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LEPTOSPIROSIS AMONG GARDENERS IN TIRUCHIRAPALLI, SOUTH INDIA: ISOLATION, SEROPREVALENCE AND MOLECULAR DETERMINATION

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ABSTRACT

Leptospirosis is a zoonotic disease that accidentally infects the humans when they exposed to contaminated environment including soil. While the first author visiting the park at Tiruchirapalli, the gardener is working in the soil for planting, at that time he handled the dead rat in his hand and threw it in the dust bin without any personal protective equipment. Thus, the objective was determined to assess the presence of leptospires in the gardeners by isolation, serology and simple molecular analysis in order to understand and prove the environmental and occupational risk of acquiring leptospirosis. A battery of 47 gardeners from various levels (from General Park to school gardens) and the same number of age and gender matched control subjects without gardening occupation in Tiruchirapalli were included. Blood samples of all the gardeners and control were inoculated in EMJH semisolid medium and serology including ELISA and MAT were performed to assess the seroprevalence. Further 30 MAT positive samples were examined for the presence of leptospiral DNA using specific primers for 16S rRNA gene. Among the 47 gardeners' and control samples, 26 and 4 were found to be positively confirmed for MAT serology respectively. Dinger's ring and culture positive were observed among 4 gardener blood samples and predominantly identified as *Leptospira interrogans* serovar Grippotyphosa. Out of 30 MAT positive samples, 12 samples were found to be PCR positive with the dominating serovar of Grippotyphosa, revealing the fragment of 275bp after gel electrophoresis. This study highlighted as the first case control study of leptospiral exposure among gardeners and also supported the association of leptospiral exposure with the occupation. Further studies required to confirm the lack of this association. All the gardeners are taught some awareness about the infection and its sources.

KEYWORDS: Leptospirosis, Gardeners, Serology, PCR, Tiruchirapalli.

INTRODUCTION

Leptospirosis is a spirochetal disease mainly affecting animals where humans get accidental infection that occurs worldwide, seems to be underestimated and common in temperate and tropical climates.[1] Most of the studies highlighted that this infection is an occupational hazard affects people who work outdoors and having direct contact with animals including farmers, slaughter house attenders, veterinarians, workers of fishing, mining and sewering, animal care takers, dairy workers, military persons etc.[2,3] Researchers are not much concentrating on other occupational groups like butchers, gardeners etc.

Leptospirosis in humans are defined as dead-end infection because human to human transmission is not recorded so far.[4,5,6] The clinical manifestations of leptospirosis are observed initially with fever, flu like symptoms, headache, arthralgia, myalgia and multiorgan involvement including liver, spleen, kidney leads to dysfunction to failure. In some special cases, cardiac and pulmonary involvement makes the condition very worse that leads to increase in mortality rate.[2,4,7]

While the first author visited the Government Park at Tiruchirapalli, the observations made by him initiate this work. In the garden, lot of pet animals mainly dogs are playing along with the children. Later domestic animals and rodents mainly cats and rats respectively also often visited the garden that may be infected with leptospires. Leptospirosis may shed by the urine of the infected animals that contaminating the garden soil, grass and plants, where gardeners are often exposed and contacting.

When interview with gardeners, they told that the supplied water received for watering the plants and trees in the garden are untreated or partially treated household and industrial waste water. While working, gardeners are often get mild to severe skin abrasions that may possible for the entry of leptospires inside the gardeners' body. Thus the gardeners is also having high risk group of getting leptospiral infections. According to the literature reviewed and local health data, the study in association of leptospirosis and gardener has not been recorded. Thus, an objective was determined to assess the presence of leptospires by cultural serological and molecular prints among gardeners.

MATERIALS AND METHODS

Study design and population

This study has been designed with age and gender matched case and control subjects. A total of 47 gardeners and equal number of control subjects (1:1) who are not having gardening as their occupation. The inclusion criteria of the subjects are

1. Gardeners who are working in this profession for more than 3 years
2. Age of 18 years and above
3. Both genders
4. Those who are willing to participate in this study

Those who are not willing to participate and not eligible as per the inclusion criteria were excluded.

