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RECONSTRUCTING THE EVOLUTIONARY HISTORY OF HANTAVIRUSES

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Segregation of hantaviruses into clades that parallel the molecular phylogeny of rodents (order Rodentia) in the Murinae, Arvicolinae, Neotominae and Sigmodontinae subfamilies suggests that hantaviruses may have co-evolved with their reservoir hosts. Recently, however, host switching and local species-specific adaptation have been proposed to account for the similarities between host and virus phylogenies. The demonstration that Thottapalayam virus, a hantavirus isolated from the Asian house shrew (*Suncus murinus*), represents an early evolutionary divergence from other hantaviruses has spurred a search to ascertain if small mammals having shared ecosystems with rodents, such as shrews (order Soricomorpha, family Soricidae) and moles (family Talpidae), as well as bats (order Chiroptera), may have figured prominently in the evolution and diversification of hantaviruses. Archival tissues from 1,258 shrews (47 species), 152 moles (10 species) and 329 bats (32 species), captured in Europe, Asia, Africa and North America in 1980-2012, were analyzed for hantavirus RNA by RT-PCR. The realization that newfound hantaviruses detected in soricomorphs are genetically more diverse than those harbored by rodents suggests that the evolutionary history of hantaviruses is more ancient and complex than previously conjectured and that ancestral rodents may not have been the original mammalian hosts of primordial hantaviruses.

Key words: hantavirus, shrew, mole, bat, phylogeny

РЕКОНСТРУКЦИЯ ЭВОЛЮЦИОННОЙ ИСТОРИИ ХАНТАВИРУСОВ

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Разделение хантавирусов на клады, соответствующие молекулярной филогении грызунов (порядок Rodentia) в подсемействах Murinae, Arvicolinae, Neotominae Sigmodontinae означает, что хантавирусы, возможно, ко-эволюционировали с их резервуарными хозяевами. Недавно, однако, было высказано предположение, что сходство между филогенией хозяев и хантавирусов обусловлено процессами смены хозяев и локальной видоспецифичной адаптации. Демонстрация того, что вирус Топтапалайям – хантавирус, изолированный от домового мускусной землеройки (*Suncus murinus*) – раньше всех остальных дивергировал от остальных хантавирусов, стимулировала поиск доказательств того, что мелкие млекопитающие, которые занимают те же экосистемы, что и грызуны, например, землеройки (порядок Soricomorpha, сем. Soricidae), кроты (сем. Talpidae) и летучие мыши (пор. Chiroptera), возможно, играли заметную роль в эволюции и диверсификации хантавирусов. Архивные образцы тканей 1285 землероек (47 видов), 152 кротов (10 видов) и 329 летучих мышей (32 видов), отловленных в Европе, Азии, Африке и Северной Америке в период 1980–2012 гг., были проанализированы на наличие РНК хантавирусов с помощью ОТ-ПЦР. Осознание того факта, что новые хантавирусы, обнаруженные в землеройках, более генетически разнообразны, чем хантавирусы, инфицирующие грызунов, наводит на мысль о том, что эволюционная история хантавирусов более древняя и сложная, чем ранее предполагалось, и что грызуны, возможно, не являлись исходным хозяином для предковых форм хантавирусов.

Ключевые слова: hantavirus, землеройка, крот, летучая мышь, филогения

INTRODUCTION

Virus and host gene phylogenies suggest that hantaviruses co-evolved with their reservoir rodent hosts. That is, phylogenetic analyses, based on full-length viral genomic sequences and host mitochondrial DNA (mtDNA) sequences, indicate that antigenically and genetically distinct hantaviruses segregate into clades, which parallel the evolution of rodents (order Rodentia) in the Murinae, Arvicolinae, Neotominae and Sigmodontinae subfamilies [18]. Recently, however, on the basis of the disjunction between evolutionary rates of the reservoir rodent host and hantavirus species, host switching and local species-specific adaptation, rather than co-divergence, have been proposed to account for the similarities between host and virus phylogenies [20]. When viewed within the context that Thottapalayam virus (TPMV), a hantavirus isolated from the Asian house shrew (*Suncus murinus*) [24, 25], represents a separate and divergent

