Molecular characterisation and disease severity of leptospirosis in Sri Lanka

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Leptospirosis is a re-emerging zoonotic disease all over the world, important in tropical and subtropical areas. A majority of leptospirosis infected patients present as subclinical or mild disease while 5-10% may develop severe infection requiring hospitalisation and critical care. It is possible that several factors, such as the infecting serovar, level of leptospiraemia, host genetic factors and host immune response, may be important in predisposition towards severe disease. Different Leptospira *strains circulate in different geographical regions contributing to variable disease severity. Therefore, it is important to investigate the circulating strains at geographical locations during each outbreak for epidemiological studies and to support the clinical management of the patients. In this study immunochromatography, microscopic agglutination test and polymerase chain reaction were used to diagnose leptospirosis. Further restriction fragment length polymorphism and DNA sequencing methods were used to identify the circulating strains in two selected geographical regions of Sri Lanka.* Leptospira interrogans, Leptospira borgpetersenii *and* Leptospira kirschneri *strains were identified to be circulating in western and southern provinces.* L. interrogans *was the predominant species circulating in western and southern provinces in 2013 and its presence was mainly associated with renal failure.*

Key words: *Leptospira* - molecular characterisation - Sri Lanka

Leptospirosis is an endemic, zoonotic disease of public health importance in Sri Lanka (Victoriano et al. 2009). Seasonal outbreaks of leptospirosis occur annually and in 2013, 4,276 cases were reported to the Epidemiological Unit of Sri Lanka. Since Sri Lanka is predominately an agricultural country with a heavy rain fall, exposure to *Leptospira* is a major occupational hazard (Brenner et al. 1999). *Leptospira interrogans*, *Leptospira santarosai*, *Leptospira kirschneri*, *Leptospira borgpetersenii* and *Leptospira weilli* have been reported from several geographical locations in Sri Lanka at different time periods with varying disease severity (Brenner et al. 1999, Agampodi et al. 2012, 2014, Nwafor-Okoli et al. 2012).

Due to the highly endemic nature and associated morbidity and mortality of this disease, it is important to investigate the circulating strains at geographical locations during each outbreak for epidemiological studies and to support the clinical management of the patients.

SUBJECTS, MATERIALS AND METHODS

This was a prospective hospital based study in western and southern provinces in Sri Lanka between January 2013-January 2014. All the patients more than 18 years of age, presenting with clinically suspected leptospirosis according to the World Health Organization (WHO) guideline admitted to the medical wards were included in the study.

Informed consent was obtained from all suspected patients and sociodemographic data and risk factors were gathered using a pre-tested interviewer administered questionnaire. A venous blood sample of 5 mL was collected following standard procedures and aliquoted into a plain tube for serum separation and the rest added to an ethylenediamine tetraacetic acid (EDTA) tube for DNA extraction. All samples were transported at 4ºC to the Department of Microbiology, University of Sri Jayewardenepura, Sri Lanka.

IgM immunochromatographic assay and microscopic agglutination test (MAT) - Leptospira infection was presumptively diagnosed by detecting *Leptospira* specific IgM using a rapid immunochromatographic assay kit (Leptocheck WB; Zephyr Biomedicals, India) following the manufacturer's instructions. MAT was done in order to obtain single MAT antibody titres using the genus specific *Leptospira biflexa* serovar Patoc 1 strain (Medical Research Institute, Sri Lanka) and ≥ 400 titre was considered as positive for MAT (WHO 2010).

DNA extraction - EDTA blood samples (200 µL) were used for *Leptospira* DNA extraction using QIAamp DNA blood mini kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. Eluted DNA was quantified and purity was checked using Nanodrop 2000/200C spectrophotometer (Thermo Fisher Scientific, USA).

