Studies on rodent-borne zoonotic diseases circulating among small mammals and humans in urban and rural settings in Sri Lanka: hantavirus infection and leptospirosis

(スリランカ都市部および農村部で小型哺乳類とヒトの間で循環しているげっ歯類媒介性人獣共通感染症に関する研究：ハンタウイルス感染症とレプトスピラ症)

Muthusinghe Bungiriye Devinda Shameera

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Abbreviations

adk	adenylate kinase
ANJZV	Anjozorobe virus
BHK	baby hamster kidney
BLAST	basic local alignment search tool
cDNA	complementary deoxyribo nucleic acid
CKDu	chronic kidney disease of unknown etiology
cytb	cytochrome b
DNA	deoxyribo nucleic acid
DOBV	*Dobrava orthohantavirus*
EP	end-point
ER	endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
flaB	flagellin b
fmol	femto-mol
G	gamma distribution
GFP	green fluorescent protein
GP	glycoprotein
GPC	glycoprotein precursor
GTR	general time reversible
HCPS	hantavirus cardiopulmonary syndrome
HEK	human embryonic kidney
HFRS	hemorrhagic fever with renal syndrome
HTNV	*Hantaan orthohantavirus*
I	invariable sites

icdA	isocitrate dehydrogenase
IgG	immunoglobulin G
IU	infectious unit
km	kilometer
L	large
lipL32	lipoprotein L32
lipL41	lipoprotein L41
M	medium
mAb	monoclonal antibody
MEM	minimum essential medium
mg	milligram
mL	milliliter
µg	microgram
µL	microliter
MUSCLE	multiple sequence comparison by log-expectation
N	nucleocapsid
NE	nephropathia epidemica
OPD	o-phenylenediamine dihydrochloride
ORF	open reading frame
pAb	polyclonal antibody
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PED	pairwise evolutionary distance
PHV	*Prospect-Hill orthohantavirus*
PUUV	*Puumala orthohantavirus*

RdRp	RNA-dependent RNA polymerase
rGn	recombinant glycoprotein Gn
rGP	recombinant glycoprotein
rN	recombinant nucleocapsid
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
rrs2	16S ribosomal RNA
rVSV	recombinant vesicular stomatitis virus
S	small
SANGV	*Sangassou orthohantavirus*
secY	protein translocase subunit secY
SEOV	*Seoul orthohantavirus*
ST	sequence type
THAIV	*Thailand orthohantavirus*
THAIV-IFA	*Thailand orthohantavirus*-indirect immunofluorescent assay
TPMV	*Thottapalayam thottimvirus*
trN	truncated recombinant nucleocapsid

Notes

This thesis contains three chapters, and the first chapter has been published as follows.

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Preface

Zoonotic diseases are recognized as an important aspect of public health worldwide. They are spread all over the globe with different dynamics and patterns. There is a diverse collection of different zoonoses caused by numerous pathogens representing both unicellular and multicellular organisms and also viruses. Zoonotic pathogens have put the lives of both humans and animals at stake by causing illnesses of different magnitudes, while the associated economic burdens are also immense. Therefore, the controlling of zoonoses have become a hot topic among the scientific community, with one-health concept has proven to be one of the best approaches to achieve the ultimate goals in zoonotic infection control.

Hantavirus infection and leptospirosis are two such important zoonotic diseases caused by a bunyavirus and a spirochete bacterium respectively. Both infections were classically identified as rodent-borne zoonoses. But, with the current knowledge, it is evident that pathogenic *Leptospira* could be harbored and transmitted by a wide range of mammals between each other and to humans. Leptospirosis has been described as the most wide-spread zoonosis in the world due to its incidence in mammals and humans mainly in tropics across almost all the continents apart from polar regions. Over 250 serovars belonging to many serogroups in pathogenic and intermediate pathogenic *Leptospira* species could cause the disease in humans and animals. The adverse economic burden mainly occurs due to the disease in livestock animals, which can cause abortions, still-birth, reduction in produce and death.

On the other hand, hantavirus infection could be considered as a rodent-borne zoonosis as human pathogenic hantaviruses are solely carried by rodents. The initial symptoms caused by hantavirus infection is similar to the symptoms seen in other infections causing febrile illnesses, making it harder to diagnose clinically at its early stages.

Therefore, hantavirus infections are largely misdiagnosed or underdiagnosed specially in tropical regions where similar symptom causing infections such as leptospirosis and Dengue virus infection are present.

Sri Lanka is a such tropical country with an agricultural and export-based economy. The agricultural communities are usually affected by poverty along with low living standards over the last century due to reasons such as lack of government support, not getting fair prices for their products, etc. More recently, over the last 30 years, Sri Lankan agricultural communities residing in the dry zone of the country are experiencing the spreading of a devastating renal disease having no established etiology. The Sri Lankan and international research community has been working on the quest of finding the possible etiologies and risk factors associated with this renal disease. Serological evidence of hantavirus infection has been found in these regions providing the evidence of association of it with the renal disease in humans. On the other hand, both hantavirus infection and leptospirosis has been documented in the urban regions in the country mostly associated to the domestic transmissions.

Regarding the above situations, this study aimed at providing detailed molecular and serological evidence from humans and small animals on these two infections in several different regions in the country. Under this objective, this thesis covers three topics divided into three chapters. Chapter 1 describes the identification of novel hantavirus species from rodents captured from an area endemic for the above-mentioned renal disease; chapter 2 focuses on developing specific diagnostics based on the Sri Lankan hantavirus genomes for the definitive serotyping of hanta virus infection among humans in several such endemic regions. Third chapter is related to the serological and molecular detection of *Leptospira* infection and hantavirus infection in small mammals and humans associated to an urban market in the country.

Chapter I

Identification of novel rodent-borne orthohantaviruses in an endemic area of chronic kidney disease of unknown etiology (CKDu) in Sri Lanka.

Introduction

A previously unexplained form of renal disease, referred to as a chronic kidney disease of unknown etiology (CKDu), has been increasingly diagnosed over the past three decades in dry zone areas of Sri Lanka, becoming an overwhelming public health burden [1]. This disease has become more prevalent among rural agricultural communities [2], where males are more often affected than females [3]. Affected individuals show no symptoms until the disease progresses into its late stages. Areas in 13 out of 25 districts in the country have been identified as high-risk regions for the occurrence of CKDu. North Central Province alone has reported approximately 20,000 CKDu patients with a population prevalence rate of 4.7% [4]. The scarcity of recent incidence data has made it difficult to understand the current prevalence of CKDu in the country. Moreover, despite many studies conducted over the past few decades, the etiology of CKDu remains obscure.

Hantaviruses are a group of zoonotic pathogens belonging to the family *Hantaviridae* of the order *Bunyavirales*. The spherical enveloped viral particles consist of a tri-segmented negative-strand RNA genome. The large (L), medium (M), and small (S) genome segments encode an L-protein, a glycoprotein precursor (GPC) of two envelope glycoproteins Gn and Gc, and a nucleocapsid protein (N), respectively [5]. Hantaviruses currently have a diverse host range, with rodents, shrews, moles, and bats being the common hosts. Interestingly, all medically important human pathogenic hantaviruses are carried by rodent hosts [6]. Hemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus cardiopulmonary syndrome (HCPS) represents two severe forms of human infections caused by hantaviruses. HCPS shows a higher fatality rate (25–35%) than HFRS in Asia (5–15%) [7]. East Asia accounts for approximately 90% of HFRS cases caused by Old World orthohantaviruses, such as

the *Hantaan orthohantavirus* (HTNV) and *Seoul orthohantavirus* (SEOV) [8]. Southeast Asia, South Asia, and the Indian oceanic region are home to the *Thailand orthohantavirus* (THAIV) [9] and its genetic variants (the Anjozorobe (ANJZV) [10], Serang [11], Jurong [12], and Mayotte [13] viruses). The pathogenicity of these viruses remains unexplained. Although several sero-epidemiological reports have described human infections involving THAIV in Thailand, India, and Sri Lanka [14–16] and ANJZV in Madagascar [17], there are no confirmed clinical cases of HFRS or HCPS documented in South Asia or Southeast Asia. Epidemiological information on hantaviruses and their hosts is limited, particularly in South Asian countries [8].

Hantavirus infection was first documented in Sri Lanka as early as 1988 by Vitarana and colleagues [18]. Since then, very few reports have been published on individuals with suspected leptospirosis who have been found to possess anti-hantavirus antibodies [19,20]. It was recently reported by Gamage et al. that 72 (54.5%) out of 132 CKDu patients from the CKDu endemic area of Girandurukotte, Sri Lanka harbored antibodies against hantaviruses [21]. The existence of THAIV- or THAIV-related hantavirus infections was confirmed by serotyping 89 anti-hantavirus antibody-positive human serum samples obtained from the same area [22]. Similarly, high levels of antibodies against the hantavirus were reported among CKDu patients from a CKDu hotspot in Polonnaruwa District in the North Central Province of Sri Lanka [23]. In addition, a cross-sectional study carried out with case-control comparisons in two geographically distinct CKDu endemic areas vs. a nonendemic area in Sri Lanka demonstrated that exposure to the hantavirus was an independent risk factor associated with renal disease in the CKDu endemic regions [24]. An ecoepidemiological study in Girandurukotte serologically confirmed that THAIV-like hantavirus species were highly prevalent among the *Rattus rattus* lineage [25].

Serological findings from both humans and rodents in the CKDu areas supported the hypothesis that exposure to hantaviruses is a risk factor for the possible development of CKDu in Sri Lanka [26]. However, no studies have provided the genomic evidence from hantavirus rodent hosts circulating in Sri Lanka. Viral genomic information is essential in developing specific diagnostics to detect hantavirus infections in CKDu patients. The results will add further insights into the relationship between exposure to a hantavirus and CKDu etiology. Therefore, the current study aimed to address this knowledge gap. Hence, this report describes a genetic analysis of small mammals captured from a CKDu endemic area in Sri Lanka to determine the hantavirus species and possible natural hosts.

Materials and Methods

Sample collection

Small mammal samples were collected in September 2018 and July 2019 from the Polonnaruwa, Welikanda, and Sinhapura areas in Polonnaruwa District, where CKDu is highly prevalent (Figure 1). The study protocol was approved by the Ethics Committee of the Faculty of Veterinary Medicine and Animal Sciences of the University of Peradeniya, Sri Lanka (VER-16-007). In September 2018, rodent trapping was performed using cage-type traps to capture the first 98 rodents. Most of the traps used in July 2019 were Sherman traps (H. B. Sherman Traps, Inc., Tallahassee, FL, USA), and 18 additional rodents and shrews were collected. The captured species were initially identified based on their morphology. The animals' body weight, sex, and other body parameters were recorded. The lungs, liver, kidneys, and blood samples from a heart puncture were collected from each animal. Parts of the lung and kidney tissues were preserved in RNAlater (Qiagen, Hilden, Germany), and a portion of the kidneys were preserved in 99.5% ethanol (Sigma-Aldrich, Burlington, MA, USA).

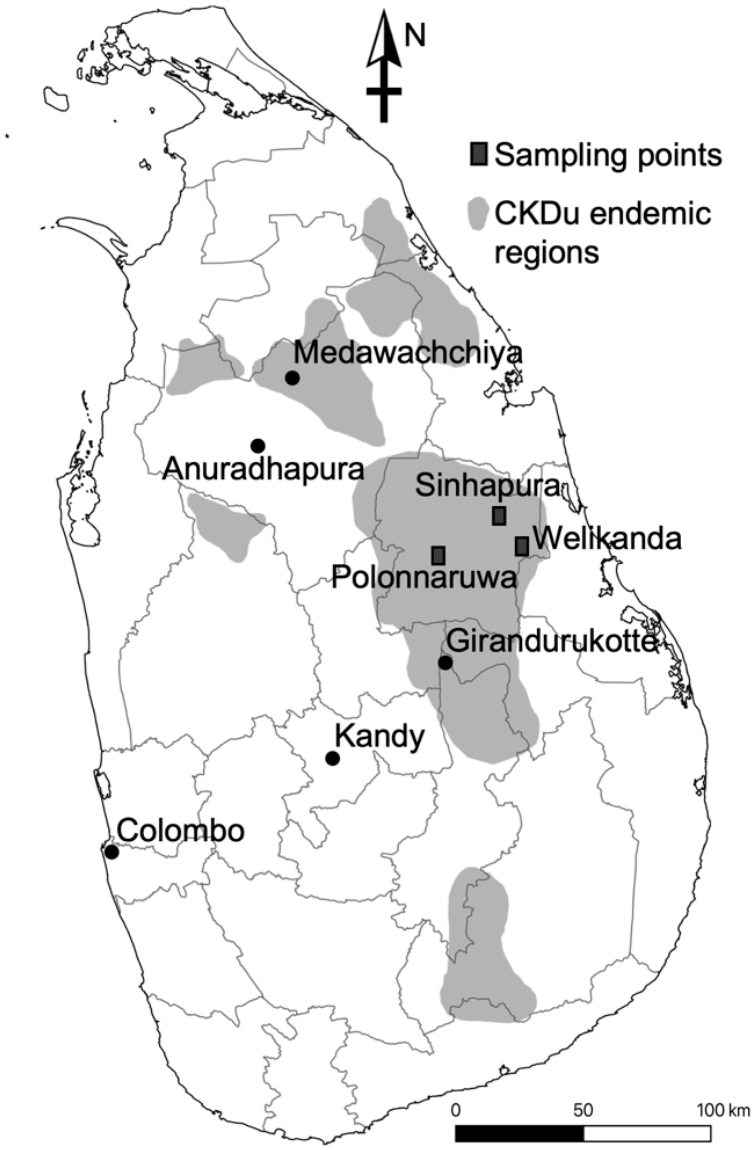


Figure 1. Map of Sri Lanka showing the CKDu endemic regions and sampling points of the study.

DNA extraction and rodent species identification

The DNA was extracted from small mammal kidney tissues preserved in ethanol using the DNAzol reagent (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's instructions. PCR was performed on kidney DNA samples to amplify a mitochondrial cytochrome *b* (*cytb*) gene using AmpliTaq Gold 360 DNA polymerase (Applied Biosystems, Life Technologies, Warrington, UK) and the primers L14115, H15300, L497A, and H655A [27,28]. The PCR program consisted of 10 min of initial denaturation at 95 ºC; 35 cycles of 95 ºC for 30 s, 55 ºC for 30 s, and 72 ºC for 30 s; and a final extension at 72 ºC for 7 min. The nucleotide sequences of the amplified *cytb* fragments were determined using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and a 3130xl Genetic Analyzer (Applied Biosystems).

Indirect Immunofluorescence Assay (IFA)

Anti-hantavirus IgG antibodies were detected in small mammal sera using IFAs based on antigens from THAIV-infected and recombinant THAIV N protein-expressing Vero E6 cells, as described elsewhere [29]. Alexa Fluor 488-conjugated goat anti-rat IgG (for rat and *Bandicota* sera), anti-mouse IgG (for mouse sera) (Invitrogen), and protein A (for shrew and gerbil sera) were used as the secondary antibodies. Each serum sample was diluted 1:100 in PBS. Scattered granular immunofluorescence patterns in the cell cytoplasm were considered to indicate positive staining.

RNA extraction, cDNA synthesis, and hantavirus screening PCR

RNA extraction was performed from lung and kidney tissues of all the small mammals preserved in RNAlater using the RNeasy Plus mini kit (Qiagen) following the

manufacturer's instructions. cDNA synthesis from the total RNA was carried out using the SuperScript IV VILO Master mix (Invitrogen). All lung cDNA samples were screened by PCR using AmpliTaq Gold 360 DNA polymerase and degenerate primers [30] targeting a conserved domain of the L genome segment of hantaviruses. The HAN-L-F2 (5′-TGCWGATGCHACIAARTGGTC- 3′) and HAN-L-R1 (5′ AACCADTCWGTYCCRTCATC-3′) primers were used for the first round, followed by hemi-nested amplification using the HAN-L-F2 and HAN-L-R2 (5′-GCRTCRTCWGARTGRTGDGCAA-3′) primers. Both amplification reactions included 10 min of initial denaturation at 95 °C; 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 7 min. Amplified PCR products with correct sizes were purified and sequenced as described previously.

Genomic sequencing

All the screening PCR-positive samples were selected for hantavirus whole-genome sequencing via either the primer walking method or Illumina MiSeq sequencing. In the primer walking method, the primers were designed for all three genomic segments based on the initial sequences obtained in this study and previously published Muridae-borne hantavirus sequences (Table 1-3) and were used to amplify segments of the genome, not including the termini. The PCR products were gel-purified and sequenced by Sanger sequencing, as described above.

For the Illumina MiSeq analysis, the RNA fractions extracted from lung tissues, as described above, were treated with the Ribo-Zero rRNA removal kit (Illumina, San Diego, CA, USA) to deplete host-derived rRNA. The treated RNAs were employed to construct sequencing libraries using the KAPA RNA HyperPrep kit (for Illumina) and the KAPA Dual-Indexed adapter kit (KAPA Biosystems, Wilmington, MA, USA).

Twenty-four libraries and other nonrelated samples were mixed in equal amounts to obtain 9 fmol of a MiSeq library, which was then sequenced on the Illumina MiSeq platform using the MiSeq reagent kit v3 (Illumina) with 2×300-bp paired-end read lengths.

Since there is no reported complete sequence of the prototype THAIV L segment available for the whole-genome comparison, the entire L segment sequence of THAIV strain-749 (LC553715) was determined using the cDNA of the virus. The primer walking method was carried out using degenerate primers designed as described above (Table 3), and the amplicons were sequenced by Sanger sequencing, as described previously. To complete the terminal sequences, the RACE method was applied as previously described [31] using the adapter sequences [31] and specific primers shown in Table 3.

Sequence alignment and phylogenetic analysis

The sequences obtained via Sanger sequencing were manually edited and aligned with reference genome sequences obtained from DNA databases. At the same time, the MiSeq reads were mapped onto reference genomes using GENETYX-MAC version 20.1.0 (Genetics Co., Ltd., Tokyo, Japan). The full-length sequences obtained from the S, M, and L segment ORFs aligned with representative sequences from other Muridae-borne hantaviruses using MUSCLE, as implemented in Geneious Prime 2020.2.2 (Biomatters, Ltd., Auckland, New Zealand). Multiple sequence alignments were edited and used to construct Bayesian phylogenetic trees using the MrBayes 3.2.6 [32] plug-in of Geneious Prime 2020.2.2 with the GTR+G+I substitutional model. Consensus cladograms were constructed using viral N protein amino acid sequences, and host *cytb* sequences were compared for the degree of

concordance using Dendroscope V3.7.2. [33] to describe the coevolutionary relationships between the hantaviruses and hosts identified in this study, along with other representative rodent-, mole-, shrew-, and bat-borne hantaviruses and their hosts.

Quantification of Viral RNA

Whole-genome-positive rodent lung and kidney cDNAs were subjected to a quantitative real-time PCR analysis. For the *Mus* cDNA samples, primers LANS_F (5′-GAGAGCATGCCAGGGGTGCAGG-3′) and LANS_R (5′-GTAGGTGGACACCTATCAGGAGC-3′) were used. For the *R. rattus* cDNA samples, primers SA108S_F (5′-GATCATGCTAGGGATGCTGG-3′) and SA108S_R (5′-GTAGGAGGACACCGATCAGGTGC-3′) were used, with the KAPA SYBR FAST qPCR master mix (KAPA Biosystems) and a Light Cycler 480 instrument II (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions.

