

# BMJ Open Study protocol: characterising the clinical, epidemiological and aetiological aspects of leptospirosis in Sri Lanka: a hospital based clinico-epidemiological study

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## ABSTRACT

**Introduction** Sri Lanka has one of the highest incidences of leptospirosis worldwide. We hypothesised that different geographical locations and patient context will have a distinct molecular epidemiology of leptospirosis, based on microgeographical characteristics related to region-specific *Leptospira* predominance. Our objective is to characterise the clinical, epidemiological and molecular aspects of leptospirosis in Sri Lanka to understand disease progression, risk factors and obtain isolates of *Leptospira*.

**Methods and analysis** We designed a multicentre prospective study in Sri Lanka to recruit undifferentiated febrile patients and conduct follow-ups during hospital stays. Patients will be recruited from outpatient departments and medical wards. This study will be conducted at two main sites (Anuradhapura and Peradeniya) and several additional sites (Awissawella, Ratnapura and Polonnaruwa). Blood and urine will be collected from patients on the day of admission to the ward or presentation to the outpatient department. Bedside inoculation of 2–4 drops of venous blood will be performed with Ellinghausen-McCullough-Johnson-Harris (EMJH) semisolid media supplemented with antibiotics. Regionally optimised microscopic agglutination test, culture and qPCR-evidence will be performed to confirm the presence of *Leptospira* in blood which in turn will confirm the presence of disease. Whole genome sequencing will be carried out for all isolates recovered from patients. Multilocus sequence typing (MLST) will be used for the genotyping of new isolates. Sri Lankan isolates will be identified using three published MLST schemes for *Leptospira*.

**Ethics and dissemination** Ethical clearance for the study was obtained from Ethics Review Committees (ERC), Medicine and Allied Sciences (FMAS), Rajarata University of Sri Lanka (RUSL) and University of Peradeniya. All genomic data generated through this project will be available at GenBank. Anonymised data will be deposited at the ERC, FMAS, RUSL.

## Strengths and limitations of this study

- This is a multicentre study involving wet and dry zones, low and highlands and high endemic and low endemic areas of the country covering different geographical and climate zones to provide a better understanding of leptospirosis in Sri Lanka.
- We will use a prospective study design focusing on culture isolation as a main aim with specific protocols for the particular objective to describe the strain diversity of *Leptospira spp.* in Sri Lanka.
- Clinical and epidemiological data will be collected as primary data to ensure high quality data using clinically trained health professionals.
- Since *Leptospira* are fastidious organisms, the culture yield will be low and linking the clinical disease with isolated *Leptospira* may be partly confounded by the growth potentials of different *Leptospira*.
- Since PCR and culture-based methods are typically valid only during the first 7–10 days of the illness and late presentations may lead to reduced sensitivity of these tests, microscopic agglutination test (MAT) is best interpreted with paired samples, and a lack of convalescent samples will impair the interpretation of MAT results.

## INTRODUCTION

Leptospirosis is a globally widespread, neglected and emerging zoonotic disease<sup>1</sup> with estimated 1.03 million cases and 58 900 annual deaths attributed to the disease.<sup>2</sup> An average of 2.9 million daily adjusted life years are estimated to be lost every year due to leptospirosis.<sup>3</sup> Emerging leptospirosis mostly affects vulnerable communities living in resource-poor settings. Often, the disease is either not suspected or is under or misdiagnosed due to the need for laboratory resources to confirm leptospirosis; typically, such resources are neither accessible

nor affordable. From the clinical perspective, a better understanding of the clinical disease associated with diverse *Leptospira* species is required and superior diagnostics are needed to prevent severe complications and death. From the public health perspective, the lack of reliable and efficient diagnostic tests makes assessing the burden of disease—whether regionally or globally—difficult.