FlaB polymerase chain reaction (PCR) assay - PCR assay was used to amplify flagella gene present in pathogenic *Leptospira* species (Kawabata et al. 2001, Natara-

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jaseenivasan et al. 2012). Amplification of isolated DNA was carried out in 50 µL volume with 0.5 µL template DNA, 5 µL 5X green GoTaq® Flexi buffer (pH 8.5) (Promega, USA), 2 mM $MgCl_2$ (Promega), 0.1 µM of each primer (F1-TCTCACCGTTCTCTAAAGTTCAAC, R1-CTGAATTCGGTTTCATATTTGCC), 0.4 mM deoxy nucleotide triphosphate (dNTP) mix (Promega) and 0.25 units of Taq DNA polymerase (Promega). *L. interrogans* DNA was used as a positive control and a negative control without the template DNA were included in each PCR assay. PCR amplification was initiated at 94ºC for 5 min followed by 45 cycles of 94ºC for 1 min, 56ºC for 1 min, 72ºC for 90 s and a final elongation step at 72ºC for 10 min with final hold at 4ºC. The resulting amplicon was 793 bp and these were stored at 4ºC until further analysis.

Restriction fragment length polymorphism (RFLP) - PCR products of flaB PCR positive patient samples were used for RFLP digestion using Hae III and Hind III restriction enzymes (Kawabata et al. 2001). The restriction digestion was carried out in 20 µL of volume in a sterile microcentrifuge tube. The reaction mixture contained 10 µL of PCR product, 2 µL of 10 X RE buffer (Multicore™ buffer, Promega), 0.5 µL restriction enzyme (10 U/µL), 0.2 µL of acetylated bovine serum albumin (10 µg/µL) and distilled water to a final volume of 20 µL. The reaction mixture was incubated in an incubator at 37ºC for 5 h. The final product was subjected to electrophoresis using 2% agarose gel in tris-acetate-EDTA buffer containing 5 µg/mL ethidium bromide (Sigma Aldrich). Each digested PCR product was mixed with 1/5 volume of the gel loading buffer (Promega) and loaded into the agarose gel. Electrophoresis was carried out at room temperature for one and half hours. At the end of the electrophoresis the gel was visualised under ultraviolet transilluminator (Biometra GmbH, Germany). RFLP was done with three reference serovars: *L. interrogans* serovar Canicola, Icterohaemorrhagiae and Pyrogenes. An undigested PCR product, where the reaction mix was prepared without Hind 111, Hae 111 restriction enzymes, was used as a control (Figs 1, 2, Lane 2).

Nested PCR - A single tube nested PCR was used to amplify 16S rDNA gene specific for pathogenic and intermediate *Leptospira* species. Amplification was carried out using PCR primers: rrs-outer F (5^1-CTCAGAACTAACGCTGGCGGCGCG-3^1), rrs-outer-R (5^1-GGTTCGTTACTGAGGGTTAAAACCCCC-3^1), rrs-inner-F (5^1-CTGGCGGCGCG T CTTA-3^1), rrs-inner-R (5^1-GTTTTCACACCTGACTTACA-3^1) (Boonsilp et al. 2011). PCR master mix consisting of 0.5 µL template DNA, 5 µL 5X green GoTaq® Flexi buffer (pH 8.5) (Promega), 4 mM $MgCl_2$ (Promega), 0.2 pmol of each outer primer, 1.2 pmol of inner F, 5 pmol of inner R, 0.2 mM dNTP mix (Promega) and 0.25 units of Taq DNA polymerase (Promega) were used in a total volume of 25 µL. PCR reaction was carried out using a thermal cycler (Techne Flexigene, UK) with an initial denaturation at 95ºC for 2 min followed by 40 cycles of 95ºC for 10 s, 67ºC for 15 s, 72ºC for 30 s, another 40 cycles of 95ºC for 10 s, 55ºC for 15 s, 72ºC for 30 s and a final elongation step at 72ºC for 10 min. The resulting amplicon size was a 547 bp. Amplicons were visualised by gel electrophoresis using an 1.5% agarose gel. *L. interrogans* Serovar Canicola and *Leptostpira fainei* BUT 6 strain were taken as positive controls and *L. biflexa* Patoc 1 strain and no template control were used as the negative controls.

