The Genesis and Spread of Reassortment Human Influenza A/H3N2 Viruses Conferring Adamantane Resistance

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A dramatic rise in the frequency of resistance to adamantane drugs by influenza A (H3N2) viruses has occurred in recent years—from ~2% to ~90% in multiple countries worldwide—and associated with a single S31N amino acid replacement in the viral matrix M2 protein. To explore the emergence and spread of these adamantane resistant viruses we performed a phylogenetic analysis of recently sampled complete A/H3N2 genome sequences. Strikingly, all adamantane resistant viruses belonged to a single lineage (the "N-lineage") characterized by 17 amino acid replacements across the viral genome. Further, our analysis revealed that the genesis of the N-lineage was due to a 4+4 segment reassortment event involving 2 distinct lineages of influenza A/H3N2 virus. A subsequent study of hemagglutinin HA1 sequences suggested that the N-lineage was circulating widely in Asia during 2005, and then dominated the Northern hemisphere 2005-2006 season in Japan and the USA. Given the infrequent use of adamantane drugs in many countries, as well as the decades of use in the US associated with little drug resistance, we propose that the globally increasing frequency of adamantane resistance is more likely attributable to its interaction with fitness-enhancing mutations at other genomic sites rather than to direct drug selection pressure. This implies that adamantanes may not be useful for treatment and prophylaxis against influenza viruses in the long term. More generally, these findings illustrate that drug selection pressure is not the sole factor determining the evolution and maintenance of drug resistance in human pathogens.

Introduction

More than 90% of influenza A viruses isolated in the 2005–2006 season from the United States are phenotypically resistant to adamantanes (amantadine and rimantadine), a class of first generation influenza antivirals commonly used during the past decade (Anonymous 2006). This represents a rise from ~15% in the 2004–2005 season and ~1–3% in all previous seasons (Anonymous 2006; Bright et al. 2005; Bright et al. 2006; Hayden 2006). In nearly every case of recent adamantane resistance in the US, the cause is a single S31N amino acid replacement in the M2 internal membrane protein which functions as an ion channel. S31N is one of 5 amino acid replacements in the M2 protein known to be linked to adamantane resistance. The same pattern of growing adamantane resistance has been described in China, with resistance rates rising from 0–5% before 2003 to reach 96% in 2005 (Bright et al. 2006; Hayden 2006), and in Japan (Saito et al. 2006; Saito et al. 2007). For this reason, the USA Centers for Disease Control and Prevention (CDC) has discouraged use of adamantanes until the frequency of this phenotype subsides (Bright et al. 2005; Hayden et al. 2006).

In theory, two evolutionary mechanisms could be responsible for the global spread of adamantane resistance. First, the S31N mutation could confer sufficient individual selective advantage to facilitate its spread in countries with high levels of adamantane use (Regoes and Bonhoeffer 2006). This hypothesis is compatible with reports of increasing over-the-counter sale of adamantanes in some countries, including China (Bright et al. 2005; Cyranoski 2005). Alternatively, the spread of S31N may be unrelated to drug selection pressure and instead result from its interaction with advantageous mutations located elsewhere in the viral genome. Such interactions could take the form of either genetic 'hitch-hiking', in which the S31N mutation is pulled to fixation because of its physical linkage to beneficial mutations, such as those in the hemagglutinin protein (HA) that facilitate immune escape (Bush et al. 1999; Smith et al. 2004), or epistasis, such that natural selection for a characteristic other than adamantane resistance favors a specific combination of genomic mutations which include S31N.

To understand the mechanisms responsible for the origin and spread of adamantane resistance we studied the evolutionary history of recent A/H3N2 viruses available in the public domain, including those generated through the Influenza Genomics Sequencing Project (Ghedin et al. 2005; http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html). Influenza is characterized by a seasonal pattern of epidemics in the Northern and Southern hemispheres in their respective winters, and a more diffuse pattern of biannual epidemics or year-round circulation in the tropics (Viboud et al. 2006). If, in the Northern hemisphere, a new strain of S31N mutants increased in incidence in the 2004–2005 season and subsequently dominated in the 2005–2006 season, it would likely have circulated in May–September 2005 in the Southern hemisphere. Unfortunately, to date, few complete genomes of viruses isolated in the Northern hemisphere during the winter of 2005–2006 are available. Therefore, to explore the evolutionary origins

Key words: influenza virus, adamantane resistance, reassortment, hitch-hiking, natural selection.

