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SEROPREVALENCE OF LEPTOSPIROSIS AMONG FEBRILE CASES WITH ELEVATED MACROPHAGE MIGRATION INHIBITORY FACTOR (MIF)

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ABSTRACT

Leptospirosis is an emerging, infectious zoonotic disease caused by pathogenic leptospires. Despite its global importance, leptospirosis remains endemic in tropical and sub-tropical regions. This study intended to explore the seroprevalence of leptospirosis in suspected patients with febrile illness in Tiruchirappalli district, Tamil Nadu, India and also evaluate the rate of seroprevalence in febrile cases with high serum Macrophage migration inhibitory factor (MIF) profile. The sera samples (253) of study population were tested by MAT assay and IgM ELISA. Among the 104 leptospirosis suspected cases, 68 patients showed the seropositivity with 65.3% of seroprevalence. Co-infection of leptospirosis was observed in 6 patients with Typhoid and one with Dengue. Out of these 74 patients, predominance in males (n=48) was observed when compared to females (n=26). A higher number of male (n=22) and female (n=13) patients was found to be in the range of age 31-40 years. The predominant clinical features observed in leptospirosis patients are fever, myalgia, chills/rigor, abdominal pain, oliguria, jaundice, breathlessness and hepatomegaly. The highly prevalent serovars are Australis (41.8%), Autumnalis (24.3%). The range of MAT titres is 1:80 and 1:2560. The leptospirosis suspected febrile cases with high MIF profile in the ranges of 16-30ng/mL and 2.6-15ng/mL showed the

significantly higher seroprevalence (61.7%), whereas the patients with low range of MIF (0-2.5ng/mL) showed the low prevalence (2.9%). Altogether, leptospirosis remains the significantly high prevalence in Tiruchirappalli especially in patients with high level of serum MIF.

Keywords: Leptospirosis, seroprevalence, Microscopic agglutination test, co-infection, prevalent serovars, clinical manifestations

INTRODUCTION

Leptospirosis is a spirochaetal zoonotic disease caused by pathogenic leptospires infecting both humans and animals. The route of transmission is either direct or indirect contact with infected animals or excretion of infected animal and contaminated water or soil respectively **[1]**. The risk of leptospirosis is influenced by the environmental and occupational conditions such as limitations in basic sanitation, poor housing, animal handling, agricultural activities etc. **[2]**. Leptospirosis has a spectrum of clinical presentations ranges from mild, nonspecific flu like illness to severe fatal conditions which including kidney injury, inflammation in liver, pulmonary hemorrhage, myocarditis, and meningitis. For past decades, pulmonary manifestations are increasingly recognized as the most severe form of leptospirosis **[3]**. Every year, 1.03 million people affected worldwide and the rate of mortality and morbidity are significantly high **[4]**. The signs and symptoms of leptospirosis are confused with that of other febrile illnesses like typhoid, dengue, malaria and acute hepatitis **[5]** which makes difficulty for physician to distinguish. Therefore, leptospirosis is mostly underdiagnosed which leads to an inadequate treatment on time **[6]**.

Although leptospirosis has distributed worldwide, it is epidemic in tropical and sub-tropical areas which provide the favorable environment of leptospiral survival **[7]**. Despite its global importance, large lacunae exist in the epidemiological and surveillance data of human leptospirosis in developing countries. Leptospirosis outbreaks were frequently reported in developing countries during natural disasters like flooding and cyclone. The studies on the seroprevalence of human leptospirosis among healthy individuals have been performed in many countries and reported that the municipal service workers, food handlers, market workers and agricultural workers are the high risk population of leptospirosis **[8]**. The case control study of leptospirosis is also conducted in several countries.

