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Research Article

Leptospira Reservoirs among Small Mammals in Sri Lanka

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Abstract

Leptospirosis is a zoonotic disease with worldwide importance. In Sri Lanka, confirmed leptospirosis cases are ever increasing. Humans contract leptospirosis through *Leptospira* infected urine-contaminated environments. While all mammals are capable of being reservoirs for this disease, feral and peridomestic rodents are known to play an important role. The objective of this study was to identify small mammal species carrying *Leptospira* in Sri Lanka. Small mammals were captured from eight localities with high leptospirosis prevalence according to hospital records in Kurunegala district. Blood, urine and kidneys were tested for lipL32 gene of pathogenic *Leptospira* using quantitative polymerase chain reaction. Of the 131 small mammals belonging to six species collected, 14.5% had *Leptospira* positive blood, urine or kidney. *Bandicota bengalensis*, *Mus cervicolor*, *Rattus rattus* and *Suncus murinus* were *Leptospira* carriers. *Leptospira* was previously reported only from *R. rattus* and *B. bengalensis* from the country, *M. cervicolor* and *S. murinus* are new carrier reports for Sri Lanka. All sampled locations had infected small mammals, indicating that the reservoirs are widespread in the district. This data could be used to control the disease and to encourage the public to take preventative measures against infection.

Keywords: Leptospirosis; *Suncus murinus*; Murine rodents; Shrews

Abbreviations

qPCR: quantitative Polymerse Chain Reaction; ELISA: Enzyme Linked Immunosorbent Assay; MAT: Microscopic Agglutination Test

Introduction

Leptospirosis has become a major threat to human health world over. Though it is most common in tropical and subtropical regions, reports of disease incidents are ever increasing from the developed world as well [1,2]. Humans contract leptospirosis through *Leptospira*-contaminated environments, bacteria entering the body through abraded skin or mucous membranes. Although Leptospirosis can be treated effectively with antibiotics, delay in diagnosis may result in serious complications that even lead to death. Most commonly, *Leptospira* infections cause asymptomatic seroconversion, but may result in severe disease conditions with jaundice, renal failure, hemorrhage, refractory shock, and myocarditis [3]. Leptospirosis is known to be an occupational disease, commonly occurring among farmers, but reported from veterinarians, abattoir workers and fishermen [4]. The disease has spread to urban areas with unhealthy sanitary practices and poor garbage disposal facilities. Recreational activities such as water sports have also been recognized as a risk factor for this disease in the recent years [1,5].

In Sri Lanka, first confirmed case of leptospirosis was reported in 1959 [6]. Since then, confirmed cases were reported from many districts in the country [7]. Leptospirosis has been reported as a major public health problem from Kurunegala, Kandy, Matele, Rathnapura, Gampaha, Matara and Kegalle districts [7]. The last three outbreaks have been reported in 2003, 2008 and 2011, 2008 outbreak being the worst ever reported from the country and the second highest leptospirosis incidence in the world during recent years [8]. In 2011, disease outbreak also extended to Anuradhapura, a region that is relatively dry and where leptospirosis is never heard of before [9]. Peak incidence of the disease is reported to be associated with the rice paddy-harvesting seasons, wherein an increase in the rodent population is observed. The majority of patients are also farmers who had been exposed in the paddy fields [4].

Animal reservoir of leptospirosis is a key factor concerning the disease control and prevention, however little attention has been given to study the reservoir animals. While any mammal is capable of carrying *Leptospira* [10], animals that live in close association with humans such as rodents, wild boars and the domesticated animals such as dogs, cattle and pigs play a major role in transmitting the disease to humans [11,12,13]. Many species of murine rodents are recorded as carriers of this pathogen around the world [14,15]. Due to their wide distribution and high abundance in rural areas with farmlands and in urban areas with high density of human population, feral and peridomestic rodents are believed to be most important reservoirs. In Sri Lanka *Leptospira* is reported only from three small mammal species; murine rodents, *B. bengalensis*, *R. rattus*, and shrew *Suncus* sp. [12,16].

The objective of this study was to identify murine rodents and shrews carrying *Leptospira* in and around paddy fields in localities with reported high prevalence of leptospirosis in Kurunegala District.