Results

Animal species identification

Morphological identification showed that the most (99/116) of the captured small mammals were *Rattus rattus*. An analysis of the *cytb* sequences from several animals confirmed that they belonged to lineage Ib, a Sri Lankan endemic lineage of *R. rattus* [25,28]. Eleven animals were identified as *Mus booduga* (Little Indian field mouse) after analyzing the *cytb* sequences (Figure 2). We identified two clusters of *M. booduga* sequences in the phylogeny, which differed from the *M. booduga* sequences from India and Nepal. The other rodent and shrew species captured in this study were *Tatera indica* (Indian Gerbil) (n = 3), *Bandicota bengalensis* (n = 1), *Bandicota indica* (n = 1), and *Crocidura horsfieldii* (n = 1) (Table 4).

Table 1. Lists of primers used for the amplification and sequencing of S segment of Lanka virus and SA108 virus.

Specificity	Primer name	Sequence (5'-3')
Lanka virus specific primers	Lanka S607F	ATGAAAGCTGAGGAAATAACCCCAGG
	Lanka S620R	TCCTCAGCTTTCATGCTTGACTGGGC
	Lanka S682F	AGGAATATGGTCAGTCCAGTGATGAG
	Lanka S707R	CTCATCACTGGACTGACCATATTCCTAGC
	Lanka S1193F	TGTTTATGGTCTCCTGGGGAAAGGAAGCAG
	Lanka S1228F	AATTTTCACTTGGGAGATGACATGGACCC
Muridae-borne and THAIV-like primers	Muridae_hanta S1F	TAGTAGTAGICTICSTRAARAGCTAC
	Muridae_hanta S43F	AAAATGGCAACIATIGARGARITMC
	Muridae_hanta S370F	ATTGAIGARCCIACAGGMCARACIGC
	Muridae_hanta S974R	CCWGCAAAIACCCAIATYGAIGAIGG
	Muridae_hanta S1226R	TCIACWGCCTCYTTICCCCAIKCAACC
	Muridae_hanta S1322R	GGCTCITGRTTIGAIATYTCYTTIAC
	Muridae_hanta S1549R	AACTTAAYTAATTAACTTRATTAGTYAAC
	Muridae_hanta S1619R	ATATGGCAGTGATAATAATCAGTAG
	Muridae_hanta S1730R	GATCAAWTGAWATCTACATCCATATATAC
	Muridae_hanta S1887R	TAGTAGTAGTAKRCTCCCTAAARAGAC
	THAIV-like S416F	TCTAYYTGACATCITTTGTIATMCC
	THAIV-like S454F	GCACTGTAIATGYTIACRACRAGAGG
	THAIV-like S508F	ATGAGAATIMGITTYAAGGATGAYAG
	THAIV-like S562F	AAGCCAAARCAYCTITAYATTTCIATGCC
	THAIV-like S917R	GCATCYCTIGCATGRTCYCTTATICC
	THAIV-like S836R	TCTGCATTTGTIGGWGRRCTIGCATG

Table 2. Lists of primers used for the amplification and sequencing of M segment of Lanka virus and SA108 virus.

Specificity	Primer name	Sequence (5'-3')
Lanka virus specific primers	Lanka M1722R	TGTGCTTTGAGCTCTTTCAAGGTCTC
	Lanka M1761R	AAGCAGTACGGACACTCAGATTGTGG
	Lanka M2090R	TGGAGCTTGAAGGAAGAGAGAAATC
	Lanka M2189F	CAGAACTTAGGGCACTGGTTTGATGC
	Lanka M2223R	TTAAGACGAGCATCAAACCAGTGCCC
	Lanka M2228F	AAAACCTCGTTCCATTGTTATGGGGC
	Lanka M2276R	AAGGATACTGGTACTTTGAACAGGC
Muridae-borne and THAIV-like primers	Muridae_hanta M1F	TAGTAGTAGACICCGCAARARAIAGCAG
	Muridae_hanta M212F	GTICCIGARAGYTCITGYAACATGG
	Muridae_hanta M483F	TICAIKCITGYAAYATGATGAAAAG
	Muridae_hanta M510R	CAICTTTTCATCATITTRCAIGMRTG
	Muridae_hanta M609R	TTITCIGGIACAAARCAYTTYCCTTC
	Muridae_hanta M903R	TCTTCICCAGIIARRTCATGRTCTTC
	Muridae_hanta M1199F	GCATCITGTGARGCITTYTCIGARGG
	Muridae_hanta M1652F	AARACWAARGGITCIATGGTITGTG
	Muridae_hanta M1754F	TACTGYTTTACICAYTGTGARCCIAC
	Muridae_hanta M1833F	GGGAIGATTTAAAIAARACWRTIAC
	Muridae_hanta M2661R	TCACCTGGITCWCCAAAITGRCAKG
	Muridae_hanta M2742R	GTTGTIGCAAAITTICAYTTYTTYC
	Muridae_hanta M3090R	GCACCITARCAIATIGCCWTRTCAC
	Muridae_hanta M3294R	CCACAYTSIGGIGCMCCATCATCATAIAC
	THAIV-like M2003F	CCACTCTGGACWGAYAATGCWCATGG
	THAIV-like M2030F	ATTGGITCWGTYCCIATGCAYACTG
	THAIV-like M2301F	TCAAAGTGRCACTTGGCTGTATGCCAAGG
	THAIV-like M2331F	GCCCAACTATTYTCATAYTCRTAATC

Table 3. Lists of primers used for the amplification and sequencing of L segment of Lanka virus, SA108 virus, and Thailand virus strain Thai-749.

	Primer name	Sequence (5'-3')
Lanka virus specific primers	Lanka L260R	TCTAAAGCAGTAATTACATTATTAGG
	Lanka L299R	AAGGTTTTCCCACTCGGATGATCTGG
	Lanka L1956R	AAACCCAGAATATAGTGATGTAACAGCAGG
	Lanka L2033R	CTTTTAATGTTATAGTAAATGAATACTTC
	Lanka L2981F	CCGCAAAATTTAGAAGATTTACAGC
	Lanka L3013F	CACAATGGGCTGTCTGATAACAAGC
	Lanka L3271R	TAGCTTTGAAGAGAAGTGACATACC
	Lanka L3313R	ATTCAAAAAAGCAATCAAGCTCAGG
	Lanka L4303F	TGGGTCACATTTAGAGAGGTGCTTGCAGC
	Lanka L4333F	GCAAACAGCTTTGCAGAGAACTATGAACC
	Lanka L4927F	TTGTGTATTGAAGTCTGGAGATGGGC
	Lanka L4978F	GAATGGTTCCATGCGTTGTGGTTTG
Muridae-borne and THAIV-like primers	Muridae_hanta L1F	TAGTAGTAGACICCIDAARIRACAA
	Muridae_hanta L37F	GATGGAKAAATAYGGAAATWCAC
	Muridae_hanta L149F	ATTGTTGACCARATGATWAARCATG
	Muridae_hanta L642F	GAAGARAGAGCTGCAYTAGAAGCMATG
	Muridae_hanta L670R	AACATKGCTTCTARTGCAGCTCTYTC
	Muridae_hanta L1440F	AAGAAGACMACWGCATGGCAYATMGC
	Muridae_hanta L1501R	GCAATCAARCTYTCWGTWATATCCC
	Muridae_hanta L1876F	TTTGYCAAAAGATGAAAYTVTGTGC
	Muridae_hanta L2134R	CCRCTTGCACCAACWGTWGATTGGTC
	Muridae_hanta L3556F	TCWACATTTTTTGARGGTTGTGCTG
	Muridae_hanta L3864R	TCCATTATTGACATWGCACCAYTYCC
	Muridae_hanta L4218F	TAACAGCWATGACHATGCARTCACC
	Muridae_hanta L4434R	TTCCATGCATAYTCTTTWGARAATG
	Muridae_hanta L4577F	GTAACAGTWGAKGAAATGTCWGATG
	Muridae_hanta L5091R	AATGCTGCACAYTGRATYTCTGGATC
	Muridae_hanta L5153F	TATTCAGGKAARCARTATGATGCATATTG
	Muridae_hanta L5184R	ACACAATATGCATCATAYTGYTTMCC
	Muridae_hanta L5192F	TATAATGAAGWRACAAAGCTWTATG
	Muridae_hanta L5489R	CACAGTTYTTRTAWACAATACATGG
	Muridae_hanta L5650F	AGTTGATGCWGTRAGTAATGTRTGG

Table 3. Continued

	Muridae_hanta L6033R	GCTTCTAYATCWATATTYTCAAAATC
	Muridae_hanta L6535R	TAGTAGTAGRCTCCGSAAAATGAAAAWG
	THAIV L301R	TTAGGGTCTTCCCACTAGGATGGTC
	THAIV L258R	TAACGCAGTTATCACATTGTTTGGG
	THAIV L6338F	AGTTTGATGCAATTGACAGGGAAGC
	THAIV L6386F	GAATCATTCCTGAGGATGTTATTCC

Table 4. Summary of the captured species and test results.

Species	No. of Captured Animals	IFA Antibody (% Positive)	PCR (% Positive)
Rattus rattus complex	99	34 (34.3%)	2 (2%)
Mus booduga	11	5 (45.5%)	5 (45.5%)
Tatera indica	3	0	0
Bandicota bengalensis	1	1	0
Bandicota indica	1	0	0
Crocidura horsfieldii	1	0	0
Total	116	40	7

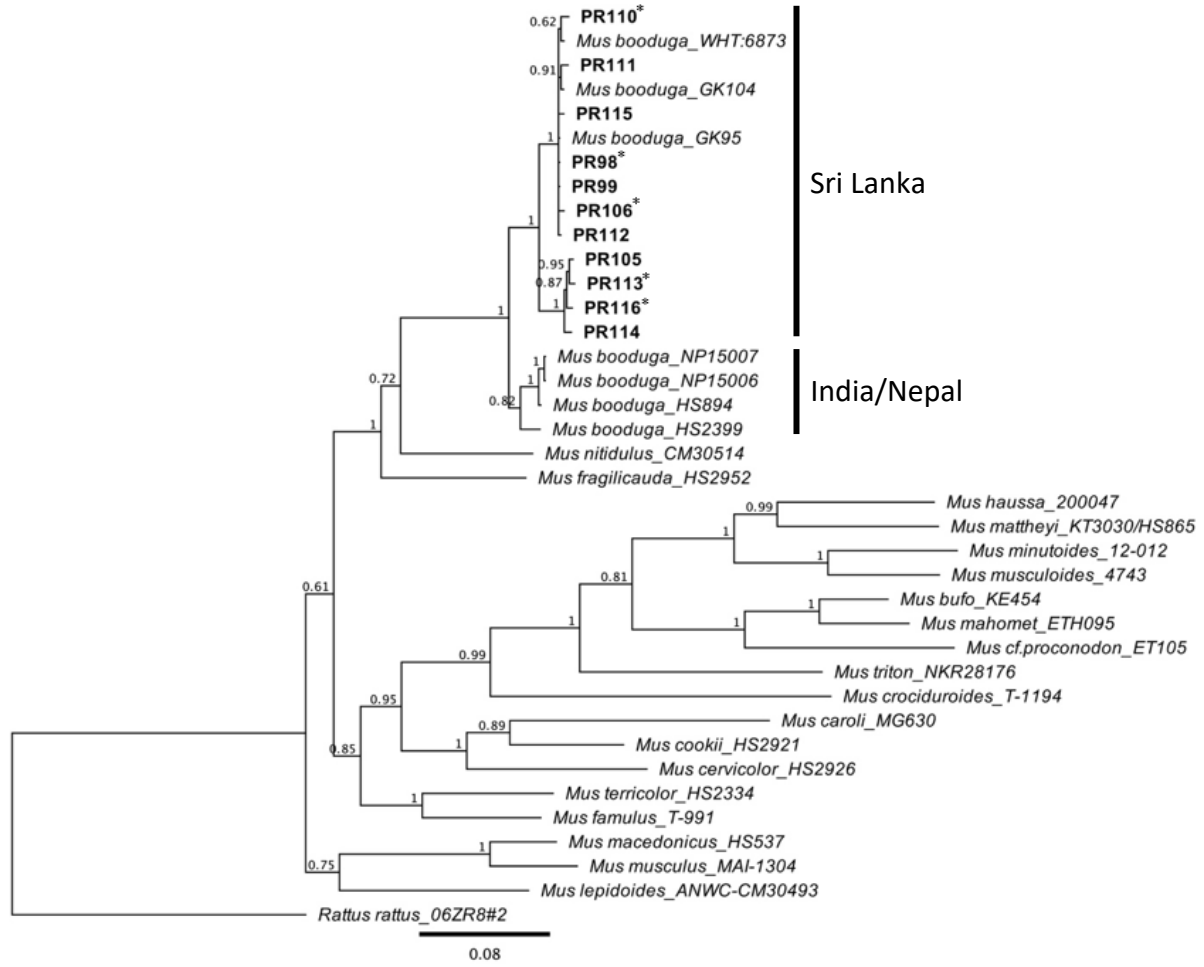


Figure 2. Phylogenetic tree based on *cytb* sequences of *Mus* species.

Mus booduga sequences obtained in this study (in boldface) are used along with the sequences of other *Mus* spp. retrieved from databases. The scale bar indicates a sequence divergence of 0.08. Numbers above nodes indicate the Bayesian posterior probability values. *M. booduga* from Sri Lanka: GK104 (LC426363), GK95 (LC426362), WHT-6873 (KY697998), *M. booduga* from Nepal: NP15007 (KY587424), NP15006 (KY587423), HS894 (AB125761), *M. booduga* from India: HS2399 (AB125760). *M. nitidulus*: CM30514 (AB269818), *M. fragilicauda*: HS2952 (AB125780), *M. haussa*: 200047 (AJ875071), *M. mattheyi*: KT3030/HS865 (AB125781), *M. minutoides*: 12-012 (LM994813), *M. musculoides*: 4743 (AJ875075), *M. bufo*: KE454 (KJ935783), *M. mahomet*: ETH095 (KJ935795), *M. cf. proconodan*: ET105 (KJ935769), *M. triton*: NKR28176 (KJ935746), *M. crociduroides*: T-1194 (AJ698878), *M. caroli*: MG630 (AB109795), *M. cookii*: HS2921 (AB125767), *M. cervicolor*: HS2926 (AB125764), *M. terricolor*: HS2334 (AB125776), *M. famulus*: T-991 (AJ698872), *M. macedonicus*: HS537 (AB125770), *M. musculus*: MAI-1304 (LC325143), *M. lepidoides*: ANWCCM30493 (AB262414), and *Rattus rattus* out-group: 06ZR8#2 (AB752981).

*Virus positive rodent samples

Sero-survey and hantavirus screening PCR

As shown in Table 1, a total of 36.4% (40/116) of the captured animals were seropositive for anti-hantaviral antibodies in an IFA. Thirty-four out of 99 *R. rattus* individuals were seropositive, as were 5/11 *M. booduga* and 1/1 *B. bangelensis*. Genome screening was performed for all the small mammal lung cDNA samples. Out of 116 captured animals, seven were positive by the hantavirus genome screening PCR (Table 4). Positive amplicons were obtained from *M. booduga* (5/11) and *R. rattus* (2/99). All five seropositive *M. booduga* were PCR-positive, resulting in a high positive rate of 45.5% (5/11). Conversely, among 34 seropositive *R. rattus*, only one was PCR-positive. PCR positivity was also detected in a seronegative *R. rattus* individual. Whole-genome sequencing was carried out to determine the respective hantavirus species precisely.

Whole-genome sequencing

We determined nearly complete whole-genome sequences of six of the seven PCR screening-positive samples using the primer walking and MiSeq approaches. All *Mus*-borne hantavirus sequences were similar in the sequence identities and showed less similarity to those of all known THAIV-like viruses. Therefore, these *Mus*-borne sequences were designated as the Lanka virus. The sequences determined from *M. booduga* sample #98 (PR98) were used to represent Lanka viruses for further analyses, as it was the first *Mus* sample to obtain the whole genome of the Lanka virus. We failed to determine the whole-genome sequence from the seronegative rat (#32) that was positive according to PCR screening, and its amplicon sequence was identical to that of the Lanka virus. Sequence comparisons with other representative Muridae-borne hantaviruses revealed that PR98 was the closest to ANJZV, and its S, M, and L

segment open reading frames (ORFs) showed 62.6–80.1%, 59.4–76.9%, and 74–79.7% nucleotide identities, respectively, while the encoded N, GPC, and L proteins showed 61.4–93.2%, 53.6–87.2%, and 68.5–94.5% amino acid identities, respectively (Table 5). Another seropositive rat (#108) carried sequences differing from those of the Lanka virus. The sequence analysis of this *R. rattus*-borne virus, designated as strain SA108 (SA108; Sri Lankan ANJZV detected from rat #108) showed high similarity to ANJZV, a genetic variant of THAIV in the *R. rattus* species from the Madagascar Islands. SA108 led to a similar sequence identity range (Tables 6 – 8). The predicted GPC cleavage site, having a conserved WAASA motif, could be observed at amino acid positions 642–646 in both strains. The novel Lanka virus detected from *M. booduga* showed a high divergence from all the known THAIV-like viruses at both nucleotide and amino acid levels. The M segment nucleotide and amino acid sequences of the Lanka virus showed the lowest identity with the THAIV and THAIV-like viruses (Table 5 and 7). In contrast, those of the L segment showed the highest identity values (Table 5 and 8).

Table 5. Nucleotide and amino acid sequence identities of S, M, and L segment ORFs and their corresponding encoded proteins of the Lanka virus strains PR98 with SA108 virus and other representative Muridae-borne hantaviruses.

	Lanka Virus Strain-PR 98					
	Nucleotide Identity, %			**Amino Acid Identity, %**		
	ORF_S	**ORF_M**	**ORF_L**	**N**	**GPC**	**L-protein**
THAIV	78.1	76.6	79.2	92.1	85.4	94.0
ANJZV	79.4	76.9	79.5	93.2	86.7	94.3
SA108	80.1	76.9	79.7	93.0	87.2	94.5
SEOV	75.0	72.3	76.7	85.8	79.7	88.3
HTNV	74.0	71.2	74.0	84.1	76.8	84.8
DOBV	72.6	70.7	74.2	83.2	75.7	85.6
PUUV	62.6	59.4	66.9	61.4	53.6	68.5

THAIV strain Thai-749 (S: AB186420, M: L08756, and L: LC553715); ANJZV strain Anjozorobe/Em/MDG/2009/ATD49 (S: KC490918, M: KC490919, and L: KC490922); Seoul virus (SEOV) strain 80-39 (S: AY273791, M: S47716, and L: X56492); Hantaan virus (HTNV) strain HTN76-118 (S: M14626, M: M14627, and L: X55901); Dobrava virus (DOBV) strain Dobrava- Belgrade (S: L41916, M: L33685, and L: JQ026206); and Puumala virus (PUUV) strain Sotkamo (S: X61035, M: X61034, and L: Z66548).

Table 6. Nucleotide and amino acid identities of S segment ORF and nucleocapsid protein of novel viruses with representative Muridae-borne hantaviruses.

Nucleotide %								
	THAIV	ANJZV	SA108	Lanka	Seoul	Hantaan	Dobrava	Puumala
THAIV	-	82.3	80.3	78.1	77.2	74.8	73.9	63.8
ANJZV	96.7	-	86.3	79.4	75.1	75.1	74.0	64.2
SA108	96.7	98.4	-	80.1	75.3	74.4	74.0	62.5
Lanka	92.1	93.2	93.0	-	75.0	74.0	72.6	62.6
Seoul	86.7	86.2	86.5	85.8	-	74.6	74.5	62.0
Hantaan	83.7	84.4	84.4	84.1	83.2	-	74.0	62.8
Dobrava	82.8	83.2	83.2	83.2	81.6	83.0	-	63.4
Puumala	62.4	62.4	62.4	61.4	61.9	60.5	61.2	-
Amino acid %								

Thailand virus strain Thai-749 (AB186420), Anjozorobe virus strain Anjozorobe/Em /MDG/2009/ATD49 (KC490918), Hantaan virus strain-HTN76-118 (M14626), Seoul virus strain-80-39 (AY273791), Dobrava virus strain-Dobrava-Belgrade(L41916), Puumala virus strain-Sotkamo (X61035). S segment sequences of Lanka virus (LC553716), and SA108 (LC553722) were used for the comparison.