In India, the awareness and knowledge on leptospirosis is poorly understood, but it is one of the important causes for hospitalization in India. The limited laboratory facility to diagnose the acute leptospirosis causes the limited data on the disease prevalence which makes it remain forgotten neglected disease, which leads to the consequence of lack of awareness on leptospirosis among physicians and the importance of early diagnosis of leptospirosis. Based on the literature, leptospirosis is endemic in seven states and one union territory of India including Tamil Nadu, Karnataka, Maharastra, Kerala, Gujarat and Andaman and Nicobar Islands **[9]**. Epidemics of leptospirosis are frequently reported in urban areas of Tamil Nadu including Chennai and Mumbai **[9, 10, 11]**. Although the outbreak and prevalence of leptospirosis are significantly notable in India, the data on leptospirosis research are rare **[12]**. The frequent observation of leptospirosis prevalence is urgently required for the public health management by preventing the disease. Therefore, this study intended to estimate the seroprevalence of human leptospirosis to providing the public awareness and unravel the importance of the consideration and early diagnosis of human leptospirosis.

MIF, a pluripotent cytokine play a key role in inflammation, immune responses and fundamental biological processes **[13]**. Several evidences revealed that MIF is involved in disease pathogenesis as well as susceptibility and severity of inflammatory and autoimmune diseases. The genetic polymorphism and epigenetic regulation in MIF mediates the multifaceted effects of MIF. MIF serves as a biomarker for several diseases such as inflammatory diseases, autoimmune diseases, sepsis, cancer, and metabolic disorders such as type 2 diabetes and obesity **[14]**. Previously we performed the evaluation of serum MIF level to identify the diagnostic marker of human leptospirosis (consideration for publication). Here we evaluated the impact of elevated serum MIF in seroprevalence of leptospirosis among febrile cases.

MATERIALS AND METHODS

Study design and study site

The present study was designed to evaluate the disease burden in Tiruchirappalli during June 2018 to February 2019 by active hospital based surveillance at the Annal Gandhi Memorial General Hospital, Tiruchirappalli district, Tamil Nadu, India. The study site details are as follows: the geographical position is 10°48′18″N latitude

and 78°41′08″E longitude and the temperature ranges from 36 to 41°C.

Study subjects and case definition

A total of 253 study subjects were recruited to participate in this study. A total of 104 blood samples from leptospirosis suspected cases with clinical manifestations including fever, myalgia, Chills/ rigor, breathlessness, abdominal pain, jaundice, Conjunctival suffusion, hepatomegaly and oliguria were collected to diagnose leptospirosis. The samples were collected before any treatment was given to patients. In addition, serum samples from 95 other febrile cases were collected to evaluate the co-infection. A total of 57 seronegative healthy controls recruited from the general population in the same geographical area matching for age (±5 yr) and sex were included as controls. The healthy control subjects those had fever for previous 2 weeks are excluded from the study. The obtained sera samples were divided into aliquots and stored at -80°C until the assay was performed.

Ethical Statement

The present study was endorsed by the Institutional Ethical Committee (No: DM/2014/101/51), Bharathidasan University. Both patients and healthy controls had requested to provide the Informed consent form before the sample collection. If the cases are minor subjects, their parents signed a consent form.

Live antigens and Microscopic agglutination test (MAT)

MAT was performed to evaluate the seropositivity of leptospirosis in study population by using the panel of twelve live leptospiral serovars. EMJH medium supplemented with BSA and Tween-80 was used to maintain the live antigens of leptospires. The serogroups used as live antigen in MAT assay are as follows, Australis (serovar Australis, strain Ballico), Autumnalis (serovar Autumnalis, strain Akiyami A), Ballum (serovar Ballum, strain Mus 127), Bataviae (serovar Bataviae, strain Swart), Canicola (serovar Canicola, strain Hond Utrecht IV), Icterohaemorrhagiae (serovar Icterohaemorrhagiae, strain RGA), Grippotyphosa (serovar Grippotyphosa, strain Moskva V), Hebdomadis (serovar Hebdomadis, strain Hebdomadis), Javanica (serovar Poi, strain Poi), Pomona (serovar Pomona, strain Pomona), Pyrogenes (serovar Pyrogenes, strain Salinem), Sejroe (serovar Hardjo, strain Hardjoprajitno). The optimum density of leptospiral antigens are seven days old live cultures with the density of $1x10^8$ organisms/mL. The dilution of serum was starting from 1:20 followed by

1:40, 1:80, 1:160. 1:320, 1:640, 1:1280 and 1:2560 and incubated with appropriate volume of live leptospiral antigens for agglutination. A titre of ≥1:80 and the ≥50% of agglutination were considered as MAT positive. Phosphate buffered saline (PBS) was used as diluent in the assay.