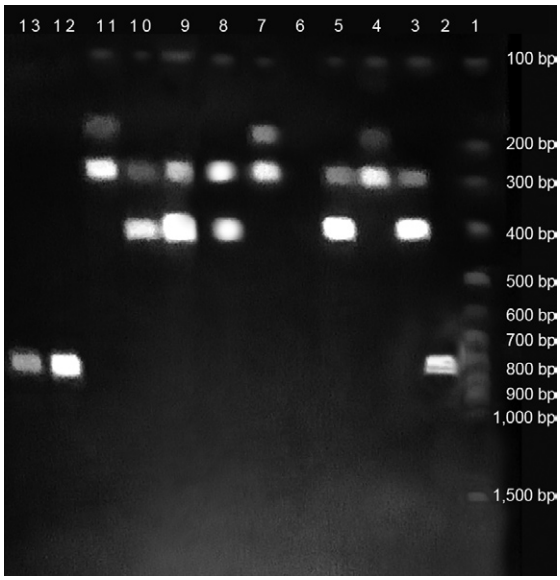

Fig. 1: hae 111 digestion of *Leptospira*. Lane 1: 100 bp DNA marker; 2: undigested polymerase chain reaction (PCR) product; 3: *Leptospira interrogans* serovar Canicola (100 bp, 300 bp, 400 bp); 4: *L. interrogans* serovar Icterohaemorrhagiae (100 bp, 200 bp, 300 bp); 5: *L. interrogans* serovar Pyrogenes (100 bp, 300 bp, 400 bp); 6; *Leptospira biflexa* Patoc 1 strain; 7-13: flaB PCR positive patient samples.

PCR products were purified using a PCR product purification kit (Promega) according to manufacturer's protocol and sequenced bidirectionally at Macrogen Inc (South Korea). DNA sequences were obtained using 3.1 Big Dye chemistry. Individual gene sequences were aligned using Bio Edit v.7.0.9.0. Consensus sequences were generated using Chromas v.5.0 and species were identified using National Center for Biotechnology Information (NCBI) BLAST. The gene sequences were deposited in the NCBI GenBank and accessions were obtained. Phylogenetic tree was developed using MEGA 6.0 (Fig. 3).

Ethics - Ethical approval was granted from the Ethical Review Committee of University of Sri Jayewardenepura (application 702/12).

RESULTS

Out of the 168 leptospirosis suspected patients 153 (91%) were males while 15 were females. Of these, 43.1% were farmers, 22.4% were outdoor laborers, 12.5% were indoor domestic workers and others included indoor office workers, housewives and school students. The mean age of the study sample was 41 years (± 20). The median duration of fever on admission was six days (± 2.5). Thirty-nine patients (23%) had been treated with antibiotics before admission to the hospital. Leptocheck rapid immunochromatographic assay for *Leptospira* IgM were

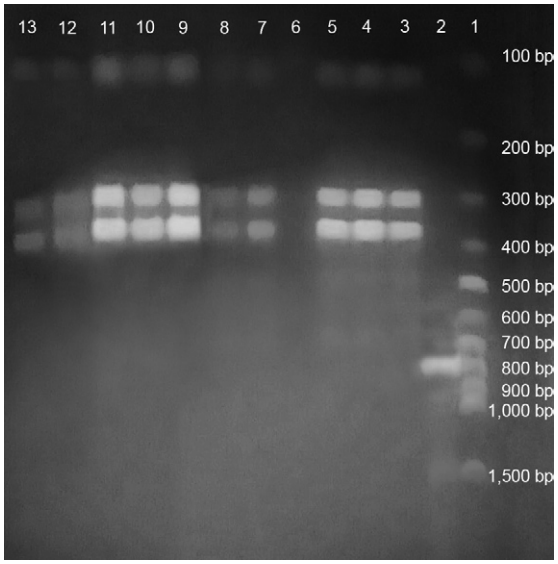

Fig. 2: hind 111 digestion of *Leptospira*. Lane 1: 100 bp DNA marker; 2: undigested polymerase chain reaction (PCR) product; 3: *Leptospira interrogans* serovar Canicola; 4: *L. interrogans* serovar Icterohaemorrhagiae; 5: *L. interrogans* serovar Pyrogenes; 6: *Leptospira biflexa* Patoc 1 strain; 7-13: flaB PCR positive patient samples.