Table 7. Nucleotide and amino acid identities of M segment ORF and glycoprotein precursor of novel viruses with representative Muridae-borne hantaviruses.

Nucleotide %								
	THAIV	ANJZV	SA108	Lanka	Seoul	Hantaan	Dobrava	Puumala
THAIV	-	79.6	79.9	76.6	74.5	71.7	71.6	59.7
ANJZV	91.4	-	87.1	76.9	74.2	71.3	71.5	59.4
SA108	91.9	96.9	-	76.9	73.9	71.2	71.2	59.4
Lanka	85.4	86.7	87.2	-	72.3	71.2	70.7	59.4
Seoul	82.0	82.9	83.3	79.7	-	72.0	71.2	60.7
Hantaan	77.1	77.5	78.3	76.8	77.1	-	71.4	60.1
Dobrava	76.7	77.2	78.2	75.7	77.2	77.4	-	60.5
Puumala	53.8	53.3	53.5	53.6	53.8	53.8	53.3	-
Amino acid %								

Thailand virus strain Thai-749 (L08756), Anjozorobe virus strain- Anjozorobe/Em/ MDG/2009/ATD49 (KC490919), Hantaan virus strain-HTN76-118 (M14627), Seoul virus strain-80-39 (S47716), Dobrava virus strain-Dobrava-Belgrade (L33685), Puumala virus strain-Sotkamo (X61034). M segment sequences of Lanka virus (LC553717) and SA108 (LC553723) were used for the comparison.

Table 8. Nucleotide and amino acid identities of L segment ORF and L-protein of novel viruses with representative Muridae-borne hantaviruses.

Nucleotide %								
	THAIV	ANJZV	SA108	Lanka	Seoul	Hantaan	Dobrava	Puumala
THAIV	-	79.3	79.5	79.2	76.3	74.2	74.9	67.0
ANJZV	94.9	-	86.5	79.5	76.2	74.8	74.8	66.6
SA108	95.0	97.8	-	79.7	76.5	74.7	75.4	67.2
Lanka	94.0	94.3	94.5	-	76.7	74.0	74.2	66.9
Seoul	89.1	88.8	88.7	88.3	-	74.5	75.0	67.1
Hantaan	85.1	84.7	84.9	84.8	85.1	-	74.7	67.0
Dobrava	85.9	86.0	86.1	85.6	85.6	85.1	-	66.9
Puumala	68.0	68.4	68.5	68.5	68.6	69.0	69.4	-
Amino acid %								

Thailand virus strain Thai-749 (LC553715), Anjozorobe virus strain- Anjozorobe/Em/ MDG/2009/ ATD49 (KC490922), Hantaan virus strain-HTN76-118 (X55901), Seoul virus strain-80-39 (X56492), Dobrava virus strain-Dobrava-Belgrade (JQ026206), Puumala virus strain-Sotkamo (Z66548). L segment sequences of Lanka virus (LC553718), and SA108 (LC553724) were used for the comparison.

Sequence alignment and phylogenetic analysis of hantaviruses and small mammals

A phylogenetic analysis based on the ORFs of all three genomic segments of the SA108 virus and Lanka viruses clustered them with THAIV-like viruses (Figure 3-5). The Lanka virus showed a quite divergent topology in the phylogenetic trees, following the sequence identity results. The Lanka virus formed the basal clade in the S and M trees, where THAIV and its genetic variants seemed to diverge from the virus later. The tanglegram illustrating the host–virus evolutionary relationships clearly showed the grouping of *M. booduga*, the Lanka virus host, with *Apodemus* and *Hylomyscus* species, which are the hosts of the Hantaan, Dobrava, and Sangassou orthohantaviruses found in Eurasia and Africa (Figure 6). The results revealed a notable difference since all the other THAIV-like hantavirus reservoir hosts were clustered into the *Bandicota* and *Rattus* groups (i.e., THAIV (*Bandicota indica*), ANJZV and Mayotte virus (*R. rattus*), Serang virus, and Jurong virus (*R. tanezumi*)).

Quantification of viral RNA

Higher viral RNA copy numbers were detected in lung tissues than in kidney tissues in all the rodent samples. *R. rattus* (PR108) had a notable difference in the viral RNA copy numbers between the two tissue types. All the *M. booduga* kidney tissues showed viral copy number values higher than 10^5 copies/mg, while the single SA108 virus-infected *R. rattus* kidney tissue sample showed a lower value (Figure 7).

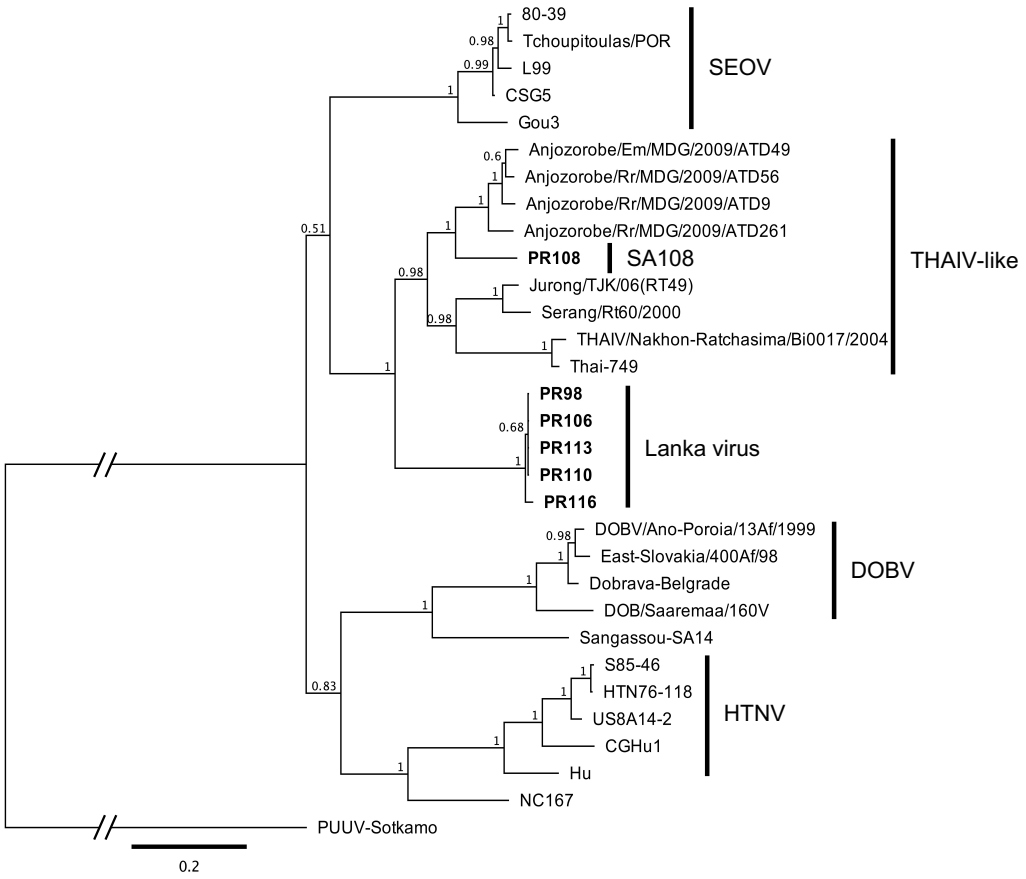


Figure 3. Phylogenetic tree of Muridae-borne hantaviruses based on S-segment ORF sequences.

Representative Muridae-borne hantavirus sequences retrieved from databases were used to compare with newfound viruses (in boldface) in this study The scale bar indicates a sequence divergence of 0.2. The numbers above the nodes indicate the Bayesian posterior probability values. Hantaan (HTNV): S85-46 (AF288659), HTN76-118 (M14626), US8A14-2 (KU207208), CGHu1 (EU092218), and Hu (AB027111); Dabieshan: NC167 (AB027523); Seoul (SEOV): Gou3 (AF184988), L99 (AF288299), CSG5 (AB618112), Tchoupitoulas-POR (KU204960), and 80-39 (AY273791); Dobrava (DOBV): DOBV/Ano-Poroia/Afl9/1999 (AJ410615), Dobrava-Belgrade (L41916), East Slovakia/400Af/98 (AY168576), and DOB/Saaremaa/160V (AJ009773); Sangassou: SA14 (JQ082303); THAIV: Nakhon Ratchasima/Bi0017/2004 (AM397664), Thai-749 (AB186420), ANJZV strain Anjozorobe/Em/MDG/2009/ATD49 (KC490918), ANJZV strain Anjozorobe/Rr/MDG/2009/ATD56 (KC490916), ANJZV strain Anjozorobe/Rr/MDG/2009/ATD9 (KC490915), ANJZV strain Anjozorobe/Rr/MDG/2009/ATD261 (KC490914), Jurong strain TJK/06/RT49 (GQ274940), and Serang strain Serang/Rt60/2000 (AM998808); and Puumala (PUUV):Sotkamo (X61035).

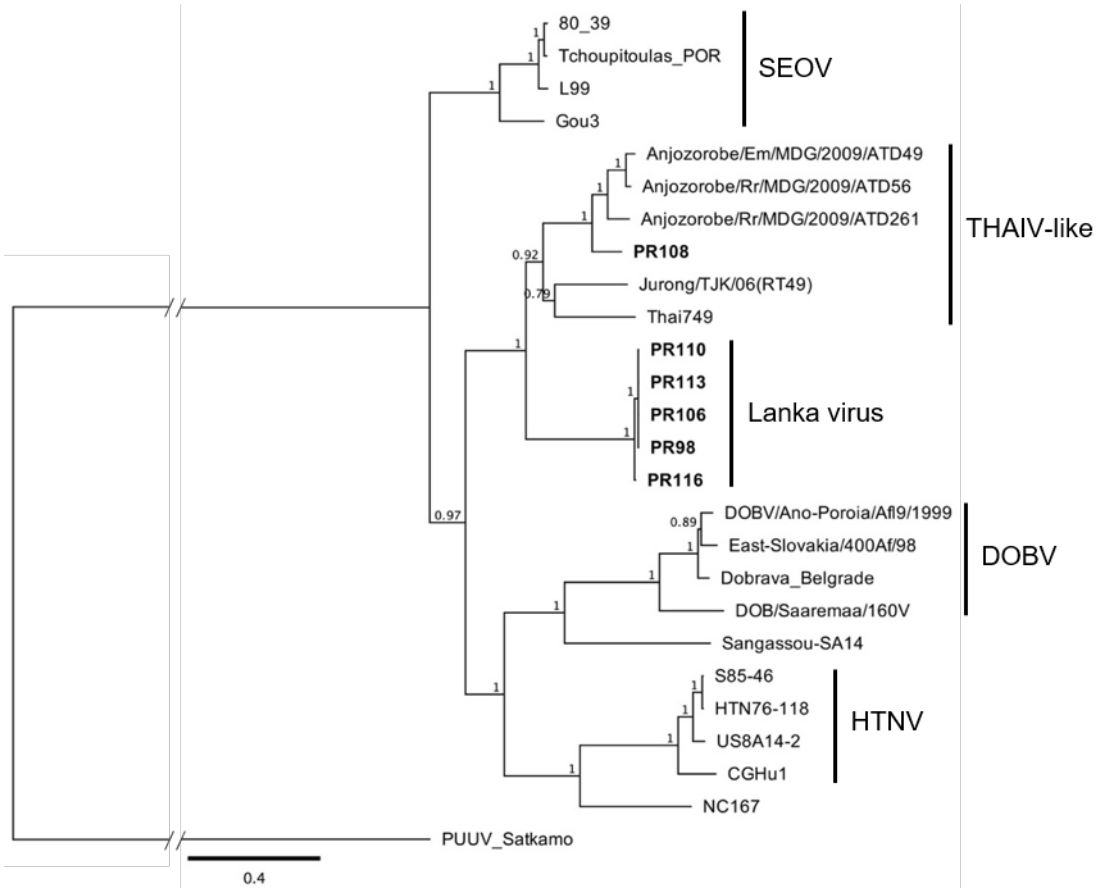


Figure 4. Phylogenetic tree of Muridae-borne hantaviruses based on M-segment ORF sequences.

Representative Muridae-borne hantavirus sequences retrieved from databases were used to compare with newfound viruses (in boldface) in this study. The scale bar indicates a sequence divergence of 0.4. Numbers above nodes indicatethe Bayesian posterior probability values. Hantaan: S85-46 (AF288658), HTN76-118 (M14627), US8A14-2 (KU207204), CGHu1 (EU092222), Dabieshan: NC167 (AB027115); Seoul: 80-39 (S47716), Tchoupitoulas-POR (KU204959), L99 (AF035833), Gou3 (AF145977); Dobrava: DOBV/Ano-Poroia/Afl9/1999 (AJ410616) Dobrava-Belgrade (L33685), DOB/Saaremaa/160V (AJ009774). Sangassou: SA14 (JQ082301); THAIV: Thai-749 (L08756), ANJZV-strain Anjozorobe/Em/MDG/2009/ ATD49 (KC490919), ANJZV-strain Anjozorobe/Rr/MDG/2009/ ATD56 (KC490921), ANJZV-strain Anjozorobe/Rr/MDG/2009/ATD261 (KC490920), Jurong-strain TJK/06/RT49 (GQ274938); Puumala: Sotkamo (X61034).

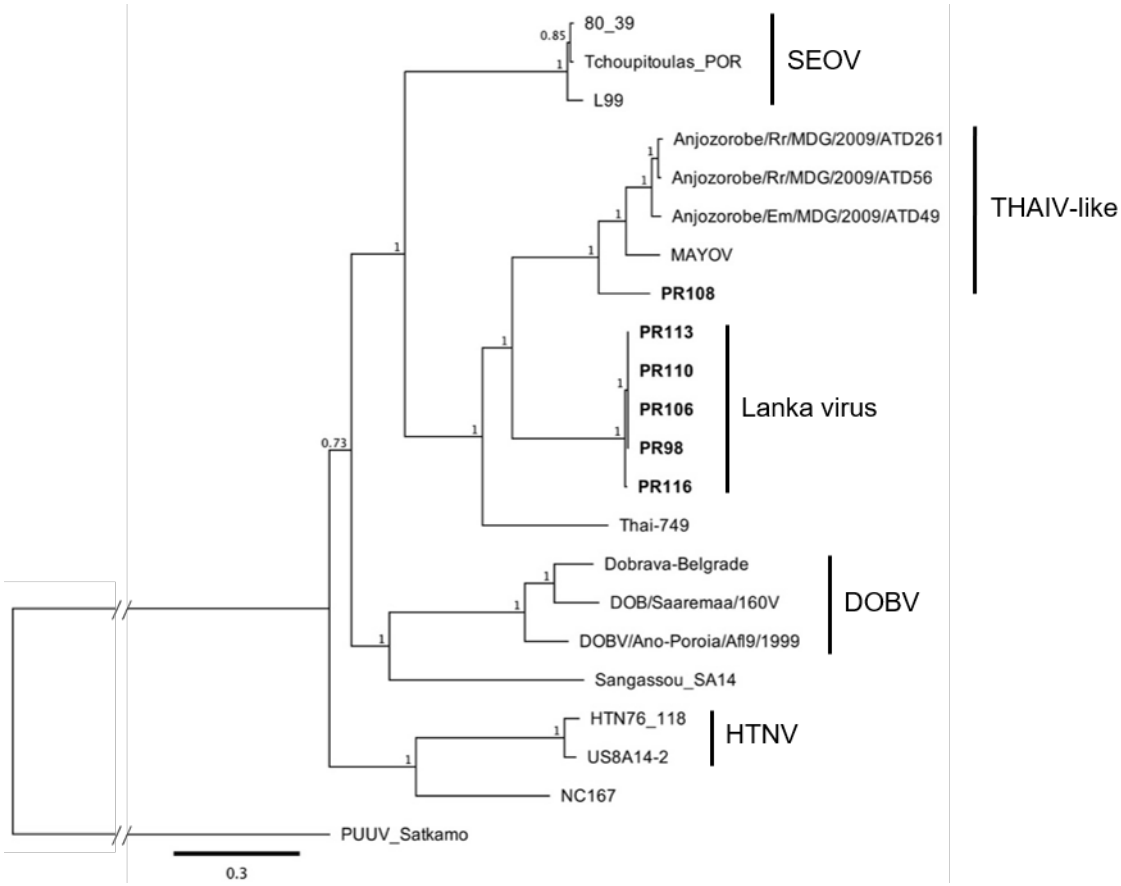


Figure 5. Phylogenetic tree of Muridae-borne hantaviruses based on L-segment ORF sequences.

Representative Muridae-borne hantavirus sequences retrieved from databases were used to compare with newfound viruses (in boldface) in this study. The scale bar indicates a sequence di-vergence of 0.3. Numbers above nodes indicate the Bayesian posterior probability values. Hantaan: HTN76-118 (X55901); Dabieshan: NC167 (DQ989237); Seoul: 80-39 (X56492), Tchoupitoulas-POR (KU204958), L99 (AF288297); Dobrava: DOBV/Ano-Poroia/Afl9/1999 (AJ410617) Dobrava-Belgrade (JQ026206), DOB/Saaremaa/160V (AJ410618). Sangassou: SA14 (JQ082302); THAIV: Thai-749 (LC553715), ANJZV-strain Anjozorobe/Em/MDG/2009/ ATD49 (KC490922), ANJZV-strain Anjozorobe/Rr/MDG/2009/ ATD56 (KC490923), ANJZV-strain Anjozorobe/Rr/MDG/ 2009/ ATD261 (KC490924), Mayotte-strain MAYOV (KU587796); Puumala: Sotkamo (Z66548).

