

LEPTOSPIRA INFECTION AT THE UNIVERSITY OF PERADENIYA TEACHING HOSPITAL, SRI LANKA: CLINICAL AND LABORATORY INVESTIGATIONS

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Abstract. To help formulate a local intervention for leptospirosis in Sri Lanka, we determined the serogroups of leptospiral species among 97 patients diagnosed with leptospirosis at the University of Peradeniya Teaching Hospital, Sri Lanka. Ninety-two point eight percent of the patients were men; nearly two-thirds were ≥ 35 years old; the majority had secondary or higher education level, half were farmers or laborers; and 57.7% presented in the acute-phase of the illness. Twenty-five patients (25.8%) were confirmed to have leptospirosis by a positive laboratory method; 17 and 8 cases were confirmed with a positive test by quantitative MAT and nested PCR, respectively. Of the 17 MAT positive cases, infection occurred in a variety of serogroups, but the predominant groups were Sejroe and Tarassovi. Of the 8 nested PCR positive cases, 7 were seen among those with a MAT titer < 200 and 1 occurred in a patient with a MAT titer ≥ 200 but < 400 . Of the 8 PCR positive cases, 7 were infected with the leptospiral species *L. interrogans*. Approximately 26% of the clinically diagnosed patients were confirmed by the two laboratory methods. Laboratory positivity was based on the time of blood collection after the onset of fever. Further studies are warranted to refine the clinical diagnostic criteria and to develop more efficient and accurate diagnostic tests for leptospirosis in resource limited settings.

Keywords: leptospirosis, emerging infectious disease, epidemiology, febrile patients, Sri Lanka

INTRODUCTION

Leptospirosis is a globally important zoonotic, emerging infectious disease (Meslin, 1997; Levett, 2001; Katz *et al*, 2002; Meites *et al*, 2004; Jansen *et al*, 2005; Ellis *et al*, 2006; WHO, 2010) caused by spirochetes of the genus *Leptospira* comprised of over 200 serovars in 26 serogroups

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(Vinetz, 2001). A variety of wild and domestic animals are a natural reservoir for pathogenic *Leptospira* (Levett, 2001) which is transmitted to humans by having a direct contact with reservoir animals (eg, rats) or by being exposed to water or soil contaminated with the urine of the infected animals. Leptospire penetrate abraded skin or mucous membranes, enter the bloodstream and then spread throughout body tissues (Levett, 2001). Infection with *Leptospira* produces a wide range of clinical manifestations (Levett, 2001; Ellis *et al*, 2006). Early leptospirosis is characterized by fever, chills, headache, and myalgia; however, fever may be the only symptom in some cases (Faine, 1993; Farr, 1995). The infection can progress during the late "immune" phase to cause multiple systemic manifestations, such as hepatic dysfunction and jaundice, acute renal failure, pulmonary hemorrhage, myocarditis, and meningoencephalitis (Levett, 2001; Ko *et al*, 2009).

Little public health effort has been given to the control and prevention of leptospirosis, even though it places a great burden on impoverished populations in developing countries and in tropical regions (McBride, 2005). The variety of clinical presentations makes a clinical diagnosis challenging and the availability of diagnostic laboratory techniques is often low in resource limited settings (Faine, 1993; Levett, 2001). Despite these challenges, Sri Lanka has embarked on control and prevention efforts.

Since 1991, Sri Lanka has documented leptospira infections. It is one of the 28 notifiable diseases in the country. The cases are reported by the National Disease Reporting System and the Sentinel Surveillance System to the Epidemiology Unit of the Ministry of Health. The data are used to guide the control and prevention program.

In 2008, the incidence of leptospirosis in Sri Lanka was 35.7 per 100,000 population, with a case fatality rate of 2.9% (Epidemiology Unit, 2008). In Kandy District, where this study was conducted, the number of cases increased from 147 in 2007 to 501 in 2008 (Agampodi *et al*, 2009). The number of cases also increased in other parts of the country, including the western, southern and central provinces (Epidemiology Unit, 2008). Leptospirosis is a serious public health problem throughout Sri Lanka (Epidemiology Unit, 2008; Agampodi *et al*, 2009).

