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# DETECTION, ISOLATION AND MOLECULAR CHARACTERIZATION OF LEPTOSPIRA FROM SOIL AND WATER SAMPLES OF CENTRAL KERALA

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## ABSTRACT
Leptospirosis has been repeatedly reported from the State of Kerala since 1989 and is one of the commonest diseases among the 14 notifiable diseases in the district level communicable disease surveillance system. As there are no field studies on human leptospirosis in Kerala, we have proposed this sero-epidemiological study of leptospirosis in the Ernakulam district in Kerala to identify locally prevalent serogroups, and to understand local epidemiological features of the disease. In spring of 2015, 75 samples, which includes water samples (50), soil samples (25), were collected from the sewage and paddy field of central part of Ernakulam district located in Kerala. Some parts of agricultural land, which had stagnant waters such as: streams, water channels, were focused to collect sample. Leptospira spp. is to be isolated using culture method and then identified by phenotyping tests. Identification of Leptospiras is possible through culture in the Leptospira Enrichment medium. PCR technique is used to confirm the Leptospira species in all the collected samples. This method is able to differentiate between the saprophytic and pathogenic Leptispiras. The results revealed the ubiquity of Leptospira in the environment and highlight the need to develop formal approaches for systematic monitoring. This study concluded that the collected water and soil samples have only limited significance in the distribution of Leptospira. This study also provides important information regarding the identification of the pathogenic serovars and saprophytic serovars.

**KEYWORDS:** Leptospirosis, sero-epidemiological, agricultural land, saprophytic, pathogenic.

## 1. INTRODUCTION
Leptospirosis is a disease caused different strains of bacteria of the genus Leptospira which are related with animals.[1] It is more widespread in the tropical countries. Of all the diversity that causes disease, *Leptospira icterohaemorrhagiae* is the most serious type. If not treated properly, it could lead to severe complications.[2-4] Leptospirosis is a disease of animals that can spread to humans. Rats are common carriers of leptospirosis. Soil contaminated with urine of infected animals can also transmit the disease to humans. All people working as Sewage workers, agricultural workers, butchers, meat inspectors or workers in contact with contaminated waters are generally prone to this disease. Leptospirosis can also be spreaded due to contact with urine, blood or tissues of infected persons.[5,6]

Leptospirosis is a serious emerging disease in Kerala but also there is no technical publication on this.[1-3] Leptospira interrogans is infective as long as it is moist and can remain outside the host in water or moist soil for six months or more. The primary hosts are rodents especially rats.[7,8] Common rats in Kerala are *Rattus rattus*, *Rattus norvegicus*, *Mus musculus*, *Bandicoota indica*, *B. malabaricus*, *B. bengaliensis*, *Nosokia bengaliensis* and *Tetera sp*. *Bandicoots* usually visit drains, sewage canals and roadside water collections after rains and are responsible for urban infections.[9-11] *Mus musculus* are generally found in residential area's store rooms or warehouse infection through edible items. *Rattus rattus* is peridomestic and domestic and has great epidemiological bearing.

Kerala is in the wet tropical area with annual rainfall of above 3000 mm and optimum temperature from 21 to 35°C throughout the year. April and May experience the temperature up to 36°C to 38°C.[12] This mixed type of climate encourage group II epidemiology prototype among residential area, where many hosts act as carriers with multiple serogroups in an limited area and cause disease throughout the year.[13] Industrialized area in contrast has group l epidemiology prototype, where exposure is occupational /recreational, the animal act as reservoir or cause of infecting. Here serogroups are

limited except at the time of any natural calamities. Often every year April to October Kerala gets heavy rains and intermittent floods. Irregular flooding of geographically lower areas show the way to repeated wash out of the forests and farmlands soil, healthier and nonhealthy waste like rodent burrows, wastes there into all water resource including streams, ponds, canal and rivers where microbes like *leptospires* can survive for days. This leads to not only monsoon outbreaks but also periodic disease cases throughout the year.

Many reports from Kerala have reported the Epidemics of *leptospirosis* during monsoon months.[14-16] Ernakulam district is situated in central Kerala lies between the Western Ghats and the coastal strip of Arabian Sea. Ernakulam district have many streams, ponds and irrigation canals which flowing together during monsoon flooding. These canals are nourishing during dry months by the irrigation project namely the Periyar Valley Irrigation Project. Uninterrupted cultivation of food crops like rice and pineapple provide rodents with food enable them to survive.[17]

## 2. MATERIALS AND METHODS

### 2.1. Sample Collection

A total of 75 samples were collected from the rice farms of central part of Ernakulam district located in Kerala, September to December 2015. The collected samples were grouped as 50 water samples and 25 soil sample from stagnant sewage water and paddy field of selected area. Then the samples were transferred to laboratory.