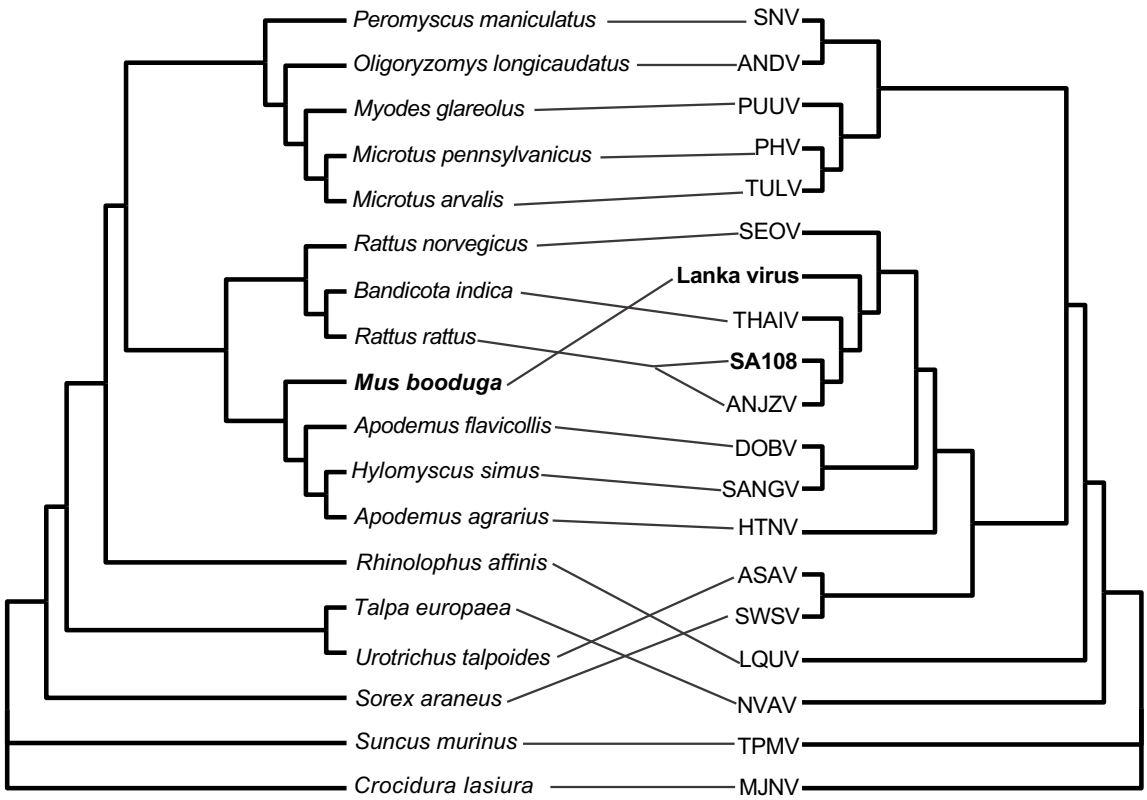


Figure 6. Tanglegram comparing the phylogenies of the representative hantaviruses and their hosts.

The phylogeny of viruses based on amino acid sequences of the N protein (on the right) is compared with the *cytb* sequence-based phylogeny of their hosts (on the left). The newfound viruses are designated as the Lanka virus (LC553716) and SA108 virus (LC553722) in boldface, and the Lanka virus host *Mus booduga* (LC556235) is also shown in boldface. The other viruses used in the analysis include the shrew-borne thottimviruses Imjin virus (MJNV, KJ420559) from *Crocidura lasiura* (KJ004674) and Thottopalayam virus (TPMV, AY526097) from *Suncus murinus* (JF784171); the mole-borne Nova mobatvirus (NVAV, KR072621) from *Talpa europaea* (KF801566); the bat-borne Longquan loanvirus (LQUV, JX465422) from *Rhinolophus affinis* (DQ297582); the shrew-borne Seewis orthohantavirus (SWAV, KY651020) from *Sorex araneus* (AJ245893); the mole-borne Asama orthohantavirus (ASAV, EU929072) from *Urotrichus talpoides* (AB033611); the rodent-borne orthohantaviruses Seoul virus (SEOV, AY273791) from *Rattus norvegicus* (AB033713), the Thailand virus (THAIV, AM397664) from *Bandicota indica* (KJ592790), the Dobrava-Belgrade virus (DOBV, AJ410615) from *Apodemus flavicollis* (AY158445), the Sangassou virus (SANGV, JQ082300) from *Hylomyscus simus* (JX893846), the Hantaan virus (HTNV, M14626) from *Apodemus agrarius* (AB032851), the Sin Nombre virus (SNV, L25784) from *Peromyscus maniculatus* (JF489123), the Andes virus (ANDV, AF291702) from *Oligoryzomys longicaudatus* (KR822254), the Tula virus (TULV, Z49915) from *Microtus arvalis* (GU187363), the Prospect Hill virus (PHV, Z49098) from *Microtus pennsylvanicus* (KF948531), the Puumala virus (PUUV, X61035) from *Myodes glareolus* (FJ881480); and the THAIV genetic variant Anjozorobe virus (ANJZV, KC490918) from *Rattus rattus* (AB033702).

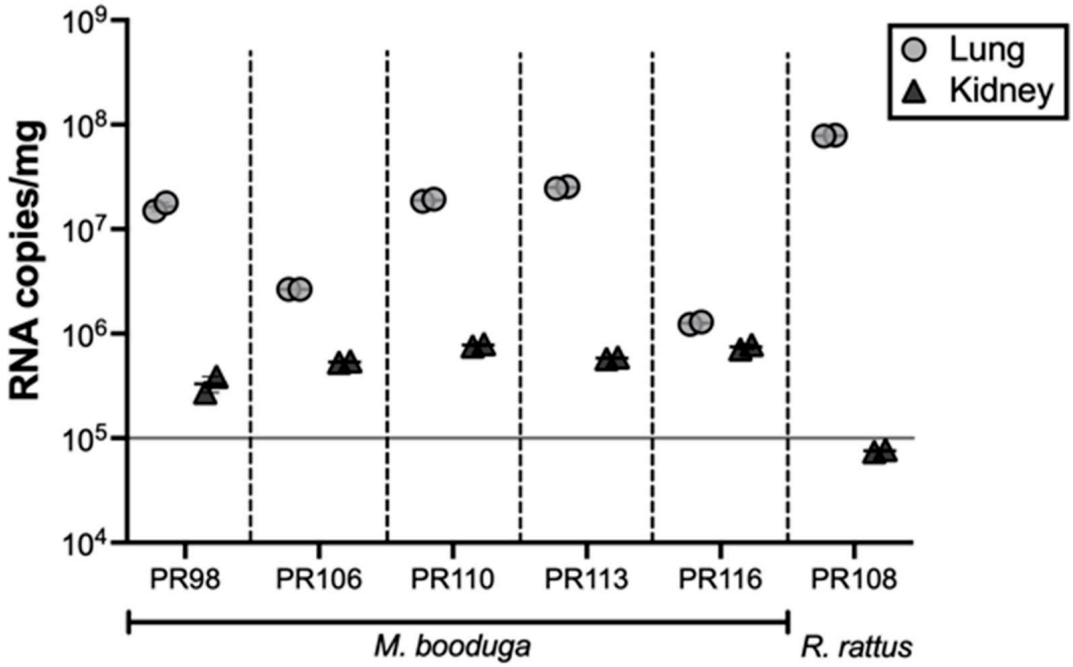


Figure 7. Viral RNA copy numbers in lung and kidney tissues of hantavirus genome-positive rodents.

Tissues from *M. booduga* and *R. rattus* were examined by quantitative real-time PCR using the Lanka virus primer set and the SA108 virus primer set, respectively. The two markers of each sample show the two replicated runs of the same cDNA sample, and the error bars representing the standard error and the median of the duplicates are shown for each sample.

Discussion

In this study, we report the detection of two novel hantaviruses, the Lanka virus and an ANJZV variant from Sri Lanka. The Lanka virus detected from *M. booduga* shows notable differences from all known THAIV genetic variants and from the THAIV prototype. The differences identified in the Lanka virus S and M segments and their corresponding proteins suggest the unique adaptation of this virus to its host, *M. booduga*. The S and M genomic sequences of the Lanka virus are placed as the basal branches of the THAIV-like clades in the corresponding phylogenetic trees, indicating that the Lanka virus might be the most ancient lineage of the THAIV-like hantaviruses. Based on the most recent proposed taxonomy guidelines, hantaviruses showing pairwise evolutionary distance (PED) values for the N protein and GPC concatenated amino acid sequences greater than 0.1 are considered distinct orthohantavirus species [34]. The corresponding values for the Lanka virus are 0.1344, 0.1214, and 0.1159 compared with the THAIV, ANJZV, and Jurong virus, respectively, suggesting that the Lanka virus is a novel, distinct orthohantavirus species (Table 9).

The tanglegram analysis further supported this hypothesis by accommodating the Lanka virus host in a different group of rodents from the usual THAIV-like virus hosts. THAIV and its genetic variants, such as the ANJZV and Mayotte, Jurong, and Serang viruses, are carried primarily by *Bandicota* and *Rattus* species. Initially, rats were the targets of trapping, and we used only cage-type traps. As a result, many seropositive rats were captured, but a virus genome was not identified from any of them [25]. A partial Lanka virus genome was first detected from a seronegative rat (#32) after heminested PCR. This rat was thought to be in the early phase of infection. Seropositive but genome negative rats were considered to have recovered from a spillover infection rather than representing a reservoir of the Lanka virus or SA108

virus. Additionally, the fact that the rats can easily experience a spillover infection directly explains why humans living in the same field exhibit the same chance of infection as rats. A single mouse, #98, was captured in a cage-type trap, and this mouse seemed to be a hantavirus reservoir. After that, we switched to traps for capturing mice and ultimately succeeded in identifying the Lanka virus. All the genome-positive mice were captured within the Sinhapura area and had a relatively dispersed origin. *M. booduga* sample PR116 was collected from a location relatively far from the area where other genome-positive *M. booduga* samples were collected. The differences in the PR116-borne Lanka virus nucleotide sequences suggest the possible diversity among the Lanka viruses distributed in CKDu endemic areas and possibly in other regions as well.

To our knowledge, this is the first report providing genetic evidence of *Mus* species acting as hantavirus reservoir hosts. Several studies describing hantavirus genome detection in *Mus musculus* appear to represent spillover infections from reservoir hosts found in the same environmental habitats [35–39]. A perusal of the available literature revealed that the *M. booduga* species is distributed in East Pakistan, India, Southern Nepal, Sri Lanka, Bangladesh, and Myanmar [40]. The finding that mitochondrial *cytb* sequences of Sri Lankan *M. booduga* are distinct from those found in India and Nepal (Figure 2) indicates that the Sri Lankan *M. booduga* evolved as a distinct group, which segregated a long time ago from other strains in the Indian Peninsula. Strong coevolutionary relationships with natural hosts are often observed among the hantaviruses [41,42]. However, the identification of *M. booduga* as a host for a THAIV-like virus suggests that a host-switching event occurred long ago, resulting in the coevolution of the Lanka virus with the Sri Lankan *M. booduga* lineage. The detection of high viral RNA copy numbers in genome-positive rodent tissues suggest

a high possibility of shedding viruses in their excreta. Yasuda et al. reported that the Seoul orthohantavirus was excreted in feces when showing more than 10^5 genome copies/mg of lung tissues of *R. norvegicus* [43]. The Lanka virus and SA108 virus may pose elevated risks of human infections.

The two hantaviruses described herein were detected in CKDu endemic areas of Sri Lanka. It is of utmost importance to understand the epidemiological relationship between the virus infection and the prevailing human CKDu in the region. It is evident that young adult males engaged in farming activities year-round are significantly affected by CKDu in Sri Lanka. Male farmers working in agricultural fields are exposed to many external risk factors. We hypothesize that these individuals are exposed to hantaviruses in their working environments rather than in their homes. However, the infective virus and its source should be confirmed to determine possible interventions. Our study identified two candidate viruses and their distinct rodent hosts, which may transmit these viruses to humans in different habitats. *Rattus* species are well-adapted to peri-domestic environments and are thus distributed in both urban and rural areas. Their habitats are generally within houses or nearby neighborhoods, where they primarily feed on harvested crops stored inside homes or garbage dumps. On the other hand, *M. booduga*, commonly known as the little Indian field mouse is most commonly found in agricultural fields, shrublands, and forest areas [39]. Therefore, the habitat of *M. booduga*, rather than that of *R. rattus*, is consistent with our hypothesized site of the acquiring a virus infection by humans. The geographic distribution of *M. booduga* includes India, where some regions are affected by CKDu. Hence, it is important to study whether Indian *M. booduga* strains can also carry hantaviruses that may confer a risk of CKDu.

CKDu has emerged as a significant public health problem in countries other than Sri Lanka, such as Nicaragua, El Salvador, and Costa Rica in Central America; some parts of India; and Egypt. Although the etiology has yet to be confirmed, extensive research has suggested some risk factors, such as heat/dehydration, infection/inflammation, and pesticides in Central American countries and water contamination/metals, pesticides, and infections in South Asian countries [44]. However, the hantavirus infection has, thus far, only been identified as a possible risk factor in CKDu patients from Sri Lanka [24,45,46]. Previous sero-epidemiological evidence indicated that THAIV-like hantaviruses infect both humans and rodents in CKDu hotspot regions. However, the unavailability of genetic information on hantaviruses circulating in the country has hindered the understanding of the relationship between these viruses and CKDu in Sri Lanka. Therefore, the current study aimed to fill the knowledge gap by identifying hantavirus genomes from rodent populations from a CKDu endemic region in Sri Lanka and further added a novel species to the list of hantavirus rodent hosts.

In conclusion, the current study revealed the genomic basis of hantaviruses in Sri Lanka. Our findings provided new insights for further investigations based on specific diagnostics for detecting the hantavirus species circulating among rodents and humans in other areas of Sri Lanka. These findings may contribute to better characterizing the exposure in CKDu patients to understanding the involvement of hantavirus infections in the context of pathophysiology of CKDu in Sri Lanka.

Table 9. Pairwise evolutionary distance (PED) values of based on concatenated amino acid sequences of N protein and glycoprotein of Lanka and SA108 virues in comparison with those of other representative orthohantaviruses.

	Lanka	THAIV	ANJZV	SA108	SEOV	HTNV	DOBV	PUUV
Lanka								
THAIV	**0.134**							
ANJZV	**0.121**	0.073						
SA108	**0.118**	0.068	0.027					
SEOV	**0.207**	0.183	0.177	0.173				
HTNV	**0.240**	0.239	0.233	0.225	0.242			
DOBV	**0.255**	0.245	0.241	0.231	0.247	0.241		
PUUV	**0.643**	0.640	0.645	0.641	0.639	0.648	0.651	

Brief Summary

This study was carried out to identify the circulating hantavirus species and their animal hosts in Sri Lanka. As previous studies have provided serological evidence for the presence of THAIV-related hantavirus among the rodents and humans in Sri Lankan CKDu hotspots, the small mammal collection for this study was carried out in CKDu endemic Polonnaruwa district. Of the 116 small mammals collected, 40 were seropositive to THAIV IFA and 6 hantavirus genome positive rodents were also identified. One *Rattus rattus* was carrying a genome similar to THAIV genetic variant ANJZV, designated as SA108 virus, and 5 *Mus booduga* animals were carrying a genome with the features of a novel orthohantavirus species designated as Lanka virus. The Lanka virus gene sequences S, M and L segments showed 62.6–80.1%, 59.4–76.9%, and 74–79.7% nucleotide identities, respectively, while the encoded N, GPC, and L proteins showed 61.4–93.2%, 53.6–87.2%, and 68.5–94.5% amino acid identities, respectively with other Muridae-borne hantavirus species. The viral genome copy numbers in infected rodent lung tissues were found to be higher than that in kidney tissues. The tanglegram analysis placed *M. booduga*, the Lanka virus host in a different group of host rodents than those of THAIV and ANJZV. This is the first confirmed report of a *Mus* species acting as a hantavirus reservoir host and the first hantavirus genome detection in Sri Lanka. The findings of the study will contribute to the development of specific diagnostic methods to determine the definite hantavirus serotypes in Sri Lankan CKDu communities.

Chapter II

Widespread human infections by a *Mus*-borne orthohantavirus in chronic kidney disease of unknown etiology (CKDu) endemic regions in Sri Lanka

Introduction

In Southeast Asia, South Asia, and the Indian oceanic region, THAIV [9] and its genetic variants (Anjozorobe (ANJZV) [10], Serang [11], Jurong [12], and Mayotte [13] viruses) have been identified as hantavirus species to circulate among human and rodent populations. Although several seroepidemiological reports have described human infections involving THAIV in Thailand, India, and Sri Lanka [14-16] and involving ANJZV in Madagascar [17], there are no confirmed clinical cases of HFRS, or HCPS documented in South Asia or Southeast Asia. Therefore, the pathogenicity of these viruses on humans is yet to be clarified.

Chronic kidney disease of unknown etiology (CKDu) is a new form of renal disease that has been first identified in 1990s and has been increasingly diagnosed over the past three decades [1]. The disease is primarily distributed in north central and northwestern provinces that falls to the dry-zone of Sri Lanka and continues to spread to new regions specially in southern dry-zone areas. High prevalence of CKDu is observed mostly among rural agricultural communities [2], where males are more often affected than females [3]. The early diagnosis of the disease has become difficult as the affected individuals show no symptoms until the disease progresses to its late stages. Moreover, the etiology of CKDu remains questionable, despite of many studies conducted over the past few decades. Areas in 13 out of 25 districts in the country have been identified as high-risk regions for the occurrence of CKDu [4] and the disease is becoming an overwhelming public health burden. The current prevalence of CKDu in the country is poorly known due to the gaps in recent incidence data updating.

In Sri Lanka hantavirus infection in humans and rodents was first reported by Vitarana and colleagues in 1988 [18]. Several studies describe the circulation of THAIV or THAIV-related hantavirus infections among humans and rodents in CKDu-endemic

areas in Sri Lanka [21,22,24,25]. In Girandurukotte, one such endemic area the CKDu patients were reported to have seropositivity to THAIV with a rate as high as 50 – 54.5%, while healthy residents reported a prevalence rate of 17.4% [22,24]. A rodent study in Girandurukotte serologically confirmed that there was a high prevalence of THAIV-like hantavirus species among the *Rattus rattus* lineage [25]. Recently, the first genomic evidence of hantaviruses in the country was reported with the detection of two orthohantavirus genomes from rodents in a CKDu endemic area [47]. One of the two genomes detected from *R. rattus* designated as SA108 virus, was closely related to ANJZV a THAIV genetic variant, while the other genome detected from *Mus booduga* designated as Lanka virus showed the features of a novel orthohantavirus species. In the same study a seropositivity rate of 34.5% was detected among the captured rodents.

The serological findings from both humans and rodent along with the newly detected hantavirus genome data supported the hypothesis that exposure to hantaviruses is possible a risk factor for the development of CKDu in Sri Lanka [26]. However, the determination of the precise serotype of the hantaviruses infecting the humans in the country was hindered due to the absence of viral genomic information. With the novel genome information in hand the development of specific diagnostics to detect hantavirus infections in CKDu patients is feasible, and it would provide insights into the relationship between exposure to hantavirus and CKDu etiology. Therefore, the current study was designed to fulfill this knowledge gap by developing specific serotyping assays and determining the infective hantavirus species among human residents in CKDu-endemic areas of Sri Lanka.

Materials and methods

Cells

Vero E6 cells (ATCC C1008) were maintained in Eagle's minimum essential medium (Gibco, Life Technologies Corporation, NY, USA) supplemented with 5% heat-inactivated fetal bovine serum (Biowest, Nuaillé, France), 1% MEM non-essential amino acids (Gibco), 1% Insulin-Transferrin-Selenium (Gibco), 1% penicillin (50 units/mL) and streptomycin (50 µg/mL; Sigma-Aldrich Co., St Louis, MO, USA), and 1% gentamicin (100 µg/mL; Sigma-Aldrich, St Louis, MO, USA). HEK 293T cells were maintained in Dulbecco's Modified Eagle's medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin, and streptomycin. BHK/T7-9 (RRID: CVCL_A8V7) cells that stably express the T7 RNA polymerase gene under control of the beta-actin promoter were kindly provided by Dr. Naoto Ito and Dr. Makoto Sugiyama of Gifu University. The cells were maintained in Eagle's minimum essential medium supplemented with 5% heat-inactivated fetal bovine serum, 1% penicillin (50 units/mL), streptomycin (50 µg/ mL), and 10% tryptone phosphate broth. All cells were cultured in a 5% CO_2 incubator at 37°C.