Sri Lanka has emerged as a country with a high incidence of leptospirosis since 2007<sup>4</sup> and in 2008, the total number of clinically suspected cases reported to the surveillance system was 7406 with 204 deaths.<sup>5</sup> In 2009, 4980 cases and 145 deaths were reported,<sup>6</sup> and the outbreak persisted until 2013 with more than 4000 reported cases each year.<sup>7</sup> The probable case incidence during this time period was more than 20 per 100 000 population, indicating that Sri Lanka has one of the highest reported incidences of leptospirosis worldwide.<sup>8</sup> Our extensive work on the 2008 outbreak suggested that clinical diagnosis of leptospirosis is highly inaccurate, since as much as 40% of the leptospirosis patients are misdiagnosed in hospitals.<sup>9</sup> However, all Sri Lankan studies, including our previous study, have the same drawback, as observed in most of the published reports in global literature: hospital admission bias, where a large number of patients with leptospirosis may have been treated as outpatients which leads to a severe underestimate of the actual disease burden. A precise disease burden estimate requires seroprevalence studies coupled with population-based incidence studies.

Our studies on the 2011 outbreak of leptospirosis in Anuradhapura showed that the molecular epidemiology, disease transmission and clinical manifestations of this outbreak were different from those observed in Central Province (wet zone) in 2008.<sup>10</sup> One of the main concerns regarding these marked variations in the *Leptospira* strain and the clinical disease was whether this was due to microgeographical variations, environmental conditions or reservoir mammal infection. It has been shown for other diseases such as malaria<sup>11</sup> and schistosomiasis,<sup>13–15</sup> that microgeography may have a major influence on disease epidemiology. Geochemistry is a well-known and major contributory factor in human health.<sup>16</sup> Despite the extensive literature available on leptospirosis, studies on the microgeographical variation of *Leptospira* are scarce.

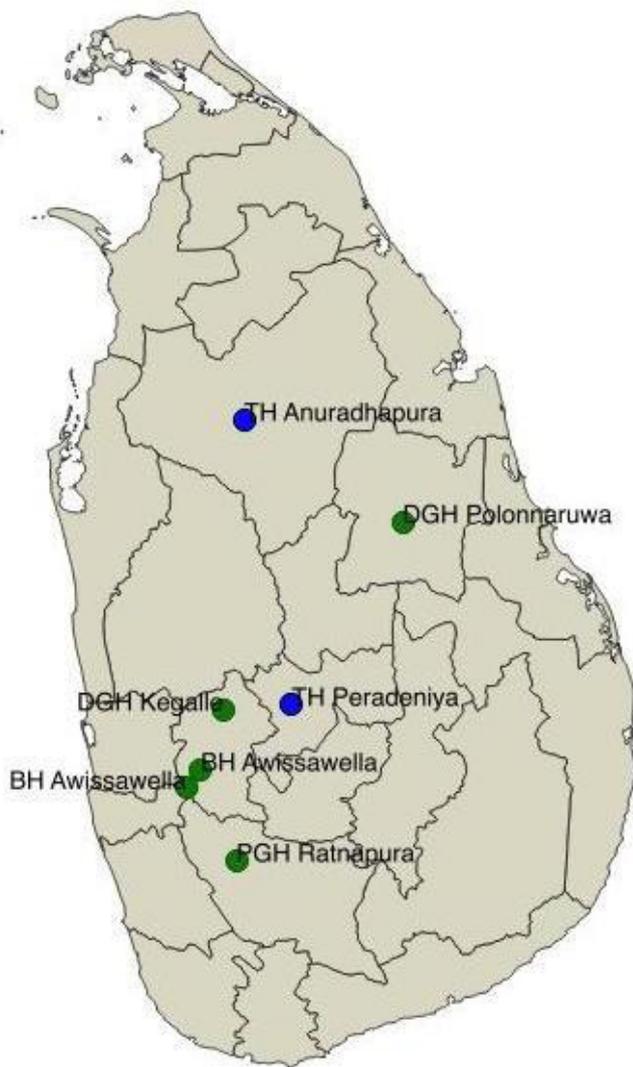
A systematic review performed on Sri Lankan isolates showed that more than 40 strains from 20 serovars of *Leptospira* have been reported from Sri Lanka.<sup>17</sup> Our previous studies on the 2008 and 2011 outbreaks of leptospirosis in Sri Lanka expanded the knowledge of circulating *Leptospira* and were consistent with a diversity of *Leptospira* infection in Sri Lanka. We showed that in the 2008 outbreak, *L. interrogans* was the predominant species, compared with the post-flood outbreak in 2011, in which *L. kirschneri* was the predominant species. However, the molecular epidemiology of *Leptospira* species in endemic settings remains unknown in