IgM ELISA (Enzyme-linked immunosorbent assay)

IgM ELISA was performed to further confirm the leptospiral infection in suspected cases by assess the presence of antibodies against leptospires in patients serum. Heat extracted leptospiral antigens were prepared as per described earlier **[15]**. 96 well microtiter plates were coated with 0.2µg of leptospiral antigens with using carbonate coating buffer (pH 9.6). The prepared plates were stored at 4ºC for 12h. Each wells were washed thrice with PBST (PBS+0.1% Tween-20) for 10 minutes each. 3% blocking solution (Non- fat milk) was added to each wells and incubated at 37º for 1h. Washing was performed as mentioned previously. Test serum were added to appropriate wells at a dilution of 1:100 and incubated at 37º for 1h. After washing the wells, bounded IgM antibody was detected by adding peroxide conjugated anti-human IgM antibody (1:1000) and incubated at 37º for 1h, followed by developed with o-phenylenediamine dihydrochloride (OPD). 50µL of 1N H_2SO_4 was added to stop the reaction and the optical density was measured at 490nm by microtiter plate reader.

MIF ELISA

Serum MIF in sera samples of study subjects was measured by MIF immunoassay using Human MIF ELISA kit (Sigma-Aldrich, St. Louis, Mo, USA). The procedures used for this assay was followed as per manufacturer's instructions with minor modifications. In short, 100µl of sera samples were added to the precoated microtiter wells and incubated for 3h at room temperature. Wash the wells with 1X wash buffer for 4 times. Then, 100µl of biotinylated antibody was added and incubated for 1.5 hour. Washed the wells and add 100µl of horseradish peroxidase (HRP)-streptavidin solution and incubated for 1 hour. After washing, 1003′ of TMB (3,3',5,5'-tetramethylbenzidine) was added and incubated at dark for 30 minutes. 50µl of stop solution was added the plates were read at 450nm.

Statistical analysis

Data from triplicate experiments were quantified and expressed as Mean ± SE, n=3. The data were calculated either with either GraphPad Prism version 9.2.0 or SigmaPlot

11.0 software. Two-tailed paired Student's t-test or Mann-Whitney *U* test was performed to analyze the difference between the study groups. The p-value of ≤0.05 was considered as significant.

RESULTS

Seroprevalence of leptospirosis

The sera samples (253) of study population in wide range of age range (10- 65) were tested by MAT assay. All the MAT positive sera samples showed the positive for IgM ELISA in the titre of 1:100. Among the 104 leptospirosis suspected cases, the anti-leptospiral antibodies against the leptospiral serovars were found in 68 sera samples and accounted for 65.3% of overall seroprevalence, whereas the overall prevalence rate of co-infection in other febrile cases were 6.3% and no leptospiral antibodies were found in sera of healthy controls. The grouping of study population and seroprevalence was reported in **Table 1**.

Co-infection of leptospirosis

Co-infection of leptospirosis was observed in 6 patients of other febrile illnesses. Interestingly, the co-infection with typhoid fever (8.3%) was found to be higher, and followed by with dengue fever (3%). Therefore, no co-infection was observed in malaria and hepatitis patients.

Age and sex wise distribution of leptospirosis patients

The patient's age was ranged from 10 to 65 years. Out of these 74 patients, predominance in males (n=48) was observed when compared to females (n=26). The higher number of male patients was under the age range of 31-40 followed by 10-20, 41-50 etc, whereas a higher number of female patients were under the rage range of 31-40 followed by 41-50, 21-30. The detailed age and sex wise distribution of leptospirosis patients was given in **Table 2**.

Clinical characteristics of leptospirosis patients

All the leptospirosis patients were accounted with fever. The predominant clinical features of leptospirosis are myalgia (97.2%), Chills/rigor (83.7%), abdominal pain (81%), and oliguria (68.9%) followed by jaundice (43.2%), breathlessness (41.8%) and hepatomegaly (47.2%). The least common feature reported in leptospirosis patients is conjunctival suffusion (5.4%). The number of patients characterized with clinical features and the percentage values are presented in **Table 3**.

