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Detection of *Leptospira* DNA in environmental water samples collected from the Gampaha District by qPCR.

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Abstract

Leptospirosis is a widely spread zoonotic disease in the world, caused by 11 pathogenic *Leptospira* belonging to the family Leptospiraceae. Sri Lanka, particularly the Gampaha District has been identified as an area with a high prevalence of leptospirosis. Wild and feral animals, livestock, and domestic pets are the main reservoirs. Sampling environmental water samples for the detection of Leptospira is used as a predictor of disease transmission. Therefore, the study aimed to detect the presence of Leptospira as evidence of environmental contamination.

In this study, we tested Leptospira DNA in water samples collected from the disease prevalent Gampaha district, using real-time polymerase chain reaction (PCR). The water samples were collected from 10 various locations in the Gampaha District. The processing was done using a pre-validated protocol for DNA extraction and the extracted DNA was then subjected to real-time PCR targeting specific genetic markers of Leptospira. No Leptospira DNA was detected from the tested samples, most probably due to the period that the study was conducted. The samples were collected in the month of April, which is a month the country reports a minimum number of cases.

Leptospirosis prevalence varies with geographical location, climate, and seasonality. Temperature, pH, and the presence of other microorganisms can all influence the survival and identification of leptospirosis bacteria in water samples. Therefore, further studies need to be done in a periodic manner to establish transmission dynamics in the Gampaha district.

Keywords: Leptospirosis, *Leptospira* DNA, water samples, DNA extraction real-time PCR

1. Introduction

Leptospirosis is a widely distributed zoonotic disease that affects humans and animals living in regions with humid, tropical, and subtropical climates.[1] It is a well-established infectious disease in Sri Lanka and is associated with high morbidity and mortality. The disease is caused by 11 pathogenic species of Leptospira belonging to the family Leptospiraceae. Leptospira have a helix-shaped body and an internal locomotor apparatus, the endo flagellum, which allows them to move freely even in the most viscous conditions (Figure 1). Wild and feral animals, livestock, and domestic pets are the main reservoirs that maintain disease in the environment. In Sri Lanka, minimum data is available on reservoir animals of leptospirosis.[2]

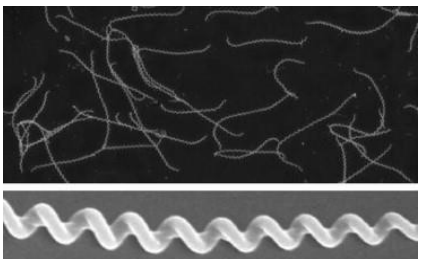

Figure 1: Electron micrographs of Leptospires.[3]

The disease leptospirosis is distributed throughout the country. In the previous two decades, the highest number of leptospirosis cases have been reported from the districts of Colombo, Kurunegala, Gampaha, Matale, and Kegalle.[4] The disease is endemic in some parts of the country and, in 2008, the country experienced its greatest outbreak of leptospirosis, with 7423 suspected cases and 204 deaths.[5] Leptospirosis is a well- known contributor to infectious disease epidemics caused by meteorological disasters in Sri Lanka.[6] Leptospirosis was first documented in Sri Lanka in 1953, among caught rats in Colombo. The country has experienced three major outbreaks in years 2003, 2008, and 2011 with significant death tolls. The 2008 epidemic was the worst ever reported in the country and the second- highest leptospirosis outbreak in the globe. Leptospirosis primarily affects agricultural populations such as paddy farmers and Chena cultivators in Sri Lanka and is linked to certain environmental conditions, mainly to monsoons.[7]

The infection manifests as mild to severe life-threatening diseases. The initial clinical presentation of leptospirosis is non-specific, and mimics other common infective and non-infective diseases in the country. The non-specific presentation challenges clinicians to diagnose the infection clinically. Therefore, knowing the transmission dynamics of the local geographical regions is essential to obtain exposure. The usual practice is to manage patients clinically and to obtain laboratory confirmation by serology retrospectively. Obtaining laboratory confirmation is a major challenge in tropical and subtropical regions due to limited laboratory facilities. However, the classic Weil's disease, which manifests as jaundice, renal failure, and bleeding, can be identified clinically but is associated with high morbidity and mortality.

There are two stages of leptospirosis. There are two phases: leptospiremic and immune. During the leptospiremic phase, you will have symptoms similar to the flu. They will begin a few days to weeks after you are first exposed to leptospira bacteria. Symptoms can persist for up to ten days. Once you enter the immunological phase, leptospira bacteria are present in your organs, particularly your kidneys. Urine tests will reveal the bacteria, and your body will develop immunity to them. During this phase, you may become ill with Weil's syndrome

The study of infectious diseases encompasses both the course of the disease in its host and how it spreads between hosts. Understanding illness transmission is critical for establishing effective interventions, protecting healthcare workers, and guiding a successful public health response.[8] Identifying the elements that contribute to disease spread is crucial for effective control and prevention actions.[9] Sampling the environment water samples for the detection of pathogens transmitted via contaminated water including leptospirosis is an important public health measure that allows to understand the transmission dynamics.