TABLE I

Results of the laboratory diagnosis of leptospirosis based on microscopic agglutination test (MAT)[a], polymerase chain reaction (PCR) and immunochromatographic assay (Leptocheck) identification methods

Category (*Leptospira* case definition)	Method	Result	Patients n (%)
Definitive cases	MAT	+	61 (36)
	PCR	+	14 (8.3)
	MAT and PCR	+	7 (4.2)
	MAT or PCR	+	66 (39.2)
	MAT, PCR and Leptocheck	+	6 (3.6)
Presumptive cases	Leptocheck	+	84 (50)
Unconfirmed cases	MAT, PCR and Leptocheck	-	73 (43.4)
Total	-	-	168 (100)

a: single sample MAT ≥ 1:400; -: negative; +: positive.

positive in 84 (50%) while 13 (7.7%) were positive by flaB PCR. Of the 168 suspected patients, 61 (36%) had MAT titre of ≥ 1:400 (Table I) among them, 90% had a MAT titre of ≥ 800.

When the flaB PCR products were subjected to restriction enzyme digestion by Hae III, the DNA of reference strains, *L. interrogans* serovar Canicola and Pyrogenes (Fig. 1, Lanes 3, 5) resulted in three bands (100 bp, 300 bp and 400 bp). When the patient samples were tested by digestion with Hae III, three patients (Fig. 1, Lanes 8-10) had a restriction digestion pattern corresponding to *L. interrogans* serovar Canicola or Pyrogenes. Hae III restriction digestion was not able to differentiate between serovars Canicola and Pyrogenes. The reference DNA from *L. interrogans* serovar Icterohaemorrhagiae (Fig. 1, Lane 4) resulted in 3 bands (100 bp, 200 bp and 300 bp). Two patients in our study had a similar RFLP pattern corresponding to serovar Icterohaemorrhagiae (Fig. 1, Lanes 7, 11). A single band of 700 bp was observed in two patients (Fig. 1, Lanes 12, 13) and they were identified as *L. borgpetersenii* by DNA sequencing.

Hind 111 digestion resulted in three DNA fragments 100 bp, 300 bp and 350 bp in all reference strains; *L. interrogans* serovar Canicola, Icterrohaemorrgiae and Pyrogenes. All patient samples tested gave the same banding pattern (Fig. 2). Therefore Hind III was found to be less discriminative in the identification of *Leptospira* serovars.

Of the 84 *Leptospira* IgM positive patients, 12 were confirmed as leptospirosis using the nested PCR targeting the 16S rDNA gene. Interestingly, two IgM negative patients also gave positive results by rrs PCR. Therefore, 14 patients had confirmed leptospirosis by rrs PCR.

When risk factors were considered among the 14 leptospirosis confirmed patients, being a farmer ($p = 0.017$), outdoor laborer ($p = 0.046$) and contact with contaminated water ($p = 0.007$) showed a significant association with having leptospirosis. All the confirmed leptospirosis patients had an exposure history prior to the onset of the disease. Of these, nine patients reported exposure to contaminated water sources (paddy/agricultural land and flood), five reported animal exposure (cattle, rats and dogs) and three had either cracked heels or wounds on their feet.

Based on sequence analysis, *L. interrogans* was the most common cause of disease in this study (n = 11, 78.57%) followed by *L. borgpetersenii* (n = 2, 14.28%) and *L. kirschneri* (n = 1, 7.14%). The consensus sequences were submitted to GenBank and accessions were obtained as shown in Table II. A BLAST search revealed 99-100% identity of our isolates to *L. interrogans*, *L. borgpetersenii* and *L. kirschneri* (Table II).