### 2.2. Preparation of Samples to Culture[18]

Each one of the water samples into the tube was mixed completely and transferred to 4 sterile short test tubes. Then, the tubes were centrifuged at 4000 rpm for 10 minutes. Surface contents of the tubes were extracted and the supernatant was poured away through a dish containing the savlon. Then, we took out the surface contents of few tubes by sterile syringe and passed them through a 0.45 µm filter and transferred them to the sterile 1.5 ml Micro tube. Again we took out the contents from 1.5 ml microtube by another sterile syringe and inoculated against the liquid Leptospira Enrichment medium. The soil sample was first transformed into suspension with aid of phosphate buffer solution and transferred into the Leptospira Enrichment medium after passage from the mentioned filters and incubated in 30°C within 15 days. After incubation time, contents of liquid Leptospira Enrichment medium were sampled with the aid of sterile loop and inoculated in to the solid Leptospira Enrichment medium. Again, plates were placed in the 30°C incubator for 15 days.

### 2.3. Identification of Pathogenic Species of *Leptospira* with Beta Hemolysis of Blood Agar

The production of hemolysin by some strains of pathogenic *Leptospira* sp., were also studied using blood agar. After streaking of culture, the plates were sealed and incubated aerobically at 30°C until colonies became visible (2 to 3 weeks). A blood agar overlay method was used due to the long incubation period required for obtaining colonies. After the overlay solidified, plates were incubated at 30°C until small, clear zones of hemolysis appeared surrounding individual colonies (24 to 48 h). The plates were transferred to the cold (4°C) for 12 h and then reexamined for zones of hemolysis. This procedure will be valuable to investigators interested in isolation of nonhemolytic mutants, in correlation of *in vitro* hemolysin production with virulence and in isolation of hemolytic and nonhemolytic serovars from mixed *leptospiral* infections.

### 2.4. Identification of Pathogenic Species of *Leptospira* with PCR Technique

Genomic DNA was extracted from the selected clinical isolate utilizing bacterial Genomic DNA Isolation Kit; integrity of the isolated DNA was analysed on 0.8% agarose gel. 16s rDNA specific primer set [18S 5'-AGATTAGAGCAATTTAAAAA -3' and 18S 5'-AAGTAAATAGTTTGTGGTAT -3'] was synthesized; PCR amplification of 16S rDNA gene was performed in a total volume of 100 µL reaction mixture containing 10 µL of 10X *Taq* DNA Polymerase Assay Buffer, 4µl dNTPs (10mM), 1 µl *Taq* DNA polymerase (3 U/ µl), 0.4 µM of each primer and 1 ng template DNA. The amplification reaction was performed with a thermal cycler (ABI2720) and the PCR amplicons were resolved by electrophoresis in 1% (w/v) agarose gel to confirm the expected size (1.5 kb) of the product. 10 µl Sequencing reactions were set with Big Dye Terminator Ready Reaction Mix 4µl, Template (100ng/ul) 1µl, Primer (10pmol/λ) 2µl and Milli Q Water 3µl. The PCR product with 16s rDNA gene was sequenced using ABI 3500 Genetic Analyzer containing POP_7 polymer and 50 cm capillary array and BDTv3-KB-Denovo_v 5.2 protocol were examined, whereas data were analyzed by software Seq Scape_v 5.2 and reaction were examined in Applied biosystem micro Amp optical 96-well reaction plate. The sequence data was analyzed by similarity search using the BLAST tool available at the website of the NCBI.

#### 2.4.1. The Thermal Cycling Conditions

| | | |
|---|---|---|
| Initial denaturation | : | 96 °C for 1 min |
| Denaturation | : | 96°C for 10 sec |
| Annealing | : | 55 for 30 sec |
| Extension | : | 72 for 1 min 30 sec |
| Final extension | : | 72 for 15 min |
| Number of Cycles | : | 35 |
| Hybridization | : | 50 °C for 5 sec |
| Elongation | : | 60 °C for 4 min |

