High Rates of Molecular Evolution in Hantaviruses

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Hantaviruses are rodent-borne Bunyaviruses that infect the *Arvicolinae*, *Murinae*, and *Sigmodontinae* subfamilies of *Muridae*. The rate of molecular evolution in the hantaviruses has been previously estimated at approximately 10^{-7} nucleotide substitutions per site, per year (substitutions/site/year), based on the assumption of codivergence and hence shared divergence times with their rodent hosts. If substantiated, this would make the hantaviruses among the slowest evolving of all RNA viruses. However, as hantaviruses replicate with an RNA-dependent RNA polymerase, with error rates in the region of one mutation per genome replication, this low rate of nucleotide substitution is anomalous. Here, we use a Bayesian coalescent approach to estimate the rate of nucleotide substitution from serially sampled gene sequence data for hantaviruses known to infect each of the 3 rodent subfamilies: Araraquara virus (*Sigmodontinae*), Dobrava virus (*Murinae*), Puumala virus (*Arvicolinae*), and Tula virus (*Arvicolinae*). Our results reveal that hantaviruses exhibit short-term substitution rates of 10^{-2} to 10^{-4} substitutions/site/year and so are within the range exhibited by other RNA viruses. The disparity between this substitution rate and that estimated assuming rodent–hantavirus codivergence suggests that the codivergence hypothesis may need to be reevaluated.

Introduction

Hantaviruses are negative-sense single-stranded, enveloped RNA viruses, with a genome comprising 3 segments: S (small), M (medium), and L (large), encoding the nucleocapsid (N) protein, the envelope glycoproteins (G1 and G2), and the RNA-dependent RNA polymerase, respectively (Schmaljohn 1996). Hantaviruses are associated with rodents of the family *Muridae* and, unlike the rest of the *Bunyaviridae*, are not vector borne. Each hantavirus species associates closely with one primary rodent species, where the virus establishes a persistent but asymptomatic infection with long term but sporadic shedding of the virus in saliva, urine, and feces (Hutchinson et al. 1998; Kuenzi et al. 2005). Transmission between rodents can occur directly during aggressive interactions between animals or indirectly through inhalation of infectious aerosol generated by contaminated urine and feces (Plyusnin and Morzunov 2001). Hantaviruses have a global distribution and are responsible for 2 different forms of human disease: 1) hemorrhagic fever with renal syndrome primarily in the Old World and 2) hantavirus pulmonary syndrome (HPS) exclusively in the New World (Peters et al. 1999). Human cases of hantavirus infection are almost exclusively the result of human–rodent interactions, with only a single epidemic in Argentina showing conclusive evidence of person-to-person transmission (Padula et al. 1998).

Phylogenetic studies of the genus have consistently found that hantaviruses cluster into 3 primary clades associated with the rodent subfamily each virus infects: *Arvicolinae*, *Sigmodontinae*, and *Murinae*. This association has been the basis of the hypothesis that hantaviruses have codiverged with their rodent hosts since the common ancestor of the 3 rodent subfamilies, an estimate that places the age of hantaviruses to be tens of millions of years (Hjelle et al. 1995; Plyusnin et al. 1996; Morzunov et al. 1998; Monroe et al. 1999; Vapalahti et al. 1999; Hughes and Friedman 2000; Plyusnin and Morzunov 2001; Jackson and Charleston 2004). Based on this assumption of codivergence, the rate of molecular evolutionary change in hantaviruses has been estimated between 2×10^{-6} and 3×10^{-7} nucleotide substitutions per site, per year (2.41×10^{-7} to 2.68×10^{-7} substitutions/site/year, Hughes and Friedman 2000; 2.2×10^{-6} to 7.0×10^{-6} substitutions/site/year, Sironen et al. 2001). These substitution rates are a substantial departure from those estimated for other RNA viruses, which generally fall within the range of 10^{-3} to 10^{-4} substitutions/site/year (Jenkins et al. 2002; Hanada et al. 2004) and which are evidently a function of high intrinsic rates of mutation coupled with rapid replication. If substantiated, the rodent hantaviruses would therefore be among the most slowly evolving of all RNA viruses.