Plasmids

The GP coding regions of Lanka and SA108 viruses were amplified from infected rodent lung cDNA by PCR using primers: Lanka_M47F_ClaI and Lanka_3448R_NheI and SA108_M47F_SacI and SA108_M3448R_XhoI (Table 10). Amplified DNAs were cloned into a mammalian expression plasmid vector, pCAGGS/MCS [48]. Plasmids containing the M segment ORFs of Lanka virus and SA108 virus were designated as pCLNK-M and pCSA-M, respectively. Similarly, the Gn glycoprotein portion of GP gene of the two viruses were amplified as above using

the following primer pairs: Lanka_M47F_ClaI and Lanka_M1965R_XhoI for Lanka virus Gn, and SA108_M47F_SacI and SA108_M1965R_XhoI for SA108 virus Gn (Table 10). The amplified Gn coding regions were cloned into pCAGGS/MCS to obtain pCLNK-Gn and pCSA-Gn recombinant plasmids for Lanka virus and SA108 virus respectively.

All the plasmids were verified by sequence analysis and plasmid stocks of 1 µg/µl concentration were prepared for subsequent transfection purposes.

Expression of the viral envelope glycoproteins (GP)

Recombinant GP (rGP) of the viruses were expressed in Vero E6 cells by transfecting the pCLNK-M and pCSA-M plasmids using TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI, USA) according to the manufacturer's instructions. The expression of novel GPs was checked by IFA using Gc-directed monoclonal antibody 1G8 and was compared with that of prototype THAIV.

Subcellular localization analysis of novel viral rGPs

To understand the sub cellular localization of rGPs of novel viruses with respect to the cellular compartments such as, Endoplasmic reticulum (ER), Golgi apparatus and Endoplasmic Reticulum-Golgi Intermediate Compartment (ERGIC) following experiments were carried out.

Vero E6 cells transiently expressing the GPs of Lanka and SA108 viruses were seeded on 24-well glass slides (Matsunami Glass Ind., Osaka, Japan) and fixed with 2% paraformaldehyde in PBS. The fixed cells were treated with Triton X100 to permeabilize and were incubated for 1 hour at 37°C with anti-Gc mAb 1G8 followed by the staining with Alexa-488 conjugated goat anti-mouse IgG (Invitrogen) at a

dilution of 1:1000. For the staining of Golgi apparatus Rabbit mAb GH130 Alexa 647 was used at a dilution of 1:200. To stain ER and ERGIC, rabbit mAb anti-protein disulfide isomerase (DL-11) at 60-fold dilution and rabbit pAb ERGIC-53 at 100-fold dilution were used as the primary antibodies respectively. For both ER and ERGIC the fluorescent staining was done with Alexa-594 conjugated anti-rabbit IgG (Invitrogen) at 1:1000 dilution as the secondary antibody. For nuclear counterstaining, the transfected cells were incubated for 10 mins with Hoechst 33342 (Dojindo Laboratories, Japan) at 1:2000 dilution prior to cell fixation.

Antigenic characterization of rGPs

Antigenic profiling of rGPs was performed by IFA with 22 different mouse monoclonal antibodies raised against GP of the prototype Hantaan virus [49]. Alexa-488 conjugated goat anti-mouse IgG (Invitrogen) at a dilution of 1:1000 was used as the secondary antibody for fluorescent staining.

Preparation of pseudotype viruses

For the preparation of pseudotype viruses bearing Lanka virus and SA108 virus GPs, a recombinant vesicular stomatitis virus (rVSV) derived from a full-length cDNA clone of the VSV genome (Indiana serotype) in which the coding region of the G protein was replaced by the coding region of the GFP gene and the G protein was expressed in trans [50] designated as rVSVΔG*G was used as the stock virus. At 36 hours after transfection of 293T cells with pCLNK-M and pCSA-M, the cells were infected with rVSVΔG*G at a multiplicity of infection of 1 for 1 hour at room temperature. The 293T cell monolayer was then washed with 1% heat-inactivated FCS-PBS three times, and culture medium was added. After 48 hours of incubation at

37°C in a CO_2 incubator, the culture supernatant was clarified by low-speed centrifugation and stored at -80°C. The pseudotype viruses bearing Lanka virus and SA108 virus GPs were designated as rVSVΔG*LNK and rVSVΔG*SA, respectively.

Titration of pseudotype viruses

For pseudotype virus titration, Vero E6 cell monolayers grown on 96-well plates were infected with 50 µL of serially diluted virus stock. After a 1 hour adsorption period, the inoculum was removed, fresh culture medium was added, and the cells were incubated at 37°C in a CO_2 incubator. At 16 hours post infection, the cells were fixed with 2% paraformaldehyde in PBS for 10 minutes at room temperature, washed with distilled water, and air dried. GFP-expressing cells were counted under a fluorescence microscope. Since pseudotype rVSVs are unable to produce infectious progeny virus, the numbers of GFP-positive cells were regarded as infectious units (IU).

Neutralization of rVSV pseudotypes

A total of 30 µL of medium containing 100 IU of prepared rVSV pseudotypes was incubated with an equal volume of serially diluted rodent sera for 1 hour at 37°C. Then, 50 µL of the mixture was inoculated onto Vero E6 cell monolayers in 96-well tissue culture plates. After adsorption for 1 hour, the mixture was replaced with Eagle minimal essential medium. After a 20 hours incubation period, the cells were fixed with 2% paraformaldehyde for 10 minutes, washed with distilled water, and air dried. Cells infected with rVSV pseudotypes were examined and counted based on GFP expression under a fluorescence microscope. 80% reduction of GFP expressing cells were considered for the titer determination.

Use of recombinant GP and Gn proteins as IFA antigens for screening and serotyping

In this study rGP and recombinant Gn (rGn) were used as the screening and serotyping antigens respectively. All four plasmid constructs (pCLNK-M, pCSA-M, pCLNK-Gn and pCSA-Gn) were transfected and expressed separately in BHK/T7-9 cells, followed by the preparation of IFA slides. Both the IFA assays based on rGP and rGn were verified by using infected rodent sera. Briefly, for GP screening, rodent sera diluted at 1:100 in PBS was applied to the antigens and incubated for 1 hour at room temperature, while for the Gn serotyping, serially diluted sera were applied to the antigens and incubated at 4°C for 1 hour. Then the binding antibodies were detected by Alexa Fluor 488-conjugated goat anti-rat IgG (for rat sera), anti-mouse IgG (for mouse sera) at 1:1000 dilution. Positive reactions and endpoint titers (EPs) against both antigens were determined by the characteristic fluorescent patterns in the cell cytoplasm.

Human samples

Human samples collected from several areas in the CKDu hotspot regions in Sri Lanka during the period of 2010–2019 [51, 21, 24] were used for the study (Figure 8). Both CKDu patient and healthy control sera that were confirmed positive for anti-hantavirus IgG antibodies were included in the study to determine the infected virus type. Ethical approval was obtained from the Institutional Ethical Review Committee (ERC), Faculty of Medicine, University of Peradeniya, Sri Lanka (Project No: 2015EC/SP/15) (Project No: 2016/EC/64 (2016)), the ERC of Kyoto University, Japan (G0313-5), and the ERC of the Institute for Genetic Medicine, Hokkaido University, Japan (21-001).

To assess the sensitivity of the assays we used forty-eight sera from confirmed HFRS and nephropathia epidemica (NE) patients that were infected with HTNV, SEOV and PUUV [52] form several countries as there were no confirmed individuals with a disease attributed to the novel viruses. Also, another group of THAIV IFA negative 120 febrile patient sera collected from Thailand [14] were used to validate the specificity of the GP assays.

Serological analyses of human samples from CKDu hotspots

A total of 373 seropositive human sera to both THAIV-infected Vero E6 cell antigens and recombinant THAIV N protein-expressing Vero E6 cell antigens were screened by the rGP-based IFA as described above using Alexa Fluor-488 conjugated protein-A at 1:500 dilution as the secondary antibody. Finally, 336 anti-GP antibody-positive sera were applied to the rGn-based IFA assay. The incubation conditions for serially diluted human sera added to the rGn antigen coated wells were set to overnight at 4°C. EPs against the two rGn antigens were determined for each serum sample.

Sensitivity and specificity of the GP screening assay

To assess the sensitivity of the GP IFA assay, forty-eight confirmed HFRS human sera were allowed to react with the antigens as described previously.

A set of 120 anti-THAIV antibody negative sera from febrile patients from Thailand were used for the specificity determination of rGP assay following the same protocol.

Calculations and analyses

Using the EP data obtained by Gn-IFA assay, the ratio of EP against Lanka virus rGn antigen (EP_{Lanka}) over EP against SA108 virus rGn antigen (EP_{SA108}) was determined

for all the tested Sri Lankan serum samples. Samples showing four times or higher titer to Lanka antigen than to SA108 antigen were considered as suspected Lanka virus infection. Conversely, samples showing four times or higher titer against the SA108 antigen than the Lanka virus antigen were considered as suspected SA108 virus infection. Samples with an antibody titer difference of less than 2 times were considered as "inconclusive".

Proportions of Lanka virus infection among CKDu patients and healthy controls were calculated, and a comparison of proportions was performed using an online calculator (https://www.medcalc.org/).

The sensitivity and specificity of GP screening assay were calculated based on the data obtained by the respective experiments.

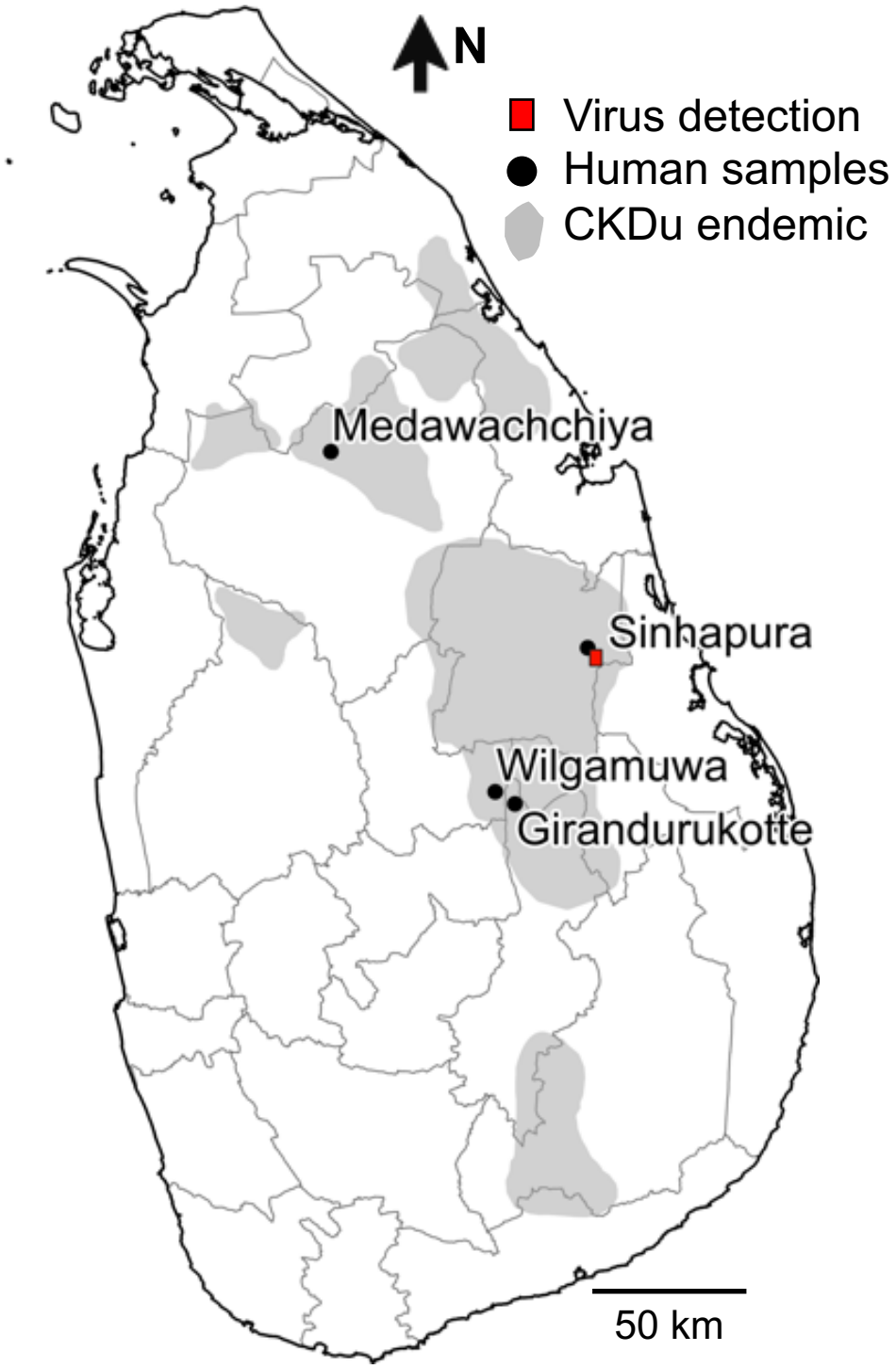


Figure 8. Map of Sri Lanka with human sample collection points and the place where the novel viruses used in the study were detected.

Table 10. Primers used for the PCR amplifications.

Target	Primer name	Sequence (5'-3')
Lanka virus M	Lanka_M47F_ClaI	AACATCGATATGTGGGGTTTACTAGCTTGGCTGC
	Lanka_M1965R_Xho1	GGACTCGAGTTATGCACTAGCTGCCCATAA GACTG
	Lanka_M3448R_NheI	GCTGCTAGCTTATGACTTCTTATGCTTACGCACAGGG
SA108 virus M	PR108_M47F_SacI	TCATTTTGGCAAAGAATTCGAGCTCATGTGGGGTTTAC TAGCTATTGCTGTTTTG
	PR108_M1965R_XhoI	GGACTCGAGTTATGCACTTGCAGCCCACATTATAGAC
	PR108_M3448R_XhoI	AAAAAGATCTGCTAGCTCGATTATGATTTCTTGTGCTT GCGAAC

Results

Expression of the viral envelope glycoproteins (GP)

The localization of Lanka virus GP in cells was notably different from prototype THAIV and SA108 virus GPs (Figure 9A) while SA108 virus GP localization pattern was comparable with that of THAIV. Prototype THAIV GP is observed to be tightly concentrated to the perinuclear region of the cells and SA108 virus GP also follow the same pattern. But interestingly, Lanka virus GP showed a distinct localization pattern where GP was observed to be dispersed in the whole cell cytoplasm (Figure 9A). Also, the transfection efficiency between the two rGPs in Vero E6 cells were different from each other where Lanka virus rGP had a low transfection efficiency than that of SA108 virus rGP.

Subcellular localization of novel viral rGPs

Novel viral rGPs were assessed for their localization with cellular organelles. As shown in Figure 9B, the viral rGPs shows no localization with the ER complex of the cells. The viral rGPs showed the yellow colocalization signal with both the Golgi and ERGIC organelle markers. Apparently, Lanka virus rGP observed to be colocalized with ERGIC markers, while SA108 virus rGP was localized both in Golgi and ERGIC as can be seen in Figure 9B. Localization of GPs in Golgi and ERGIC is a common feature in the *Hantaviridae* family.

Antigenic characterization of rGPs

Based on the reactivities towards the panel of mAbs SA108 virus rGP showed a very similar reactivity profile to prototype THAIV. In contrast, Lanka virus rGP had a distinct mAb profile particularly in Gn glycoprotein. As shown in Figure 10, none of

the mAbs directed to Gn could react with Lanka virus rGP. In the dendrogram based on the reactivities of representative hantaviruses against the GP mAb panel, Lanka virus appears in a different branch when compared to SA108 virus and THAIV which are showing a close similarity. The main reason for this difference is the unreactive Gn glycoprotein of Lanka virus as the reactivity of Lanka rGP with Gc-directed mAbs are very similar to that of THAIV and SA108 virus rGP.

Preparation and titration of pseudotype viruses

The pseudotype viruses were prepared using the rGPs of novel viruses expressed in HEK 293T cells and titer of produced viruses were determined by infecting Vero cells. The titers of the viruses were found to be 1.3×10^5 IU/ml & 2.3×10^5 IU/ml for rVSVΔG*LNK and rVSVΔG*SA, respectively.

Neutralization of rVSV pseudotypes

In the neutralization assay, sera from hantavirus genome positive rodents were used as the anti-hantavirus antibody positive samples and genome and THAIV-IFA negative rodent sera were used as the anti-hantavirus antibody negative samples for the initial analysis. As a control for the pseudotype viruses, SEOV GP bearing rVSVΔG* (rVSVΔG*SEO) was used.

As shown on the table 11, rVSVΔG*LNK and rVSVΔG*SA were neutralized by their respective homologous virus infected rodent sera; PR106 and PR108, with high titers. In addition to that, Lanka virus infected PR106 serum could neutralize rVSVΔG*SA and SA108 virus infected PR108 serum could neutralize rVSVΔG*LNK with similarly high neutralization titers. Both infected and non-infected rodent sera showed no neutralization towards rVSVΔG*SEO control.

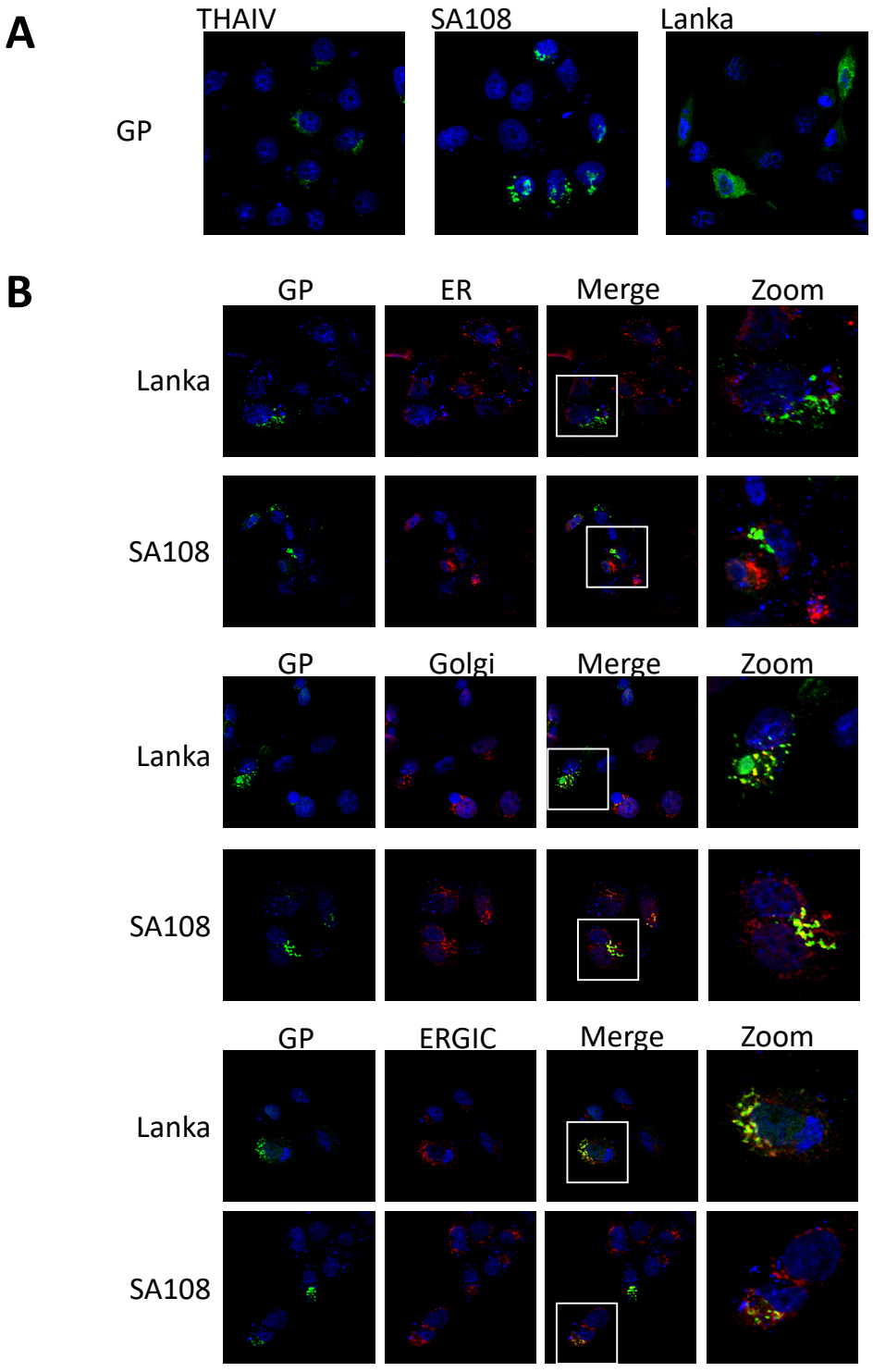
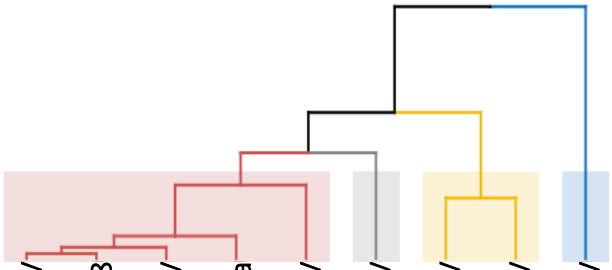


Figure 9. Expression and subcellular localization of novel virus GPs.