Nature of the subjects included

After getting the institutional ethical clearance of this study, the socio-demographic data including age, gender, birthplace and education; occupational data including duration of the work, contact with sewage, soil and water, eating in working place, water used drinking and washing hands, history of mild to severe abrasions, usage of personal protective equipments (PPEs); clinical data including fever in the last 6 months with headache and conjunctival suffusion; Behavioural data including animal contacts, handling dead animals in the garden, consumption of meat and its products, degree of meat cooking, travelling etc. A separate questionnaire was prepared with the above said all details and was assessed.

Blood culturing

After getting written consent from the subjects, 5ml of blood sample was collected in the garden itself with proper aseptic precautions and 0.5ml of blood was inoculated in the McCartney bottles which is incorporated with EMJH semisolid medium, sealed properly and transported to laboratory and incubated at room temperature for one to six weeks. Every week it was sub-cultured and presence of Dinger's ring formation was observed. The tubes that are having Dinger's ring are microscopically determined for the presence of leptospires. Further, all the cultures that satisfy the criteria of positive both to formation of Dinger's ring and dark field microscopy were sent to national reference centre for serovar level determination.

Serology

The serum samples of all the gardeners were subjected to serological studies including genus and serovar specific Enzyme linked immunosorbent assay (ELISA) and Microscopic agglutination test (MAT) respectively. In ELISA, an absorbance reading of ≥ 0.5 optical density units was used as a cut off for positivity. All tests were performed according to the instructions of the manufacturer, positive and negative controls in each run. Further all the sera were subjected to the MAT procedure against battery of live leptospiral antigens including Australis, Autumnalis, Bankinang, Canicola, Grippotyphosa, Hardjo, Hebdomadis, Icterhaemorrhagiae, Javanica, Pyrogens, Patoc and Shermani obtained from National Leptospirosis Reference centre, RMRC, Andaman and Nicobar Islands (Table 1). Results were considered when 50% or more agglutination of leptospires at the titre of 1:80 and above. A titre of equal to or above 1:160 and 1:80 are considered as cutoff for endemic and non-endemic study areas.[8]

Table 1: Battery of live leptospiral strains used for MAT.

Serogroup	Serovar	Strain
Australis	Australis	Ballico
Autumnalis	Bangkinang	Bangkinang I
Canicola	Canicola	H. Uterecht IV
Grippotyphosa	Grippotyphosa	Moskva V
Hebdomadis	Hebdomadis	Hebdomadis
Icterohaemorrhagiae	Icterohaemorrhagiae	RGA
Javanica	Poi	Poi
Pomona	Pomona	Pomona
Pyrogens	Robinsoni	Robinsoni
Semaranga	Patoc	Patoc I
Sejroe	Sejroe	M84
Sejroe	Hardjo	Hardjoprajtno

PCR analysis

Genomic DNA was extracted from the blood samples that are positive to serology (both ELISA and MAT). Further the concentration of the DNA was measured using quantitative spectrophotometry and the absorbance provides the estimate of DNA purity (OD of 1.8 to 2.0; below and above considered as protein and RNA). The integrity of the DNA was tested by 0.8% agarose gel electrophoresis followed by visualizing in ethidium bromide staining.[9]

The 16S rRNA gene of leptospires was amplified using specific primers and performed with mixture of blood sample, template DNA, forward and reverse primers, $MgCl_2$, PCR buffer and Taq polymerase A master cycler gradient was done with initial denaturation at 95°C for one minute, annealing at 58°C for one minute, extension at 72°C for one minutes and final extension at 72°C for 5 minutes.

The PCR product was analyzed by running the electrophoresis by using 1% agarose gel. DNA ladder of 1kb size was used as a molecular marker. The expected size of the amplicons was 275bp.

RESULTS

The majority of cases included in this study were male (39/47; 83%) and females were accounted as 8 (17%). The maximum cases were observed in the age group of 36 to 45 with 21 cases (44.7%) followed by 17 cases (36.2%). The detailed description about the gender and agewise distribution among the subjects are depicted in figure 1.