Given that all RNA viruses replicate using an RNA-dependent RNA polymerase that does not possess proofreading or error correction, the most likely mechanistic explanation for an anomalously low rate of molecular evolution in the hantaviruses is that replication rates (generation times) have been greatly reduced in these viruses. Specifically, because hantaviruses generate persistent infections in their reservoir hosts, it has been widely assumed that they are latent within hosts, undergoing little to no viral replication following acute infection. Indeed, a reduced rate of replication has been proposed to reduce long-term evolutionary rates in the retrovirus human T-cell lymphotropic virus (HTLV), producing substitution rates in the order of $\sim 10^{-7}$ substitutions/site/year (Salemi et al. 1999; Hanada et al. 2004), although unlike hantavirus HTLV is able to integrate into host genomes and therefore replicate with higher fidelity DNA polymerases. However, recent work suggests that hantavirus infection may not be latent because viral RNA can be detected sporadically by polymerase chain reaction throughout the course of infection (Botten et al. 2003; Kuenzi et al. 2005).

Critically, all estimates of rates of molecular evolution in the hantaviruses undertaken to date have assumed a codivergence between the viruses with their rodent hosts. Although we do not test the hypothesis of codivergence

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explicitly here, an independent and direct estimate of the rate at which hantaviruses evolve is a necessary first step toward validating this widely accepted view of hantavirus evolution. Indeed, one of the key factors that must be true for any proposal of host–parasite codivergence to be plausible is that the timescales over which the host and parasite groups have diverged are congruent (Page 1996). To this end, we estimate rates of nucleotide substitution in each of the 3 major clades of rodent hantavirus using serially sampled data, in which the extent of genetic divergence among viruses sampled at different times is used to infer fundamental evolutionary dynamics.

Materials and Methods

Data Sets

All hantavirus sequences for which the date (day or year) of sampling was available were downloaded from GenBank, and each hantavirus with greater than 20 available sequences was retained for analysis. Under these criteria, the nucleocapsid genes from 3 hantaviruses were chosen for further analysis: Dobrava virus ($n = 30$, 1,302 bp, sampled from 1985 to 2006) which infects *Murinae* rodents, along with the Puumala ($n = 59$, 1,302 bp, sampled from 1979 to 2004) and Tula viruses ($n = 23$, 1,293 bp, sampled from 1987 to 1996) which infect *Arvicolinae* rodents. As there was no data set available from GenBank for those hantaviruses that infect *Sigmodontinae* rodents, we obtained sera samples from patients diagnosed with HPS or wild rodents collected near human outbreak sites in the states of Sao Paulo, Minas Gerais, Santa Catarina, and the Federal District, Brazil (table 1). From these samples, 312 bp of G1 sequences ($n = 32$), 302 bp of G2 sequences ($n = 13$), and 261 bp of N sequences ($n = 33$) were recovered from whole genomic RNA (Figueiredo LM, Moreli ML, de Sousa RLM, Borges AA, de Figueiredo GG, Machado AM, Bisordi I, Nagasse-Sugahara TK, Suzuki A, Pereira LE, de Souza RP, de Souza LTM, Braconi CT, Zanotto PM de A, and the VGDN consortium, in preparation). These Brazilian hantavirus sequences were identified as Araraquara through phylogenetic comparison with North and South American hantavirus sequences taken from GenBank (Figueiredo et al., in preparation—trees available on request). Prior to estimating the substitution dynamics of these 5 hantavirus data sets, all sequences were aligned manually using Se-Al (v2.0a11 Carbon, http://evolve.zoo.ox.ac.uk) and examined for evidence of recombination using the RDP3 program (Martin et al. 2005).

Rates of molecular evolution (substitutions/site/year) were estimated for each taxon (and gene) individually using the Bayesian Markov chain Monte Carlo (MCMC) method available in the BEAST package v1.4.6 (Drummond and Rambaut 2007). Modeltest v3.7 (Posada and Crandall 1998) was used to determine the model of nucleotide substitution that best fit the data, and all data sets were subsequently run using the HKY85 + Γ_4 model. Sequences were dated according to the year of sampling for Dobrava, Puumala, and Tula viruses and the day of sampling for Araraquara virus. Coalescent analyses were run until all parameters converged, with confidence intervals given by the 95% highest probability density (HPD). Data sets were analyzed using both a strict and relaxed molecular clock with an uncorrelated lognormal rate distribution, using a range of prior values for the substitution rate, and under demographic models of 1) a constant population size, 2) exponential population growth, and 3) logistic population growth.