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Mol. Biol. Evol. 24(8):1811–1820. 2007
doi:10.1093/molbev/msm103
Advance Access publication May 23, 2007

of adamantane resistance, we first utilized a data set of whole genomes of influenza A/H3N2 viruses sampled from New Zealand during the 2004 and 2005 seasons. We then expanded our analysis to a wider set of genome sequences sampled from Australia, New York State, USA, and New Zealand, as well as an expansive and global data set of HA1 sequences isolated between 2004 and 2006.

Materials and Methods

Influenza Viruses Used in this Study

Complete genome sequences of 104 influenza A/H3N2 viruses from New Zealand sampled during the years 2004–2005 were downloaded from the NCBI Influenza Database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html; see Supplementary Material). These data were analyzed separately and combined with equivalent whole genome sequence data sampled from New York State, USA, and Australia during the period 2003–2006, to produce a final data set of 485 complete genomes of influenza A/H3N2 viruses (note that because this analysis uses viruses sampled from both hemispheres, viruses were collected by year, rather than by season). Separate sequence alignments were then compiled for the complete coding region sequence of each segment. The size of each data set was as follows (with regions of overlapping reading frame deleted in the case of M1–M2 and NS1–NS2): PB2—2277 bp, PB1—2271 bp, PA—2148 bp, HA—1698 bp, NP—1494 bp, NA—1407 bp, MP—979 bp, NS—835 bp. Because of the large number of closely related isolates that adds little to the phylogenetic analysis at the scale of circulating lineages, we constructed a second data set of 100 genome sequences that are representative of the global genetic diversity of each segment (that is, of each of the lineages circulating during this time period; see Supplementary Material) following an initial neighbor-joining phylogenetic analysis (trees not shown; available from the authors on request).

To infer the evolutionary relationships of the HA1 domain in more detail we compiled a data set comprising those A/H3N2 HA1 sequences available on GenBank covering the period 2004–2006 and sampled on a global scale (and including the isolates from Australia, New York State and New Zealand described above). This resulted in an HA1 data set of 393 sequences, 987 bp in length.

Phylogenetic Analyses

We used MODELTEST (Posada and Crandall 1998) to identify the model of nucleotide substitution that was the best-fit for each data set—individual genome segments and HA1 in isolation. In most cases these were found to be derivatives of the GTR + I + G_4 model (full results and relevant parameter values available from the authors on request). With these models in hand, we then inferred phylogenetic trees using the maximum likelihood (ML) method available in the PAUP* package (Swofford 2003). In all cases TBR (tree bisection-reconnection) branch-swapping was utilized. A bootstrap re-sampling analysis was also undertaken in each case, involving the inference of 1000 replicate neighbor-joining trees using the ML substitution model inferred for each data set.

To identify the genomic mutations that uniquely defined the adamantane resistant viruses we mapped all nucleotide and amino acid changes onto the ML trees inferred above using a parsimony approach (Maddison and Maddison 2000). To determine the subset of these mutations most likely to confer a fitness advantage, we identified those in the HA1 domain that both defined the adamantane resistant viruses and fell at known antigenic sites (Bush et al. 1999). In addition, we consulted the immune epitope database (http://www.immuneepitope.org) and studies on influenza epitopes in humans (Voeten et al. 2000; Rimmelzwaan et al. 2004; Rimmelzwaan et al. 2005) to identify those mutations that fell in known B- and T-cell epitopes.