## 3. RESULTS AND DISCUSSION

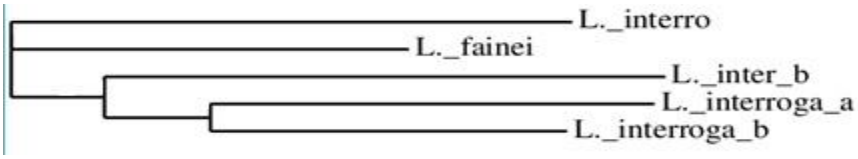
Out of 75 samples processed total of 12 colonies of *leptospira* sp., were isolated. Out of 50 water samples, 39 samples showed positive results towards presence of *Leptospira* sp. From 25 soil samples, 19 samples showed positive results (Table 1). From the positive sample, Four different types of colonies were isolated for soil sample

and 8 different types of colonies were isolated from water sample were isolated from the plates of leptospira medium. Out of 12 isolates, 1 isolate were found to possess beta haemolysis and the remaining 11 culture possessed gamma haemolysis. All the beta haemolysis isolated colonies were processed for 16s rDNA identification and gene sequencing. The microbe was found to be most similar to *Leptospira interrogans* strain 16s ribosomal DNA gene, partial sequence (Figure 1). Phylogenetic Tree Builder uses sequences aligned with System Software aligner. A distance matrix is generated using the Jukes-Cantor corrected distance model. When generating the distance matrix, only alignment model positions are used, alignment inserts are ignored and the minimum comparable position is 200. The tree is created using Weighbor with alphabet size 4 and length size 1000.

**3.1.Aligned Data (500bp)**

TCTAATCTCGTTAAATTTTTGCAGTAGTTCCACA
TTTAAAGAATCGATCTTAAAGTTTAGATTCCAAT
TTTTTTTCAGAATCATGGGTTTGACCTGGTAACA
TAAAAGTGGACATTTTTTTCGCCAAAAATTGAG
GTAAAAGATAAAGGTGAGAATGAAAAGAAGTA
ACTATCAATACGTCTGTTTCAAAACTTATAATAT
AACATTTCTTTTACAAAGGCCAACAATTTTGAAT
TAGGTGCAATTTGTAAGGTCTTGATGAATTGTTT
TATTTAAAAATTATGAACCGAATTTTCAAAATTT
AGGATTTGTTTTAAAATTCATAATGCTCTATTAC
ACAAGGATCAATAAAATTCCTAAACTTAAATTT
GACTTTTATAATCATTTTCAATAATTCTAAAGCT
CGAAGTACTTTTAAATAAATCACCTTTTATATAG
CAGTATTTAATTTTATCAGCACTTAGAATTTCTA
AACCTGAGTTCATTTATCAAACACCATA

**3.2.Phylogenetic tree**




**Phylogenetic tree analysis**

**3.3. PCR Amplification**

Leptospirosis is the most commonly reported notifiable communicable disease in Kerala. Since 1989 several cases of *leptospirosis* have been diagnosed among the local people here.[15] The survey reports made in local hospitals of Ernakulam district have reported 155 cases of human leptospirosis in 1993 and 888 cases in 1994. Monthly reports of the District Medical Officer, Ernakulam, had reported 20 confirmed leptospiral deaths in his district alone in 2006.[16,17] This study also evident that, in which cases of death as a result of Leptospirosis are observed obviously locate in the mild and moist region from viewpoint of geographical and climatic region and there are plentiful rainfalls in most seasons of year in the mentioned provinces, it can be said that it has provided an appropriate habitat for survival of Leptospiras because rainfalls of the seasonal rains has led to the soil shedding phenomenon.[18] There might be many more unreported and unconfirmed cases too. Most of the patient history of subjects says that the major occupations of these families are agriculture and animal rearing. Although the prevalence of Leptospira is low, there is still a risk of infection to those who are involved in outdoor activities and paddy fields. Control and preventive measures are therefore important in tackling preventable diseases related to pathogenic Leptospira. Hence, the villagers must take preventive measures such as wearing protective clothing and rubber boots to minimise contact with a contaminated environment as suggested by Koay et al.[19]

Depending on the level of exposure of organism, Leptospiral infection may be subclinical or clinical depending. *Leptospira interrogans*, causes human leptospirosis, consists of over 24 serogroups. This wide range of Serogroups and their member serovars is primary cause the difference in leptospirosis from region to region. This factor contributes a way for endemicity for leptospirosis. Since leptospirosis is a Zeonosis disease, it is suggested that the required actions are to be taken in order to control the rodents and drain the water of streams and swamps. Also, because of occurrence of disease in the region during the agriculture seasons, it is suggested that exposed to risk individuals are placed under the profilaxy with antibiotic or vaccination so that occurrence of disease is to be prevented.