Table 1
Origin of Sera Sample (rodent or human HPS patient), Location, and Date of Sampling for the Sequences of Araraquara Virus Used to Determine the Rate of Nucleotide Substitution in Brazilian Hantaviruses

Gene	Date of Sampling (month)	Location (State)	Origin of Sera
GP1, N	August 2003	Sao Paulo	*Akodon* sp.
GP1, GP2, N	June 1999	Sao Paulo	HPS patient 1
GP1, GP2, N	May 2001	Sao Paulo	HPS patient 2
GP1, GP2, N	August 2002	Sao Paulo	HPS patient 3
GP1, GP2, N	May 2002	Sao Paulo	HPS patient 4
GP1, GP2, N	August 2002	Sao Paulo	HPS patient 5
GP1, GP2, N	August 2002	Sao Paulo	HPS patient 6
GP1, GP2, N	March 2004	Sao Paulo	HPS patient 7
GP1, GP2, N	June 2003	Sao Paulo	HPS patient 8
GP1, GP2, N	February 2002	Minas Gerais	HPS patient 9
GP1, GP2, N	February 2002	Minas Gerais	HPS patient 10
GP1	September 2003	Sao Paulo	HPS patient 17
N	August 2004	Federal District	HPS patient 23
GP1, N	August 2004	Sao Paulo	HPS patient 26
GP1	October 2004	Goiás	HPS patient 33
GP1	April 2005	Sao Paulo	HPS patient 90
N	March 2005	Sao Paulo	HPS patient 94
GP1, N	March 2004	Sao Paulo	HPS patient 95
GP1, N	August 2004	Sao Paulo	HPS patient 96
GP1, N	August 2004	Sao Paulo	HPS patient 97
GP1, N	June 2004	Sao Paulo	HPS patient 98
GP1, N	August 2005	Minas Gerais	HPS patient 101
GP1, GP2, N	June 2003	Sao Paulo	*Necromys lasiurus* 1
GP1, N	July 2003	Sao Paulo	*N. lasiurus* 2
GP2, N	July 2003	Sao Paulo	*N. lasiurus* 3
GP1, GP2, N	July 2003	Sao Paulo	*N. lasiurus* 4
GP1, N	June 2004	Federal District	*N. lasiurus* 5
N	July 2003	Minas Gerais	*N. lasiurus* 12
GP1, N	July 2003	Minas Gerais	*N. lasiurus* 19
N	June 2004	Federal District	*N. lasiurus* 32
GP1, N	June 2004	Sao Paulo	*N. lasiurus* 47
GP1, N	June 2004	Federal District	*N. lasiurus* 55
GP1, N	June 2004	Federal District	*N. lasiurus* 56
GP1, N	June 2005	Sao Paulo	*N. lasiurus* 65
GP1, N	June 2005	Sao Paulo	*N. lasiurus* 66

NOTE.—The identity of the gene sequenced from each sample is also given: GP1 = glycoprotein 1, GP2 = glycoprotein 2, and N = nucleocapsid.

Results and Discussion

As we observed no recombinant sequences in any of the data sets, all available sequences were used to estimate the evolutionary dynamics of Araraquara, Dobrava, Puumala, and Tula viruses. The mean rate of molecular evolution estimated for these hantaviruses across all clocks and demographic models in our Bayesian coalescent analyses ranged from 2.10×10^{-2} to 2.66×10^{-4} substitutions/site/year (table 2). Importantly, these values are several orders of magnitude higher than any previous estimates given for the evolutionary dynamics of hantaviruses based on the assumption of host–parasite codivergence. Further, similar mean substitution rates were recovered

Table 2
Bayesian Estimates of the Rate of Nucleotide Substitution in Araraquara, Dobrava, Puumala, and Tula Hantaviruses