Sri Lanka. In addition to the single tube nested PCR (STNPCR) method, for the first time, we showed that previously published multilocus sequence typing (MLST) schemes could be used with modifications for direct patients samples to study the genetic diversity of infecting *Leptospira* species in resource-poor settings, where the facilities needed to perform cultures and isolations are minimal.<sup>18</sup>

One of the most important conclusions we draw from our published work is that qPCR is promising as a clinical diagnostic tool in the diagnosis of leptospirosis during the acute phase, with a wider window of positivity than previously noted.<sup>19</sup> The editorial by Katz<sup>20</sup> endorsed the qPCR approach but also emphasised the need for a prospective evaluation of this method. However, most countries with high leptospirosis burdens still find it prohibitively expensive to have molecular based methods for rapid diagnosis, in terms of both the costs and logistics.

Disease burden assessment, public health interventions and clinical management of leptospirosis are challenges due to the lack of diagnostic facilities. Any population-based attempt to estimate the disease burden is often limited by the unavailability of diagnostic tests or the poor performance of the available diagnostic tests. The development of diagnostic tests that are both global and country-specific requires knowledge on circulating serovars. Furthermore, well-characterised samples are required with species and subspecies level identification of infecting *Leptospira* backed by culture isolations. In Sri Lanka, a culture isolation of *Leptospira* has not been reported since the 1970s. Even though we showed the microgeographical changes of leptospirosis in 2014,<sup>10</sup> no prospective studies have been conducted in a Sri Lankan cohort to primarily identify the disease diversity with specific research design. We hypothesised that a different study site and patient context—inpatient vs outpatient—will have a distinct molecular epidemiology of leptospirosis, based on microgeographical characteristics related to region-specific *Leptospira* predominance, clinical characteristics related to pathogenesis/virulence potential of specific *Leptospira* species, strains, serovars and the acquisition of infection related to occupational and activities of daily living, reflecting different environmental exposure contexts. Based on these background analyses and hypotheses, the objectives of the present study were as follows:

1. To determine the prevalence of leptospirosis among undifferentiated febrile patients (inpatient and outpatient) in contrasting geographical settings in Sri Lanka.
2. To describe the full clinical spectrum of leptospirosis in patients infected with *Leptospira*.
3. To determine the predictors of leptospirosis progression using a nested case control approach among febrile patients, both inpatient and ambulatory.
4. To determine the *Leptospira* species and type diversity in different geographical settings in Sri Lanka.
5. To characterise Sri Lankan *Leptospira* using MLST and whole genome sequencing methods.



**Figure 1** Locations of study sites.

6. To have a well-characterised collection of serum, whole blood and urine samples for the future development and validation of leptospirosis diagnostics.