Although leptospirosis has increased in Kandy District, the number of recent studies evaluating the epidemiology of leptospirosis is small (Epidemiology Unit, 2008; Agampodi *et al*, 2009). Effective control and preventive measures must be based on accurate epidemiological factors, such as risk factors and modes of transmission (WHO, 2010). Clinical and epidemiological data are important in understanding the disease dynamics, but these must be conducted using evidence derived from laboratory examinations. There are a number of leptospirosis cases that have been reported to the Ministry of Health but few of these have laboratory confirmation. Laboratory confirmation is crucial since the clinical diagnosis is not always accurate (WHO, 2003). For control and prevention measures, accurate information on leptospirosis (eg, serogroups and infecting species) is needed.

This study evaluates evidence derived from clinical, epidemiological and laboratory investigations among patients with febrile illness at the University of Peradeniya Teaching Hospital, Kandy District, Sri Lanka. The purpose of the study was to identify circulating leptospiral species and serogroups through laboratory confirmed cases.

MATERIALS AND METHODS

Kandy District, Central Province, Sri Lanka has a total land area of 1,940 km² with a population of 1,279,028 and a population density of 667 /km². Kandy is 115 km east from Colombo, the capital, and is 465 m above sea level.

The University of Peradeniya Teaching Hospital (UPTH) is a tertiary teaching and referral center situated in Kandy District with 19 wards and 500 beds. Ten thousand patients are admitted to UPTH annually, of whom 10% are admitted due to fever. In the medical wards of UPTH patients were screened by attending physicians using a symptom check list during 1 April 2009 to 31 March 2010 for leptospirosis. The national criteria for a case definition of leptospirosis are the presence of fever, headache, myalgia, exposure to a contaminated environment, and/or contact with reservoir animals. Patients who had at least fever, headache and myalgia were included in the study and were subsequently diagnosed as having clinical leptospirosis. Ninety-seven patients were included in the study. Ethical approval for the study was obtained from the Committee on Research and Ethical Review, Faculty of Medicines, University of Peradeniya (clearance no. 009/EC/26).

After obtaining written informed consent from all participants, face-to-face interviews were conducted and 3-5 ml of blood was obtained from each participant. Demographic information and symptomatology were obtained by interview. The blood was sent to determine the presence of leptospirosis and the serotype.

The blood samples were centrifuged, then the serum was frozen and sent to the National Institute of Infectious Diseases in Tokyo, Japan, for serological analysis for leptospiral antibodies. We performed

the microscopic agglutination test (MAT) on all serum samples to detect leptospiral antibodies using a battery of representative leptospiral serogroups as described previously (Faine *et al*, 1999; Koizumi *et al*, 2009). Each sample was centrifuged at 13,000g for 20 minutes; the resultant pellets were used for DNA extraction with a DNeasy Tissue Kit (Qiagen, Valencia, CA). Extracted DNA was subjected to nested PCR to detect the leptospiral *flaB* gene (Koizumi *et al*, 2008). The nucleotide sequences of the amplicons were determined using the dideoxynucleotide chain termination method by means of the BigDye terminator V1.1 Cycle sequencing Kit (Applied Biosystems, Foster City, CA). The *flaB* sequences were aligned in a MEGA4 (Oliveria *et al*, 1995) with a CLUSTAW and the phylogenetic distance was calculated in the MEGA4 using the neighbor-joining method. The numbers on the nodes are the bootstrap support after 100 replicates.

Cases were classified as either laboratory-confirmed positives or confirmed negatives. Laboratory confirmation of leptospirosis was made using at least one of the following laboratory criteria: 1) a microscopic agglutination reciprocal titer equal to or greater than 400 from a single serum sample, or 2) the detection of the leptospiral *flaB* gene from extracted DNA samples (Oliveria *et al*, 1995; Koizumi *et al*, 2008).

