars which were in the panel

Table 4: Serovars which were common causes of acute leptospirosis in Guilan Province in 2003

Order	Serovars	Frequency
1	Sejroe	20
2	Grippotyphosa	15
3	Mini,saxkoebing	10
4	Copenhageni, Celledoni	5
5	Icterohaemorrhagiae,Rachmat,Cynopteri	4
6	Proechimys	3
7	Canicola,Hadjotypebovis,Shermani,Poi,Panam,	2
8	Ballum, ,Tarasovi,Hebdomadis	1

Discussion

In Iran, there are some regions with ecological and socioeconomic conditions, which are highly favorable for prevalence of leptospirosis. Animal leptospirosis is widespread in main parts of the country, where traditional animal husbandries are common but Human leptospirosis is mostly prevalent in two Northern provinces, which have temperate climate, e.g. Guilan province. This area has two different ecological regions: flat and mountain areas. Flat area is located along Caspian Sea with temperate climate, lots of surface waters mainly rivers and ponds, and lots of rodents and wild animals specially jackals and boars that live closely to villages. Rice farming is main activity of villagers followed by cattle breading. Rice paddies must always be wet. Rivers and ponds are the main source of irrigation of rice farms.

Leptospira is fastidious and its isolation from clinical samples is difficult, time consuming and usually unsuccessful. Interpretation of MAT results is not easy because cross reaction between different serogroups especially those collected from clinical specimens of acute phase of disease (25, 32). Patients usually have high titers against most serovars of a serogroup. Paradox reactions is another problem meaning that we will have highest titer of a serogroup unrelated to the disease, sometimes high rate of cross reaction in acute phase will follow relative specialized reaction in convalescence phase. In MAT, we measure IgM and IgG simultaneously and there are common antigens between different kinds of Leptospires (31, 33).
Paired serum assays increases accuracy of diagnosis. Minimum two-fold increasing titers of the second specimen, which is taken at least 10 d

later, to a serogroup (seroconversion), will show the recent *Leptospira*. High titer of a single specimen also can be indicative of acute infection but rate of it depends on the casual exposure background to causative agent or seroprevalence in any population. A titer ≥ 1:200 have diagnostic value if accord with clinical symptoms but not for endemic regions. For tropical and endemic regions, higher titers are indicative (33-35).

In this study, we had taken specimens from patients with clinical compatible and similar symptoms related to leptospirosis. They are all screened by a sensitive ELISA method and also by MAT only specimens with titer ≥ 160 in IgM -ELISA, and Mat are analyzed for determination common causative Leptospires. So we had 2 standard criteria. We had abandoned 11 specimens, which had high IgG but Low IgM titers that indicate previous infection because all patient with history of previous infection with one or more than one serogroup, have memory immune response. In these cases, titers to those serovars will increase higher and faster than recent causative serovars with usually broad spectrum cross reaction with other serovars that takes usually a few weeks to decrease. It causes difficulty in diagnosis and need second specimen with at least 10 d interval, which can have increasing titer to recent causative serovar but not others (34-37). MAT is suitable for sero-epidemiological studies (38, 39). It is usual to use a titer of ≥ 1:100 as evidence of past exposure. MAT data can give a general impression and show which serogroups are present in a population (40-42).

In conclusion, we could find that nine serogroups including sejroe, grippotyphosa, mini, ictero haemorrhagiae, celledoni, autumnalis, cynopteri,
Pomona, and javanica were more responsible for acute leptospirosis in Guilan Province.

Ethical Considerations

Ethical issues including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc. have been completely observed by the authors.

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