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Novel strains of Limestone Canyon virus detected in *Peromyscus boylii* from
southwestern New Mexico

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Abstract

Peromyscus boylii is a dominant species in rodent communities in southwestern New Mexico and is a known carrier of Limestone Canyon virus (LSCV) in Arizona. Five species of *Peromyscus* were collected from Hidalgo and Grant County, most of which were *P. boylii*. All mice were serologically tested for antibodies to Sin Nombre virus and Rio Mamore virus. Lung tissue from all seropositive and negative mice was used for RNA extraction, amplification, and sequencing using newly designed primers specific to LSCV for both the S and M segments. A phylogenetic analysis of the virus was performed and showed a about a 5.5% divergence from LSCV in Arizona, and a 4.5% divergence among *P. boylii* representing two clades found in southwestern New Mexico. Also, viral RNA was obtained from a seronegative mouse emphasizing the importance of PCR testing in addition to serology testing to determine the presence of hantaviruses.

Key word: hantavirus, Limestone Canyon virus, New Mexico, *Peromyscus boylii*, *Peromyscus maniculatus*, Sin Nombre virus.

Introduction

Hantaviruses make up a genus within the family Bunyaviridae. All members of this family are enveloped and contain a trisegmented negative sense RNA genome that includes the large (L), medium (M), and small (S) segments. Segments code for the viral transcriptase, 2 viral glycoproteins, and a nucleocapsid protein respectively (8).

Hantaviruses were first discovered in Europe and Asia and are recognized as Old World hantaviruses. They are responsible for hemorrhagic fever with renal syndrome (HFRS). This disease possibly occurred as early as 1,000 years ago in China but did not gain recognition until the Korean War when it led to the hospitalization of more than 3,000 United Nations soldiers (15). New World hantaviruses were first recognized in May 1993, with the outbreak of hantavirus cardiopulmonary syndrome (HCPS) in the four corners region of New Mexico (18).

Hantaviruses, unlike the other 4 genera in the family, are transmitted via a rodent host rather than an arthropod vector (8). Hantaviruses are reported to have co-evolved with their murid rodent hosts, a pattern probably due to the chronic nature of this viral infection, which may contribute to the observed pattern of co-evolution (11). Generally, each hantavirus is carried by a primary rodent host, and these hantaviruses may or may not be pathogenic to humans.

Recognition of this co-evolutionary process stimulated the rapid identification of suspected hosts of novel hantaviruses based on the known phylogenetic relationships of the hosts. According to Morzunov et al. (17), at higher taxonomic levels hantaviruses carried by rodents in different subfamilies, Murinae, Arvicolinae, and Sigmodontinae, fall into three phylogenetically distinct groups, irrespective of their geographic location. However, host switching may occur in rodent communities where closely related taxa occur in sympatry. Although demonstrated with New York virus and Monongahela virus (17) and with Topografov virus, Khabarovsk virus, and Puumala virus (28), host switching is uncommon. Another source of novel viruses may come as a result of

reassortment and/or recombination of viral RNA or from simple genetic drift which appears commonly in hantavirus (20).

Hantaviruses may undergo recombination when closely related viruses come into contact within the same individual host (19). Indicating contact zones between closely related species should be productive areas to survey for recombinant viruses. Therefore, co-evolutionary histories of viruses and their hosts should be examined in more detail, especially in areas of potential contact between hosts and associated viral strains. Delineation of these viral and rodent phylogenies at interspecific and intraspecific levels should provide a framework for discovery of novel viruses (29) and hosts (22).

The deer mouse, *Peromyscus maniculatus*, a member of the family Muridae, is known to be the primary host of Sin Nombre virus (SNV; 6). Dragoo et al. (7) examined DNA sequence for deer mice collected from sites throughout North America to provide a foundation for studies of spatial structure and evolution of this ubiquitous host. That study found six largely allopatric lineages, some of which may represent unrecognized species. Zones of contact among divergent viral elements found in different hosts may increase the possibility of formation of recombinant variants. Those findings have been supported by other sources. For example, Schmaljohn et al. (24) reported new hantavirus variants from *P. maniculatus* in northern California in a zone identified by Dragoo et al. (7) as potential region of contact between distinctive deer mice lineages.

Within the deer mouse complex, distinct geographic populations of mice (and their associated viruses) have different evolutionary trajectories. The focus of new hantavirus discovery to date has been at the interspecific level. However, new viral strains may emerge within currently recognized species that span broad geographic

ranges and cross ecological boundaries, such as were identified in the large complex of deer mice (7).