The cut-off value for the MAT was set at ≥ 400 , based on previous studies (Koizumi *et al*, 2009; Herrmann-Storck *et al*, 2010) that utilized single serum samples.

RESULTS

Table 1 summarizes the sociodemographic profiles of the 97 suspected cases of leptospirosis. Ninety-two point eight

Table 1
Socio-demographic characteristics of patients clinically diagnosed with leptospirosis at the University of Peradeniya Teaching Hospital, Sri Lanka.

Characteristics	n=97	%
Gender		
Male	90	92.8
Female	7	7.2
Age (years)		
<35	36	37.1
≥35	61	62.9
Marital status		
Married	75	77.3
Single	22	22.7
Education level		
Low (less than secondary school)	37	38.1
High (secondary school or higher)	60	61.8
Occupation		
Farmer or laborer	48	49.5
Other	49	50.5
Monthly income (Rs) (USD1 = 111Rs)		
Low (<15,000)	54	55.7
High (≥15,000)	43	44.3
Residence in community (years)		
<35	51	52.6
≥35	46	47.4
Location of patient by district		
Kandy	79	81.4
Kegalle	8	8.2
Matale	5	5.2
Ratnapura	2	2.1
Nuwara Eliya	1	1.0
Polonnaruwa	1	1.0
Vavuniya	1	1.0

percent were men; 62.9% were aged ≥35 years. Half the suspected cases were farmers or laborers, while the other half belonged to a wide range of occupational categories (eg, students). Fifty-five point seven percent had a monthly income of ≤15,000 rupees (1USD = 111 rupees).

Table 2
Cases by MAT and nested PCR among patients clinically diagnosed for leptospirosis at the University of Peradeniya Teaching Hospital, Sri Lanka.

MAT (titer)	MAT positives	PCR positives
≥1,600	1	0
≥800	6	0
≥400	10	0
≥200	16	1
≥100	5	0
<100	10	0
Negative with MAT primary screening	49	7
Total	97	8

MAT, microscopic agglutination test; PCR, polymerase chain reaction.

Fifty-six of the suspected cases (57.7%) had an acute presentation, having experienced symptoms for less than seven days before presentation. Of the 97 suspected cases 17 (17.5%) had a MAT ≥400 for anti-*Leptospira* antibodies (Table 2). Twenty-one had equivocal results (a MAT titer of 100-400) and 59 had a negative result with the MAT. Fifty-six cases (5 of 17 (29.4%) MAT positives, 9 of 21 (42.9%) with equivocal results and 42 of 59 (71.2%) MAT negatives were obtained from acute samples (data not shown). The PCR test was positive in only 8 patients, all of whom had either equivocal or negative results with the MAT test. No positive PCR results were found among patients with positive MAT results.

Table 3 shows the distribution of the *Leptospira* serogroups. The predominant serogroups obtained were Sejroe (9/17, 52.9%) and Tarassovi (6/17, 35.3%). Three of the 17 MAT positive patients had multiple serovars.

Table 3
Distribution of *Leptospira* serogroup, serovar and strains by MAT among patients clinically diagnosed with leptospirosis at the University of Peradeniya Teaching Hospital, Sri Lanka.

Serogroup	Serovar	Strain	No. of samples by MAT titer ^a					No. of samples with MAT $\geq 400^b$
			≥ 100	≥ 200	≥ 400	≥ 800	$\geq 1,600$	
Sejroe	Sejroe	M84		5 ^c	3	4		7
Tarassovi	Tarassovi	Perepelitsin	1	4	4	1		5
Hebdomadis	Hebdomadis	Akiyami B		4	3	1		4
Pyrogenes	Pyrogenes	Salinem	3	3	3			3
Sejroe	Wolffi	3705	1	6	2			2
Cynopteri	Cynopteri	3522C	1			2		2
Icterohaemorrhagiae	Icterohaemorrhagiae	Ictero No.1	1		1			1
Canicola	Canicola	Hond Utrsch IV	2		1			1
Javanica	Javanica	Veldrat Batavis 46		2			1	1
Autumnalis	Autumnalis	Akiyami A	1	3				0
Australis	Australis	Akiyami C		3				0
Ballum	Castellonis	Castellon 3	1					0
Bataviae	Bataviae	Van Tienen	4	3				0
Icterohaemorrhagiae	Copenhageni	Shibaura	5	2				0
Panama	Panama	CZ214		3				0
Pomona	Pomona	Pomona		2				0
Grippotyphosa	Grippotyphosa	Moskva V	0					0
Sejroe	Hardjo	Hardjoprajitno	0					0