Travelers are more likely to contract leptospirosis if they visit flooded or recently flooded areas, swim, wade, kayak, or raft in possibly contaminated fresh water, such as lakes and rivers. Infection can spread by contact with infected animals, such as mice, cows, sheep, goats, pigs, horses, dogs, and wildlife, especially in metropolitan settings with poor sanitation. There is no vaccine licensed in the United States to prevent leptospirosis in humans.

Travelers should avoid touching fresh water or soil that may have been contaminated with animal urine. Avoid direct contact with materials potentially contaminated with animal urine, such as animal bedding. Refrain from wading, swimming, or submerging the head in floodwater or natural bodies of water including lakes, rivers, and swamps, particularly following periods of flooding or heavy rainfall. Minimize exposure to freshwater sources during and after such events. When contact with floodwater or freshwater is unavoidable, wear appropriate protective clothing, ensure all cuts and abrasions are securely covered with waterproof dressings, and consume only water that has been properly treated or boiled to

ensure safety.[10] The study aimed to detect the presence of *Leptospira* as evidence of environmental contamination.

2. Methodology

2.1 Sample collection. The water collections close to paddy fields, and human dwellings are well known to be contaminated with pathogenic and intermediate Leptospira. Ten replicates of water samples (50 ml) possibly contaminated with Leptospira were collected into sterile polypropylene centrifuge tubes. The collection sites were decided based on the published literature as source of transmission of Leptospirosis such as water retain in the paddy field, natural water collections in rat dwellings areas and marshy lands. The samples were transported in room temperature to the Molecular Medicine Unit, Faculty of Medicine, Ragama for detection of Leptospira DNA by a pretested and validated qPCR protocol.

2.2 DNA extraction from water samples. DNA from the collected water (200 µl) samples were extracted using the QIAamp viral RNA mini kit according to the manufactured instructions. All buffers (AVL, AW1, and AW2) were prepared according to the manufacturer's instructions. If precipitate had formed in the AVL buffer, it was dissolved by incubating at 56°C. Preprocessed water in 1XPBS (200 µl) was added to a 1.5 ml microcentrifuge tube. Then, 560 µl of prepared buffer AVL containing carrier RNA added to the sample and mixed by pulse vortexing for 15 seconds. Then the homogenate was incubated at room temperature (25°C) for 10 minutes. Thereafter, the sample was briefly centrifuged to remove drops from the inside of the lid. Following that 560 µl of absolute ethanol was added to the sample and mixed by pulse vortexing for 15 seconds. After mixing, the sample was briefly centrifuged to remove drops from the inside of the lid, and 630 µl of the homogenate was added to the QIAamp mini spin column (in a 2 ml collection tube) without wetting the rim. Subsequently, it was centrifuged at 8000 rpm for 1 minute, and the tube containing the filtrate was discarded. Then 500 µl of buffer AW1 was added to the column without wetting the rim and centrifuged at 8000 rpm for 1 minute. The filtrate was discarded, and buffer AW2 (500 µl) was added to the column and centrifuged at 13400 rpm for 4 minutes. The column was placed again in a new collection tube and centrifuged at the same speed for 1 minute to remove any residual binding buffers from the DNA. Then the column was placed in a new 1.5 ml microcentrifuge tube, 60 µl of buffer AVE was added to the column, and it was incubated at room temperature for 1 minute. Finally, the column was centrifuged at 8,000 rpm for 1 minute, and the filtrate (DNA) was stored at -20°C until it was analyzed by PCR. The same procedure followed for all ten samples to extract DNA.

2.3 Primer design. Pathogenic Leptospira specific primers were selected from the fragment of the *secY* gene homologous to the L. interrogans S10-spc-a locus (Genbank accession number AF115283). The primers amplify 202 bp fragment between the locus positions 15 744 and 15 946 of *secY* gene that is located within the S10-spc-α locus containing genes for ribosomal proteins and it encodes preproteintranslocase for Leptospira.[11]

2.4 Reagents and reaction conditions for real time PCR. To detect leptospirosis, real-time PCR was performed. Before preparing the master mix, calculations were made to determine the exact volumes of the components required to prepare 10 samples, The amplification process was carried out after initial denaturation at 950°C for 10 minutes. The thermal process of amplification for 40 cycles was carried out including steps of denaturation at 950°C for 15 seconds, annealing at 540°C for 30 seconds, extension at 720°C for 30 seconds and with an extended final incubation at 720°C for 8 minutes. After cooling 300°C for 1 minute, melting curve (Tm) analysis from 65- 940°C with readings every 0.50°C was performed according to the manufacturer's instructions. The cut-off for the analysis was set at Threshold Cycle (Ct) value 35 and the approximate total time taken to run the entire program was about 2 hours. All samples were repeatedly tested at least twice for maximum reproducibility. All the data was

analyzed using the software provided by the Esco e Swift Spectrum 48 fluorescence qPCR detection system.[12,13]

Table 2. Optimal reaction conditions for real time PCR

Reagents	Optimized concentration range	Final Concentration
Double distilled water		
Forward primer SecY IV F	80-250 nM	2.5 mM
Reverse primer Sec Y IV R	80-250 nM	2.5 mM
SYBR Green qPCR Master mix	1X-5X	1X
Template DNA	<100 ng	Variable

Table 3. Optimal thermal conditions for real time PCR

Program	Cycles	Target Temperature	Incubation time
Initial Denaturation		950°C	15 minutes
Denaturation	40 Cycles	950°C	15 seconds
Annealing		540°C	30 seconds
Extension		720°C	30 seconds
Final Extension		720°C	8 minutes
Melting Temperature		650°C-950°C	

3. Results and Discussion/Analysis and Findings

Only the two positive controls show the amplification curves. All 10 samples did not get any amplification curves. All the 10 samples were negative.