## METHODS AND ANALYSIS

### Study sites

This study will be performed in four primary subject enrolment/data collection centres in two main contrasting sites: Kandy and Anuradhapura. In addition, a small component of the study will be carried out in Sabaragamuwa, Western, North Central and Southern provinces for species diversity (figure 1). Kandy and Anuradhapura was selected based on our previous observations of *Leptospira* diversity in these two settings, supported by historically distant identification of diverse *Leptospira* in Sri Lanka (1960s).<sup>21</sup> Since no recent studies have been conducted, this project will yield up-to-date information at the molecular and genomically-determined taxonomical levels in addition to conventional methods of identifying *Leptospira*. The initial focus is on Anuradhapura, located in North Central province of Sri Lanka, in the

dry zone of the country. The annual rainfall is 1200–1600 mm, with a mean annual temperature of ~30°C. In Anuradhapura, paddy farming is carried out by traditional, full-time farmers and constitutes the main mode of income. Cattle, water buffalo, certain rodent species—well-known sources of *Leptospira* transmission—are found in and around the paddy fields. Paddy fields are large and well maintained. There are no wetlands or marshy lands in these areas, except the paddy fields during working seasons. Paddy field work depends on irrigation systems, such that, between farming seasons, the paddy fields become completely dry. Other risk factors for acquiring leptospirosis include daily activities involved in living in economically poor conditions, for example, living in proximity to dogs and rodents in houses and neighbourhoods and the wearing of shoes that are often no more than sandals.

The soil structure, water quality/hardness and ecological systems in Anuradhapura contrast with those of the wet zone of Sri Lanka. Kandy (another historic site in Sri Lanka/Ceylon, the cultural capital of the country since ancient times) is situated in the wet zone, with over 3000 mm of rainfall, and is located in the middle, hilly parts of the country. Paddy farming in this area is based on rainwater and the paddy fields remain wet throughout the year. These are small fields adjacent to wildlife and the soil and ecological structure is different from that in Anuradhapura. Nonetheless, the economic conditions of residents in this region are poor and rural, such that rodent-transmitted and dog-transmitted *Leptospira* in the context of activities of daily living is likely common but completely unexplored.

The purpose of including additional sites is primarily to identify differences in leptospirosis outbreaks, especially during floods and unsuspected outbreaks. Only selected components of the study will be performed in those areas if there are outbreaks.

### Study population

The study population will include all undifferentiated fever cases either presented to the outpatient department (OPD) or admitted to medical wards/intensive care units (ICUs) in teaching hospitals (TH) Anuradhapura and TH Peradeniya (main sites) and selected hospitals in Western, Sabaragamuwa and Southern provinces.

### Inclusion criteria

- ▶ Age ≥12 years.
- ▶ Temperature ≥38°C.
- ▶ Self-reported fever ≥2 days.

### Exclusion criteria

- ▶ Fever with running nose without any other symptom or sign.
- ▶ Any patient with confirmed diagnosis/foci of infection as a cause for the fever.
- ▶ Traumatic or post-operative fever per physician discretion.

- Fever due to nosocomial infections.

### Patient recruitment

Graduates with a Bachelor of Medicine and Bachelor of Surgery awaiting their internship appointment and established registrars will be assigned to all four centres and directly supervised on a daily basis. They will screen all febrile patients and perform directed physician examinations in the OPD, as well as on inpatients admitted to the medical wards. Once the diagnostic facilities are established, we will inform all clinicians in the selected districts about the available facilities. If they express interest, diagnostic facilities, including cultures to determine species diversity will be provided.

### Procedure

All possible undifferentiated fever cases will be interviewed and examined by the on-site pre-intern medical officers and suspected leptospirosis cases (fulfilling inclusion and exclusion criteria) will be referred to the study project. In the OPD, once the medical officers complete the history taking and examination, the data collector will collect the data. For inward patients, the pre-intern medical officer will screen all patients with fever on admission to the ward, for the selection of eligible patients. In Anuradhapura, four medical units are available and the study will be carried out in all four medical wards. In Peradeniya, a single medical ward is available. Data collectors will visit all wards and ICUs daily for the purpose of data collection. Once the routine ward procedures are completed, data will be collected. All eligible patients will be provided an explanatory statement of the study. The data collector will also explain the benefits and data confidentiality, as well as the right to withdraw from the study at any given time. Once a patient is well informed, written consent will be obtained before enrolment. Data will be collected in the ward, at the patient bed side to ensure the patient's privacy and confidentiality, as a routine clinical procedure. In the OPD, a separate location will be used to collect the data. Data will be collected using a fully structured, interviewer-administered questionnaire. Once the initial data collation is completed, all biological sample will be collected (see the section on sample collection, procession and storage procedure). An additional clinical data check list will be used to assess the clinical features. A daily follow-up will be performed for all hospitalised patients to observe the clinical progress of the disease using a clinical checklist. The questionnaires and the checklists will be adopted from previously published time-tested protocols.<sup>22</sup>

