Short Report: Genetic Diversity of Thottapalayam Virus, a Hantavirus Harbored by the Asian House Shrew (*Suncus murinus*) in Nepal

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Abstract. Despite the recent discovery of genetically divergent hantaviruses in shrews of multiple species in widely separated geographic regions, data are unavailable about the genetic diversity and phylogeography of Thottapalayam virus (TPMV), a hantavirus originally isolated from an Asian house shrew (*Suncus murinus*) captured in southern India more than four decades ago. To bridge this knowledge gap, the S, M, and L segments of hantavirus RNA were amplified by reverse transcription polymerase chain reaction from archival lung tissues of Asian house shrews captured in Nepal from January to September 1996. Pair-wise alignment and comparison revealed approximately 80% nucleotide and > 94% amino acid sequence similarity to prototype TPMV. Phylogenetic analyses, generated by maximum likelihood and Bayesian methods, showed geographic-specific clustering of TPMV, similar to that observed for rodent- and soricid-borne hantaviruses. These findings confirm that the Asian house shrew is the natural reservoir of TPMV and suggest a long-standing virus–host relationship.

A previously long-unclassified virus, isolated from an Asian house shrew (*Suncus murinus*) captured in Thottapalayam, near Vellore in Tamil Nadu, India, in 1964,¹ is now known to be a member of the *Hantavirus* genus (Family *Bunyaviridae*) by virtue of its morphological characteristics and overall genomic organization as well as its antigenic and evolutionary relationship with well-characterized rodent-borne hantaviruses.^{2,3} The assumption that Thottapalayam virus (TPMV) represented a spillover event from a rodent reservoir has been thoroughly discredited by full-genome analysis, showing strong support for an ancient non-rodent host origin and an early evolutionary divergence from rodent-associated hantaviruses.^{3,4} Moreover, the realization that shrews of multiple species (Order *Soricomorpha*, Family *Soricidae*), including the Therese's shrew (*Crociodura theresae*),⁵ Ussuri white-toothed shrew (*Crociodura lasiura*),⁶ northern short-tailed shrew (*Blarina brevicauda*),⁷ Chinese mole shrew (*Anourosorex squamipes*),⁸ Eurasian common shrew (*Sorex araneus*),⁹ masked shrew (*Sorex cinereus*),¹⁰ dusky shrew (*Sorex monticolus*),¹⁰ and flat-skulled shrew (*Sorex roboratus*),¹¹ harbor hantaviruses that are far more genetically diverse than those viruses carried by rodents suggests that shrews, being evolutionarily more ancient than rodents, may have served as the primordial hosts of ancestral hantaviruses.

Although the isolation of TPMV predates the isolation of all other hantaviruses, including Hantaan virus (HTNV),¹² the prototype virus of hemorrhagic fever with renal syndrome (HFRS),¹³ data about its genetic diversity and phylogeography are unavailable. To address this gap in knowledge, we analyzed tissues originally collected for studies on the prevalence and genetic diversity of bartonellae.¹⁴ Archival lung and kidney tissues from 134 Asian house shrews, trapped around

densely populated human habitations in Bhaktapur (Pode Tole, Bhelkhel, and Bhelukhel), Kathmandu (Kalimati, Lagan Tole, Sabal Bahal, Teku, Hyumata, and Inakha Tole), and Lalitpur (Bakhar, Bakhar Thati, Lagankhel, and Patan) districts of Nepal (Figure 1) from January 31 to April 10, 1996 and July 22 to September 3, 1996, were tested for hantavirus RNA by reverse transcription polymerase chain reaction (RT-PCR) (Table 1).

Initially, total RNA was extracted from frozen tissues using the PureLink Micro-to-Midi Total RNA Purification Kit (Invitrogen, San Diego, CA), and cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen) and random hexamers as well as a universal primer (OSM55: 5'-TAGTAGTAGACTCC-3') designed from the genus-specific conserved 3' end of the S, M, and L segments of all hantaviruses. Oligonucleotide primers designed for amplification and sequencing of hantaviruses are shown in Table 2. PCR was performed in 20-μL reaction mixtures containing 250 μM deoxyribonucleotide triphosphate, 2 mM MgCl₂, 1 U AmpliTaq polymerase (Roche, Basel, Switzerland), and 0.25 μM each primer. Initial denaturation was at 94°C for 5 minutes followed by two cycles each of denaturation at 94°C for 40 seconds, 2° step-down annealing from 48°C to 38°C for 40 seconds, elongation at 72°C for 1 minute, 32 cycles of denaturation at 94°C for 40 seconds, annealing at 42°C for 40 seconds, and elongation at 72°C for 1 minute in a GeneAmp PCR 9700 thermal cycler (Perkin-Elmer, Waltham, MA). Amplicons were separated by electrophoresis on 1.5% agarose gels and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and DNA was sequenced directly using an ABI Prism 377XL Genetic Analyzer (Applied Biosystems, Foster City, CA).⁸