MAT: microscopic agglutination test; ≥ 400 identified laboratory confirmed positive cases.

^a Highest reactive serovars of each positive sample are described.

^b 3 of 17 positive samples had multiple serovars.

^c 1 of 5 were nested PCR positive.

The nested PCR test detected the leptospiral *flaB* gene in 8 of 97 serum samples, all of whom had equivocal or negative MAT results. Fig 1 shows the leptospiral species detected by PCR were: *L. interrogans* in 7 samples and *L. kirschneri* in 1 sample.

DISCUSSION

This study was carried out in Kandy District, Sri Lanka to obtain laboratory evidence of leptospiral infections, species

and serogroups. Our data are intended to help formulate strategies for the local control and prevention measures. Local data germane to control and prevention is sparse (Gamage *et al*, 2011a).

As in other developing countries (Evangelista *et al*, 2010), the number of reported leptospirosis cases in Sri Lanka has been rising, compelling the government to focus greater attention on control and prevention. Our research suggests leptospirosis is not as common as reported in Kandy District (Epidemiology Unit,

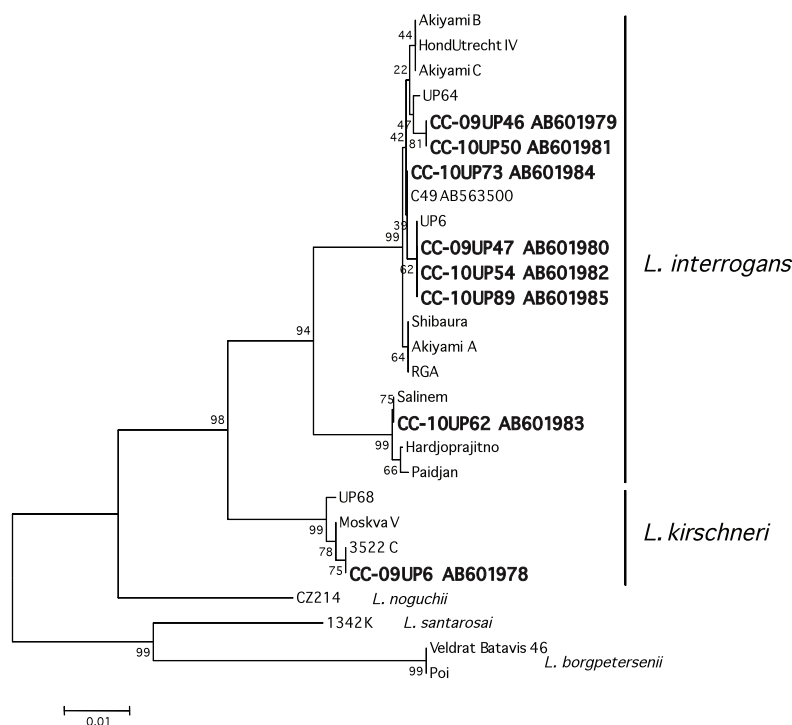


Fig 1—Phylogenetic tree based on the leptospiral *flaB* gene sequence. The sequences obtained in this study are indicated in bold type and have been deposited in the DDBJ/GenBank/EMBL with accession numbers as indicated. The sequences of UP6, 64 and 68 are derived from human serum samples from a previous study (Koizumi *et al*, 2009) and a cattle urine sample C49 (accession no. AB563500), derived from a recent study (Gamage *et al*, 2011a). The sequences were aligned in MEGA4 using CLUSTALW, and phylogenetic distances were calculated in MEGA4 using the neighbor-joining method. The numbers on the nodes are bootstrap support after 100 replicates.