All patients will be given an appointment for follow-up 3 weeks after the initial assessment. To maximise patient follow-up, reminders and telephone calls will be used to contact participants before their appointment, and the fee for transportation will be paid. The main purpose of the follow-up would be to obtain a sample for disease confirmation.

### Sample size

Assuming that at least 70% of patients are infected with same infective *Leptospira* strain in a specific setting, at least 24 patients with leptospirosis with molecular identification are needed from each site. This calculation was performed with a power of 80% and an alpha value of 0.05. Based on our previous studies, the interpretable sequencing data are available for around 50% of confirmed cases of leptospirosis<sup>19</sup> and leptospirosis accounts for 15% of acute undifferentiated fever.<sup>23</sup> Based on these assumptions, the total number of febrile patients that is required from one sample collection site for this study is 320. This number of patients is conservatively estimated and feasible to obtain within the project period.

### Variables and epidemiological data

We will be looking at socio-demographic, exposure and clinical data for the clinic-epidemiological components of the study. The sociodemographic data will include age, sex, ethnicity, religion, occupation, income and residence. These variables are usually the distance determinants of leptospirosis and considered as important in determining the social determinants of the disease. Exposure variables will be looking at several types of exposure. First set of variables will include the residential and working environment and hosts. Next set will include actual individual exposure during last 3 weeks. Clinical data will be obtained initially to describe the presenting complaints. All symptoms will be recorded as described by the patients. We will be using a set of probing questions to clarify the presenting complaints. Signs will be documented after the physical examination by the clinically qualified data collector. If a sign is documented after the admission by a treating physician it will also be included, even if it is not demonstrable at the time of examination.

### Sample collection, procession and storage procedure

Blood and urine samples will be collected from patients on the day of admission to the ward or on presentation to the OPD. A total of 7 mL of blood will be collected and a bed side inoculation of 2–4 drops (100–500 µL) will be performed using two tubes containing 9 mL of EMJH semisolid media with added antibiotics (5-fluorouracil and neomycin). Collected blood added to EMJH media for culture will be incubated at 30°C in an incubator.

Venous blood (2.5 mL) will be collected in an EDTA tube and the remainder will be placed into plain tubes for serum separation. For serum separation, the blood in the plain tubes will be centrifuged at 1300 rpm for 10 min. The blood in the EDTA tubes will be used for PCR assay and the serum samples for serological assays. Aliquots (500 µL) will be prepared from the whole blood and serum samples. These will then be frozen at –20°C or –80°C for short-term or long-term storage, respectively. This procedure will be completed within 2 hours.

Clean catch mid-stream (10–15 mL) urine will be collected into a sterile wide mouth container and processed within 2 hours of collection. Initially, the urine

**Table 1** Criteria for the diagnosis of confirmed and probable cases of leptospirosis

Case	Definition
Confirmed	<b>Clinical signs and symptoms consistent with leptospirosis with any one of the following criteria:</b> <ol style="list-style-type: none"> <li>1. Fourfold increase in MAT titre in acute and convalescent serum samples.</li> <li>2. MAT titre <math>\geq 1:400</math> in single or paired serum samples.</li> <li>3. Isolation of pathogenic <i>Leptospira</i> species from normally sterile sites.</li> <li>4. Pathogenic <i>Leptospira</i> species DNA detected by PCR.</li> </ol>
Probable case	<b>Clinical signs and symptoms consistent with leptospirosis with any one of the following criteria:</b> <ol style="list-style-type: none"> <li>1. Presence of IgM antibodies by ELISA or dipstick.</li> <li>2. MAT titre <math>\geq 1:100</math> in single acute-phase serum sample (with no convalescent serum).</li> </ol>