As determined by RT-PCR and sequencing, TPMV RNA was detected in lung tissues of 12 Asian house shrews (Tables 1 and 3). Of these samples, TPMV RNA was detected in kidney tissues of only four shrews, suggesting lower viral burden; 11 of 12 TPMV-infected shrews were captured in the Kathmandu district. Overall prevalence of TPMV infection was essentially the same during the January to April (6/72 or 8.3%) and

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FIGURE 1. Map of Nepal showing metropolitan centers (black dots) in Kathmandu, Bhaktapur, and Lalitpur districts where Asian house shrews were captured in 1996.

the July to September (6/62 or 9.7%) time periods in 1996 (Table 1).

Although studies comparing the prevalence of hantavirus infection using serologic and genetic assays are few, hantavirus RNA is frequently undetectable in tissues of rodents and shrews, when their sera contain anti-hantavirus immunoglobulin G (IgG) antibody,^{15,16} possibly because of the insensitivity of RT-PCR or the failure of some rodents and shrews to develop persistent infections. In this study, shrew sera were not available for anti-TPMV antibody testing. Therefore, the frequency of TPMV infection, as determined by RT-PCR, likely represents the minimum prevalence. Conceivably, if serologic evidence of infection had been used instead, the prevalence of TPMV infection might be much higher than the 8.3% or 9.7% noted above.

Pair-wise alignment and analysis of 359–1,199 nucleotides of the nucleocapsid-encoding S segment, 377–404 nucleotides of the envelope glycoprotein-encoding M segment, and 1,436–2,484 nucleotides of the RNA-dependent RNA polymerase-

TABLE 1
Prevalence of TPMV infection in Asian house shrews captured in Nepal in 1996

District	Month and year	Number trapped	TPMV RNA
Kathmandu	January to April 1996	43	5
Bhaktapur		10	1
Lalitpur		19	0
Kathmandu	July to September 1996	61	6
Lalitpur		1	0

encoding L segment revealed that TPMV strains from Asian house shrews, captured in two adjacent districts in Nepal, exhibited very low nucleotide sequence variation ($S = 0$ –1.7%, $M = 0$ %; $L = 0.1$ –0.7%), but these samples differed in all three segments from the prototype TPMV strain VRC66412 from India by approximately 20% (Table 4). However, the amino acid sequences of the encoded proteins were highly conserved, with 2.8–5.3% variation between TPMV strains from Nepal and India. Compared with soricine shrew-borne hantaviruses (shown in Figure 2), the genetic distances of TPMV strains from Nepal were approximately 30–50% at the nucleotide and amino acid levels for each segment.

To determine the molecular diversity of TPMV strains, DnaSP 5.10¹⁷ was used for haplotype diversity, nucleotide diversity, Tajima's D test, and Fu and Li's D and F tests (Table 5). The level of diversity in TPMV strains was significantly lower than the diversity of all other hantaviruses in three segments. TPMV strains gave negative values for the Tajima's D and Fu and Li's D and F tests, and most of the values were not statistically significant except for the S segment.

Maximum likelihood (ML) and Bayesian methods, implemented in Phylogenetic Analysis Using Parsimony, 4.0b10 (PAUP*),¹⁸ RAxML Blackbox webserver,¹⁹ and MrBayes 3.1²⁰ under the best-fit GTR+I+Γ model of evolution using jModeltest 0.1.1,²¹ were used to generate phylogenetic trees. Two complementary and not redundant ML methods were used to gain additional analytical rigor; RAxML is a very useful and

TABLE 2
Oligonucleotide primers for amplification and sequencing of TPMV

Primer	Segment	Polarity	Sequence (5' to 3')
S437F	S	+	SWG GTC ARA CTG CHR AYT GG
Han-S1260R	S	–	CDG GRT CCA TRT CAT CHC CAN
Han-S694F	S	+	CCN GTN ATG RGN GTN ATH GGN TT
Cro2R	S	–	ANG AYT GRT ARA ANG ANG AYT TYT T
Han-M1190F	M	+	GGN CCN GGD GCW NVH TGT GA
Han-M2219R	M	–	CAT KCN CCA TAR CAR TGR AA
HTM-1490F	M	+	TGT GTN CCW GGN TTY CAT GGN T
ULU-M2178R	M	–	GTA TCM ANC CAR TGN CCT AG
Han-L1900F	L	+	ATG AAR NTN TGT GCN ATN TTT GA
Han-L3000R	L	–	GCN GAR TTR TCN CCN GGN GAC CA
Han-L2520F	L	+	ATN WGH YTD AAR GGN ATG TCN GG
Han-L2970R	L	–	CCN GGN GAC CAY TTN GTD GCA TC