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Table 1: Positive Blood, urine and kidney samples collected from eight localities in Kurunegala district.

Locality	Small mammal species	Ct values for positive samples		
		Blood	Urine	Kidney
Kiwlegedara (07˚23'N, 80˚12'E, elevation 75m)	*Bandicota bengalensis*		36.046	
	Suncus murinus		35.886	
Malliyagoda (07˚24'N, 80˚28'E, elevation 170m)	*B. bengalensis*		35.198	
	S. murinus		27.845	18.531
Udawela (07˚33'N, 80˚02'E, elevation 40m)	*Mus cervicolor*	34.694		35.1
	Rattus rattus	34.861		
		34.937		
			27.106	
			27.943	
Polgahawela (07˚19'N, 80˚17'E, elevation 75m)	*R. rattus*	35.084		
Ipalawa (07˚34'N, 80˚27'E, elevation 145m)	*R. rattus*		35.106	
		36.835		
Bogollagama (07˚47'N, 80˚10'E, elevation 80m)	*R. rattus*		25.67	
			31.826	
Herathgama (07˚52'N, 80˚25'E, elevation 155m)	*R. rattus*		35.612	
		33.662		
Minhettiya (07˚35'N, 80˚18'E, elevation 100m)	*S. murinus*		32.208	33.321
				23.571
	R. rattus			33.559

Materials and Methods

Small mammals were trapped from eight sites in Kurunegala District (Table 1). Traps were placed in and around paddy fields. Sampling was carried out for 4-6 days at each collection site. A sample of voided urine (500-1200 µL/ 100-500 µL from *M. cervicolor* and *S. murinus*) and a sample of blood (100-300 µL) from saphenous vein were collected from each small mammal captured. Ethical clearance for sample collection and handling was obtained from the ethical clearance committee of the Postgraduate Institute of Science, Peradeniya, Sri Lanka. DNA was extracted from blood cell pellets, collected by centrifugation at 12000rpm for 10min, using Wizard® Genomic DNA Purification Kit, according to the manufacture's protocol with few modifications. Volume of each blood pellet was adjusted to 300µl by adding PBS. Following modifications were done to the original protocol; Step 3: After adding cell lysis solution samples were centrifuged at 14,000rpm for 1min.; Step 6: samples were incubated at 80˚C for 5min. in nuclei lysis solution; Step 15: samples were kept at room temperature over night to rehydrate the DNA after adding DNA rehydration solution; Step 16: DNA Samples were stored at -20˚C. Sample processing and DNA Extraction from blood and urine samples were done within one week of collection. Urine samples were pelleted by centrifugation at 12000rpm for 20min at room temperature, pellet was washed once with PBS. DNA was extracted from the pellet using "QIAamp DNA Mini Kit™" (Qiagen Sciences, Maryland, USA) according to manufacturer's protocol for "Isolation of bacterial DNA from biological fluids". Two hundred and forty two BP fragment of lipL32 gene present only in pathogenic *Leptospira* spp. [17] were amplified in Step one Real Time PCR system (Applied Biosystems) using Go Taq probe qPCR master mix (Promega) and Taq Man probe. Primers and probe used were; Forward-48F (5' -AAG CAT TAC CGC TTG TGG TG-3'), Reverse-286R (5' -GAA CTC CCA TTT CAG CGA TT-3') and the Probe-189P (FAM-5' -AA AGC CAG GAC AAG CGC CG-3' -BHQ1). Kidneys were collected only from accidental kills. DNA from kidneys was also extracted employing the same method used for blood pellet. Quantitative PCR mixture consisted of 13µl of Go taq probe qPCR master mix, 2µl of 6.25µM forward and reverse primer and 1µl of 2µM probe, 4.5µl of nuclease free water and 2.5µl of template. Final volume of the PCR mixture was 25µl. The thermal profile of the assay composed of initial holding temperature of 60˚C for 30s for Pre PCR read and 95˚C for 5min for heat activation, 45 cycles of amplification at 95˚C for 15sec and 60˚C for 1min and final post PCR read stage of 60˚C for 30s. The reactions were performed in Step one Real Time PCR System (Applied Bio systems, USA) and analyzed using Step One software version 2.2.2. All samples were tested in duplicates and an additional run was performed for samples with one positive or one negative result. Samples, which gave positive results with Ct values less than 40 were considered positive and the smaller Ct values of the two positives were used in analysis. A standard curve was generated using tenfold dilution series of 200µl DHL vaccine (Merial) extracted and eluted to 200µl. According to the standard curve we report positives with 88.1% efficiency, R^2=0.99, slope=-3.6, Y intercept=40.248.