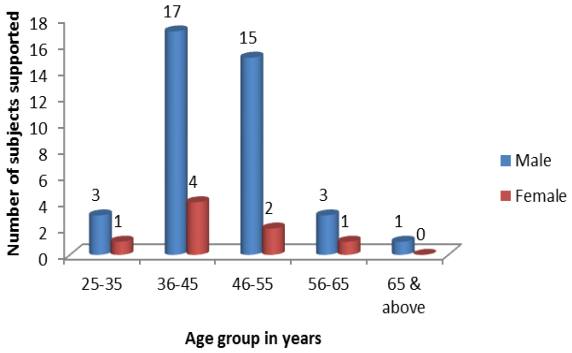

Figure 1: Age and gender wise analysis.

The detailed socio-demographic, occupational, clinical and behavioural data also suggested the risk of getting leptospirosis among subjects. The answers given in the questionnaire were statistically analyzed and impregnated in table 2.

Table 2: Analysis of socio-demographic, occupational, clinical and behavioural data.

Characteristics	Description	Number
Residential status	Rural Urban	37 (78.7) 10 (21.3)
Education	Illiterate Primary Secondary Graduate	04 (8.5) 24 (51.1) 17 (36.2) 02 (4.2)
Duration of work experiences	Below 5 years 5 to 10 years 11 – 20 years Above 20 years	07 (14.9) 28 (59.6) 10 (21.3) 02 (4.2)
Contact with soil, water and sewage	Yes No	47 (100) 0
Eating in working place	Yes No	43 (91.5) 04 (8.5)
Water using for drinking	Available in working place Take from home Others	12 (25.5) 31 (66) 04 (8.5)
Washing hands	Never Frequently Only after work get over	03 (6.4) 12 (25.5) 32 (68.1)
History of mild to severe abrasions	Mild Severe Deep wound Never	42 (89.4) 41 (87.2) 12 (25.5) 02 (4.2)
First Aid availability in work place	Yes No	11 (23.4) 36 (76.6)
Usage of PPEs Gloves	 Yes	 03 (6.4)

Masks	No Yes No	44 (93.6) 00 47 (100)
Fever in last 6 months	Yes No	37 (78.7) 10 (21.3)
Fever with additional syndrome	Yes No	35 (74.5) 12 (25.5)
Animal contact	Yes No	34 (72.3) 13 (27.6)
Handling dead animals	Yes with gloves Yes without gloves Never touch	02 (4.2) 44 (93.7) 01 (2.1)
Consumption of meat & its products	Never Frequent Rare	03 (6.4) 12 (25.5) 32 (68.1)
Degree of meat cooking	Complete Partial	42 (89.4) 05 (10.6)

[Figure in parenthesis denoted percentages]

Blood culture and Dinger's ring

After appropriate incubation, the EMJH semisolid culture media tubes were observed for the presence of Dinger's ring (marker of leptospiral growth). Out of 94 (47 gardeners and 47 control) blood samples inoculated, 4 gardeners' samples (8.5%) supported the growth of leptospires in both Dinger's ring formation and positive to screening under dark field microscopy (Figure 2). None of the control samples have the leptospiral culture prints. Out of 4 positive samples, the serovar Grippotyphosa predominates with 2 samples followed by Icterohaemorrhagiae and Javanica among one samples each.

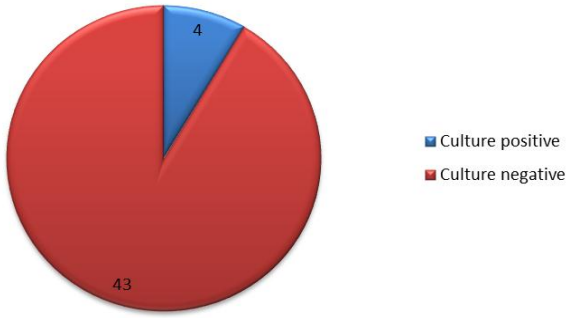

Figure 2: Analysis of blood culture among subjects included.