Phylogenetic analysis shows that *L. interrogans* strains in our study were similar to the *Leptospira* identified in the 2008 outbreak in the central province of Sri Lanka (Fig. 3). Specimens SLUSJ_1, 2, 16, 111, 160 and 181 in our study were identified as *L. interrogans* which were closely related to isolate 68-JF910147 identified in the 2008 outbreak while specimen SLUSJ_3, 4, 19, 23 and 119 were closely related to *L. interrogans* isolate 229-JF910145 and isolate 109-JF910144 which were also identified during this outbreak (Agampodi et al. 2011). Specimen SLUSJ_12 and 70 were identified as *L. borgpetersenii* and specimen SLUSJ_176 was identified as *L. kirschneri* strains (Table II).

When clinical symptoms were analysed almost all patients were febrile on admission and had prostration. Headache (57%), myalgia (57%) and muscle tenderness (43%) were the common symptoms found in all confirmed cases. Conjunctival haemorrhage was seen in 35.7% of the confirmed leptospirosis patients. Elevated blood urea was seen in 14.2% whilst serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase were

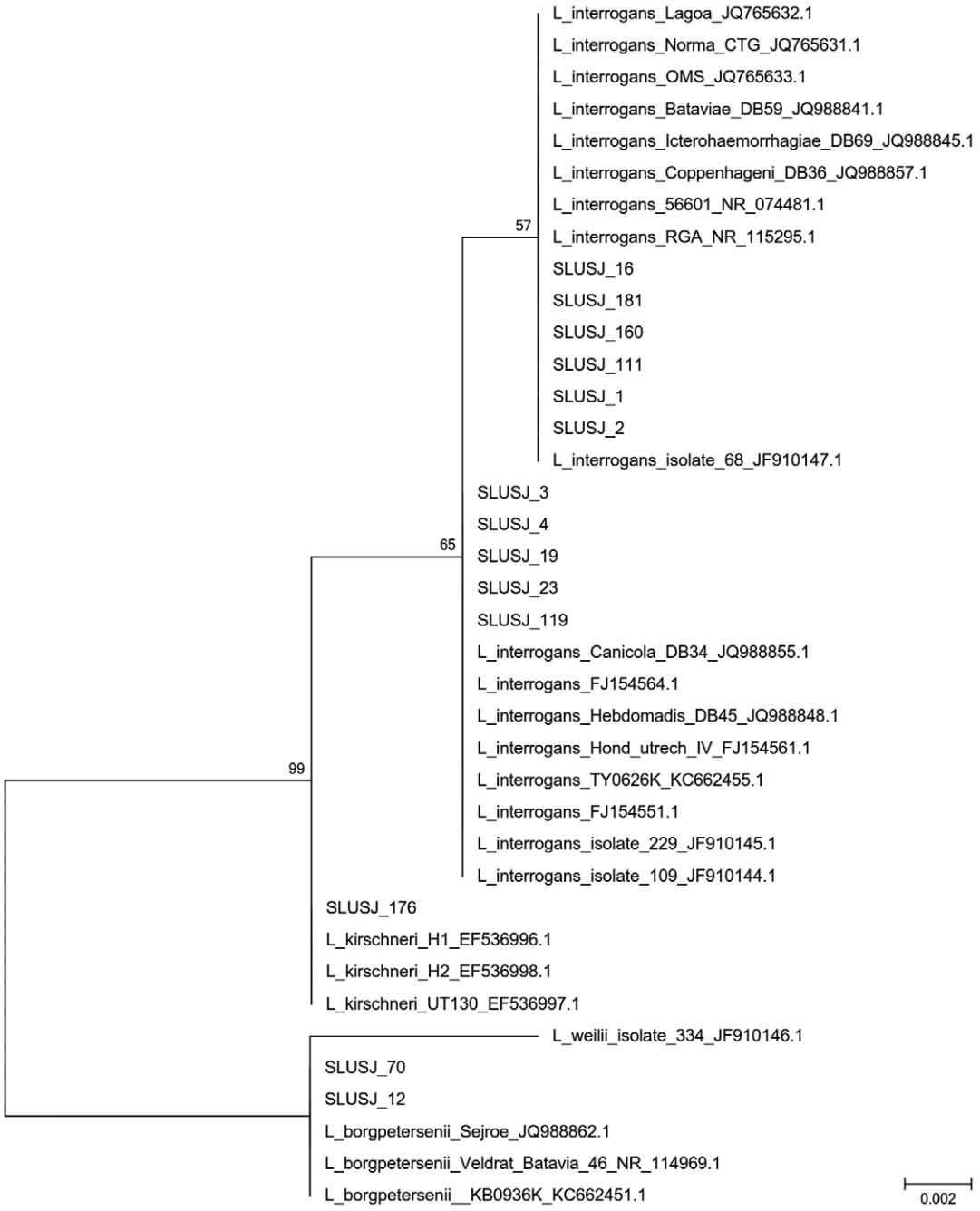

Fig. 3: phylogenic analyses were conducted with MEGA 6.0, the phylogenetic tree being drawn based on 1,000 bootstrap replicates with Kimura 2-parameter. The numbers on the nodes are the bootstrap support after 1,000 replicates. The specimens identified in the study are denoted by SLUSJ_ 1, 2, 3, 4, 12, 16, 19, 23, 70, 111, 119, 160, 176 and 181.

raised in 28.5% patients. Of these patients, 35.7% had leucocytosis and 57.5% had neutrophilia whilst haematuria (> 5 red blood cells per high power field) was seen in 35.7%. Serum creatinine levels were elevated in 7.14%. Electrocardiography changes were seen in 14.2%. Among the leptospirosis confirmed patients 28.5% required ICU treatment. Of these patients, 75% had infection due to *L. interrogans* and 25% had *L. borgpetersenii* infection. Renal failure was seen in 35.7% of the confirmed cases out of them, 80% were due to *L. interrogans*.

DISCUSSION

Leptospirosis is a widespread zoonotic infection gaining rapid importance in Sri Lanka due to the fact that the disease is associated with high morbidity and mortality (Agampodi et al. 2011, 2014, Nwafor-Okoli et al. 2012). In this study population, 50% were presumptively identified as leptospirosis, whilst 36% were confirmed by MAT (titre ≥ 400) (WHO 2010) (Table I). Of the total suspected patients, 13 were confirmed as leptospirosis by flaB PCR and 14 by rrs PCR, respectively,