Highly prevalent serovars and MAT titres

Out of these 12 leptospiral serovars tested, 7 were reported among the study population. The highly prevalent serovars are Australis

(41.8%), Autumnalis (24.3%) followed by the prevalent serovars Icterohaemorrhagiae (9.45%), Grippotyphosa (9.45%), Pomona (8.1%), Ballum (5.4%) and Canicola (4%). The range of MAT titres demonstrated was between 1:80 and 1:2560. The prevalent serovars and MAT titres were listed in **Table 4**.

Association of elevated serum MIF with leptospirosis seroprevalence

Serum MIF level of leptospirosis suspected cases and other febrile cases was estimated by MIF ELISA. The leptospirosis suspected febrile cases with elevated MIF (16-30ng/mL) showed the significantly higher seroprevalence (61.7%) whereas febrile case with 2.6-15ng/mL showed the seroprevalence of 20.5% and the cases with lower MIF level showed only the 2.9% of seroprevalence. Interestingly, in other febrile cases, only the patients with elevated MIF (16-30ng/mL) showed the seropositivity (100%) of leptospirosis. In healthy control cases, there is no seropositivity was reported. The MIF profiling of study subjects and the associated seroprevalence was mentioned in **Table 5**. Therefore, the seroprevalence of leptospirosis among febrile cases with elevated serum MIF are significantly high.

Table 1: Seropositivity of leptospirosis among study populations

Study groups	No. of subjects (n=253)	No. of seropositivity, n (%)	95% CI
Leptospirosis suspected febrile cases	104	68 (65.38)	0.65 (0.55 – 0.75)
Other febrile cases	95		
Typhoid	60	5 (8.3)	0.08 (0.02 – 0.18)
Dengue	33	1 (3)	0.03 (0.0008 – 0.15)
Malaria	7	-	-
Hepatitis	5	-	-
Healthy controls	54	-	-

Table 2: Age and sex wise distribution of leptospirosis patients

Age (in years)	Male, n= 48 (%)	Female, n= 26 (%)
10-20	9 (18.7)	1 (3.8)
21-30	6 (12.5)	5 (19.2)
31-40	22 (45.8)	13 (50)
41-50	7 (14.5)	6 (23)
51-60	3 (6.2)	-
61-65	1 (2)	1 (3.8)

Table 3: Distribution of clinical features of leptospirosis patients

Clinical features	No. of seropositive cases	95% CI
Fever	74 (100)	1 (0.95 - 1)
Myalgia	72 (97.2)	0.97 (0.9 – 0.99)
Chills/ rigor	62 (83.7)	0.83 (0.73 – 0.91)
Breathlessness	31 (41.8)	0.41 (0.3 – 0.53)
Abdominal pain	60 (81)	0.81 (0.7 – 0.89)
Jaundice	32 (43.2)	0.4 (0.31 – 0.55)
Conjunctival suffusion	4 (5.4)	0.05 (0.01 – 0.13)
Hepatomegaly	35 (47.2)	0.47 (0.35 – 0.59)
Oliguria	51 (68.9)	0.68 (0.57 – 0.79)

Table 4: Serovar distribution and MAT titres in leptospirosis patients

Serovar	Frequency, n (%)	1:80 n (%)	1:160 n (%)	1:320 n (%)	1:640 n (%)	1:1280 n (%)	1:2560 n (%)
Australis	31 (41.8)	4 (12.9)	8 (25.8)	10 (32.2)	4 (12.9)	3 (9.6)	2 (6.4)
Autumnalis	18 (24.3)	0	6 (33.3)	8 (44.4)	3 (16.6)	0	1 (5.5)
Icterohaemorrhagiae	7 (9.45)	1 (14.2)	3 (42.8)	0	2 (28.57)	0	1 (14.2)
Grippotyphosa	7 (9.45)	0	4 (57.1)	1 (14.2)	2 (28.57)	0	0
Pomona	6 (8.1)	1 (16.6)	2 (33.3)	2 (33.3)	0	1 (16.6)	0
Ballum	4 (5.4)	0	0	1 (25)	1 (25)	2 (50)	0
Canicola	3 (4)	1 (33.3)	1 (33.3)	1 (33.3)	0	0	0