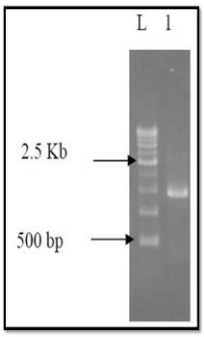



**Figure 1: PCR amplification of DNA fragments from bacterial sample. The size of PCR amplified product is ~500bp (Lane1 :Sample-1, Lane L: ladder).**

**Table 1: Results of isolates obtained from the collected samples.**

| Name of the Samples | No. of samples collected | Positive samples | No of isolates obtained | % acheived |
|---|---|---|---|---|
| Water | 50 | 39 | 8 | 78 |
| Soil | 25 | 19 | 4 | 76 |

## 4. CONCLUSIONS

In Kerala, there are an increasing number of reported leptospirosis cases which led to mortality. The infection is usually spread through the urine of infected animals and may contaminate the environmental soil and water. This study was conducted to conclude the frequency of leptospira species in selected environmental soil and water samples. In conclusion, this study suggests important information concerning the infection levels and identification of the pathogenic serovars and saprophytic serovars. The results obtained suggests that water and soil have only limited significance in the persistence and dissemination of Leptospira in central part of Kerala. Increased awareness, continuous monitoring and efficient preventive measures should be taken by concerned authorities to control the occurrence of leptospirosis.

### Author Contributions

The authors have no competing interests with the work presented in this manuscript.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## REFERENCES


1. John TJ, Rajappan K, Arjunan KK. Communicable diseases monitored by Disease Surveillance in Kottayam district, Kerala state, India. Indian J Med Res, 2004; 120: 86-93.
2. Speelman P. Leptospirosis. 16th ed., In: Kasper D L, Braunwald E, Hauser S, Lougo D, Jameson L, Fauci AS, (eds) Harrison's principles of internal medicine, McGraw-Hill Professional, USA, 2005; 988-991.
3. W. H. O. International Leptospirosis. 2003; 23-24.
4. Faine S, Adler B, Bolin C, Perolat P. Leptospira and leptospirosis. 2nd ed., Victoria: Medisci press, Melbourne, Australia, 1999; 138-139p.
5. Kariv R, Klempfner R, Barnea A, Sidi Y, Schwartz E. The changing epidemiology of leptospirosis. Emerg Infect Dis, 2001; 7(6): 990-992.
6. Pappachen MJ, Sheela M, Aravindan KP. Relation of rainfall pattern and epidemic leptospirosis. J Epidemiol Community Health, 2004; 58(12): 1054-1055.
7. Bal AE, Gravekamp C, Hartskeerl RA, De Meza-Brewster J, Korver H, et al. Detection of leptospires in urine by PCR for early diagnosis of leptospirosis. J clin Microbiol, 1994; 32(8): 1894-1898.
8. Ridzlan FR, Bahaman AR, Khairani-Bejo S, Mutalib AR. Tropical biomedicine. detection of pathogenic Leptospira from selected environment in Kelantan and Terengganu, Malaysia. 2010, Trop Biomed; 27(3): 632-638.
9. Laurichesse H, Gourdon F, Smits HL, Abdoe TH, Estavoyer JM, et al. Safety and immunogenicity of subcutaneous or intramuscular administration of a monovalent inactivated vaccine against *Leptospira interrogans* serogroup Icterohaemorrhagiae in healthy volunteers. Clin Microbiol Infect, 2007; 13(4): 395-403.
10. Ananthanarayan R and Panicker CKJ. Textbook of Microbiology. 8th ed. Orient Longman, Hyderabad, 2009; 389-394.
11. Heymann DL. Control of Communicable Diseases (20th edition), Washington: APHA press, USA, 1980; 433 p.
12. Levett PN. Leptospirosis. Clinical Microbiology Review, 2001; 14(2): 296-326.
13. Cohen ML. Resurgent and Emerging Diseases in a changing world. Br Med Bull, 1990; 54(3): 523-532.
14. NICD. Communicable Diseases CD, Alert Newsletter, 1997; Vol 14.
15. W.H.O. The Veterinary Contribution to Public Health, Tech. 1975, Report Series No.573.
16. W.H.O. Bacterial and Viral Zoonosis, Tech. 1982, Report Series No.682.
17. W.H.O. Human Leptospirosis Guidance for Diagnosis, Surveillance and Control, 2003; 92: 4.
18. Yasouri SR, Moghadam RG and Ghane M. Identification of Pathogenic and Saprophytic Leptospira spp from the Rice Fields of Tonekabon Township Using PCR Technique. Adv Stud Biol, 2013; 5(10): 437–445.
19. Koay TK, Nirmal S, Noitie L and Tan E. An epidemiological investigation of an outbreak of leptospirosis associated with swimming, Beaufort, Sabah. Med J Malaysia, 2004; 59(4): 455–459.