TABLE II

Leptospira sequence identity related to disease complications

Specimen number (SLUSJ_)	Identity	Sequence similarity (%)	GenBank accession	Disease complication
1	*L. interrogans*	100	KP732501	Myocarditis
2	*L. interrogans*	100	KP732502	Acute renal failure
3	*L. interrogans* strain Canicola	100	KP732503	Acute renal failure
4	*L. interrogans* strain Canicola	100	KP732504	No complications
12	*L. borgpetersenni* strain *sejroe*	100	KP732506	Liver insufficiency
16	*L. interrogans*	100	KP732508	No complications
19	*L. interrogans* strain Canicola	100	KP732507	Liver insufficiency
23	*L. interrogans* strain	100	KP732509	Liver failure
70	*L. borgpetersenii* strain	99	KP732510	Liver failure
111	*L. interrogans*	99	KP732511	Myocarditis
119	*L. interrogans* strain Canicola	100	KP732512	Acute renal failure
160	*L. interrogans*	100	KP732513	Acute renal failure
176	*L. kirschneri* H2	100	KP732514	Acute renal failure
181	*L. interrogans*	99	KP732515	No complications

TABLE III

Comparison of selected features of leptospirosis outbreaks in Sri Lanka reported in 2008 and 2011 with the current study

Feature	2008[a]	2011[b]	2013[c]
Outbreak	Central province	North central province	Western and southern provinces
Period	Throughout the year	Following heavy rains and floods in first quarter of the year	Throughout the year
Predominant species	*Leptospira interrogans* (20/26)	*Leptospira kirschneri* (26/32)	*L. interrogans* (11/14)
Median duration of fever (IQR)	6 (4-8)	6 (2-8)	6 (4-8)
Renal failure (%)	13.8	21.9	35.7
Myocarditis (%)	10.3	15.6	14.3

a: Agampodi et al. (2011); *b*: Agampodi et al. (2014); *c*: current study; IQR: interquartile range.

according to the LERG guideline (WHO 2010). The rapid immunochromatographic assay (Leptocheck) used in this study had a sensitivity of 93% (Bandara et al. 2014) while the PCR was less sensitive. The high sensitivity of rapid immunochromatographic assay may have been associated with false positives. Similar observations were seen in a study done in India (Panwala et al. 2011). In this study the low PCR positivity may be explained by limited survival of the organism in the collected blood sample, immune system responses, prior use of antibiotics, DNA degradation during transportation and varied level of bacteraemia (Smythe et al. 2002).