Virus (gene)	N	Sequence Length (bp)	Date Range	Molecular Clock	Nucleotide Substitutions per Site, per Year (95% HPD)		
					Constant	Logistic	Exponential
Araraquara (N)	33	261	1999–2005	Strict	2.49×10^{-3} (2.11×10^{-6} to 4.48×10^{-3})	2.43×10^{-3} (3.25×10^{-4} to 4.19×10^{-3})	3.23×10^{-3} (8.88×10^{-4} to 6.15×10^{-3})
				Relaxed lognormal	2.48×10^{-3} (8.85×10^{-4} to 4.26×10^{-3})	2.63×10^{-3} (1.05×10^{-3} to 4.34×10^{-3})	2.84×10^{-3} (1.29×10^{-3} to 4.61×10^{-3})
Araraquara (G1)	32	312	1999–2005	Strict	8.65×10^{-3} (2.01×10^{-3} to 1.54×10^{-2})	9.05×10^{-3} (2.04×10^{-3} to 1.55×10^{-2})	1.08×10^{-2} (3.57×10^{-3} to 1.77×10^{-2})
				Relaxed lognormal	2.68×10^{-3} (9.28×10^{-4} to 4.68×10^{-3})	2.62×10^{-3} (8.38×10^{-4} to 4.52×10^{-3})	3.01×10^{-3} (1.23×10^{-3} to 5.06×10^{-3})
Araraquara (G2)	13	302	1999–2005	Strict	2.67×10^{-3} (7.38×10^{-4} to 6.37×10^{-3})	2.52×10^{-3} (3.30×10^{-4} to 5.69×10^{-3})	6.26×10^{-3} (3.38×10^{-4} to 1.22×10^{-2})
				Relaxed lognormal	3.01×10^{-3} (2.49×10^{-4} to 5.68×10^{-3})	2.98×10^{-3} (5.80×10^{-4} to 5.48×10^{-3})	3.69×10^{-3} (8.12×10^{-4} to 6.62×10^{-3})
Dobrava (N)	30	1,302	1985–2006	Strict	2.99×10^{-4} (1.00×10^{-6} to 6.87×10^{-4})	2.80×10^{-4} (7.38×10^{-6} to 6.85×10^{-4})	4.74×10^{-4} (2.78×10^{-5} to 1.02×10^{-3})
				Relaxed lognormal	2.66×10^{-4} (3.15×10^{-8} to 5.87×10^{-4})	2.90×10^{-4} (3.31×10^{-5} to 6.12×10^{-4})	3.74×10^{-4} (4.29×10^{-5} to 7.13×10^{-4})
Puumala (N)	59	1302	1979–2004	Strict	6.09×10^{-4} (1.27×10^{-4} to 1.08×10^{-3})	5.41×10^{-4} (7.44×10^{-5} to 9.81×10^{-4})	6.22×10^{-4} (1.59×10^{-4} to 1.06×10^{-3})
				Relaxed lognormal	5.20×10^{-4} (9.10×10^{-5} to 9.38×10^{-4})	5.51×10^{-4} (6.46×10^{-5} to 9.28×10^{-4})	6.14×10^{-4} (1.66×10^{-4} to 1.08×10^{-3})
Tula (N)	23	1,293	1987–1996	Strict	1.99×10^{-2} (6.93×10^{-3} to 3.50×10^{-2})	2.10×10^{-2} (8.23×10^{-3} to 3.19×10^{-2})	1.84×10^{-2} (5.25×10^{-3} to 3.28×10^{-2})
				Relaxed lognormal	8.07×10^{-3} (1.81×10^{-3} to 1.60×10^{-2})	6.77×10^{-3} (1.44×10^{-3} to 1.33×10^{-2})	8.87×10^{-3} (3.26×10^{-3} to 1.50×10^{-2})

when far lower (1.0×10^{-8} substitutions/site/year) prior probability values were used. By including all models regardless of their likelihood and posterior probability, our evolutionary rates are conservative in their estimates and have 95% HPD values that vary widely between models. These values ranged from 3.15×10^{-8} under the least optimal model for Dobrava virus to 3.28×10^{-2} substitutions/site/year for Tula virus (table 2). Although values in the range of 10^{-8} are consistent with those previously estimated under the hypothesis of codivergence, it is important to note that this value is a clear outlier across the analysis as a whole and is distinct from the mean rate estimated for this virus ($\sim 3 \times 10^{-4}$ substitutions/site/year). However, the wide distribution of sampling error in our estimates highlights both the inherent difficulties in working with the small data sets that are available for hantaviruses and the clear need for larger data sets of dated sequences.

An additional issue of importance, when inferring evolutionary dynamics from sequence data sampled over a relatively short time period and from closely related taxa, is the difficulty in separating the relative contributions of the mutation and substitution rates. In particular, the Araraquara data set was sampled over only 6 years so that the number of nucleotide substitutions measured may in fact include slightly deleterious mutations that would later be purged by purifying selection, thereby artificially inflating estimates. However, our Dobrava and Puumala data sets included many more sequences sampled over longer time intervals and hence many more viral generations, providing more time for selective effects to be observed. As such, the mean rates measured for these 2 taxa ($\sim 3 \times 10^{-4}$ and $\sim 5.5 \times 10^{-4}$ substitutions/site/year for Dobrava and Puumala viruses, respectively) may more accurately represent the true substitution rates for the Hantavirus genus. Additional sampling over longer time periods would further clarify the long-term evolutionary rates of these viruses.