MAT, microscopic agglutination test.

will be centrifuged at 3000 rpm for 5 min to sediment the white cells, epithelial cells and other crystals. Next, the supernatant will be separated and further centrifuged at 15 000 rpm for 10 min. The sediment obtained will be used for PCR studies and stored at  $-20^{\circ}\text{C}$  until further use.<sup>24–26</sup> The available protocols for qPCR based urine analysis will be tested and optimised. If the urine cannot be processed during the specified time period, the pH of the urine will be adjusted to approximately 7.4 using 1 M NaOH solution. Three weeks after the onset of fever, a follow-up blood sample (2 mL) will be collected in plain tubes for paired sera and 10–15 mL of urine will be collected for PCR assay.

### Disease confirmation

Disease will be confirmed using a regionally optimised microscopic agglutination test (MAT), culture and qPCR-evidence of the presence of *Leptospira* in the serum. The criteria for confirmed and probable cases of leptospirosis is presented in table 1.<sup>27</sup>

### Microscopic agglutination test

For this purpose, we will use the US Center for Disease Control's MAT panel with five already available local strains obtained from the Royal Tropical Institute, Amsterdam (KIT). Once the Sri Lankan isolates are obtained, a validation of best panel suitable for Sri Lanka will be determined. MAT will be carried out in two main steps. First, the serum samples will be screened at a titre of 1/50 using the MAT panel of 24 strains, which includes the five local strains. Positive sera from the screening test will be subjected to a run out test with the serial dilution of sera from 1/50 to 1/3200. MAT evidence for seropositivity will be defined as one or more of the following: seroconversion from negative to positive ( $<1/50$  to  $>1/50$ ); fourfold increase in titre between acute-phase and convalescent-phase (follow-up) samples; or a single titre of  $\geq 400$ .

### Culture isolation of *Leptospira*

For the culture isolation, we adopted the previously validated protocol published by Wuthiekanan *et al.*<sup>28</sup> EMJH semisolid media will be prepared by adding 2.3 g of EMJH base, 1.5 g of bacteriological agar and 100 mg of sodium pyruvate into 785 mL of distilled water, then adjusting

the pH to 7.4. The media will be autoclaved and cooled to around  $50^{\circ}\text{C}$ . Next, 100 mL of *Leptospira* enrichment media and 100 mL of fetal bovine serum will be added to the media.<sup>29–32</sup> To suppress the growth of possible contaminants, 5-fluorouracil and neomycin will be added, at concentrations of 100  $\mu\text{g}/\text{mL}$  and 25  $\mu\text{g}/\text{mL}$ , respectively. The media inoculated with blood will be inspected using dark field microscopy for the presence of motile *Leptospira*, initially after 3 weeks and then on a monthly basis. When a positive growth is detected, sub cultures will be transferred into liquid and semisolid media. An aliquot will then be obtained and fixed with 5% DMSO (dimethyl sulfoxide) and stored in  $-80^{\circ}\text{C}$ . To maintain live cultures, the isolates will be subcultured in liquid media on a bi-weekly basis and on semisolid media every 3 months. Serotyping of the newly isolated *Leptospira* strains will be carried out at the Pasteur Institute, France.