Serology

Out of 47 gardeners' serum samples, 34 showed positive to genus specific ELISA with the maximum and minimum OD units of 1.3 and 0.6 respectively. In the case of serovar specific MAT, 26 samples considered as positive with minimum and maximum titre value of 1:80 and 1:1280, where leptospiral serovar Grippotyphosa dominated with 13 samples followed by Icterohaemorrhagiae and Javanica with 6 and 2 samples respectively. Further, 5 samples were supported by poly-serovar determination with the combinations of 2 to 4 serovars (Table 3). Among 47 control samples, Comparative seroprevalence of leptospirosis among gardeners by using ELISA (72.3%) and MAT (55.3%) is impregnated in table 4. Out of 47 control samples, 12 and 4 samples supported positive to ELISA and MAT respectively.

Table 3: Serovars involved in 26 MAT positive cases.

Serovar	Number of positive cases (n=26)	Highest titre values
Mono-serovar determination (n=21; 80.8)		
Grippotyphosa	13 (61.9)	1:640
Icterohaemorrhagiae	6 (28.6)	1:1280
Javanica	2 (9.5)	1:320
Poly-serovar determination (n=5; 19.2)		
Grippotyhosa + Australis	1 (20)	1:640
Grippotyphosa + Australis + Icterohaemorrhagiae	2 (40)	1:320
Australis + Javanica + Canicola + Autumnalis	2 (40)	1:160

[Figure in parenthesis denoted percentages]

Table 4: Seroprevalence comparison of ELISA and MAT.

Samples and Percentages	ELISA		MAT	
	Positive	Negative	Positive	Negative
Serum samples (n=47)	34	13	26	21
Percentage	72.3	27.7	55.3	44.7
Control samples (n=47)	12	35	4	43
Percentage	25.5	74.5	8.5	91.5

PCR analysis

Out of 30 MAT positive samples (26 gardeners and 4 controls), 12 samples were found to have DNA of leptospires. All the PCR positive cases are gardeners and

none are from control samples. Primers designed with 275 bp fragment were visualized by agarose gel electrophoresis (Figure 3). All the 12 samples are supported well with positive control of the PCR kit.

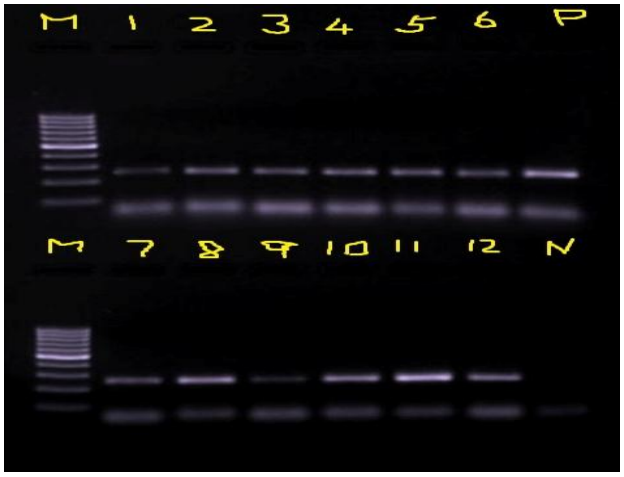

Figure 3: Agarose gel electrophoresis of blood PCR.

[M – Marker; 1 – 12 – Blood samples; P – Positive control; N – Negative control]

The complete description of the results of culture, serology and molecular analysis of 12 PCR confirmed samples were compared, thereby Grippotyphosa dominated with 8 (66.6%) isolates followed by Icterohaemorrhagiae and Javanica with 3 (25%) and 1 (8.4) samples respectively (Table 5).

Table 5: Results of serological and molecular analysis of 12 PCR confirmed cases.