Rodent communities in southwestern New Mexico contain four distinct clades of *P. maniculatus* that potentially come into contact (7), making this an area of high interest to search for novel hantaviruses. *Peromyscus* from southwestern New Mexico were collected during the summer of 2006. In this collection of mice a predominance of the brush mouse, *P. boylii* was found.

Peromyscus boylii is of particular interest in this community because in Arizona the species was shown to carry a unique hantavirus, Limestone Canyon virus (LSCV; 23). The virus was found to be distinct from other hantaviruses, but its phylogenetic placement was difficult to interpret. The S segment was more closely aligned with *Reithrodontomys*-like viruses; whereas, the M segment aligned at the base of the *Peromyscus/Reithrodontomys* viruses. This relationship suggests that viruses from different primary hosts could have reassorted in the past. It was important to determine whether LSCV also occurred in *P. boylii* in southwestern New Mexico. Because there may be incomplete taxonomic delineation of hosts, the potential for host switching among taxonomic groups, and the possibility of reassortment/recombination among viruses in the same host, it is important to investigate the presence of virus in other members of the rodent community (especially, *Peromyscus*) in transition zones.

Materials and Methods

Specimen collection. The specimens were all collected during the summer of 2006 in the areas of Granite Gap and a number of different trapping sites in the Burro Mountains of the Gila National Forest in Hidalgo and Grant Counties, New Mexico.

These collections were done following the protocol approved by the Office of the Animal Care and Compliance Committee (Animal Welfare Assurance Number A4023-01). The mice were trapped using Sherman live traps set in transects at various trapping localities. Six to 10 transects of 40 traps per location were used. All traps were set at dusk. Traps were baited using oats, bird seed, sunflower seeds, and peanut butter. Traps were checked early the next morning. Non-target animals were released at site of capture. All *Peromyscus* were taken to a central processing area. Rubber gloves and powered air purifying respirators (PAPR) with high efficiency particulate air (HEPA) filters were used when processing mice. *Peromyscus* were removed from the traps and placed into clear plastic bags and euthanized using an overdose of Isoflourene. To prevent cross contamination each mouse was given a separate new plastic bag. Once euthanized, the animal was removed, numbered, sexed, measured, and weighed. These measurements taken were for total body length, tail length, length of ear, and length of hind foot. Reproductive condition was also recorded.

Tissues were collected for identification of animals and detection of viruses. The organs collected included liver, spleen, heart and kidney, and lung, each in a separate cryotube. Embryos also were collected when present. Tissue and embryo tubes were placed in liquid nitrogen in the field and stored at -80° Celsius in the lab until they were analyzed. Skeletons were removed, tagged, and skins were stuffed with cotton and dried. All voucher material was accessioned into the collections and are housed in the University of New Mexico, Museum of Southwestern Biology, Division of Mammals and Division of Genomic Resources.

Rodent identification. Identification was done by sequencing the cytochrome *b* gene of the mitochondrial DNA. Approximately 10 mg of liver were used to extract DNA using a salt extraction (1). Verification of quality and quantity of DNA extraction was done by visualization on a 0.8% agarose gel. PCR was performed using primers L14724 and H15915 (12). Cleaned PCR products were sequenced using BigDye Terminator Cycle Sequencing Ready Reaction mix v1.1 (Applied Biosystems) and the forward primer L14724. Sequence reactions were run on an ABI 3100 automated DNA sequencer in the Molecular Biology Facility, in the University of New Mexico Biology Department. Completed and cleaned sequences were compared to known sequences of mice currently available as voucher specimens and sequenced in lab used by Dr. Dragoo.

Serology. Serology was done by using blood extracted from the heart. The presence of antibodies to SNV and Rio Mamore virus (RMV) hantaviruses then were determined using strip immunoblot assay (SIA) following the protocol described in Yee et al. (30). A brief summary of the procedure is as follows: each SIA strip was prepared using a model SB 10 mini slot blot apparatus. Each strip had an orientation band at the top of coomassie blue dye, a 3+ intensity control band, and SNV-N and an RMV-N antigen band, and a 1+ intensity control band. Components were vacuum blotted onto nitrocellulose membrane. Blood was applied to the strips in separate wells and incubated overnight. Strips were washed and then developed in NBT/BCIP to visualize bands. The bands were rated for seropositivity from 0 to 3+ including trace-, trace, 0.25, 0.5, 0.75, 1, 2, and 3.