RFLP has been used by several researchers to differentiate genotypes of *Leptospira* (Kawabata et al. 2001, Zakeri et al. 2010). The two restriction enzymes, Hae III and Hind III, used in our study were unable to differentiate between *L. interrogans* serovar Canicola and Pyrogenes. However, Hae 111 digestion was more discriminative than Hind 111 digestion for differentiating *L. interrogans* from *L. borgpetersenii*. Thus, its use in *Leptospira* genotyping is limited which is in line with studies done globally (Kawabata et al. 2001). Therefore, we used a more discriminative 16S rDNA sequencing method. Phylogenetic analysis of *Leptospira* indicates the presence of three clades namely, the pathogenic serovars, nonpathogenic serovars and intermediate group. While the rrs primer is able to identify both pathogenic and intermediate *Leptospira* species, flaB primers amplify only the pathogenic strains of *Leptospira* (Agampodi et al. 2011, Boonsilp et al. 2011, Natarajaseenivasan

et al. 2012). In the current study, SLUSJ_111 gave a positive PCR with rrs, but was negative with the flaB PCR. This can occur as a result of an intermediate strain or due to varying degree of sensitivity of the two assays. In the blast search of the amplified rrs sequence of SLUSJ_ 111 revealed an identity of 99% with *L. interrogans*. However, there is still a possibility of this being an intermediate strain because in the current study only a segment of *rrs* gene was subjected to sequencing. Intermediate species of *Leptospira* such as *Leptospira broomii, Leptospira inadai, Leptospira licerasiae, Leptospira wollfi* and *L. fainei* has been reported to cause acute febrile illness (Levett 2001). However there is no documented report of intermediate strains causing leptospirosis in Sri Lanka thus far.

In this study *L. interrogans* strains were the most common cause of disease followed by *L. borgpetersenii* and *L.kirschneri* strains. Circulating *L. interrogans* strains showed a 100% similarity to the 2008 strain which was isolated from central province in Sri Lanka (Agampodi et al. 2011). The strains isolated in this study showed 100% similarity to *L. interrogans* which was found to be the predominant strain in the current study and had been reported in Sri Lanka in 2008 outbreak. This strain was identified as a highly virulent strain (Agampodi et al. 2013). Moreover it has been reported from China and the Andaman Islands and seems to be associated with both severe and nonsevere disease (Agampodi et al. 2013).

Among 14 confirmed leptospirosis patients, only 11 developed complications whilst four were managed in intensive care units. Renal failure was the most common (45%) complication seen in the current study as seen in 2008 study (Agampodi et al. 2011) (Table III). Further in the current study, *L. interrogans* was the main cause of renal failure followed by hepatic insufficiency and myocarditis. *L. borgpetersenii* and *L. kirschneri* were not detected in the 2008 outbreak, but they have been reported previously during the 1960s and in the recent past from human and animal sources in Sri Lanka (Brenner et al. 1999, Koizumi et al. 2009, Agampodi et al. 2011, 2014). However, circulation of *L. borgpetersenii* among humans has not been well documented previously although it has been found among dairy cattle (Gamage et al. 2014). Cattle may be the source of infection in these two patients.

This study was conducted in the western and southern provinces of Sri Lanka having a different climatic, geographical and socioeconomical conditions when compared to the previous studies done in central and mid central provinces. This study highlights the evolutionary pattern of circulating strains in different time frames in Sri Lanka. In conclusion, *L. interrogans* was the predominant circulating strain in western and southern provinces in 2013 in Sri Lanka. The current data will contribute to determining molecular epidemiological diversity both in Sri Lanka and globally.

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