### Quantitative PCR

#### Diagnosis

For the diagnosis, DNA will be extracted from whole blood, serum and urine samples using the QIAamp DNA Blood Mini Kit (Qiagen, USA), according to the manufacturer's instructions. The extracted DNA will be quantified using an Invitrogen Qubit 4 Fluorometer. Two previously published quantitative PCR protocols targeting 16s ribosomal RNA and LipL32 genes will be used for the detection of pathogenic *Leptospira* DNA based on SYBR Green Chemistry (table 2).<sup>33</sup>

#### Speciation

To determine the *Leptospira* species in the positive cultures, a previously published quantitative PCR protocol based on SYBR Green Chemistry (table 3) will be used (before sequencing).<sup>34</sup> This will facilitate the identification of *L. interrogans*, *L. borgpetersenii*, *L. kirschneri* and *L. noguchii*.

#### Molecular studies

For newly isolated *Leptospira*, whole genome sequencing will be performed using the following two technologies: MinION Nanopore sequencing in the Leptospirosis Research Laboratory (Faculty of Medicine and Allied Sciences, Rajarata University of Sri Lanka) and PacBio third generation sequencing at the Institute for Genomic

**Table 2** Primers for the detection of *Leptospira* DNA in clinical samples

Primer/probe sequence	Annealing temperature	Complementary target species
Forward: 5'-AAG CAT TAC CGC TTG TGG TG-3' Reverse: 5'-GAA CTC CCA TTT CAG CGA TT-3'	60°C	lipL32 gene of pathogenic <i>Leptospira</i> spp.
Forward: 5'-TAA AGG CTC ACC AAG GCG AC-3' Reverse: 5'-TTA GCC GGT GCT TTA GGC AG-3'	60°C	16s gene of pathogenic <i>Leptospira</i> spp.

Medicine (University of California, San Diego, USA) or in a place where it is available.

BAS and BAX files containing all the DNA sequence information will be converted into FASTA files. Prior to assembly, the quality (coverage) of the raw data will be assessed. Using the PacBioEDA software, the quality summary of the data will be obtained. The CanuPacBio assembly programme will be used for genome assembly. The raw data of BAS/BAX files will be converted into bam files using the bax2bam software. These bam files will be aligned to our references using the Pbalngn software. The software Quiver will be used to polish the data. The assembled data will be annotated using Prokka<sup>35</sup> and Roary,<sup>36</sup> and annotations will be inspected and curated using Artemis. The functional annotation will be performed using the eggNOG and eggNOG MAPPER online tools.<sup>37</sup> The genomes of the Sri Lankan isolates will be compared using MAUVE software. The organism will be roughly identified from the direct raw data using KRAKEN.<sup>38</sup>

MLST will be used for the genotyping of the new isolates. Sri Lankan isolates will be identified using three published MLST schemes for *Leptospira*.<sup>39–41</sup> Genetic similarity between the isolates will be determined using SPLITSTREE.

### Taxonomgenomics, pan-genome and phylogenetic analyses

To establish how this strain was related to other *Leptospira* strains, we will use several genomic analysis tools. First, the 16S rRNA sequences will be used to construct a phylogeny. High sequence identities ( $\geq 97\%$ ) between the newly sequenced strain and previously identified *Leptospira* strains will be preliminarily used as suggestive of phylogenetic relationships. Accordingly, the 16S rRNA gene sequences of *Leptospira* spp. deposited in the GenBank database will be acquired. Multiple sequence alignment based on 16S rRNA will be performed using ClustalX

V.1.81. These results will be then used to construct the maximum likelihood tree using MEGA V.5.05. To evaluate clade support, we will perform a bootstrap analysis with 1000 replicates. The relationship of each sequenced genome to the previously described or novel *Leptospira* species will be determined according to the Overall Genetic Relatedness Indices, Average Nucleotide Identity (ANI) and the Average Amino Acid Identity. The indices will be automatically calculated using two-way BLA blastn and blastp. For the ANI calculation, the default parameters shall be as follows: sequence identity cut-off, 30%; alignment cut-off, 70%; query length, 1020 bp.