TABLE 3
TPMV strains detected in lung tissues of Asian house shrews captured in Nepal

TPMV strain	District	Capture date	GenBank accession number		
			S segment	M segment	L segment
H-1091/96	Bhaktapur Kathmandu	March 22, 1996	HQ831358		
H-0274/96		February 2, 1996	HQ831359		
H-0570/96		February 20, 1996	HQ831360	HQ831376	
H-0898/96		March 10, 1996	HQ831361		
H-1779/96		April 7, 1996	HQ831362	HQ831375	HQ831374
H-1863/96		April 9, 1996	HQ831363	HQ831377	HQ831373
H-3753/96		August 20, 1996	HQ831364		
H-3758/96		August 20, 1996	HQ831365		HQ831372
H-3858/96		August 26, 1996	HQ831366		
H-4066/96		August 30, 1996	HQ831367		
H-4111/96		September 3, 1996	HQ831368		HQ831371
H-4116/96		September 3, 1996	HQ831369		HQ831370

powerful method for doing rapid ML bootstrap analysis, but because RAxML is a relatively new method, the topologies were backed up with PAUP*, which is more traditional and well-accepted in the field. Parameters were reestimated during successive rounds of ML heuristic searches using the tree bisection reconnection (TBR) and subtree-pruning-regrafting (SPR) algorithms implemented in PAUP*.²² Nearly identical tree topologies, well-supported by bootstrap analysis (> 70%) and posterior node probabilities (> 0.70), were estimated from analysis of the partial S-, M-, and L-segment sequences of TPMV from Nepal, which formed a monophyletic group with the prototype TPMV strain from India (Figure 2).

Phylogenetic networks of each genomic segment, as determined by the median-joining algorithm using Network 4.6,²³ indicated clear division between TPMV and Imjin virus (MJNV) strains (Figure 3). Although TPMV strains from Nepal showed closer network relationships with prototype TPMV (VRC66412) from India, they clearly formed separate branches.

In this regard, phylogenetic segregation of TPMV sequences according to the geographic origin of the reservoir host species resembled that shown recently for Seewis virus in *Sorex araneus*,^{24,25} and MJNV in *Crocidura lasiura*.¹⁶ These data were also consistent with the geographic-specific lineages shown previously for rodent-borne hantaviruses, including HTNV in *Apodemus agrarius*,²⁶ Soochong virus in *Apodemus peninsulae*,²⁷ Puumala virus in *Myodes glareolus*,^{28,29} Muju virus in *Myodes regulus*,³⁰ and Tula virus in *Microtus arvalis*.³¹ Overall, these data were suggestive of long-standing associations between hantaviruses and their reservoir rodent and soricid

hosts, but the basis for these relationships through evolutionary time is unclear.

A distinct weakness of this opportunistic study is that our analysis was based on a very limited number of TPMV strains from a restricted geographic region in Nepal. Phylogeographic studies of TPMV throughout the vast geographic range of the Asian house shrew are necessary to obtain clearer insights into the biogeographic origin and radiation of hantaviruses and their soricid hosts as well as to clarify whether other crocidurine shrews in Asia harbor TPMV or TPMV-related hantaviruses.

Molecular identification of TPMV-infected shrews was determined by amplification and sequencing of the entire 1,140-base pair cytochrome *b* gene of mitochondrial DNA using previously described universal primers.²² All TPMV-infected shrews from Nepal were confirmed as Asian house shrews by phylogenetic analysis of cytochrome *b* sequences of multiple shrew species available in GenBank.³² This seemingly trivial finding firmly established that the Asian house shrew is the natural reservoir of TPMV and dismissed lingering doubts that another shrew or rodent species might be involved. That said, the issue of host sharing with other crocidurine shrew species is now being pursued by testing tissues from the Anderson's shrew (*Suncus stoliczkanus*), which is found near watercourses and grassy embankments at lower elevations, occasionally sharing habitats with the Asian house shrew in Nepal.