A. Expression patterns of rGPs of Lanka and SA108 viruses in Vero cells compared to the GP expression of Thai-749 infected cells. **B**. Subcellular localization of novel virus GPs with respect to ER, Golgi and ERGIC of Vero cells. The cells were stained with antibodies against GP (green) and ER, Golgi or ERGIC organelle markers (red), and the nuclei were stained with Hoechst 33342 (blue). The yellow areas in the merged images show the cellular localization of GPs with organelle markers. Magnification of the merged area is shown on the right side of the merged image.



Specificity	Epitope	mAb	THAIV	rGP-SA108	DOBV	rGP-Lanka	HTNV	SEOV	TPMV	PUUV	PHV
Gn	a	8B6	+	+	+/-	-	+	+/-	-	+/-	-
		6D4	+	+	+	-	+	+	-	-	-
		10F11	+	+	+	-	+	+	-	+	-
	b	2D5	-	+/-	-	-	+	-	-	-	-
		3D5	-	-	-	-	+	-	-	-	-
		16D2	-	-	-	-	+	-	-	-	-
Gc	a	HCO2	+	+	-	+	+	+	-	-	-
		16E_6	+	+	+	-	+	+	+/-	+/-	-
	b	EBO6	-	+	+/-	+/-	+	+	-	-	-
	c	11E_10	-	+	+	+	+	+/-	-	+	+
	e	17G6	+	+	+	+	+	+/-	+	+	+/-
		3D7	+	+	+	+	+	+	+	+	+/-
		5B7	+	+	+	+	+	+	+	+	+/-
		20D3	+	+	+	+	+	+/-	+	-	-
	f1	8E_10	+	+	+	+	+	+	+	+	+/-
		IC6	+	+	+	+	+	+	+	+	+/-
		IG8	+	+	+	+	+	+	-	+	+/-
		23G10-2	+	+	+	+	+	+	+	+	+/-
		3B6	+	+	+	+	+	+	+/-	+	+/-
	f2	23G10-1	+	+	+	+	+	+	+	-	-
		7G6	+	+	+	+	+	+	-	-	-
		18F5	+	+	+	+	+	+/-	-	-	-

Figure 10. The mAb profiles of rGPs of Lanka and SA108 viruses with other representative hantaviruses.

The dendrogram shows the clustering of viruses based on the reactivity profiles against 22 mAbs.

Table 11. Results of rVSVΔG*-based neutralization assay.

Rodent species	Serum ID	Neutralizing antibody titers to rVSVΔG* variant		
		Lanka	SA108	SEOV
Mus booduga	#PR106†	640	640	<40
Mus booduga	#PR99‡	<40	<40	<40
Rattus rattus	#PR108§	640	640	<40
Rattus rattus	#PR107‡	N/A	N/A	N/A

†Lanka virus genome positive ‡Virus genome and IgG negative §SA108 virus genome positive
N/A: Not available

Use of recombinant GP and Gn proteins as IFA antigens for screening and serotyping

The GPs of Lanka and SA108 viruses were expressed in BHK/T7-9 cells to serve as screening antigens. Based on the previous observations in mAb reactivity difference of novel virus rGPs, the Gn glycoproteins of both Lanka and SA108 viruses were also expressed in BHK/T7-9 cells to serve as the serotyping antigens. Both rGP and rGn antigens were allowed to react with homologous and heterologous virus infected rodent sera to assess their reactivities. The rGP antigens showed cross reactivities with heterologous virus infected sera as expected (Figure 11). IFA profiles and EPs of virus infected sera were comparable for both homologous and heterologous rGP antigens. In contrast the IFA profiles of virus infected rodent sera against heterologous rGn antigens were greatly reduced in fluorescent intensity and frequency. And the heterologous EPs of rodent sera were also clearly reduced (Figure 11 and Table 12).

Sensitivity and specificity of the GP screening assay

To check the sensitivity of rGP IFA, 48 clinically confirmed HFRS patient sera infected with HTNV(n=20), SEOV (n=8) and PUUV (n=20) collected from several countries were employed. These patient sera showed a high cross-reactivity with rGP antigens of both Lanka and SA108 viruses. Thirty-four sera were positive to Lanka rGP while 30 sera were positive to SA108 virus rGP antigens. All the SEOV virus infected sera were positive to both rGPs while 16 and 15 sera out of 20 HTNV infected sera were positive for rGP-Lanka and rGP-SA108 respectively. PUUV infected patient sera showed comparatively low cross-reactivity with 10 and 7 out of 20 against rGP-Lanka and rGP-SA108 respectively. Overall sensitivity of rGP-Lanka was found to be 70.8% while that of rGP-SA108 was 62.5% against these heterologous virus infected

serum samples. All 48 samples were subjected to rGn IFA to check whether there is a cross reactivity to the assay. Interestingly only three samples showed an EP of >400 against rGn antigens. Majority of the sera had EPs ≤100 for both rGns.

In the specificity assessment, 4 of 120 anti-hantavirus antibody negative febrile patient sera from Thailand gave non-specific reactions to both rGP antigens. The specificity of the rGP assay therefore was 96.7%.

Serological analyses of human samples from CKDu hotspots

A total of 373 human serum samples belonging to two groups, i.e., CKDu patients (n=242) and healthy controls (n=131) were used in this study. The screening assay based on rGP antigens resulted 336 positives (210 CKDu patients and 126 healthy controls) which were then used for rGn based serotyping. As shown in the Figure 12, it was identified that Lanka virus infection was predominant among both groups of sera. Among CKDu patient sera group of 210, 184 (87.6%) and two (1.0%) were positive for Lanka and SA108 viruses, respectively. And in healthy control sera group 110 (87.3%) of 126 were positive for Lanka virus while no SA108 virus positive sera were identified. There is no significant difference between CKDu patients and healthy controls in Lanka virus infection. When taken as a whole, 294 (87.5%) of 336 are suspected Lanka virus infection while two (0.6%) are suspected infection with SA108-relating virus. Forty (11.9%) human serum samples showed inconclusive pattern in total (Table 13).

Table 12. Results of rGP- and rGn-based IFA assays with virus infected and non-infected rodent sera.

Rodent species	Serum ID	IFA end point titers to following antigens[1]				
		rGP-Lanka	rGn-Lanka	rGP-SA108	rGn-SA108	w/o exp.
Mus booduga	#PR106†	25600	12800	25600	800	<400
Mus booduga	#PR99‡	<400	<400	<400	<400	<400
Rattus rattus	#PR108§	12800	<400	25600	6400	<400
Rattus rattus	#PR107‡	<400	<400	<400	<400	<400

†Lanka virus genome positive ‡Virus genome and IgG negative §SA108 virus genome positive

[1]Recombinant IFA antigens were transiently expressed in BHK/T7-9 cells and used for the determination of endpoint titers.

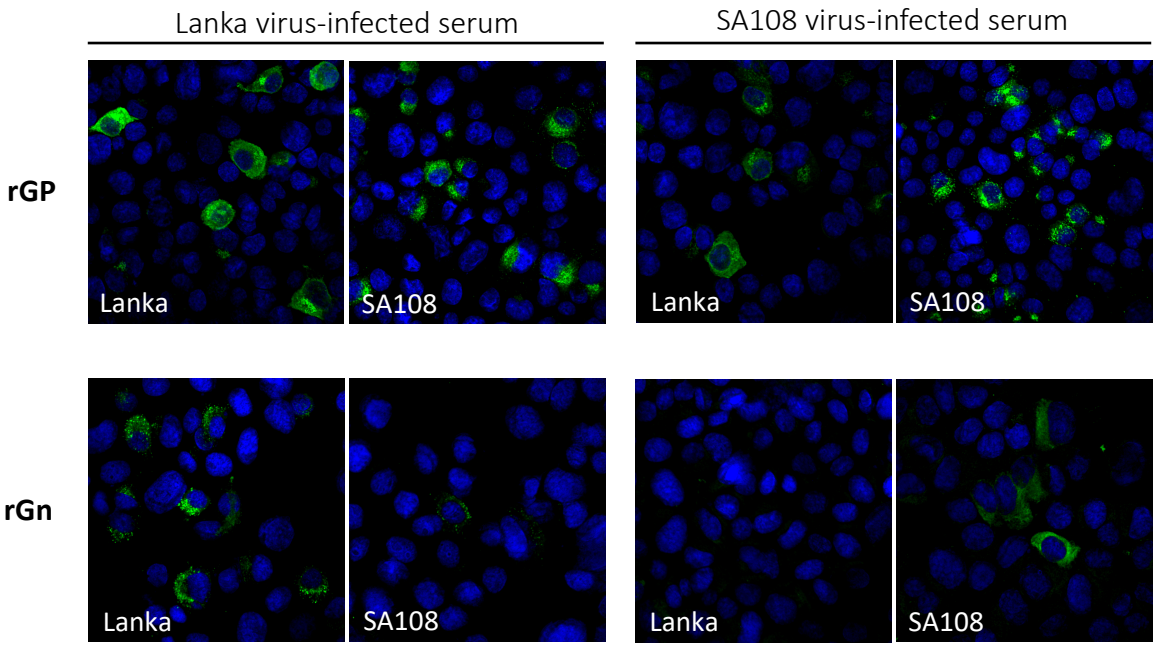


Figure 11. IFA profile of sera from host rodents.

Images taken at rodent serum dilution of 1:1600 are shown. Lanka virus-infected serum from genome-positive IgG positive *Mus booduga* #PR106. SA108 virus-infected serum from genome-positive IgG positive *Rattus rattus* #PR108.

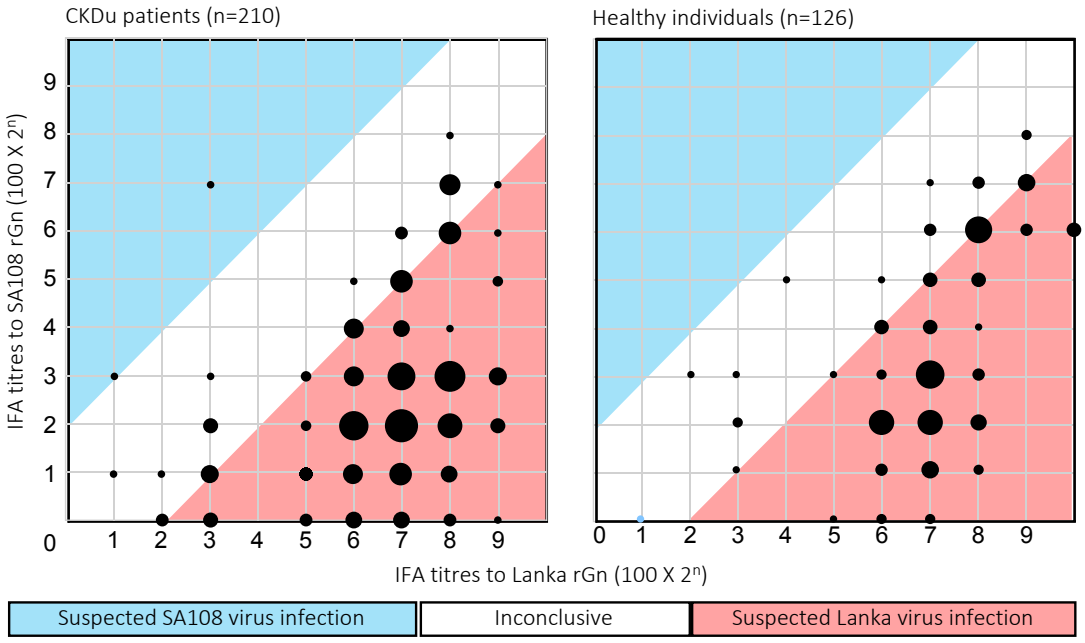


Figure 12. Plot of rGn-IFA antibody endpoint titers of Sri Lankan human samples.

The area of the points reflects the number of samples. Samples showing four-fold or higher titers to the Lanka virus antigen than to the SA108 virus antigen are displayed in red and are suspected Lanka virus infections. Conversely, samples showing four-fold or higher titers against the SA108 virus antigen than the Lanka virus antigen are shown in blue and are suspected SA108 virus virus infections. Samples with an antibody titer difference of less than 2-fold are shown in white areas as "inconclusive".

Table 13. Details of the sample collections and test results.

Condition	Year	Area	Collected Number	THAIV IFA positive	rGP IFA positive	rGn IFA typing		
						Lanka	SA108	Inconclusive
CKDu	2010	Girandurukotte	151*	63 (42·0%)	62	53	··	9
		Medawachchiya	160*	65 (40·6%)	64	55	··	9
	2015	Girandurukotte	132	62 (49·2%)	51	47	··	4
	2017	Girandurukotte	104	52 (50·0%)	33	29	2	2
CKDu total	··	··	547	242 (44·2%)	210	184 (33.6%)	2 (0.4%)	24 (4.4%)
Healthy	2010	Girandurukotte	150*	28 (18·7%)	28	26	··	2
		Medawachchiya	156*	24 (15·4%)	24	19	··	5
	2017	Girandurukotte	242	42 (17·4%)	42	38	··	4
	2019	Welikanda	59	12 (20·3%)	8	8	··	··
	2019	Wilgamuwa	143*	25 (17·5%)	24	19	··	5
Healthy total	··	··	750	131 (17·4%)	126	110 (14.7%)	··	16 (2.1%)
Total	..	..	1297	373	336	294 (22.7%)	2 (0·2%)	40 (3·1%)

*Samples were collected from male subjects due to the inclusion criteria of the respective studies.
Percentages are given in respect to the total sample number (CKDu: 547, Healthy: 750) of each group and the combined total sample count (n=1297).

Discussion

There are previous reports on detecting large number of orthohantavirus antibody-positive individuals from CKDu endemic areas in Sri Lanka [21,22,24]. Although there is a statistical association between orthohantavirus antibody positivity and being a CKDu patient [24], the full association with the disease has not been unclear yet. The first question to be solved was to clarify virus species that infects the people. In this study, the establishment of a diagnostic method to distinguish the virus serotype in humans was carried out using the genetic information of hantaviruses previously identified from a CKDu endemic area of the country [47].

The viral glycoprotein was selected for the development of the specific diagnostic assays due to the lowest amino acid identity of it between the two viruses. The lack of response of Lanka virus glycoprotein against the Gn-specific mAbs suggest the presence of unique antigenic sites on the Gn glycoprotein. The difference in the distribution pattern of Lanka virus rGP is interesting feature to be studied further and also the subcellular localization should also be clarified more by further experiments. Because the golden standard assay for serotyping of hantavirus infection is a neutralization test, we developed alternative neutralization test based on the pseudotype-rVSV system using the recombinant GPs of novel viruses. Although, high titers of neutralizing antibodies were detected from both animals, equal degree of cross-neutralizations were observed. Therefore, it was difficult to differentiate two virus infections by neutralization assay. A similar observation was obtained when rGPs were used as IFA antigens. It appears that Gc-specific antibodies in the sera are more responsible for the high cross reactivities observed during neutralization and rGP-IFA assays. The high cross reactivity of the rGPs suggest its ability to function as screening antigens in future surveillance studies. In this study we utilized the rGP-

IFA to screen and select the human serum samples having enough level of anti-hantavirus antibodies to generate a detectable signal with glycoprotein antigens.

Next, we used Gn glycoprotein as a novel IFA antigen to distinguish the two virus infections based on their difference in mAb reactivity. At the verification stage, the rGn antigens showed almost no cross reactivity at relatively high serum dilutions of rodent sera indicating their ability to use as serotyping antigens. The incubation period for primary antibody reaction was shortened for rodent sera because chronically infected reservoir rodents possess very high antibody titers resulting very high EPs. But when using human sera 1hour incubation period was not enough for the low levels of Gn-specific antibodies to bind with rGn antigens to produce a detectable fluorescent signal. By performing the serotyping rGn-IFA, the corresponding infections could be clearly distinguished by the comparison of EPs against Lanka virus and SA108 virus antigens. This Gn-based IFA method showed that the around 80-90% of seropositive humans had antibodies to Lanka virus. To our knowledge, this is the first report providing evidence for a *Mus*-borne hantavirus corresponding to a large-scale hantavirus transmission to humans.

The human serum samples were originated from several CKDu hotspot regions where majority of the CKDu cases are reported annually. Sample collection consists of sera that had been collected during 2010-2019 by different groups. Therefore, the study samples represent the CKDu affected community in Sri Lanka as a whole. The results of Gn based IFA clearly shows that *Mus*-borne Lanka virus was the predominant virus type. By obtaining similarly high positive rates in all the regions we can understand that Lanka virus spreads without any regional difference. The capturing place of virus positive rodents, Sinhapura, is situated 69 km and 98 km from Girandurukotte and Medawachchiya areas respectively, where most of the human samples were collected.

Studies are needed to investigate distribution of *Mus booduga* and Lanka virus in whole island of Sri Lanka including CKDu non-endemic areas.

On the other hand, only two cases were categorized as SA108-related virus infection. Despite the widespread distribution of its natural host *R. rattus* in these areas, this positive rate is very low. In addition, only one rat carrying the ANJZV-like virus was found in the study described in the previous chapter, even though a very high seroprevalence among the black rats was observed [47]. Epizootiological analysis suggest that many black rats might have been infected with Lanka virus by spillover and then the virus was eliminated. This observation may suggest that Lanka virus is excreted from *M. booduga* mice into the environment, providing many opportunities of infection in rats and humans. The data suggest that the distribution of SA108 virus might be limited. Further studies for SA108-related viruses are required in the future.

We reported a sex difference in the seroprevalence to hantavirus in CKDu endemic regions [21,22,24]. Based on that observation, we hypothesize that people are exposed to the hantaviruses in their work environment rather than at home, making men who work in paddy field more susceptible to the infection. *Rattus* species are well adapted to peridomestic environments, and their habitats are generally within houses or nearby areas, where they mostly feed on harvested crops or garbage dumps. On the other hand, *M. booduga*, commonly known as the little Indian field mouse, is most commonly found in agricultural fields, shrublands, and forest areas [40]. Due to its habitat without any invasion to human houses, *M. booduga* is a plausible source of infection for CKDu patients than *R. rattus*. This is an important information when considering public health measures to control this zoonotic virus.

