

Original Research Article

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Occurrence of *Leptospira* spp. in Paddy Field Water Samples in and around Thrissur District of Kerala, India

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ABSTRACT

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Leptospirosis is an acute zoonotic infection occurring worldwide caused by a pathogenic spirochete belonging to the genus *Leptospira*. It is estimated that 1.03 million cases of leptospirosis occur every year worldwide. Leptospirosis has become an isolated public health problem in some of the waterlogged areas of Thrissur. The changing rainfall pattern in the Western Ghats has reportedly affected normal climatic condition of Kerala. According to reports of Directorate of Health Services 2017, Kerala, from January 1st to June 30th, a total of 717 confirmed cases of Leptospirosis were reported with 1.25 per cent mortality. In the present study, a total of 85 paddy field water samples were collected in both monsoon and summer seasons. The samples were directly observed under dark field microscope after centrifuging at high speed but none of the samples showed the presence of viable organisms. Further, the identification of organisms were done by polymerases chain reaction by targeting genus specific *16Sr RNA* and virulence genes *i.e. lipL 21, lipL 41 and lipL 32* respectively. The presence of *16Sr RNA* was observed in 20 per cent of the samples and three samples showed presence of all genes except *lipL 32*. The study reveals the role of environment in maintenance of the infection in the area under study.

Introduction

Leptospirosis is an infectious zoonotic disease affecting wide population, currently there are 20 recognized species of with 300 serovars were identified (Mayer-Scholl *et al.*, 2014). The survival and reproduction of these

bacteria is particularly occurred in renal tubules of maintenance hosts (Lim *et al.*, 2011). It is estimated that 1.03 million cases of leptospirosis occur every year worldwide (Costa *et al.*, 2015). These bacteria can also exist in the natural environment such as water and soil contaminated with urine of infected

hosts (Rawlins *et al.*, 2014). Humans usually get the infection by coming in direct contact with the infected animal urine via mucous membrane and wound on the body surfaces or indirect contact with contaminated soil, water, and food. According to reports of Directorate of Health Services 2017, Kerala, from January 2016 to August 2017 a total of 2599 confirmed cases of *Leptospira* were reported with 47 deaths. The development of different molecular technique has helped to identify *Leptospira* from water samples. The PCR technique helped to identify the specific organism with rapid amplification of pathogenic *Leptospira* in paddy field water samples.

The outer membrane proteins of pathogenic leptospire consist of *lipl 32*, *lipl 41* and *lipl 21* genes which can be identified by PCR assay. The commonly used technique for confirming the disease is by cultural isolation but the procedure is time consuming. The endemicity of the disease in Kerala with highest number of cases occurring due to climate change augments the need to study the role of environment in maintaining the organism in paddy field water sources.

Materials and Methods

Collection of river water samples

For the study, paddy field water samples from different areas of Thrissur were collected. The sampling was divided into two seasons of the year *i.e.* monsoon (June to September 2016) and summer (February to May 2017). A total of 85 paddy field water samples were collected in both monsoon (45) and summer (40) seasons. Paddy field water samples (100ml each) were collected in sterilized bottle (Ridzaln *et al.*, 2010). The pH and temperature of these samples were recorded at the time of collection. For finding the dissolved oxygen of the samples, 50 ml of

sample was separately taken in amber coloured bottle. All the samples were aseptically collected and transferred to laboratory under normal temperature *i.e.* 28-30°C. The processing of the samples was done on the day of collection.

Processing of the samples

The collected samples were processed in the Biosafety level - II laboratory facility available in the Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Mannuthy.

All the paddy field water samples were centrifuged at high speed and a drop was examined under dark field microscope (100X). All the water samples were subjected to identification of *Leptospira* by the modified procedure given by Ridzlan *et al.*, (2010). The samples were centrifuged at 14000×g for 25min and isolation of DNA was carried out by using nucleospin water DNA isolation kit.

Molecular detection of *Leptospira* spp.

Four sets of primers were used *i.e.* *16Sr RNA*, *lipl 32*, *lipl 21* and *lipl 41* (Accession code:- AY688410) for amplification as shown in table 1 as per the procedure described by Tansuphasiri *et al.*, (2006). Different cyclic conditions used for different primers and standardization of PCR for *lipl 41* was carried out as shown in the Table 3.

The samples were analyzed for its pH, temperature, salinity, conductivity, turbidity and dissolved oxygen (Multiparameter, Singapore). The cyclic conditions for PCR targeting *16Sr RNA* and *lipl 32* genes were carried out by the procedure given by Tansuphasiri *et al.*, (2006) whereas for *lipl 21* gene the modified procedure given by Vishak, 2015 with annealing temperature of 54°C for 45 sec. with 36 cycles were used.

Results and Discussion

The agarose gel image for representative PCR amplicons in this study is shown in Fig 1. The genus specific *16Sr RNA* was found with an amplicon size of 430 bp whereas virulence gene *i.e. lipl 21* with an amplicon size 507 bp. The results revealed that 16.47 per cent (14/85) of the river water samples were positive for pathogenic and 3.5 per cent (3/85) were positive for saprophytic *Leptospira* in both the seasons as shown in table 2. The virulence gene *lipl 21* was detected in three samples in summer season. However, *lipl 32* and *lipl 41* genes were not detected in any of the samples analyzed.