phylogenetic clade, the long-forgotten, historical accounts of hantavirus antigens in non-rodent small mammals captured in HFRS-endemic regions, such as the Eurasian common shrew (*Sorex araneus*), alpine shrew (*Sorex alpinus*), Eurasian water shrew (*Neomys fodiens*) and common mole (*Talpa europea*) in Russia and the former Yugoslavia [7, 11, 19], and the greater white-toothed shrew (*Crocidura russula*) and Chinese mole shrew (*Anourosorex squamipes*) in China [3], suggest that rodents may not be the primordial or principal reservoir hosts of hantaviruses. An opportunistic search was conducted to ascertain the role of other small mammals in the evolution and diversification of hantaviruses.

MATERIALS AND METHODS

Archival frozen tissues from 1,258 shrews (47 species) and 152 moles (10 species), captured in Europe, Asia, Africa and North America in 1980–2009, and

either frozen, ethanol-fixed or RNAlater®-preserved tissues from 323 bats (32 species), collected in Asia, Africa and the Americas in 1981 – 2012, were analyzed for hantavirus RNA by RT-PCR. Total RNA was extracted from tissues, using the PureLink Micro-to-Midi total RNA purification kit (Invitrogen, San Diego, CA), then reverse transcribed, using the SuperScript III First-Strand Synthesis System (Invitrogen) and random hexamers. A two-step PCR was performed in 20-mL reaction mixtures, containing 250 μM dNTP, 2 mM MgCl₂, 1 U of AmpliTaq polymerase (Roche, Basel, Switzerland) and 0.25 μM of each primer. Initial denaturation at 94 °C for 5 min was followed by two cycles each of denaturation at 94 °C for 40 sec, two-degree step-down annealing from 48 to 38 °C for 40 sec, and elongation at 72 °C for 1 min, then 32 cycles of denaturation at 94 °C for 40 sec, annealing at 42 °C for 40 sec, and elongation at 72 °C for 1 min, in a GeneAmp PCR 9700 thermal cycler (Perkin-Elmer, Waltham, MA). DNA was sequenced directly using an ABI Prism 377XL Genetic Analyzer (Applied Biosystems, Foster City, CA). Nucleotide sequences of the newfound hantaviruses were aligned and compared with representative rodent- and soricomorph-borne hantavirus sequences, using the ClustalW method (TranslatorX server and BioEdit 7.0.5). Phylogenetic trees, based on

the coding regions of the S, M and L segments, were generated using the maximum likelihood (ML) method implemented in PAUP* (Phylogenetic Analysis Using Parsimony, 4.0b10) and RAxML Blackbox webserver, as well as a Bayesian approach using MrBayes 3.1. The optimal evolutionary model was estimated as the GTR + I + Γ model of evolution, as selected by jModelTest version 0.1. ML topologies were evaluated by bootstrap analysis of 1,000 neighbor-joining iterations (in PAUP*) or 1,000 ML iterations (in RAxML). Bayesian analysis consisted of two million Markov Chain Monte Carlo generations sampled every 100 generations to ensure convergence across two runs of four chains each, with average standard deviations of split frequencies less than 0.01 and effective sample sizes over 100, resulting in consensus trees supported by posterior-node probabilities. The taxonomic identities of the hantavirus-infected mole shrews were verified by sequence analysis of the complete 1,140-nucleotide cytochrome *b* gene, amplified by PCR. Host phylogenies based on mtDNA cytochrome *b* sequences, along with published sequences for shrews for this gene region, were generated, using ML and Bayesian methods described previously.