In conclusion, the findings of this study reveal the first definitive serotyping evidence of hantavirus species infecting humans in Sri Lanka. These findings contribute to

better characterizing the etiology of CKDu and will ultimately lead to an understanding of the involvement of these hantavirus infections in the pathophysiology of CKDu in humans.

Brief summary

The above study was carried out with the objective of determining the serotype of the hantavirus infecting the residents in CKDu endemic regions in Sri Lanka. In the previous study we described the identification of two novel hantaviruses, i.e., Lanka virus and SA108 virus from rodents in Polonnaruwa district of Sri Lanka. In this study, we used the virus-infected rodent cDNA samples to amplify and clone viral gene sequences and used their protein products as antigens to establish specific diagnostic methods that allowed the distinguishing of two virus infections serologically. The pseudotype virus-based neutralization assay found to be ineffective to distinguish two infections due to high cross reactivity. Due to this rGP antigens were used as screening IFA antigens to select the serum samples with GP specific IgG antibodies. The sensitivity of rGP-IFAs were found to be 60-70% by using HFRS confirmed human sera, with a sensitivity rate of 96.6%. The use of Gn glycoproteins of two viruses which had different antigenic characters as IFA antigens, successfully distinguished the two viral infections in rodent sera. This assay was then used to serotype the hantavirus infection in Sri Lankan human samples. According to the results, Lanka virus infection was found to be predominant by far, compared to the SA108 virus infection among both CKDu patient and healthy control groups. The analysis of data showed that out of 336 sera serotyped by rGn-IFA, 294 (87.5%) were Lanka virus infected, 2 (0.6%) were SA108 virus infected and 40 (11.9%) were inconclusive. The results of this study demonstrate that Lanka virus had been infecting the humans in several CKDu endemic areas at least for a decade and warrants the need of further in-depth studies to understand the epidemiological link between Lanka virus and CKDu.

Chapter III

Zoonotic pathogen survey in an urban public market in Sri Lanka: *Leptospira* and hantavirus infection among small mammals and market workers.

Introduction

Zoonotic diseases play a major role in public health around the globe where one-health approaches are increasingly recognized for their high potential in addressing them effectively [53]. Leptospirosis and hantavirus infection are two such neglected zoonotic diseases that are being increasingly diagnosed among humans and animals in many countries. The incidence of human leptospirosis is mostly seen in tropics with seasonal outbreaks mostly related to natural disasters such as floods [54,55]. On the other hand, hantavirus infection in humans, having two different severe presentations, i.e., HFRS in Eurasia and hantavirus cardio-pulmonary syndrome (HCPS) in Americas, could be identified to occur as sporadic outbreaks that are mostly attributed to the out-door activities that allows the exposure to the excreta of infected rodents and insectivores [56].

In Sri Lanka, human leptospirosis is endemic and has been identified as a notifiable disease by the government. Seasonal or regional outbreaks occur with approximately 3,000-5,000 suspected cases reported every year with a case fatality rate of 1-2% [57]. Several leptospiral species; *Leptospira interrogans*, *L. santarosai*, *L. kirschneri*, *L. borgpetersenii* and *L. weilli* have been detected from human cases in the country and a number of them has been isolated [58 – 62]. In the case of animals, anti-*Leptospira* antibodies has been detected in cattle, pigs, water buffaloes, dogs and rodents. A few studies have described the detection of pathogenic leptospiral DNA from kidney tissues of cattle and cattle urine [63,64]. Analysis of *Leptospira* diversity along with their association with other animals and microbiome through environmental DNA metabarcoding in Kandy district, Sri Lanka has shown that pathogenic *Leptospira* DNA is present in high levels and are associated with a wide range of animal DNA in the region [65]. In Sri Lanka, the reservoir animals that contaminate environments by

excreting the spirochetes have not been clarified well because of the limited molecular evidence of *Leptospira* infections in mammals. Leptospirosis is called as “rat fever” in Sri Lanka because typically rats are believed as the most common reservoir of leptospires. However, the available molecular evidence is not enough to conclude that rats are the major *Leptospira* reservoir in Sri Lanka.

Multilocus sequence typing (MLST) is one of the genotyping methods provided thorough a freely accessible public database sharing accumulated strains data from all over the world [66 – 69]. The use of IFA for the detection of anti-*Leptospira* antibodies in animals or humans has not been widely documented. One report has shown that utilization of IFA as an initial leptospirosis diagnostic test has a moderate sensitivity and specificity. Also, the report suggests that it could replace microscopic agglutination test (MAT), which is been increasingly questioned for its low sensitivity and complexity [70].

Hantavirus infection in Sri Lanka was first described by Vitarana and colleagues in 1988 [18]. Few reports have shown that individuals with suspected leptospirosis have been found to possess anti-hantavirus antibodies [19,20]. One of these reports describes the detection of anti-hantavirus antibodies from 8 of 105 leptospirosis suspected patients from Peradeniya teaching hospital in Kandy district of Sri Lanka [16]. This article demonstrated that these patients had elevated levels of antibodies against THAIV using serotyping and FRNT assays. These findings suggest that human hantavirus infection is prevalent in Kandy district of Sri Lanka and the virus is antigenically related to THAIV. In recent past several reports describing the presence of antibodies against THAIV-related hantavirus among humans and rodents in CKDu endemic areas of the country and there is a significant association of this virus infection with the renal disease [21,22,24,25]. Such serological evidence compels the

need of further research to understand the distribution of hantaviruses in the country which is poorly understood.

As there are comparatively more evidence on such zoonotic infections in rural settings of Sri Lanka and less information on urban areas, we selected an urban public market to carry out a survey on zoonotic pathogens among small mammals infesting the place to understand their carrier status and among the market workers to understand if they have exposed to these pathogens. Kandy municipal market is situated in the city of Kandy in the central province of Sri Lanka. It is the second largest city of the country with a population of about 125,000. Every day several hundreds of people visit this market to purchase food and other daily needs and they are at risk of acquiring the infections if the market is contaminated with the pathogens. Therefore, the current study will provide the information on circulation of two zoonotic infections in an urban public setting of Sri Lanka to fill the research need on the distribution and incidence of zoonotic diseases in the country.

Materials and methods

Ethics statement

The research protocol was approved by the Institutional Animal Care and Use Committee of University of Peradeniya, Sri Lanka (VER-15-007). All animal procedures carried out in this study were performed in accordance with Protocols for field and laboratory rodent studies [71]. Ethical clearance for the human blood collection was obtained from the ERC, Faculty of Allied Health Science, University of Peradeniya, Sri Lanka (AHS/ERC/2017/007), and the ERC of the Institute for Genetic Medicine, Hokkaido University, Japan (21-001).

Small mammal sample collection

A total of 116 rodents and 16 shrews were captured using traps placed at the Kandy municipal market in December 2014 and October 2015 (Table 15). Both cage type traps and Sherman traps (H. B. Sherman Traps, Inc., Tallahassee, FL, USA) were used for the collections. All the captured animals appeared to be healthy without any jaundice or internal bleeding. Sera and kidneys were collected from animals and stored at -20^{o}C and -80^{o}C until further use. Portions of fresh kidneys were used for the isolation of *Leptospira* immediately. Sera were not collected during the sampling in December 2014.

Human serum sample collection

Venous blood samples of 94 trade-personnel working in the Kandy municipal market were collected in February 2018. Blood was collected only from workers who are above 18 years old. The demographic data of the participants were also collected for

analysis purposes. Sera were separated from the blood samples and stored at -20ºC until further analyses.

Leptospira isolation and identification

During the dissection of the small mammals, 250 mg of kidneys were aseptically homogenized and suspended into 1.5 ml of *Leptospira* storage medium (10% bovine serum albumin in 25 mM phosphate buffer pH 7.4) [72]. Fifty µl of the kidney-suspension was inoculated to 3 ml of Ellinghausen-McCullough-Johnson-Harris (EMJH) medium that comprised Difco *Leptospira* Medium Base EMJH and Difco *Leptospira* Enrichment EMJH (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Cultures were kept at room temperature (25-30ºC). Severely contaminated cultures were filtrated through a 0.45 µm-filter before a passage into fresh media. This isolation trial was confined to animals captured in October 2015 due to the limitation of materials and equipment. The cultures were visually checked for growth of leptospires at every 2 weeks for 6 months by dark field microscopy. After 6 months, 500 µl of each positive culture medium was boiled and screened by PCR for *flaB* gene using the primers listed on table 14 and Platinum® *Taq* DNA Polymerase High Fidelity (Thermo Fisher Scientific, Waltham, MA, USA). The *flaB* PCR-positive DNA samples were subjected to PCRs targeting the leptospiral genes; *rrs2, secY, lipL32, lipL41, adk* and *icdA* using the primer sets listed on table 14. Positive PCR amplicons were sequenced by BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and a 3130xl Genetic Analyzer (Applied Biosystems).

PCR detection of *Leptospira* genes from small mammal kidneys

A portion of each kidney was homogenized, and total DNA was extracted by using Mighty Prep reagent for DNA (Takara Inc., Otsu, Japan) according to the manufacturer's instructions. The extracted DNA were screened by PCR for *Leptospira flaB* gene as described above. Next, *flaB* PCR-positive DNA samples were subjected to PCRs targeting the leptospiral genes; *rrs2, secY, lipL32, lipL41, adk* and *icdA* and positive amplicons were sequenced as described above.

***Leptospira* species identification, phylogenetic analysis and MLST analysis**

Obtained sequences were aligned in Geneious Prime 2020.2.2 (Biomatters, Ltd., Auckland, New Zealand) using MUSCLE. Then aligned sequences of each targeted gene were submitted to BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenic trees were constructed by the neighbor-joining method in the Geneious Prime 2020.2.2. Reference sequences for each targeted gene were selected from BLAST research results and MLST was performed to identify *Leptospira* genotype of samples using MLST scheme 3 primers [66]. A set of primers for *icdA* was optimized in this study. Sequences of *rrs2, secY, lipL32, lipL41, adk* and *icdA* were submitted to the query database in *Leptospira spp.* locus/sequence definitions database at PubMLST to identify sequence type (ST) from a combination of allelic profiles. (http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_leptospira_seqdef)

Table 14. Primers used for the PCR analyses in the study.

Gene	Primers	Sequence (5' - 3')
flaB	flaBF1	TCTCACCGTTCTCTAAAGTTCAAC
	flaBR1	CTGAATTCGGTTTCATATTTGCC
	flaBF2	TGTGCACAAGACGATGAAAGC
	flaBR2	AACATTGCCGTACCACTCTG
rrs2	rrs2Fd	CATGCAAGTCRAGCGRAGTA
	rrs2Rd	AGTTRAGCYCRCAGTTTTC
secY	secYFd	ATGCCGATCATYTTYGCTTC
	secYR3	TTCATGAAGCCTTCATAATTTCTCA
	secYF4	GCTTCTTCCTTGATCCTGTTTC
	secR4d	TTCATRAAGCCTTCRTAATTTCTCA
lipL32	lipL32Fd	ATCTCCGYTGCACTCTTTGCA
	lipL32Rd	TCACCATCATCATCATCGTYCAA
lipL41	lipL41F	TAGGAAATTGCGCAGCTACA
	lipL41Rd	GCATCGAGAGGRATYARCATCA
adk	adkFd	GGGCTGGAAAAGGYACRCAA
	adkF3d	CCTCAGATTTCYACRGGMGATA
	adkR3d	GCCACTCTTACRCAAGCTCC
icdA	icdA_F	GGGACGAGATGACCAGGAT
	icdA_R	CTTTTTTGAGATCCGCAGCTTT
	inner icdAF	GAACTGGAYTATTATGATTTAGG
	inner icdAR	TCYTTTGTIGCRAACCAAAGATC
cytb	L14115	GACATGAAAAATCATCGTTG
	Mmin-cyt-H	GGTGGAATGGGATTTTATCT

Small mammal species identification

Species of *Leptospira* PCR positive animals were initially identified by a morphological assessment. An additional *cytb* gene sequence analysis was carried out to determine the species of PCR positive shrew samples. Primers used for the PCR amplification are listed on table 14 [27]. Obtained sequences were analyzed and species were identified by Geneious Prime 2020.2.2 and BLAST search as described before.

***Leptospira* IgG detection in small mammals and humans**

To detect the anti-*Leptospira* antibodies in animal and human sera, an IFA was established. *Leptospira interrogans* strain MMC grown in EMJH liquid medium for 7 days were diluted appropriately to obtain the suitable cell numbers. Twenty-four well glass slides (Matsunami Glass Ind.) were spotted with 3 µl/well of diluted live-*Leptospira* culture and air-dried at room temperature before the fixation with acetone. Fixed slides were washed once with distilled water and air-dried before store in -20ºC until use.

To perform the IFA, 20 µl/well of animal and human sera diluted 1:200 in 1×PBS was added to the prepared slides and incubated for 1 hour at room temperature followed by the addition of fluorescent secondary antibodies for staining. Alexa Fluor 488-conjugated goat anti-rat IgG (for rat and *Bandicota* sera) (Invitrogen), and protein A (for shrew and human sera) were used as the secondary antibodies at 1:1000 and 1:500 dilutions respectively. Clear fluorescence signals of *Leptospira* cells were considered as a positive.

Anti-hantavirus IgG detection IFA for small mammals and humans

For the hantavirus IFA, authentic and recombinant IFA antigen slides were prepared. Thailand orthohantavirus (THAIV) was inoculated to the overnight grown Vero E6 cell monolayers on 6-well plates and incubated for 24 hours at 37 ^{o}C in a 5% CO_2 incubator to serve as authentic antigens. For recombinant antigen slides, THAIV Nucleocapsid (N) protein [25] was transiently expressed in Vero E6 cells and incubated described above. Then 24-well glass slides (Matsunami Glass Ind.) were separately spotted with 20 µl/well of infected and transfected cell suspensions and incubated for another 24 hours under the same conditions. Finally, the slides were fixed with acetone and washed once with distilled water and air-dried before store in -20^{o}C until use.

To detect the anti-hantavirus IgG antibodies in small mammal and human samples, 20 µl/well of 200-fold dilutions of animal and human sera were added to the prepared IFA slides and incubated for 1 hour at room temperature followed by the addition of fluorescent secondary antibodies for staining. Alexa Fluor 488-conjugated goat anti-rat IgG (for rat sera) (Invitrogen), and protein A (for shrew and human sera) (Invitrogen) were used as the secondary antibodies at 1:1000 and 1:500 dilutions respectively. Clear scattered fluorescent pattern in cell cytoplasm was considered as a positive staining for authentic THAIV IFA while a clear uniform fluorescent pattern in the cell cytoplasm surrounding the nucleus was considered as a positive staining for recombinant THAIV IFA.

Hantavirus serotyping ELISA for human samples

To determine the serotype of hantavirus infecting humans, both authentic and recombinant IFA positive samples, along with one sample negative for both IFAs were selected for the serotyping ELISA.

The ELISA procedure was carried out as described previously [73]. Briefly, ELISA plates were coated with ascites E5/G6 hantavirus N protein monoclonal antibodies for overnight at 4^{o}C followed by blocking with 3% BSA in PBS for 1 hour at room temperature. Chromatographically purified THAIV truncated rN (trN) protein and SEOV trN protein expressed in Baculovirus system were then added to separate wells as the antigens and incubated for 1 hour at room temperature. After washing with PBST, human serum samples diluted at 1:500 in 1×PBS was added and incubated for 1 hour at room temperature. Bound antibodies were detected by using horseradish peroxidase-labeled anti-human IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) secondary antibodies. O-phenylenediamine dihydrochloride (OPD) solution (OPD 1 tablet, 6 ml DDW, 4 µl 30% H_2O_2) was added to initiate the coloring reaction and OD values at 450 nm were determined.

Hantavirus serotyping IFA for human samples

Using the rGP and rGn antigens of Lanka virus and SA108 virus, IFAs were carried out to determine the definite serotype of hantaviruses infecting the THAIV IFA positive humans. The assays were carried out as described in chapter 2, in which rGP antigens were used to detect the presence of GP reactive antibodies and rGn antigens to determine the serotype based on the EPs. The serotype of infected virus by comparing the EPs against Lanka-rGn and SA108-rGn antigens separately.

Results

Isolation of *Leptospira* from animal kidney tissues

Three isolate candidates from kidneys of SLRa15_13, SLRa15_35 and SLSu15_43, were found in cultures as filamentous spiral bacteria under dark field microscopic observation after six months. PCR for *flaB* gene was positive for all three cultures. Also, all three isolates were positive for *rrs2, secY, lipL32, lipL41, adk* and *icdA* genes. Obtained sequences were analyzed by BLAST search.

Detection of leptospiral genes from kidneys

First, 132 DNA samples were screened by PCR targeting *flaB* gene of pathogenic *Leptospira* species. A total 6 (4.5%) DNA samples were positive and proceeded to amplify *rrs2, secY, lipL32, lipL41, adk* and *icdA* genes by PCR (Table 15). Five out of 6 DNA samples from kidneys, SLSu14_43, SLRa15_13, SLRa15_35, SLSu15_43 and SLRa15_86, could amplify all targeted genes. The other *flaB* positive sample, SLRa14_41 showed a weak amplification of *lipL32* and *lipL41* genes only.

Species identification of *Leptospira*-positive animals

Morphological features allowed the identification of *Leptospira*-positive animals SLSu14_43 and SLSu15_43 as shrews and SLRa14_41, SLRa15_13, SLRa15_35 and SLRa15_86 as rats. The species of shrews were determined as *Suncus murinus* by *cytochrome b* sequence analysis. The species of rats were determined as *Rattus rattus* complex by comparing the partial *cytb* sequences data of this study and the sequence data with the morphological assessment from previous reports [27].

Identification of isolated *Leptospira* species

The BLAST analysis showed that all the obtained sequences had an identity of 99 to 100% to *Leptospira borgpetersenii* sequences registered in GenBank. The neighbor-joining tree of *Leptospira* spp. based on *flaB* partial sequences showed that all the detected sequences clustered together with other *L. borgpetersenii* sequences (Figure 13).

All six gene loci of *Leptospira* MLST scheme 3 were successfully amplified and sequenced from the three isolates, two from *R. rattus* complex (SLRa15_13 and SLRa15_35) and one from *S. murinus* (SLSu15_43). The obtained sequences from SLRa15_35 and SLSu15_43 were identical and the MLST analysis resulted in a new allelic profile, (*adk/ icdA/ lipL32/ lipL41/ rrs2/ secY)*: (65/ 61/ 10/ 51/ 20/ 49), while SLRa15_13 had a different allelic profile of (65/ 60/ 10/ 51/ 20/ 49). Two other *flaB* PCR positive samples also allowed the amplification and sequencing of all the above loci. MLST analysis of those sequences resulted another two allelic profiles, i.e., SLSu14_43 (65/ 60/ 10/ 51/ 20/ 49) which was similar to that of isolate SLRA15_13 and SLRa15_86 (65/ 60/ 35/ 51/ 20/ 49) which was novel. The new allelic profiles showed a close identity with ST133 and ST189 (Table 16). All the reference STs defined *L. borgpetersenii* with isolate records from Tanzania (ST133), New Zealand, the Netherlands, Brazil, China and Guadeloupe (ST189).

***Leptospira* IFA for small mammal and human sera**

Animal sera were screened by IFA to detect the antibodies against whole cell leptospiral antigens in captured animals. Fifteen (13.8%) out of 108 small mammals were antibody positive for IFA. Four of the PCR positive animal sera were positive in the assay (Table 15). Of the 15 *Leptospira* IgG positive animals 7 and 8 were male

and female, respectively. This serological examination was limited for the samples collected in October 2015 since serum samples were not available from June 2014 collection.