The detection of Leptospira DNA from suspected environmental water samples is widely used as a predictor in surveillance to confirm the spreading of pathogens beyond the geographical locations and importantly as a predictor of an outbreak. However, such a procedure has its limitations. The sample testing is only a representation based on the existing knowledge. Low pathogen levels and inadequate volumes used may lead to false negative results.

Figure 2: Fluorescence Curves of the amplified product of the secY gene

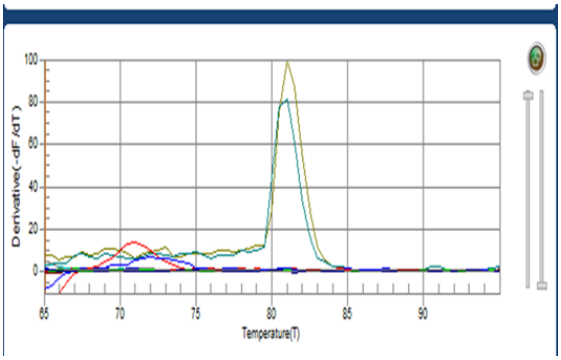

Figure 3: Melting Curves of the amplified product of the secY gene

In this study, all 10 water samples tested were negative for Leptospira DNA. The most possible reasons for a such result are the absence of Leptospira in water during the study period. The study was done in the month of April which was dry enough to have no reservoir water collections. The timing was just before the main monsoon of the country and were experiencing very high temperatures. All these climatic conditions probably impacted negatively on the study. If the area where the water samples were collected has a low prevalence of leptospirosis during the study period, it's possible that none of the samples contained the bacteria although the area is a highly prevalent region.[13] Environmental factors such as temperature, pH, and the presence of other microorganisms can affect the survival and detection of leptospirosis bacteria in water samples. The study indicates that

health authorities need not continue with prescribing prophylaxis to risk people during such time of the year.

The presence of carrier RNA may have contributed to improved DNA recovery from low-concentration samples. This is in agreement with Levesque-Sergerie *et al*., who reported that carrier RNA enhances the yield and stability of nucleic acids during extraction, especially when working with small sample volumes.[14]

The PCR results further validated the quality of the extracted DNA. Positive control samples produced clear and specific amplification bands, while the negative controls showed no amplification, confirming the absence of contamination.

In the DNA extraction process buffer AVL was a viral lysis buffer designed to isolate viral nucleic acids. It can be given with or without carrier RNA. When purifying small amounts of DNA, adding carrier RNA was improved DNA recovery.10 Carrier keeps the minimal amount of target nucleic acid in the sample from being irretrievably bound. The centrifuge process was used to harvest the bacterial sample, and this separates the sample into solids and liquids. After adding the sample to the carrier RNA and AVL mixture and incubating it at room temperature for ten minutes, carrier RNA is added to the sample to aid in the effective binding of DNA to the silica membrane of the spin column in future phases. Incubating at room temperature allows carrier RNA to bind to the DNA molecules, increasing their stability and avoiding destruction. Ethanol is frequently used in DNA extraction processes because of its ability to precipitate DNA from solution. Ethanol is added to the DNA-containing solution to cause precipitation. When ethanol is added to a solution, the solubility of DNA molecules decreases, leading them to cluster together and become insoluble in the aqueous solution. This precipitation separates the DNA from the other dissolved cellular components, such as proteins and lipids.[15] Buffers AW1 and AW2 function as a washing agent for DNA, ensuring that it is free of other bimolecular components.[16] Buffer AW1 washes away pollutants and impurities from the spin column's DNA-bound silica membrane. After centrifugation, the filtrate with these contaminants is discarded. Using a new spin column keeps the DNA bonded to a clean membrane, free of impurities that could interfere with downstream uses. Buffer AE elutes DNA from the spin column membrane into the microcentrifuge collection tube, allowing for stable DNA storage.

Positive controls are reviewed to ensure that the method is capable of amplifying the target nucleic acid, and negative controls are examined to ensure that no contamination occurs. Taq DNA Polymerase is important in Polymerase Chain Reaction (PCR) because of its role in synthesizing and amplifying new strands of DNA.[17] It is important to optimize PCR to avoid a non- specific amplification.

4. Conclusion

The study indicates the absence of Leptospira in environmental water samples during January, February, March and April of the year. The risk groups need not provide with antibiotic prophylaxis during such times of the year. Thus, this indicates a need for an island-wide study, to decide on appropriate control measures.

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