The pH, temperature, salinity, conductivity, turbidity and dissolved oxygen of the samples was found to be in a range from 6.5 to 6.9, 29-30 °C, 0.04- 0.07 psu, 90-120 µs/sec, 60-70 NTU and 5.5-6.5 mg/L respectively. Statistical analysis of physicochemical parameters of paddy field water samples in both the season showed no significant difference in turbidity but values of all the other parameters in both the seasons differs significantly.

The correlation between the observed range of physicochemical parameters detected in the present study and the presence of *Leptospira* in paddy field water in both the seasons were assessed. It was found that the pH and dissolved oxygen of water samples in both the season showed positive correlation in the observed range with the presence of organism.

In the present study, 26.67 and five per cent of saprophytic leptospire were identified in monsoon and summer season respectively,

whereas none of the samples collected in monsoon showed positive results for pathogenic bacteria. In summer season the occurrence of pathogenic *Leptospira* was found to be only 7.5 per cent. Ryu *et al.*, (1966) in their study revealed that *Leptospira* were able to maintain their viability at a range of temperature 0 to 30°C and at a pH value ranged between 7 to 8 in paddy field water, which may be the reason attributed to lower positivity of pathogenic *Leptospira* in paddy field samples collected in present study.

According to Pui *et al.*, (2017) cultivated paddy field with more rodent inhabitation was found to be contaminated with an occurrence of 6.5 per cent, this statement supports the finding of present study, as 7.5 per cent occurrence of pathogenic organism was seen in the water samples collected from cultivated paddy field from Maddakathara where large number of rodent population were noticed.

An alkaline pH, low levels of heterotrophic bacteria and high dissolved oxygen favoured survival of bacteria in paddy field environment but in this study the pH was in a range of 6.5- 6.9 which might have reduced the occurrence of organisms in the samples.

The occurrence of *Leptospira* in the present investigation reinstalls the role of transmission dynamics of *Leptospira* in Thrissur. Hence, systematic monitoring of environmental contamination is required to formulate prevention and control of strategies against the disease in the region. The study also revealed that molecular methods were able to provide quicker qualitative results rather than culture technique.

Table.1 Primers used for identification of leptospires

Primer	Sequence	Size (bp)
<i>16Sr RNA F</i>	5'GAACTGAGACACGGTCCAT3'	430 bp
<i>16Sr RNA R</i>	5'GCCTCAGCGTCAGTTTTAGG3'	
<i>lip132 F</i>	5'ATCTCCGTTGCACTCTTTGC3'	474 bp
<i>lip132 R</i>	5'ACCATCATCATCATCGTCCA3'	
<i>lip1 21 F</i>	5'CGGGTCGACTCCAGTACTGACACAGGACAAA3'	507 bp
<i>lip1 21 R</i>	5'CGGCTGCAGTTATTGTTTGGAAACCTCTTGA3'	
<i>lip1 41 F</i>	5'CTGGTTTGAAAGTGGCAGGT3'	243 bp
<i>lip1 41 R</i>	5'CCAAACCTTGTCCGAAAGAA3'	

Table.2 Number of river positive samples

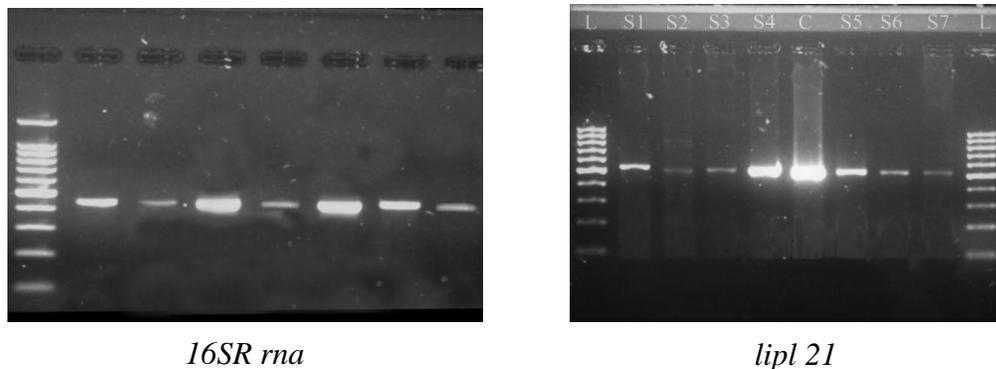
Sl no.	Source	No. of samples positive for <i>Leptospira</i> spp.	
		Monsoon	Summer
1.	Paddy field water	23.1 ^a	13.5 ^a

^{a-b} Figures in a rows bearing same superscripts do not differ significantly

Table.3 Temperature and cyclic conditions for PCR *lip1 41* genes

Steps	Conditions	No. of cycles
Initial denaturation	94° C for 5 min.	35 cycles
Denaturation	95° C for 1 min.	
Annealing	60.2° C for 40 sec	
Extension	72° C for 1min	
Final extension	72° C for 10min	

Fig.1 PCR results



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