RESULTS AND DISCUSSION

Table 1

Newfound Soricomorph-borne Hantaviruses

Family	Subfamily	Genus species	Country	Year	Virus Name
Soricidae	Crocidurinae	<i>Suncus murinus</i>	India	1963	Thottapalayam
		<i>Crocidura douceti</i>	Guinea	2012	Bowé
		<i>Crocidura lasiura</i>	Korea	2004	Imjin
		<i>Crocidura shantungensis</i>	Korea	2007	Jeju
		<i>Crocidura theresae</i>	Guinea	2004	Tanganya
		<i>Crocidura obscurior</i>	Côte d'Ivoire	2009	Azagny
	Myosoricinae	<i>Myosorex geata</i>	Tanzania	1996	Uluguru
		<i>Myosorex zinki</i>	Tanzania	2002	Kilimanjaro
	Soricinae	<i>Anourosorex squamipes</i>	Vietnam	2006	Cao Bang
		<i>Anourosorex yamashinai</i>	Taiwan	1989	Xinyi
		<i>Blarina brevicauda</i>	United States	1998	Camp Ripley
		<i>Blarina carolinensis</i>	United States	1983	Iamonia
		<i>Neomys fodiens</i>	Poland	2011	Boginia
		<i>Sorex araneus</i>	Switzerland	2006	Seewis
		<i>Sorex caecutiens</i>	Russia	2006	Amga
		<i>Sorex cinereus</i>	United States	1994	Ash River
		<i>Sorex cylindricauda</i>	China	2005	Qiandao Lake
		<i>Sorex monticolus</i>	United States	1996	Jemez Springs
		<i>Sorex palustris</i>	Canada	2005	Fox Creek
		<i>Sorex roboratus</i>	Russia	2006	Kenkeme
<i>Sorex trowbridgii</i>		United States	2003	Tualatin River	
<i>Sorex unguiculatus</i>		Japan	2006	Sarufutsu	
Talpidae	Talpinae	<i>Sorex vagrans</i>	United States	2003	Powell Butte
		<i>Talpa europaea</i>	Hungary	1999	Nova
		<i>Urotrichus talpoides</i>	Japan	2008	Asama
		<i>Neurotrichus gibbsii</i>	United States	2003	Oxbow
	Scalopinae	<i>Scaptonyx fuscicaudus</i>	China	1989	Dahonggou Creek
		<i>Scalopus aquaticus</i>	United States	1986	Rockport

Being permitted access to archival tissues in museum repositories and personal collections made possible the detection of genetically distinct hantaviruses in shrews of multiple species captured in widely separated geographic regions, including the Chinese mole shrew (*Anourosorex squamipes*) [15], Taiwanese mole shrew (*Anourosorex yamashinai*), northern short-tailed shrew (*Blarina brevicauda*) [10], southern short-tailed shrew (*Blarina carolinensis*), Eurasian water shrew (*Neomys fodiens*), Eurasian common shrew (*Sorex araneus*) [21], Laxmann's shrew (*Sorex caecutiens*), masked shrew (*Sorex cinereus*) [17], dusky shrew (*Sorex monticolus*) [17], flat-skulled shrew (*Sorex roboratus*) [16], long-clawed shrew (*Sorex unguiculatus*), Doucet's shrew (*Crocidura douceti*), Ussuri white-toothed shrew (*Crocidura lasiura*) [2], West African pygmy shrew (*Crocidura obscurior*) [13], Asian lesser white-toothed shrew (*Crocidura shantungensis*) [4], geata mouse shrew (*Myosorex geata*) and Kilimanjaro mouse shrew (*Myosorex zinki*) (Table 1). Previously unrecognized hantaviruses were also found in the Japanese shrew mole (*Urotrichus talpoides*)

[14], American shrew mole (*Neurotrichus gibbsii*) [12], European common mole (*Talpa europaea*) [6], eastern mole (*Scalopus aquaticus*) [23] and long-tailed mole (*Scaptonyx fuscicaudus*), as well as in the banana pipistrelle (*Neoromicia nanus*) [5], an insectivorous vesper bat species captured in Côte d'Ivoire. Only three of the soricomorph-borne hantaviruses listed in Table 1 (Thottapalayam virus, Tanganya virus and Qiandao Lake virus) were reported by others.