### Data management and analysis

Patient interviews and patient health records will be used as data sources. On-site data entry will be performed in real-time for all the collected data. An EpiInfo database will be prepared using cross-checks, field validation and other in-built techniques available for data quality improvement. The investigators will frequently visit the hospitals to cross-validate the data collected. All databases will be stored on password-protected computers. The main database will be stored at the Leptospirosis Research Laboratory of the Faculty of Medicine and Allied Science, Rajarata University of Sri Lanka. Personal identification data will be removed from all the databases, such that only aliquots and predesigned patient identifiers are available. Hardcopies of the questionnaires will be archived using the standard protocols for archiving hard copies of questionnaires. Only the investigators will have access to the database. The database will be continuously updated with laboratory data using a linking patient identifier (key variable). The laboratory results will be generated for all samples daily, once a fully functioning laboratory is set up, and the treating physicians will be informed about this over the phone. Separate laboratory registers will be

**Table 3** Primer pairs for detection of species

Primer/Probe sequence	Annealing temperature	Complementary target species
Forward: 5'-CTT GAG CCT GCG CGT TAY-3' Reverse: 5'-CCG ATA ATT CCA GCG AAG ATC-3'	63°C	secY gene of <i>L. interrogans</i>
Forward: 5'-GAT TCG GGT TAC AAT TAG ACC-3' Reverse: 5'-TTG ATC TAA CCG GAC CAT AGT-3'	65°C	ompL1 gene of <i>L. borgpetersenii</i>
Forward: 5'-CTG GCT TAA TCA ATG CTT CTG-3' Reverse: 5'-CTC TTT CGG TGA TCT GTT CC-3'	60°C	secY gene of <i>L. kirschneri</i>
Forward: 5'-TCA GGG TGT AAG AAA GGT TC-3' Forward: 5'-CAA AAT TAA AGA AGC AAA GA-3'	63°C	secY gene of <i>L. noguchii</i>

maintained for the receipt of samples and the delivery of the results.

For the missing data, case deletions and pairwise deletions will be carried out, based on the amount of missing data. Proportions and 5% confidence intervals will be used to describe the confirmed cases, diversity of infecting *Leptospira* and sequelae. A  $\chi^2$  test will be used to identify any trends and test significant differences in the *Leptospira* diversity in the different microgeographical and clinical settings. Predictors of severe disease will be analysed using a logistic regression model. Model building will be performed using a conceptual hierarchy of determinants, which we have previously proposed for leptospirosis. An online database for *Leptospira* MLST will be used for typing analysis.

### Patient and public involvement

The study was developed based on the patients' feedback regarding leptospirosis disease burden, its impact on their lives and in particular, the problems faced during illness. In previous studies, we specifically explored patients as well as general public views on leptospirosis disease and prevention, and used this information for the design of this study. Moreover, the results of this study will be disseminated on a continuous basis for the patients. We have already conducted several public awareness and health worker education workshops based on our findings.

### ETHICS AND DISSEMINATION

Undifferentiated fever patients eligible for the study will be given an explanatory statement of the study and written informed consent will be obtained before enrolment. Rapid diagnostic kits will be used on site to assist treating physicians. qPCR diagnostic facilities will be provided for all patients whenever the urgent tests are requested by treating physicians. All test results will be informed to the patients and the wards.

All genomic data will be deposited in Gene Bank. Characterised serum samples will be stored in  $-80^{\circ}\text{C}$  in the Faculty of Medicine and Allied Sciences, Rajarata University of Sri Lanka. Data pertaining to this study will be available for researchers and policy-makers on a reasonable request address to the corresponding author. After the completion of work, data will be deposited in a repository.

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**Contributors** JV and SA drafted the initial protocol. JW, DJ and IS are responsible for the detailed design, field/ laboratory work analysis and interpretation of objectives 1-3, 4 and 5, respectively. CG, SK and SS helped in design, field, hospital and laboratory work. MM participated in scientific design and guided all laboratory components. All authors have read and approved the manuscript.

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