Besides Nepal, the highly adaptable Asian house shrew is widely distributed throughout Afghanistan, Bangladesh,

TABLE 4
Sequence similarities of the S, M, and L segments of TPMV strain VRC66412 and TPMV strains from Asian house shrews captured in Nepal

TPMV strain	S segment (%)		M segment (%)		L segment (%)	
	nt	aa	nt	aa	nt	aa
H-1091/96	80.2 (527 nt)	96.0 (175 aa)	—	—	—	—
H-0274/96	80.1 (848 nt)	97.2 (282 aa)	—	—	—	—
H-0570/96	79.8 (550 nt)	96.2 (183 aa)	81.4 (404 nt)	94.8 (134 aa)	—	—
H-0898/96	80.0 (522 nt)	96.0 (174 aa)	—	—	—	—
H-1779/96	79.9 (536 nt)	96.1 (178 aa)	81.5 (377 nt)	95.2 (125 aa)	81.6 (1,439 nt)	95.8 (479 aa)
H-1863/96	80.2 (1,046 nt)	96.6 (384 aa)	81.1 (401 nt)	94.7 (133 aa)	79.9 (2,482 nt)	94.9 (827 aa)
H-3753/96	80.8 (359 nt)	95.8 (119 aa)	—	—	—	—
H-3758/96	80.3 (546 nt)	96.1 (182 aa)	—	—	81.4 (1,436 nt)	95.6 (478 aa)
H-3858/96	80.8 (359 nt)	95.8 (119 aa)	—	—	—	—
H-4066/96	80.3 (398 nt)	94.7 (132 aa)	—	—	—	—
H-4111/96	80.3 (1,199 nt)	97.0 (399 aa)	—	—	80.2 (2,484 nt)	95.0 (828 aa)
H-4116/96	80.7 (363 nt)	95.0 (121 aa)	—	—	80.0 (2,484 nt)	94.8 (828 aa)

TPMV = Thottopalayam virus; nt = nucleotide; aa = amino acid.

FIGURE 2. Phylogenetic trees generated by the ML and Bayesian methods under the best-fit GTR+I+Γ model of evolution based on partial sequences of the S-, M-, and L-genomic segments of TPMV strains from Nepal and other well-characterized hantaviruses. The phylogenetic positions of TPMV strains from Nepal are shown in relationship to prototype TPMV VRC66412 (AY526097, EU001329, and EU001330) from India. Also shown are Imjin virus (MJNV CI05-11: EF641804, EF641798, and EF641806) from the Ussuri white-toothed shrew (*Crocidura lasiura*), Seewis virus (SWSV mp70: EF636024, EF636025, and EF636026) from the Eurasian common shrew (*Sorex araneus*), Ash River virus (ARRV MSB73418: EF650086 and EF619961) from the masked shrew (*Sorex cinereus*), Qiandao Lake virus (QDLV YN05-284: GU566023, GU566022, and GU566021) from the stripe-backed shrew (*Sorex cylindricauda*), Jemez Springs virus (JMSV MSB144475: FJ593499, FJ593500, and FJ593501) from the dusky shrew (*Sorex monticolus*), Kenkeme virus (KKMV MSB148794: GQ306148, GQ306149, and GQ306150) from flat-skulled shrew (*Sorex roboratus*), Cao Bang virus (CBNV CBN-3: EF543524, EF543526, and EF543525) from the Chinese mole shrew (*Anourosorex squamipes*), Camp Ripley virus (RPLV MSB89863: FJ790772, EF540774, and EF540771) from the northern short-tailed shrew (*Blarina brevicauda*), Asama virus (ASAV N10: EU929072, EU929075, and EU929078) from the Japanese shrew mole (*Urotrichus talpoides*), Oxbow virus (OXBV Ng1453: FJ539166, FJ539167, and FJ539497) from the American shrew mole (*Neurotrichus gibbsii*), and Nova virus (NVAV MSB95703: FJ539168, HQ840957, and FJ593498) from the European common mole (*Talpa europaea*). Representative rodent-borne hantaviruses are shown, including Hantaan virus (HTNV 76-118: NC_005218, NC_005219, and NC_005222), Soochong virus (SOOV SOO-1: AY675349, AY675353, and DQ056292), Dobrava virus (DOBV Greece: NC_005233, NC_005234, and NC_005235), Seoul virus (SEOV 80-39: NC_005236, NC_005237, and NC_005238), Tula virus (TULV 5302v: NC_005227, NC_005228, and NC_005226), Puumala virus (PUUV Sotkamo: NC_005224, NC_005223, and NC_005225), Prospect Hill (PHV PH-1: Z49098, X55129, and EF646763), Sin Nombre virus (SNV NMH10: NC_00521, NC_005215, and NC_005217), and Andes virus (ANDV Chile9717869: NC_003466, NC_003467, and NC_003468). All tree topologies were very similar using MrBayes, RAXML, and PAUP. Therefore, trees generated by PAUP* were selected. The numbers at each node are posterior probabilities (above) based on 2 million generations with estimated sample sizes well over 100 (implemented in MrBayes) and bootstrap values (below) from 1,000 ML replicates (implemented in RAXML), respectively. The scale bars indicate nucleotide substitutions per site.