Results

Phylogenetic Analysis of Recent H3N2 Isolates

We first studied 104 complete genomes of A/H3N2 influenza A viruses sampled in New Zealand during 2004 and 2005. Although the S31N mutation was not sampled during 2004, 20 of the 46 (43%) NZ isolates sampled between March and September 2005 carried this adamantane resistance mutation. None of the other amino acid sites proposed to be associated with adamantane resistance (positions 26, 27, 30, 34 in M2) exhibited any variation. Strikingly, in the whole genome phylogeny (fig. 1), as well as the phylogenetic trees for individual genes where sufficient resolution was apparent (all segments with the exception of the NS segment; available from the authors on request), the S31N viruses comprised a distinct lineage. Following the recent observation of a lineage of S31N-associated viruses in Japan (Saito et al. 2006, 2007) we termed this phylogenetically distinct cluster of adamantane resistant isolates the "N-lineage". In total, this lineage was characterized by 86 genomic mutations relative to other A/H3N2 viruses circulating in New Zealand during 2004 and 2005, 17 of which represented amino acid replacements (table 1).

To explore the distribution of the N-lineage beyond New Zealand, we undertook a second phylogenetic analysis considering a more expansive data set of 393 hemagglutinin HA1 gene sequences from A/H3N2 viruses sampled worldwide during 2004–2006 (fig. 2). Of the 71 isolates from the Southern hemisphere sampled during 2005, 22/48 isolates from New Zealand (46%; including the 46 sequences discussed above) and 6/23 isolates from Australia (26%) had the N-lineage HA1 pattern (fig. 2). Similarly, of the 25 HA1 isolates from the Northern hemisphere 2005-2006 season, 14/16 Japanese and 9/9 USA isolates had the N-lineage HA1 pattern (92%). In Japan, the N-lineage HA1 pattern emerged in isolates from late in the 2004–2005 season (one of 2 isolates were from a traveler returning from China) and increased in frequency during the 2005–2006 season, and all but one of the Japanese isolates with the N-lineage HA1 pattern also carried the S31N mutation (the exception possessing a D at residue 31). Also of interest is isolate A/Canterbury/127/2005 from NZ (highlighted in yellow in figs 1 and 2). In the phylogenies of 6 of 8 viral segments (available on request), and the whole genome phylogeny, this isolate clusters with the N-lineage, but does *not* carry the S31N mutation. Because this isolate occupies a divergent

FIG. 1.—Phylogenetic tree for the concatenated major protein sequences of 104 A/H3N2 influenza A viruses sampled during the period 2004–2005 from New Zealand. Members of the N-lineage are shaded in red. Reassortant isolate A/Canterbury/127/2005, which clusters with the N-lineage in most genes, is shaded in yellow. The phylogeny is mid-point rooted for purposes of clarity only and all horizontal branch lengths are drawn to a scale of the numbers of substitutions per site. Bootstrap values are shown for key nodes only.

location in the MP and PB2 phylogenies, these data strongly suggest that a reassortment event has occurred between co-circulating adamantane resistant and sensitive viruses.

The increasing frequency of the N-lineage during 2005 and 2006 sits in marked contrast to earlier seasons. In the 2004–2005 season in the Northern hemisphere only 1 of 82 viral isolates (~1%) from New York State carried the S31N mutation and this isolate did not cluster in the N-lineage suggesting that this represents a background occurrence of the S31N mutation. However, the national

Table 1
Amino Acid Replacements Characterizing the N-lineage and Their Possible Phenotypic Effects.

Segment (domain)	Amino acid replacement	Possible function	Epitope position	HLA type
PB1	V113A			
PA	E101G			
PA	S208T			
PA	K256Q			
PA	D382E			
PA	I421V			
PA	Y437H			
PA	I602V			
HA1	S193F	Positively selected antigenic site in HA1	193	Human
HA1	D225N	Adjacent to key receptor-binding site	226	Human
NP	A280V	Adjacent to T-cell epitopes (CTL)	$NP_{263-271}$	HLA-B8
NP	I312V		$NP_{265-273}$	HLA-A3
NP	S377G	Adjacent to T-cell epitopes (CTL)	$NP_{380-388}$	HLA-B*0801
			$NP_{383-391}$	HLA-B*2705
NA	D93N			
M1	K174R			
M1	V219I			
M2	*S31N*	*Adamantane resistance determinant*		