2008). Only 97 of 6,987 admissions to UPTH during the study period (1.4%) had suspected leptospirosis. Of these, 57.7% were acute suspected cases, and only 25 (25.8% of suspected cases; 0.4% of total admitted cases) had laboratory confirmed leptospirosis.

The 2 diagnostic tests were employed to improve the specificity of the study results. The reason why nearly two-thirds of suspected cases had negative tests for leptospirosis is not clear but it is possible the clinical criteria for a probable case were too broad. Further studies need to be

carried out to evaluate the validity of the national case definition of leptospirosis.

Our serogroup findings (*ie*, Sejroe and Tarassovi) were similar to those of another study at same location (Koizumi *et al*, 2009). One study suggested cattle may act as a reservoir for leptospirosis in the same study area (Gamage *et al*, 2011b). All these studies revealed Sejroe was the most common serogroup among humans and in dairy cattle, suggesting dairy cattle may be the reservoir for human leptospirosis in Kandy. This information is useful for control and prevention efforts. Measures

geared toward dairy cattle owners may help to prevent transmission from animals to humans.

In our study 14 serogroups were found, indicating a wide array of leptospiral serogroups. Our data regarding leptospiral species, while limited, adds an important contribution to the knowledge regarding *Leptospira* in Kandy. In this study *L. interrogans* was the most common leptospiral species, similar to other studies in the same study area (Koizumi *et al*, 2009; Gamage *et al*, 2011a). However, the nucleotide sequence in our study was different from those among humans in another study (Koizumi *et al*, 2009) and cattle in one study (Gamage *et al*, 2011a) (Fig 1).

Blood samples were obtained within 7 days of fever onset in 56 of 97 clinically diagnosed cases. All 8 PCR-positive cases had MAT titers <400. Of the 8 PCR positive cases, 6 were collected within 7 days of fever onset and 2 were collected after 7 days of fever onset. These findings suggest PCR to detect the *flaB* gene may be capable of detecting "convalescent" phase samples among patients diagnosed with leptospirosis.

Positive MAT results also depended on time of specimen collection after fever onset, 8.9% being detected during the acute phase and 29.3% during the chronic phase. Further studies are needed to validate these findings using paired sera. The choice of a diagnostic test is vital to determining the epidemiology of leptospirosis in Sri Lanka.

The diagnosis of leptospirosis in Sri Lanka is primarily clinically based due to limited resources and inadequate laboratory facilities. However, the clinical presentation of leptospirosis is similar to other diseases in Sri Lanka, such as dengue fever (Kanakaratne *et al*, 2009)

and hantavirus infections (Gamage *et al*, 2011b). Reliance on clinical presentation alone hampers the validity of the epidemiological data. Therefore, it is essential to have laboratory confirmation of leptospirosis (Epidemiology Unit, 2008).

It is important to develop better diagnostic methods for leptospirosis, with greater sensitivity and specificity, reasonable cost and ease of use in laboratories in developing countries.

Our findings highlight some of the problems outlined above. Our clinical and epidemiological data were derived from purposively selected cases. Additionally our laboratory results regarding serogroups may not necessarily reflect the local serovars in Kandy since we do not know if the battery of antigens used for the MAT cover the serogroups in this district. Future studies need to address these limitations.

ACKNOWLEDGEMENTS

The authors are thankful to the director of the University of Peradeniya Teaching Hospital, Dr MGP Samarasinghe for the technical assistance provided during the study. The logistic support provided by Dr Y Suzuki, Department of Global Epidemiology, Research Center for Zoonosis Control, Hokkaido University is duly acknowledged. The authors would also like to thank Dr A Arai, Department of Global Health and Epidemiology, Hokkaido University Graduate School of Medicine, for support with data management.

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