Bhutan, Brunei Darussalam, Cambodia, China, India, Indonesia, Lao People's Democratic Republic, Malaysia, Myanmar, Pakistan, Singapore, Sri Lanka, Taiwan, Thailand, and Viet Nam. Possibly because their diets consist mostly of insects, Asian house shrews may have been intentionally introduced by humans as a possible means of vector control into Africa (Egypt, Eritrea, Kenya, Republic of Djibouti, Rwanda, Sudan, and Tanzania), the Middle East (Iraq, Kingdom of Bahrain, Kuwait, Saudi Arabia, Sultanate of Oman, and Yemen), islands within the Indian Ocean (Comoros,

Madagascar, Republic of Mauritius, and Réunion), and Asia and the Pacific (Japan, Guam, and Philippines). Although typically residing near human habitation, Asian house shrews have been found in diverse environments ranging from natural forests, scrubland, and grasslands to plantations, pasture, cultivated fields, and altitudes exceeding 2,000 m.

Nepal is a landlocked South Asian country sharing borders to the north with the People's Republic of China and to the south, east, and west with India. Of 75 districts, the most densely populated is Kathmandu District, with the population

TABLE 5
Neutrality tests and haplotype and nucleotide diversity analyses of TPMV

Segment	Subgroup	n	Hd (mean ± SD)	π (mean ± SD)	Tajima's D	Fu and Li's D	Fu and Li's F
S	TPMV	13	0.603 ± 0.088	0.03099 ± 0.024	-2.30339 (<i>P</i> < 0.001)	-3.03677 (<i>P</i> < 0.02)	-3.25097 (<i>P</i> < 0.02)
S	Total	33	0.875 ± 0.057	0.42197 ± 0.025	-0.41991 (NS)	0.72590 (NS)	0.39173 (NS)
M	TPMV	4	0.500 ± 0.265	0.09200 ± 0.048	-0.86982 (NS)	-0.86982 (NS)	-0.92815 (NS)
M	Total	23	0.988 ± 0.019	0.41007 ± 0.010	-0.41824 (NS)	0.66588 (NS)	0.38072 (NS)
L	TPMV	6	1.000 ± 0.096	0.17068 ± 0.068	-1.4223 (NS)	-1.33317 (NS)	-1.48874 (NS)
L	Total	26	0.991 ± 0.015	0.33466 ± 0.027	-0.69 (NS)	-0.52655 (NS)	-0.6838 (NS)

n = number of sequenced strains; Hd = haplotype diversity; π = nucleotide diversity; NS = not significant (*P* > 0.10).

FIGURE 3. Phylogenetic networks based on partial nucleotide (nt) and amino acid (aa) sequences of the S, M, and L segments constructed by the median-joining algorithm using Network 4.6. The numbers on the branches are proportional to the number of allele differences, and the sizes of the circles are proportional to the number of virus strains. Black and gray circles represent TPMV and MJNV strains, respectively. The small white circles are hypothetical median vectors created to connect existing strains within the network by maximum parsimony.

exceeding 1.5 million people. Studies of febrile patients in Kathmandu show an unrelentingly heavy disease burden of enteric fever (caused by *Salmonella* serovars Typhi and Paratyphi A), streptococcal pneumonia, dengue fever, murine typhus, scrub typhus, and leptospirosis.^{33–35} Limited and inadequate diagnostic facilities, however, still leave many febrile illnesses without a definitive microbial etiology. Although human exposure to peridomestic Asian house shrews presumably occurs with some regularity, the frequency of TPMV infection in humans is unknown. The recent detection of IgG antibodies against TPMV by enzyme immunoassay, Western blot analysis, and focus-reduction neutralization test in a febrile Laotian patient hospitalized in Thailand³⁶ is suggestive of past infection, but it falls short of showing disease causation. Intensified efforts, now underway, to test sera from residents in neighborhoods in Kathmandu, where TPMV-infected Asian house shrews have been captured, might clarify if TPMV accounts for undiagnosed febrile diseases.

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