prevalence of S31N for the entire 2004–2005 season in the USA was estimated at 15% (Bright et al. 2006). Therefore, the New York State collection available through the Influenza Genomics Sequencing Project is unlikely to represent the full diversity of influenza A/H3N2 viruses from the 2004–2005 season in the US due to geographical heterogeneity. Specifically, all of the New York State viruses were collected between September 2004 and March 2005, while peak activity on the East Coast occurred during October to January, at a time when all isolates in this region were antigenically categorized as A/Fujian/2002-like (Anonymous 2005a). Since the replacement of A/Fujian/2002-like viruses by later antigenically distinct A/California/2004-like viruses started on the West coast from around February 2005 (Anonymous 2005b), the 15% resistance prevalence estimate for the US (Bright et al. 2005) is likely based on sampling of the A/California/2004 lineage from West Coast regions not represented in the New York State sample. Similarly, none of the 11 HA1 sequences isolated in 2004 in China cluster with the N-lineage in HA1, although it has been reported that the prevalence of S31N-adamantane resistance in China had been high for several years; 58% of A/H3N2 isolates were adamantane resistant in 2002–2003, 74% in 2003–2004 and 96% in 2004–2005 (Bright et al. 2005; Bright et al. 2006). These data therefore suggest that several other viral lineages carrying the S31N replacement were circulating in China at this time, with the N-lineage only becoming dominant in the 2005–2006 season.

Taken together, our phylogenetic survey strongly suggests that the N-lineage dominated the 2005–2006 Northern hemisphere influenza season in a number of countries, and comprised a substantial proportion of A/H3N2 viruses from the 2005 Southern hemisphere in several localities. From inspection of dates of isolation we propose that the N-lineage emerged in the first months of 2005 (possibly in China) and then spread to other geographic regions.

Genesis of the N-lineage

To explore the genomic events responsible for the genesis of the N-lineage in more detail we inferred phylogenetic trees of the 8 individual segments of A/H3N2 viruses of 100 (phylogenetically) representative complete genomes sampled from 3 geographical areas – Australia, New York State and New Zealand—during the period 2003–2006. Strikingly, each segment conforms to one of 2 patterns. For the PB2, HA, NA and NS segments, the N-lineage viruses are most closely related to A/H3N2 viruses circulating globally during 2004–2005 (fig. 3*A*). Hence, in these segments, the N-lineage is clearly distinct from earlier sampled isolates, particularly those assigned to the "Clade B" identified in New York State during the 2002–2003 and 2003–2004 seasons (Holmes et al. 2005). As the HA segment from Clade B was involved in a reassortment event associated with the rise of A/Fujian/2002-like antigenic type viruses (Holmes et al. 2005), the Clade B isolates identified here are evidently remnants of the unreassorted Clade B lineage. For simplicity, we denote this as phylogenetic 'pattern A'. Although there is little resolution in the NS tree, that the N-lineage is always closely related to the viruses from 2004-2005 and clearly distinct from some clade B isolates suggests that this segment also follows pattern A.

A very different phylogenetic pattern is observed for segments PB1, PA, NP and MP (fig 3*B*). Here, isolates of the N-lineage are most closely related to viruses of unreassorted Clade B that was isolated in earlier seasons, with the viruses sampled from Australia, New York State and New Zealand during the more recent 2004-2005 season occupying a distant phylogenetic position. This may be considered as phylogenetic 'pattern B'.

Such a major difference in phylogenetic signal is strongly suggestive of a recent 4+4 segment reassortment event (fig. 4). The ancestry of those genome segments possessing pattern A clearly lies with contemporaneous viruses sampled from Australia, New York State and New Zealand. This is evident by the relative lack of genetic differentiation between the N-lineage and the other 2004–2005 viruses; in these 4 segments only 2 amino acid changes distinguish the N-lineage, both in HA1 (positions 193 and 226), and the bootstrap support for the N-lineage is often relatively low (only 39% in the case of NA). Ancestry is more opaque for the segments with phylogenetic pattern B. However, that these viruses are most closely related to isolates of the unreassorted Clade B suggests that this clade continued to circulate at low frequency from 2003 to 2005, but went largely undetected until the recent availability of relatively densely sampled whole genome sequences. This interpretation is further supported by the long branch separating the N-lineage from members of the unreassorted Clade B group, reflecting the ongoing (but hidden) accumulation of nucleotide substitutions. It is also intriguing that one of the 7 Clade B viruses sampled from New York State, isolate A/New York/204/2003, also possessed the S31N mutation. This virus was sampled from a 39-year-old male during the influenza "off-season" on August 16, 2003, indicating that it may have been acquired from a location outside of the USA where influenza is epidemic at that time of