Newly identified soricid-borne hantaviruses exhibited geographic-specific clustering, akin to that observed for rodent-borne hantaviruses. For example, distinct lineages were noted for Seewis virus in Eurasian common shrews from Switzerland, Austria, Finland, Germany, Hungary, Czech Republic, Slovakia, Poland and Russia [1, 8, 22], and for Thottapalayam virus in Asian house shrews from India and Nepal [9]. Moreover, host sharing was evidenced by genetic variants of the same hantavirus in evolutionarily related shrew species: Seewis virus in *Sorex araneus*, *Sorex daphaenodon*, *Sorex tundrensis* and *Neomys anomalus*

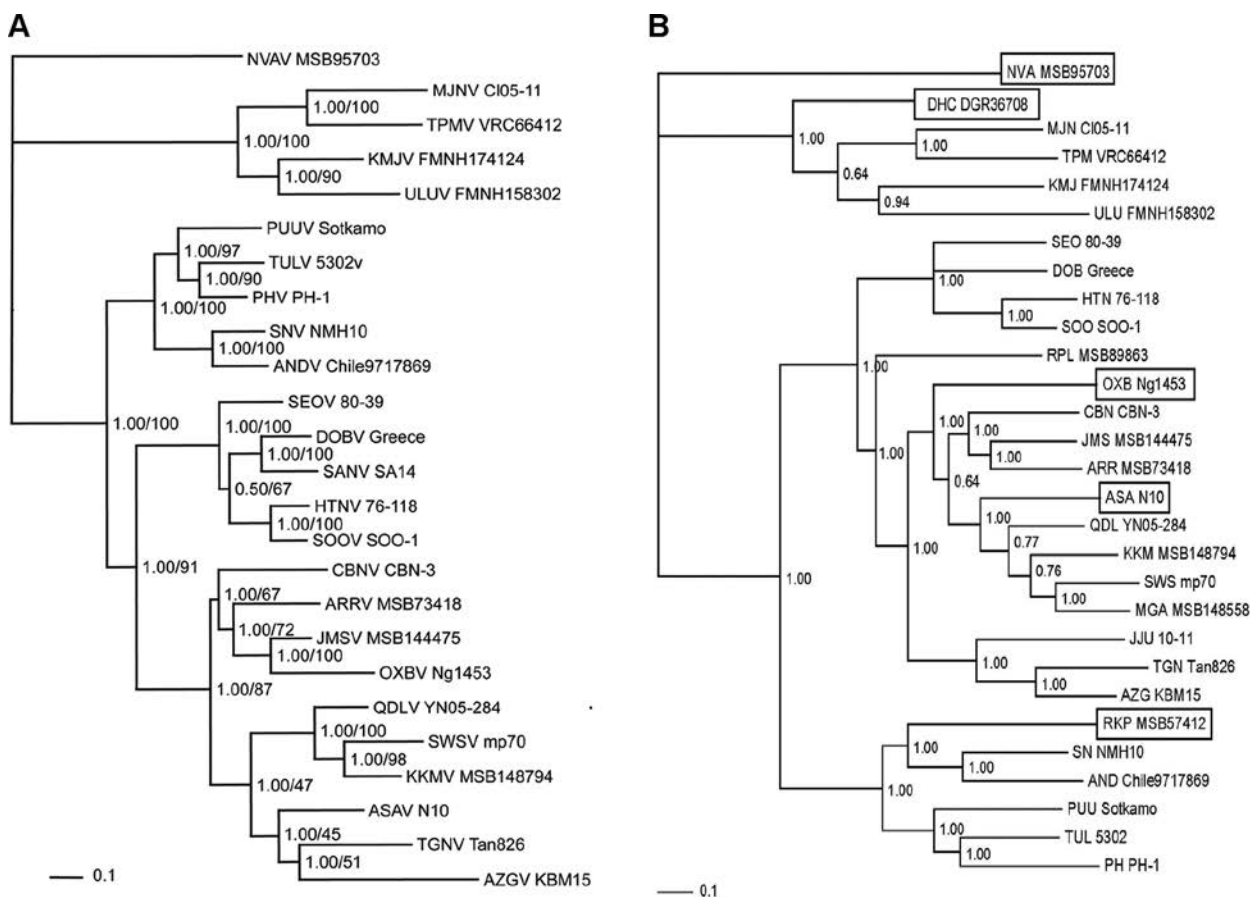


Fig. 1. Phylogenetic trees, using the best-fit GTR+I+Γ model of evolution, based on the (A) S-genomic and (B) L-genomic segments of representative rodent-, shrew- and mole-borne hantaviruses. The numbers at each node are posterior probabilities and ML bootstrap supports. The scale bar indicates the nucleotide substitutions per site. The five taxa in (B), enclosed by individual boxes, represent hantaviruses detected in moles. Abbreviations: ANDV (Andes virus), ARRV (Ash River virus), ASAV (Asama virus), AZGV (Azagny virus), CBNV (Cao Bang virus), DHCV (Dahonggou Creek virus), DOBV (Dobrava virus), HTNV (Hantaan virus), JJUV (Jeju virus), JMSV (Jemez Spring virus), KKMV (Kenkeme virus), KMJV (Kilimanjaro virus), MGA (Amga virus), MJNV (Imjin virus), NVAV (Nova virus), OXBV (Oxbow virus), PHV (Prospect Hill virus), PUUV (Puumala virus), QDLV (Qiandao Lake virus), RKPV (Rockport virus), RPLV (Camp Ripley virus), SANV (Sangassou virus), SEOV (Seoul virus), SNV (Sin Nombre virus), SOOV (Soochong virus), SWSV (Seewis virus), TGNV (Tanganya virus), TPMV (Thottapalayam virus), TULV (Tula virus), ULUV (Uluguru virus).

[22]; and Jemez Springs virus in *Sorex monticolus*, *Sorex palustris*, *Sorex trowbridgii* and *Sorex vagrans*.

Host switching, or cross-species transmission of hantaviruses, was found between reservoir hosts of the same family (Soricidae and Soricidae), of different families (Soricidae and Talpidae) and of different orders (Soricomorpha and Rodentia). Notable examples were Asama virus in the Japanese shrew mole [14] and Oxbow virus in the American shrew mole [12], and the shared ancestry of Rockport virus in the eastern mole and hantaviruses carried by cricetid rodents [23], as well as Jemez Springs virus in the Trowbridge shrew.