Of the 94 market workers' serum samples 22 (23.4%) were IFA positive for anti-*Leptospira* antibodies (Table 17A). Only one female worker was found to be harboring IgG antibodies against *Leptospira* while the rest of 21 were males. All of them were working at the market for more than 8 years.

Table 15. Sample collection and test results for small mammals.

Year	Species	Captured No.	Tests for *Leptospira*			Hantavirus IFA
			IFA	PCR	Isolation	
2014[†]	*Rattus rattus* comp.	14	N/A	1	N/A	N/A
	Suncus murinus	10	N/A	1	N/A	N/A
2015	*Rattus rattus* comp.	102	13	3	2	12
	Suncus murinus	6	2	1	1	0
Total		132	15 (13.8%)	6 (4.5%)	3 (2.8%)	12 (11.1%)

[†]Sera from this collection were not available for testing. N/A: Not available

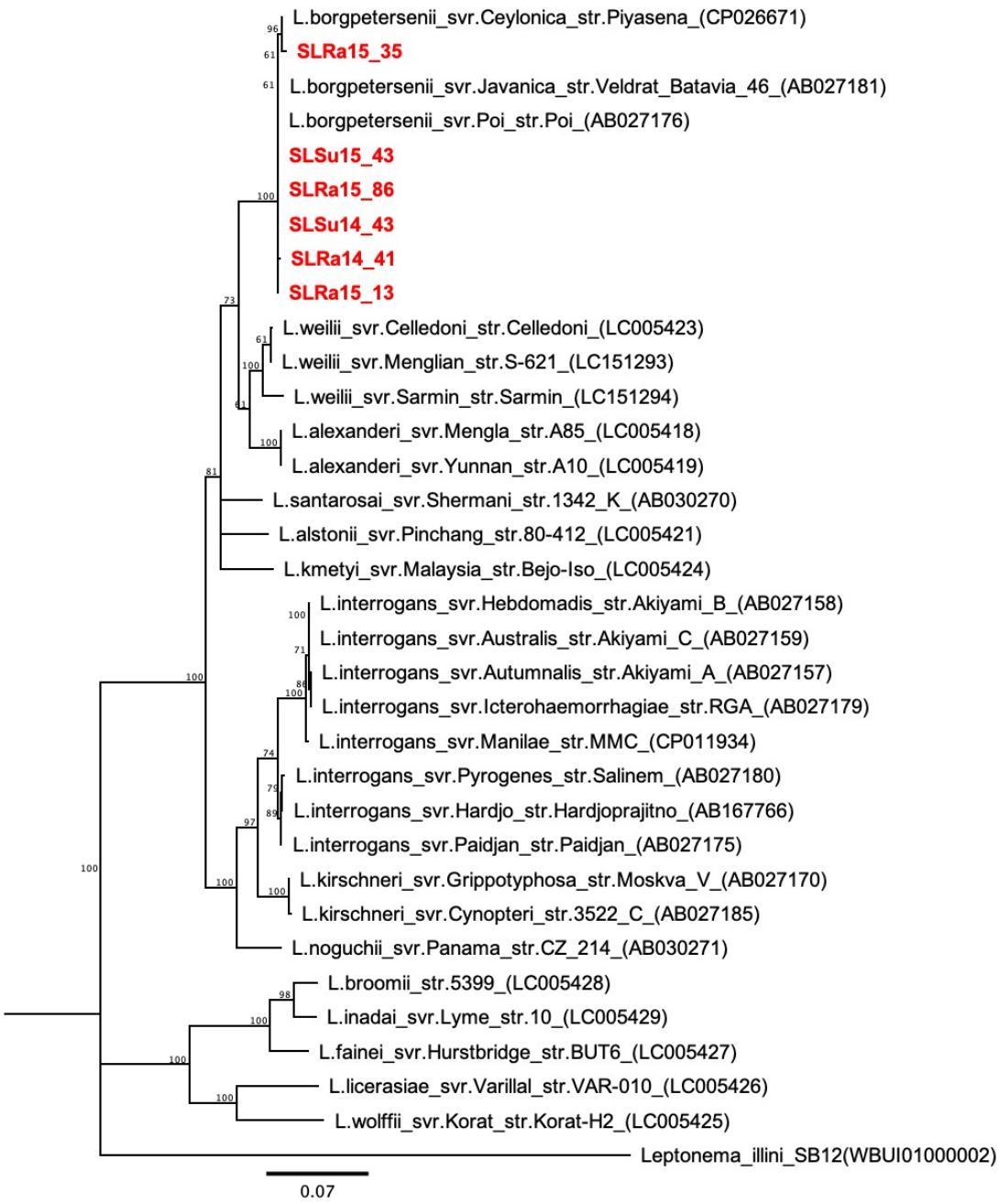


Figure 13. Neighbor-joining phylogenetic tree based on the partial *flaB* sequences. *Leptospira* spp. sequences detected from the animals in this study are shown in red color.

Table 16. *Leptospira* MLST analysis results.

Sample	Sequence Type (ST)	MLST Scheme 3 locus					
		adk	*icdA*	*lipL32*	*lipL41*	*rrs2*	*secY*
SLSu14_43 SLRa15_13		65	60	10	51	20	49
SLRa15_35 SLSu15_43		65	61	10	51	20	49
SLRa15_86		65	60	35	51	20	44
References	ST133	65	60	10	24	20	49
	ST189	75	61	10	51	20	49

Anti-hantavirus IgG detection in small mammal and human sera

Both authentic and recombinant IFA positive serum samples were considered as true positives for anti-hantavirus antibody harboring animal and human sera. Twelve (11.1%) of 108 animal sera were positive in these assays and all were found to be belonging to *Rattus rattus* complex. Out of 12 animals 10 were female rats and none of the animals having anti-hantavirus IgG were positive for *Leptospira* IgG (Table 15). Among 94 human serum samples 2 (2.1%) were positive for both IFA assays and both were males at their middle ages (Table 17A). One of them aged 37 who was detected seropositive for *Leptospira* IFA also, has been working in the market for 12 years as a helper.

Hantavirus serotyping ELISA for human samples

Two anti-hantavirus IgG positive human samples (#28 and #155) were used for the serotyping assay along with three other THAIV-IFA negative human sera. As shown in Figure 14 the OD values of IFA positive samples towards trN-THAIV antigen were higher than the OD values towards trN-SEOV. IFA negative samples showed very low signal when compared to the positive samples.

Hantavirus serotyping IFA for human samples

The two samples (#28 and #155) that were serotyped by ELISA as THAIV-like virus infected were further serotyped by more specific rGn-based IFA that was developed against Lanka and SA108 viruses as described in the previous chapter. Both sera were rGP IFA positive against both Lanka and SA108 antigens with a strong fluorescent signal. When they were subjected to serotyping IFA, both samples showed 4-fold or higher EP against Lanka-rGn antigens than against SA108-rGn antigens (Table 17B).

Demographic data analysis of seropositive market workers

Median (range) age of 23 seropositive market workers for both infections was 49 (23-71) years with 95.7% male predominance. All 23 were residing within Kandy district and 18 (78.3%) of them were residing in urban settings according to the demographic data obtained. Twenty-one (91.3%) of the seropositive individuals have been working in the market for more than 8 years.

Table 17. Test results for human sera.

A. Summary of human sample collection and test results. **B**. Serotyping IFA results of hantavirus IgG positive human serum samples.

A.

Gender	Collected No.	*Leptospira* IFA	Hantavirus IFA	Hantavirus serotype
Male	84	21	2	THAIV-like
Female	10	1	0	-
Total	94	22 (23.4%)	2 (2.1%)	-

B.

Sample ID	rGn EPs	
	Lanka	SA108
28	3200	800
155	6400	400

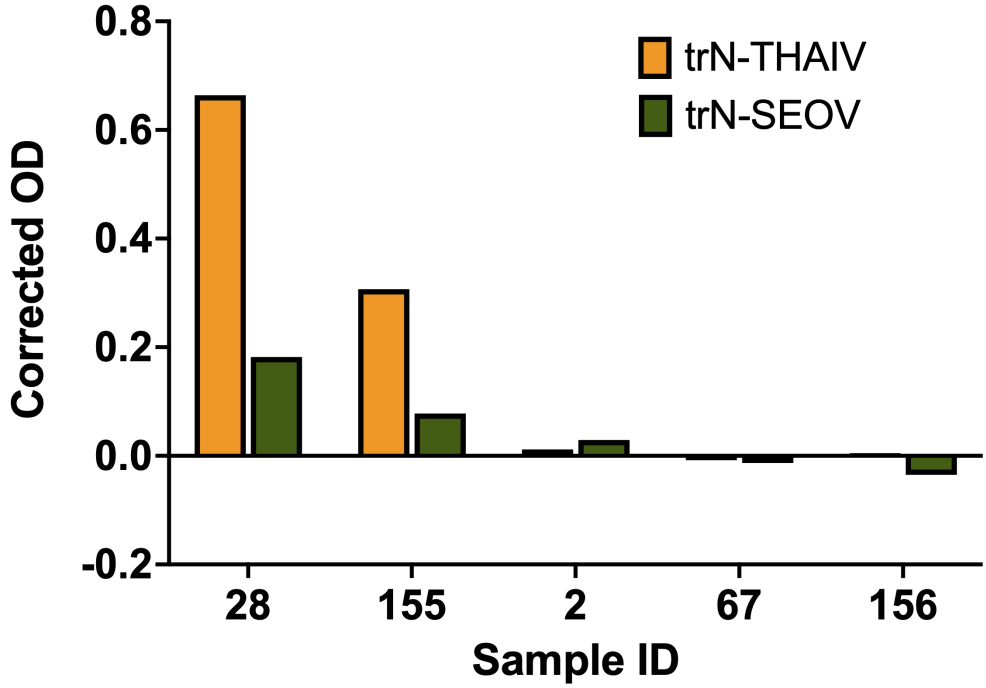


Figure 14. The serotyping ELISA results.

Human samples that were hantavirus IgG positive (#28 and #155) and representative negative (#2, #67, and #156) human sera were tested.

Discussion

The current study was aimed at obtaining molecular and serological evidence to determine the presence of two zoonotic pathogens and their infections in an urban public market in Sri Lanka. This municipal market is situated in the central area of the city of Kandy, where thousands of people come for their day-to-day work. The market is important for the public to buy fresh produce, meat, and grocery items. Due to the easy availability of food, the market premises are infested with animals such as rodents, shrews, street cats and dogs. Specially the rodents and shrews are actively searching for food during the night by crawling into the shops. Due to this the shop workers and the people who visit to buy the food are at high risk of exposing to the excreta of these animals though the contaminated items. Therefore, this study was carried out to detect the circulation of two zoonotic pathogens, i.e., hantaviruses and *Leptospira* species, primarily carried by rodents and shrews, and to assess the presence of the infection among the market workers who at the highest risk due to the continuous close contact with the contaminated goods.

The live trapping of small mammals was carried out for only a short period of time (1-2 weeks) during the nights. These rodents and shrews cause a huge pest problem to the market as they feed on the food and other materials. During the 2015 collection, 108 animals were live trapped in 9 nights indicating the high rat and shrew density in the premises. Any zoonotic disease that circulate among the rats and shrews infesting the market has the high potential of transmitting to humans and other companion animals in the market.

In the small animal survey detection of high seropositivity rates for both *Leptospira* (13.8%) and hantaviruses (11.1%) suggest the high incidence of these infections among the small mammal populations. Even though the *Leptospira* PCR detection

(4.5%) and culture isolation (2.8%) rates were low, all the detected and isolated samples were *L. borgpetersenii*, a well-documented pathogenic species. The isolation of pathogenic leptospires from rodents is an important aspect of this study because there were no records of *Leptospira* isolation from rodents in the country since 1970s until 2015 [59]. Also, by the MLST analysis of isolated and PCR detected *Leptospira*, several novel STs were identified for the first time. ST144 and ST157 listed in MLST database are *L. borgpetersenii* isolated from Sri Lanka. One of two records in ST144 is *L. borgpetersenii* serovar Ceylonica strain Piyasena, which was shown to be a close strain to the isolates obtained in this study by partial *flaB* sequence phylogeny. These leptospires are being shed in the urine of these rats and shrews that can contaminate the surrounding material (food and other grocery items), exposing not only the people who work at the shops but also the people who visit the market to buy the food, to the pathogen.

On the other hand, hantavirus infection is also found to be prevalent among the *Rattus rattus* population of the market. The hantavirus species that had been serologically identified to be present among humans in Kandy is a THAIV or THAIV-related virus and the same findings are documented from humans and rats in Girandurukotte area also. THAIV-like hantaviruses are carried by *Bandicota* and *Rattus* species. Recently, a such THAIV-like Anjozorobe virus carried by *Rattus rattus* and a novel orthohantavirus designated as Lanka virus carried by *Mus booduga* (little Indian field mouse) has been identified in Welikanda, Sri Lanka. But the prevalence of these viruses among rodents in Kandy is yet to be assessed. As all the sero-positive animals from the market were also *Rattus rattus*, we can suspect that they may be infected with THAIV or THAIV-like hantavirus. Another important fact is the association of THAIV-like viruses with the renal disease found in some regions in the country, where

the exposure to hantaviruses was found to be an independent risk factor associated with renal disease in the CKDu-endemic areas [24]. According to the seroprevalence rate among rodents, the market workers are at a high risk of acquiring the hantavirus infection from them via their contaminated excreta, especially during the cleaning and dusting of the surfaces.

The sero-survey among market workers revealed a much higher seropositivity rate towards *Leptospira* (23.4%) than towards hantaviruses (2.1%). The data obtained in this study are not enough to assess the exact place where they acquired the infections but to demonstrate that the workers are already infected with the pathogens. The data shows that more infections are detected among the male workers than in female workers, while one male individual was harboring IgG antibodies against both infections. Most of the seropositive individuals were residing in urban settings where high rodent populations are commonly found with elevated risk of infection. In a study carried out in the western province, Sri Lanka, isolated two *L. interrogans* strains from leptospirosis patients discusses that both exposures occurred in domestic settings [61] suggesting that urban *Leptospira* infections could be common in the country. Also, another hospital-based study describing the serological detection of co-infections with *Leptospira* and hantaviruses in western province authored by Sunil-Chandra and colleagues provide evidence for the circulation of both pathogens in urban areas of the country [20]. The serotyping ELISA results showing that the two hantavirus seropositive individuals were harboring specific antibodies against THAIV or THAIV-related hantavirus strengthens the previous findings from Kandy district leptospirosis-suspected febrile patients and supports the findings from CKDu endemic area residents in which the similar serotyping data were obtained [22]. More definitive serotyping information were obtained by subjecting those two sera to rGn-IFA

developed to determine whether the infection is caused by Lanka virus or SA108 virus. According to the rGn-IFA EPs, these individuals were infected with the *Mus*-borne Lanka virus. Although, this virus was first detected recently in Sinhapura area in Polonnaruwa district of Sri Lanka, serotyping information described in chapter 2 reveals its widespread distribution in several other regions in the dry zone of the country infecting human populations. The finding of Lanka virus infection among the Kandy market workers, provides the first evidence of its existence in wet zone and in a major urban community of the country. Therefore, these hantavirus serological findings collectively suggest that Sri Lankan rodents are harboring THAIV-like hantavirus in their populations and that the newly identified Lanka virus has spread to the human populations not only in rural dry zone areas but also in urban wet zone regions in the country.

The findings of the current zoonotic pathogens' survey are important in public health aspect because they demonstrate the circulation and prevalence of two infections among rodents and humans that shares the same public facility, highlighting the need of carrying out more similar studies in other major cities in the country and the implementation of strict animal control measures in public markets to ensure the safe health of workers and general public.

Brief summary

In this study, circulation of two zoonotic pathogens was assessed in an urban public market in the city of Kandy, Sri Lanka. The small mammals infesting the premises were first screened for *Leptospira* and hantavirus infections. Anti-leptospiral antibodies were detected in 13.8% of tested animals, with three *Leptospira* isolates obtained by animal kidney culture and additional 3 animals were detected with *Leptospira* genes in their kidneys by PCR. All isolates and positive PCR amplicons from animal kidneys were identified as pathogenic species *L. borgpetersenii* and MLST scheme 3 analysis of these samples resulted novel sequence types. IgG antibodies against hantaviruses were detected in 12 animals. The market workers were screened next for these two diseases using their sera. Anti-leptospiral antibodies and anti-hantavirus antibodies were detected by IFA and found that 23.4% and 2.1% were seropositive for *Leptospira* and hantaviruses, respectively. One individual was having antibodies against both pathogens. The hantavirus serotype in infected humans were identified by ELISA as THAIV-like while rGn-IFA confirmed it as Lanka virus. The study results show clear molecular and serological evidence for the presence of two zoonotic pathogens and their infections in animal and human associated to the market. The transmission of these pathogens from infected animals to humans can occur within the market or via the contaminated food, indicating a high public health risk. Therefore, implementation of strict animal control measures is suggested to the authorities to ensure the safe health of market workers and the general public.

Conclusions

This study aims at providing relevant information to understand the distribution of two zoonotic infections among selected small animal and human populations in Sri Lanka. The findings of this study will be useful for the control of these two infections in the country.

The survey on hantaviruses mainly focused on CKDu endemic regions of the country where the infection was reported to be prevalent among both rodent and human populations. The hantavirus genome information from Sri Lankan rodents are important for the implementation of control measures to stop the virus infection in humans. Also, the identification of *Mus booduga* as a novel orthohantavirus reservoir host warrants for more studies on other *Mus* species in nature to understand whether they also have the potential to carry hantaviruses.

The large scale and widespread human infections by new-found Lanka hantavirus alert the Sri Lankan public health system to implement measures to control the virus infection in humans by controlling the reservoir rodent populations particularly in CKDu endemic areas. The detection of similarly high positive rates of Lanka virus infection in human samples collected at different years from different CKDu-endemic areas in last 10 years, indicates that the virus and its natural hosts are distributed in those areas without any regional difference, and thus, control measures are very important to minimize the further spreading of the virus that can result more human infections.

The screening and serotyping assays developed in the study to assess the human infections caused by Sri Lankan hantaviruses shows promising to use them for large scale screening and serotyping assays to understand the infection status in island wide sero-surveys.

On the other hand, detection and isolation of pathogenic *Leptospira* species from rodents and shrews in Kandy market indicate the circulation of these bacteria in urban regions that are highly populated. The *Leptospira* genetic information from host animals in Sri Lanka are limited and these findings contribute to the better characterization of circulating species using tools such as MLST analysis. The MLST results with novel STs show that the diversity of this bacteria is high in the country even within a single genome species. Due to the habitat of the *Leptospira* host animals found in this study urges for strict rodent control measures and further research regarding this endemic disease in urban areas in the country. Furthermore, the hantavirus seropositive rodents suggesting the possible circulation of the virus in the city, again demands the availability of effective rodent control measures and good hygienic practices in public places.

The information on human leptospirosis in the country are well documented as the disease is considered to be hyperendemic, while the information on hantavirus infection is still being identified in the country, particularly in CKDu endemic regions. As this study revealed the presence of Lanka hantavirus infection among the market workers, which should be carefully studied further to understand whether this infection was acquired from the market premises or from their homes. Either of these possibilities shows that Lanka virus has spread to urban areas of the country also.

In this thesis, important information about hantaviruses and *Leptospira* along with their rodent and human infections in Sri Lanka are provided. This information can be used as baseline to implement effective control measures to address these public health issues. Also, this information will support the future studies on both infections to assess the status of these diseases in Sri Lanka.

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