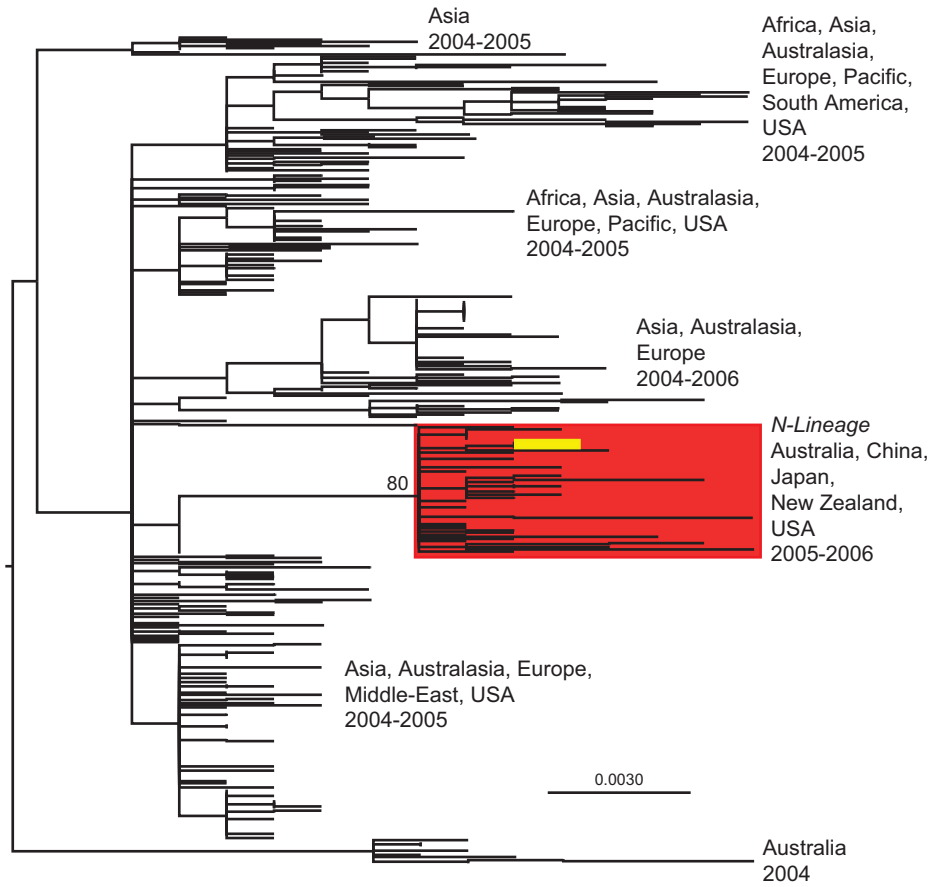

FIG. 2.—Phylogenetic tree for the HA1 protein (987 bp) for influenza A/H3N2 viruses sampled globally during the period 2004–2006 (n= 393). Members of the HA1 N-lineage (58 isolates) are shaded in red, while reassortant isolate A/Canterbury/127/2005 is shaded in yellow. The bootstrap support value is shown for the N-lineage only. The phylogeny is mid-point rooted for purposes of clarity only and all horizontal branch lengths are drawn to a scale of the numbers of substitutions per site. Five other trees had the same likelihood score, but differ only in very minor branch rearrangements.

year (the Southern Hemisphere or the tropics). It is therefore possible that the S31N mutation associated with the N-lineage first appeared during 2002–2003, but did not come to dominance until it was put into a suitable genetic background at a later date—an event achieved by reassortment. To test this hypothesis it will be necessary to undertake an extensive global survey of genetic diversity during 2002–2003.