Table 5: Leptospirosis seroprevalence on patients with elevated MIF

Range of Serum MIF levels (ng/mL)	Febrile cases, n (%)	Leptospirosis seroprevalence, n (%)
Suspected febrile cases, n=104		
0-2.5	36 (34.6)	2 (2.9)
2.6-15	21 (20.1)	14 (20.5)
16-30	47 (45.1)	42 (61.7)
Other febrile cases, n=95		
0-2.5	79 (83.1)	0
2.6-15	10 (10.5)	0
16-30	6 (6.3)	6 (100)
Healthy Controls, n=54		
0-2.5	51 (94.4)	0
2.6-15	3 (5.5)	0
16-30	0	0

DISCUSSION

Leptospirosis was widely distributed in Tiruchirappalli district and characterized with common symptoms which were similar to that of other febrile associated illnesses like dengue, typhoid, malaria and hepatitis **[16, 17]**. Leptospirosis has been correlated with occupations regarding contact with animals and contaminated environments. Agricultural workers, veterinary and animal handlers and sewage workers are the high risk population of leptospirosis **[18]**. Therefore, this study was carried out to estimate the seroprevalence of the Tiruchirappalli, where agriculture continues to be the most predominant sector of economy and the three forth population are associated with agriculture and allied activities **[19]**.

The result of this study showed high seroprevalence among suspected febrile cases. Several studies were undertaken for analyze the seroprevalence in population with high risk occupations **[20]**. Klement-Frutos *et al*., reported that age and sex determines the risk of leptospirosis. Young man with active age group is more susceptible to leptospirosis. The median age of cases was 33.6 years (range 1.1–84.8 years) and the mean age of fatality was 48.6 years (median 51.7, range 17–78). This study reported that male is predominantly infected with leptospirosis than female **[21]**. Based on the literature, the clinical features are vary

depends on the host immune response and genetic factors. Previously reported clinical features are jaundice, acute renal failure, rhabdomyolysis, pancytopenia, respiratory failure and disseminated intravascular coagulation **[22]**.

The predominant serovars detected in this study were Australis and Autumnalis followed by Icterohaemorrhagiae, Grippotyphosa, Pomona, Ballum and Canicola which constituted more than 60% of the leptospirosis confirmed cases. Out of 12 serovars tested, 7 serovars were tested positive with the blood samples from suspected cases. The predominant serovars isolated in Andaman and Nicobar Islands are Australis, Grippotyphosa, Canicola and Icteroharmorrhagiae **[23]**. Previous review on leptospirosis reported that 37 serovars had been isolated from animals and human in Malaysia **[24]**.

Based on our previous results (accepted for publication), ≤2.5ng/mL is the cutoff value/ baseline MIF concentration of human serum. Here, we evaluated the impact of serum MIF level of patients on rate of seroprevalence of leptospirosis in febrile cases. The serum level of patients determines the susceptibility and severity of several diseases Rheumatoid arthritis, Vitiligo, pulmonary tuberculosis, cancer, ulcerative colitis, coronary artery disease, pneumococcal meningitis and inflammatory bowel disease. The elevated serum MIF and overexpression of MIF mRNA was regulated by MIF gene promoter polymorphisms **[14]**. Therefore, the further studies are needed to explore the association of host genetics and immune response with susceptibility and severity of inflammatory diseases. This study generated the knowledge on seroprevalence and prevalent serovars which are helpful for physicians and technicians to diagnose and manage the leptospirosis outbreak upon natural catastrophes and also revealed the significant impact of patient's serum MIF on prevalence of human leptospirosis.

CONCLUSION

The seroprevalence of leptospirosis was found to be 65.3%. In addition to seroprevalence, this study revealed the prevalence of infecting leptospiral serovars and most common clinical features of leptospirosis. These results can make awareness to physicians for the serious consideration of leptospirosis in tropical areas and provide the list of predominant serovars to aids to set the standard panel of live antigens for MAT assay. Further studies should be carried out to determine the potential risk factors and molecular mechanism of leptospirosis.

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