Results

A total of 131 small mammals: *Rattus rattus* (98), *Bandicota indica* (9), *B. bengalensis* (7), *Mus cervicolor* (4), *Mus musculus* (2) and *Suncus murinus* (11) were collected. Fourteen point five percent (19/131) of small mammals had *Leptospira* positive blood, urine or kidney. Of these 5% (6/120) blood, 9% (11/117) urine and 28% (5/18)

kidney were positive for *Leptospira* spp. Of the small mammals, 29% (2/7) *B. bengalensis*, 25% (1/4) *M. cervicolor*, 12% (12/98) *R. rattus* and 36% (4/11) *S. murinus* were positive for any of the samples. *Bandicota indica* and *Mus musculus* were not infected. Least Ct value was from a kidney sample (18.531) collected from a *S. murinus* indicating a high infection. Urine from same *S. murinus* and 4 other *R. rattus* specimens had Ct values between 25.0-28.0, while rest of the samples had Ct values between 30.0-37.0 (Table 1). Of the sampled locations, all had at least one infected small mammal, with Udawela having the highest number. None of the specimens were positive for both blood and urine but there were 3 kidney positives with blood or urine positives. Two samples were only positive for kidney. For 9 small mammals with all three samples available, 6 were negative for all 3 sample types, 2 kidneys were positive along with urine and 1 kidney was positive along with blood.

Discussion

Evaluation of gold standard testing for detection of *Leptospira* have defined qPCR as the most sensitive test for the diagnosis of leptospirosis over other tests such as Enzyme Linked Immunosorbent Assay (ELISA) and Microscopic Agglutination Test (MAT) [18,19]. Hence, we used qPCR to detect *Leptospira*. This is the first attempt to use qPCR for detection of *Leptospira* in reservoir hosts in the country.

Here we report 29% (2/7) *B. bengalensis*, 25% (1/4) *M. cervicolor*, 12% (12/98) *R. rattus* and 36% (4/11) *S. murinus* positives with an overall infection of 14.5%. Another single study reported small mammal reservoirs in Sri Lanka, where they used MAT for serum and conventional PCR for kidney samples [16]. They reported 17.5% (13/74) serum samples collected from rodents [20.3% (11/54) of *B. bengalensis* and 10.0% (2/20) of *R. rattus*] positive for anti leptospiral antibodies but zero positives for kidney samples. Though prevalence of the pathogens is similar in the two studies they cannot be directly compared due to the different testing methods used.

Mus cervicolor and *S. murinus* are not reported as *Leptospira* carriers from Sri Lanka before. Outside Sri Lanka, *Leptospira* are reported from many rodent and shrew species [20]. *Rattus rattus*, *R. norvegicus*, *B. indica*, *B. bengalensis*, *M. musculus*, *M. cervicolor* and shrew *S. murinus* are among them, which also occur in Sri Lanka [8,9,21,22]. None of the *B. indica* or *Mus musculus* collected during this study were infected. We intended to sample small mammals nondestructively, hence kidney samples were collected only from accidental kills. From the small mammals with all three types of samples (blood, urine and kidney), it can be deduced that kidney is the best sample to detect the presence of *Leptospira* in small mammals. However, it is equally effective if both urine and blood are tested.

Detecting *Leptospira* in reservoirs is an effective environmental monitoring method [23]. In the present study, all sampled localities had *Leptospira* infected small mammals, indicating that the pathogen is widespread in the Kurunegala district. Since leptospirosis is a life threatening disease, prevention of infection is most important. This data could be used by public health authorities to control the disease by increasing public awareness of the disease and encouraging them to take preventive measures against infection.

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