Our phylogenetic analysis also provides evidence for a number of other reassortment events. Perhaps of most interest are those involving isolates A/Western Australia/55/2004 and A/Western Australia/58/2004. These viruses occupy a number of disparate phylogenetic positions, and are closely related to Clade B in PB2, indicating that they have been involved in multiple reassortment events (fig. 3). Similarly, isolate A/New York/269/2003 clusters with the Clade B viruses in PB1 and MP, although away from this group in other segments (excluding NS where phylogenetic resolution is poor), while isolate A/New York/204/2003, which clusters with the Clade B in 7 of the 8 segments (including MP as noted above), is more diverse in HA.

Possible Selection Pressures Acting on the N-lineage

We next considered the possible mechanisms for the dramatic rise of the N-lineage. A null hypothesis is that the spread of this lineage is due simply to the random action of genetic drift, with no fitness benefit conferred by S31N or any other mutation associated with the N-lineage. Although S31N has not yet reached fixation, its rise in global frequency from $<5\%$ to $>90\%$ in less than a year strongly argues against the action of genetic drift and for the action of natural selection on the N-lineage. Indeed, rapid selective sweeps on the time-scale observed here are common occurrences in HA1 evolution (Smith et al. 2004).

In theory, any of the 17 amino acid replacements across the genome that define the N-lineage could be responsible for its selective advantage. However, as the S31N mutation associated with the N-lineage was seemingly at low frequency prior to the reassortment event (following phylogenetic pattern B), it seems most likely that fitness was enhanced following the acquisition of the segments conforming to pattern A—PB2, HA, NA and NS. Of the 2 mutations associated with the N-lineage in HA1—S193F and D225N—site 193 is one of 18 positively selected sites in HA1 previously proposed to be subject to immune pressure (Bush et al. 1999). The non-conservative D225N replacement is only observed in the N-lineage, and its position adjacent to a key receptor-binding site (at position 226; Bush et al. 1999), suggests that it may impact viral fitness. While these mutations may have altered