This study has demonstrated that the mean rate of evolutionary change in hantaviruses is approximately within the range of 10^{-2} to 10^{-4} substitutions/site/year, an estimate concordant with those of the majority of other RNA viruses (Jenkins et al. 2002; Hanada et al. 2004). Substitution rates in the order of 10^{-3} are not unexpected because hantaviruses rely on RNA-dependent RNA polymerase for replication, which lacks mechanisms of proofreading and repair mechanisms and which possesses an error rate of ~ 1 mutation/replication/genome (Drake 1999). Indeed, previous work has calculated the mutational frequency for hantaviruses to be in the range of 1×10^{-3} to 3×10^{-3}, with intrahost genetic variation approaching that seen in HIV and hepatitis C (Plyusnin et al. 1995, 1996; Feuer et al. 1999). As a consequence, it does not seem unreasonable for this mutation rate to translate to the substitution rates of the order of 10^{-4} substitutions/site/year observed here. In contrast, for a mutation rate of this order to translate into a substitution rate of 10^{-6} to 10^{-7} substitutions/site/year, hantaviruses would have to replicate only once every 1.33 years (assuming a genome of 10 kb and a neutral evolutionary process). Considering the average rodent life span in the wild is likely to be only a year or 2, this replication rate would seemingly create implausible conditions for effective transmission (de Oliveira et al. 1998). A rapid mutation rate could also translate into a low substitution rate if hantaviruses became latent following the acute phase of infection, a widely held assumption for infection in rodent hosts. However, recent studies using more sensitive methods have detected viral RNA in the blood intermittently over the course of long-term infection, indicating continuous viral replication even after the acute phase of infection (Hutchinson et al. 1998; Feuer et al. 1999; Botten et al. 2003; Kuenzi et al. 2005). As such, it is extremely difficult to reconcile a mutation rate of 10^{-3} with a substitution rate of 10^{-7} within the context of hantavirus biology.

Previous estimates of evolutionary dynamics in hantaviruses were based on the critical assumption that the congruence between hantavirus and rodent phylogenies reflects codivergence between these 2 groups since the divergence of the rodent genera *Mus* and *Rattus*, approximately 10–40 MYA (Hughes and Friedman 2000; Sironen et al. 2001; Nemirov et al. 2002). However, the observation of host–pathogen phylogenetic congruence does not necessarily indicate codivergence. Phylogenetic congruence between a parasite and its host can also arise from delayed cladogenesis, where the parasite phylogeny tracks that of the host but without temporal association (Jackson and Charleston 2004). This could occur if hantaviruses largely evolve host associations by cross-species transmission and related species tend to live in the same area, in which case a pattern of strong host–pathogen phylogenetic congruence could be observed in the absence of codivergence. In contrast to previous work, our evolutionary rates were estimated directly from primary sequence data sampled at known dates so that they more closely reflect the evolutionary changes undergone by the virus, at least in the short term. At the very least, the observation that hantaviruses exhibit short-term evolutionary rates equivalent to those seen in rapidly evolving RNA viruses makes a stringent reevaluation of the codivergence hypothesis necessary (Adkins et al. 2003).

Accession Numbers

The GenBank accession numbers of the Araraquara virus sequences determined for use in this study are: EU170207–EU170239 (N), EU170162–EU170193 (G1), and EU170194–EU170206 (G2).

The GenBank accession numbers for the sequences retrieved from previously published studies are:

1. Dobrava virus: DQ305279, AJ009773, AJ009775, AF060014, AF060015, AF060016, AF060017, AF060018, AF060019, AF060020, AF060021, AF060022, AF060023, AF060024, AJ410615, AJ410619, NC_00523, EF028074, EF059978, EF059979, EF059980, AF442622, AF442623, AJ131672, AJ131673, AJ251996, AJ251997, AY168576, AY961615, and AY961618.
2. Puumala virus: AJ888751, AJ888752, PVU95306, AJ277030, AJ277031, AJ277032, AJ277033, AJ277034, AJ238791, AJ278092, AJ278093, AB010730, AB010731, AJ314597, AJ314598, AJ314599, AJ314600, AJ314601, Z21497_1, Z30702_1, Z30703_1, Z30704_1, Z30705_1, Z30706_1, Z30707_1, Z30708_1, Z46942_1,

Z69985_1, AJ223368, AJ223369, AJ223371, AJ223374, AJ223375, AJ223376, AJ223377, AJ223380, AF367064, AF367065, AF367066, AF367067, AF367068, AF367069, AF367070, AF367071, AF411447, AF411448, AF411449, AF442613, AJ238788, AJ238789, AJ238790, AJ888731, AJ888732, AJ888733, AJ888734, AJ888735, AJ888736, AJ888738, and Z48586.

3. Tula virus: U95302, U95303, U95304, U95305, U95309, U95310, U95311, U95312, NC_005227, Z30941, Z30942, Z30943, Z30944, Z30945, Z48573, Z48574, Z48741, AF063892, AF063897, AJ223600, AJ223601, Y13979, and Y13980.

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