Phylogenetic analyses showed that hantaviruses segregated into five distinct lineages (Fig. 1A). Two of these lineages, comprising hantaviruses hosted by murid and cricetid rodents, have previously been well described. The three new lineages were composed of hantaviruses harbored by soricine, crocidurine and mysosoricine shrews, with the most divergent being that of a mole-borne hantavirus, Nova virus (NVAV) from the European common mole. As stated earlier, other mole-borne hantaviruses (shown in boxes in Fig. 1B) appeared to be somewhat more catholic in their host range compared to present-day rodent-borne hantaviruses. More in-depth investigations are warranted to define the genetic diversity of hantaviruses in moles. That is, despite the overall success of the RT-PCR approach at identifying previously unrecognized hantaviruses, overcoming technical barriers of primer design and deploying more powerful applications, such as ultra high-throughput sequencing technology, are essential to accelerate the virus discovery process.

The re-discovery of genetically divergent hantaviruses in soricomorphs, and the more recent detection of a divergent lineage of hantavirus in an insectivorous bat, have forced the rewriting of textbook chapters and have necessitated the reframing of their evolutionary origin and diversification. That these newfound hantaviruses are genetically more diverse than those hosted by rodents suggests that the evolutionary history of hantaviruses is far more ancient and complex than previously conjectured. Moreover, ancestral rodents may not have been the original mammalian hosts of primordial hantaviruses. Instead, in keeping with other members of the *Bunyaviridae* family which are carried by insects and arthropods, the evolutionary history of hantaviruses might have entailed an insect-borne virus, initially infecting insectivorous ancestral hosts in the mammalian superorder Laurasiatheria, dating 50 or more million years before present, with subsequent host switching and species-specific adaptation in the distant and more recent past.

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