Leptospiral isolate	Serogroup	Serovar	Genomic species	Strain
PL04	Grippotyphosa	Grippotyphosa	*L. kirschneri*	Moskva V
PL06	Icterohaemorrhagiae	Icterohaemorrhagiae	*L. interrogans*	RGA
PL07	Grippotyphosa	Grippotyphosa	*L. kirschneri*	Moskva V
PL09	Javanica	Poi	*L. borgpetersenii*	Poi
PL12	Grippotyphosa	Grippotyphosa	*L. kirschneri*	Moskva V
PL14	Icterohaemorrhagiae	Icterohaemorrhagiae	*L. interrogans*	RGA
PL16	Icterohaemorrhagiae	Icterohaemorrhagiae	*L. interrogans*	RGA
PL17	Grippotyphosa	Grippotyphosa	*L. kirschneri*	Moskva V
PL22	Grippotyphosa	Grippotyphosa	*L. kirschneri*	Moskva V
PL34	Grippotyphosa	Grippotyphosa	*L. kirschneri*	Moskva V
PL35	Grippotyphosa	Grippotyphosa	*L. kirschneri*	Moskva V
PL42	Grippotyphosa	Grippotyphosa	*L. kirschneri*	Moskva V

DISCUSSION

The *Leptospira* strains isolated from gardeners were culturally, serologically and genetically identified using culture in EMJH semisolid medium, ELISA, MAT and RT-PCR methods. Until now, no special investigations of leptospiral isolates from gardeners have been carried out in this study area. These isolates have been caused various diseases in both humans and domestic animals. The humidity of the study region is moderate and the land is slightly alkaline, that favour the survival of leptospires in the environment. Additionally the rodents and domestic animals in the garden region are the usual reservoirs of leptospires, creating natural foci of leptospirosis, with conditions that are highly favourable for infection both in animals and humans.[3,10,11]

The diagnosis of leptospirosis is often unconfirmed, because of a lack of clinical suspicion, inappropriate sample collection and non-availability of testing facilities.[12] Leptospires in blood usually found within the first week of illness, that supported our study of maximum duration of pyrexia that was culture positive for leptospires in their blood samples was 5 days.[13,14,15]

Based on this literature review, a follow up was made for the 4 positive culture gardeners. They are not attended their work for three days due to fever, further they are asked to visit hospital. We submitted the culture and serological report to the clinician for specific therapy. Then the physician administered doxycycline antimicrobial therapy for one week. After a week, dark field microscopy and serology showed negative. The predominant serovar of *Leptospira* was Grippotyphosa followed by Icterohaemorrhagiae. The longest duration for keeping the culture tubes was 84 days, thus maintenance of the culture tubes for 3 months is mandatory.[2,6,15]

Serological determination of IgM are more sensitive than MAT and provide positivity in the acute phase of the infection.[12] Since MAT was performed with single serum samples, we cannot confirm if the presence of anti-*Leptospira* antibodies corresponds to past or recent

infections, but the finding of seropositive gardeners without clinical evidence of disease arise the question regarding the potential risk of how they get the infection either by zoonotic transmission of the bacterium or exposing to the urinary shedding of leptospires by the animals with renal disease.[16,17] Therefore, is important to determine the *Leptospira* as a source of infection and environmental contamination with the bacterium with bacteriological or molecular diagnostic tests of urine.[16,17,18]

PCR based genomic analysis were highly utilized for the characterization of leptospires and also to analyse the genomic make ups. The typing of the PCR based assay are divided into two major groups including PCR for characterization – use of repetitive elements,[19] insertion elements,[20] restriction fragment length polymorphisms (RFLPs) as amplification targets,[21] arbitrarily primed PCR[22] and randomly amplified polymorphic DNA (RAPD).[23,24]

CONCLUSION

The clinical features and routine laboratory findings are not specific, a high index of suspicion make the diagnosis very easy and earliest. The etiological agent of leptospirosis may be cultured in EMJH semisolid medium but the serological testing is more useful and immediate. Among them, MAT is considered as gold standard, but expertise and handling of live leptospiral cultures are less available in clinical laboratories. Thus early diagnosis and appropriate therapy makes this infection to be easily eradicated from the individuals who are infecting with leptospires.

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