RNA extaction/rtPCR/sequencing. Lung tissue was used to extract RNA following the RNeasy kit (Qiagen) extraction protocol. The presence of RNA was

confirmed using absorbance spectrometry. RNA was converted into a cDNA library through the use of the Omiscript Reverse Transcription kit (Qiagen). A polyT primer was used to construct cDNA. The S segment was initially amplified because it is shorter and contains diagnostic markers. An attempt to amplify the M segment was only performed in mice in which the S segment amplified.

Primer design. Primers were designed by submitting DNA sequences for LSCV to Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi - (21) for analyses and primer design. Multiple primers were designed for both the S and M segment, but only 2 worked for the S segment and 3 for the M segment (Table 1). Cleaned PCR products were sequenced, with the amplification primers, using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems). Sequences were determined by running samples on an ABI 3100 automated DNA Sequencer.

Phylogenetic analyses. Sequences of 23 other S segments and 16 other M segments of hantaviruses were obtained from GenBank and compared to the sequence of the virus obtained in this study. Sequences were aligned using ClustalX (26), followed by visual inspection using MacClade 4 (14). Phylogenetic analyses of DNA sequences was conducted using Maximum Parsimony (MP) in the software package PAUP* 4.0b10 (25). In the MP analysis all characters were weighted equally. An heuristic search option with 100 replications of random addition of taxa and TBR branch swapping was used to generate parsimony trees. Bootstrap support (9) for results of MP analyses was conducted using 1,000 repetitions of resampling data using the heuristic search option, with 10 replications of random addition of taxa.

Results

A total of 87 *Peromyscus* were collected from localities in Hidalgo and Grant Counties, New Mexico. *P. boylii* accounted for 66% of the mice captured followed by *P. eremicus* and *P. truei* accounting for 16% and 15% respectively. *P. maniculatus* and *P. leucopus* accounting for less than 1% of the mice collected. The phylogenetic analysis of *P. boylii* indicated that two clades of mice were present in the area where virus was amplified (fig. 1).

Serologically, 33 of the 87 mice were found to be seropositive for SNV and 17 of those also were found to be seropositive for RMV (table 2). The level of seropositivity did not correlate with detection of virus as seen by Botten et al. (4). Virus (S segment) was only detected in 9 specimens of *P. boylii* using RT-PCR. Of those nine, the M segment was only amplified and sequenced from seven. The S segment from virus found in southwestern New Mexico had a 5.4-6.1% sequence divergence compared to Limestone Canyon virus found in Arizona, and 5-7% sequence divergence between virus from Arizona and that found in southwestern New Mexico for the M segment. Within the New Mexico samples a 4.5% and 1-7% sequence divergence for the S and M segment respectively, was found among populations in the Burro Mountains. Phylogenetic analysis shows that the New Mexico viruses found in this study are most closely related to the Limestone Canyon virus in Arizona then to other hantaviruses in North America (fig 2).

DNA sequences from the cytochrome *b* gene from mice in this study were compared to Genbank sequences reported by Tiemann-Boege et al. (27). These mice also were found to have two distinct viral types, but the viral types did not correspond to the *P. boylii* clades. Brush mice from each of the 2 clades were found in areas were different

viral types were found. Viral types were found at different localities with the exception of locality 2 (Table 3).

Discussion

Dragoo et al. (7) established the existence of at least 4 clades of *P. maniculatus* converging in southwestern New Mexico. They hypothesized that each clade could potentially carry a distinct viral type, and that when these clades are in sympatry there is potential for the viruses to recombine resulting in new viral types. *Peromyscus boylii* is one of the species that occurs in the rodent community in southwestern New Mexico and has been shown to carry a unique hantavirus. In *P. boylii* two clades of mice were found in sympatry the area surveyed. However, Hall (10) reported only a single subspecies of *P. boylii* in New Mexico. This study, like Dragoo et al. (7), shows that the phylogeography of rodent hosts can provide a framework for interpreting geographical variability not only in hosts, but also in associated viral variants and provide an opportunity to predict potential geographical distribution of newly emerging viral strains.

Hantaviruses are a persistent infection and are known to undergo genetic drift, which is the accumulation of base substitutions, deletions and insertions. They also undergo genetic shift or reassortment (20). Heterologous reassortment has not been observed, however Li et al. (13) found reassortment between closely related virus strains. Analyses of the S and M segments of the viruses found in this study did not indicate that a rearrangement had occurred and the variation observed likely was a result of genetic drift. Even though mice from the different clades were found in sympatry there was no evidence that the viruses had undergone rearrangement. However, sample sizes in this study may have been too small to detect evidence of virus genetic shift.

Although 38 % of the mice caught were seropositive virus was only detected in 10% of the mice, and only in *P. boylii*. This could be because *P. truei*, and *P. eremicus* may harbor a different virus with antibodies that cross react with the SNV and RMV antigen for seropositivity, but the RNA may be distinct enough that the primers for LSCV were unable to bind. It could also be because these mice were not chronically infected with LSCV therefore only antibodies and not virus were present. Anti IgM tests are not currently available. This makes it hard to distinguish a recent infection from a current or past infection. Also, antibodies to hantavirus can be spread vertically but transmission of the virus is not. Therefore juveniles may have antibodies from their mother without evidence of the virus (2, 5). Another possible explanation may be that only lung tissue was used for virus detection. According to Botten et al. (4), studies on SNV in *P. maniculatus* showed that some mice have disseminated infection resulting in high levels of viral RNA where as others displayed a restrictive pattern that did not have the replicative form of RNA in the heart, lung, and kidney. They also showed a decrease in the amount of RNA present after the day 21 of infection.

We also were able to detect virus in a seronegative mouse. This could be because we captured the mouse during the period between when the host was infected but antibodies had not yet been produced. Botten et al. (3) found that mice develop detectable antibodies at 14-21 days after inoculation with hantavirus. Yee et al. (30) also stated that negative SIA does not ensure the mouse has not been infected. Camaioni et al. (5) describes two instances of seroconversion found when using the recommendation for testing captured rodents for hantavirus antibodies at the beginning and end of a 5-week quarantine period whenever potential reservoir species are used to establish laboratory

colonies (16). Only upon completion of the second test can an animal be considered uninfected by a hantavirus. This would imply that in future research regarding hantavirus it is essential to not only do serologic testing on specimens but PCR analysis as well.

Results from this study support the findings of Dragoo et al. (7). Although, few *P. maniculatus* were collected another *Peromyscus* with at least two closely related hantaviruses was found in an area where deer mice are reported to occur. Additionally, co-evolutionary histories of viruses and their hosts should be examined in more detail, especially in areas of potential contact among closely related hosts in rodent communities, and their associated viral strains. Phylogeographical and population-level analyses may provide key insight into situations that promote the emergence of novel viral elements.

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Table 1. Primers designed to amplify and sequence Limestone Canyon virus in *Peromyscus* for southwestern New Mexico.

S Segment Primers

LSCS 114 5' AGTGGACCCGGATGATGTTA 3'

LSCS 1117 5' TACGTCGGAGGTAGGATTGG 3'

M Segment Primers

LSCM 2077 5' ATCCTTGGTCATTGGATGA 3'

LSCM 3038 5' GAATGGCCTCCCTTTCCTAC 3'

LSCM 3312 5' TGTGAACGAATGGGACAGAA 3'

Table 2. Species and numbers of *Peromyscus* testing seropositive for Sin Nombre virus (SNV) and Rio Mamore virus (RMV).

Species	Number tested	SNV +	RMV+
<i>P. boylii</i>	57	28	17
<i>P. truei</i>	14	4	0
<i>P. eremicus</i>	14	1	0
<i>P. maniculatus</i>	1	0	0
<i>P. leucopus</i>	1	0	0
Total	87	33	17

Table 3. Relationships of mice and viral types in various localities.

NK number	Locality	Mouse clade	S segment clade	M segment clade
136938	1	1	2	2
136942	1	2	2	2
136944	1	2	2	2
136972	2	1	1	1
136973	2	1	1	1
136976	2	1	1	1
136983	2	1	1	
136986	3	2	2	2
136989	3	1	1	

Figure 1. Consensus tree of 18 most parsimonious trees found from parsimony analysis of *Peromyscus boylii* found in this study compared with DNA sequences from genebank.

The mice from this study (identified by NK numbers) were found in two clades.

Numbers above branches indicate percentage of most parsimonious trees in which clades were supported.

Figure 2. Parsimony analysis of the S and M segments. Only one most parsimonious tree was found for each analysis. The tree shows that virus found in this study (indicated by NK numbers) grouped into two distinct clades which were both similar to Limestone Canyon virus. Numbers associated with clades are the bootstrap support values (for Limestone Canyon virus clades). A) Parsimony analysis of S segment and B) Parsimony analysis M segment.

Majority rule