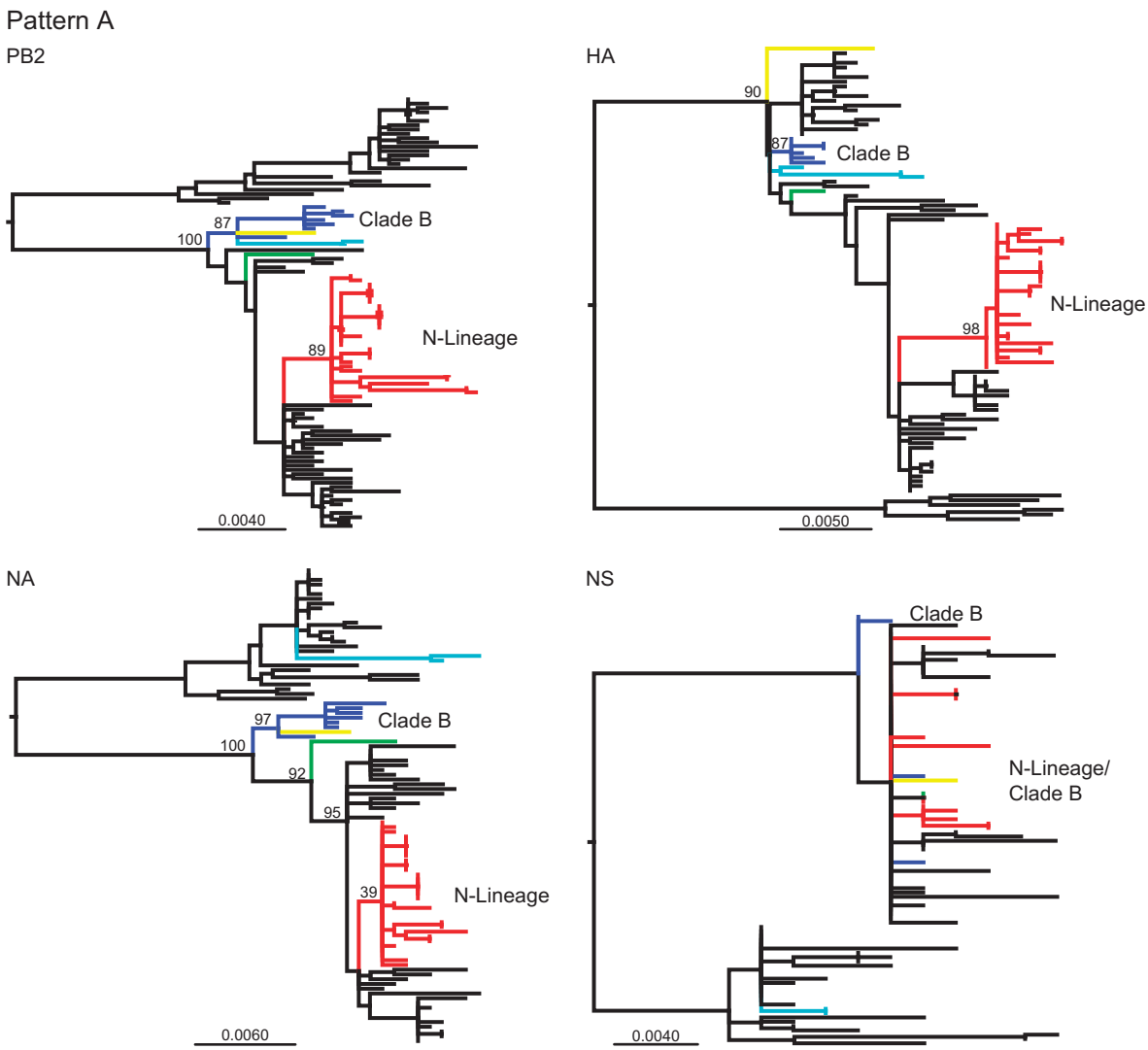

FIG. 3.—Evolutionary history of the genome segments of 100 isolates of A/H3N2 influenza A virus sampled during the period 2003-2006. (*A*) Genome segments conforming to phylogenetic "pattern A": PB2, HA, NA, NS. (*B*) Genome segments conforming to phylogenetic "pattern B": PB1, PA, NP, MP. Different colored lineages are used to identify occurrences of reassortment: In all cases members of the N-lineage are shaded in red, viruses assigned to unreassorted Clade B are shaded in blue, isolates A/Western Australia/55/2004 and A/Western Australia/58/2004 are shaded in turquoise, while isolates A/New York/204/2003 and A/New York/269/2003 are shaded in yellow and green, respectively. Isolate A/New York/204/2003 is also notable in that it is the only member of Clade B to carry the S31N mutation. Branch lengths are scaled according to the numbers of nucleotide substitutions per site and bootstrap support values are shown for key nodes. Trees are mid-point rooted for purposes of clarity only.

the fitness of the virus strains that carry them, they were insufficient to produce a measurable difference in hemagglutinin inhibition (HI) assay as all New Zealand viruses from the 2005 season were phenotypically classified as A/California/2004-like (or the antigenically similar A/Wellington/2004-like).

Although the NP segment follows pattern B, 3 mutations characterized the N-lineage in this segment, one of which—S377G—lies between an HLA-B27 CTL escape mutation R384G (Gog et al. 2003; Voeten et al. 2000) and an essential co-varying mutation E375G (Rimmelzwaan et al. 2004). More distant co-varying mutations in NP have been shown to have a partially compensatory effect (Rimmelzwaan et al. 2005). In addition, the S377G mutation lies adjacent to a known HLA-B8 epitope (Boon et al. 2002), and alteration of flanking sequences can affect epitope processing (Oldstone et al. 1997). Another NP mutation—A280V—is close to 2 other known CTL epitopes (NP263-271 and NP265-273) (http://www.immuneepitope.org). Evidently, future experimental work is needed to characterize the phenotypic consequences associated with each of the 17 amino acid replacements that characterize the N-lineage.

While the mutations that caused the apparently elevated fitness of the N-lineage are currently uncertain, it is evident that drug selection pressure is highly unlikely to explain its spread. A worldwide surveillance study reported that $<1\%$ of influenza isolates from 43 countries during 1992–1995 were resistant to adamantanes (Ziegler et al. 1999), despite the fact that in some countries like the USA this class of antivirals have been used for decades. Furthermore, US data on adamantane sales (available through a national prescription audit by IMS Health Inc; personal communication, Pamela L. Sauerwald, IMS

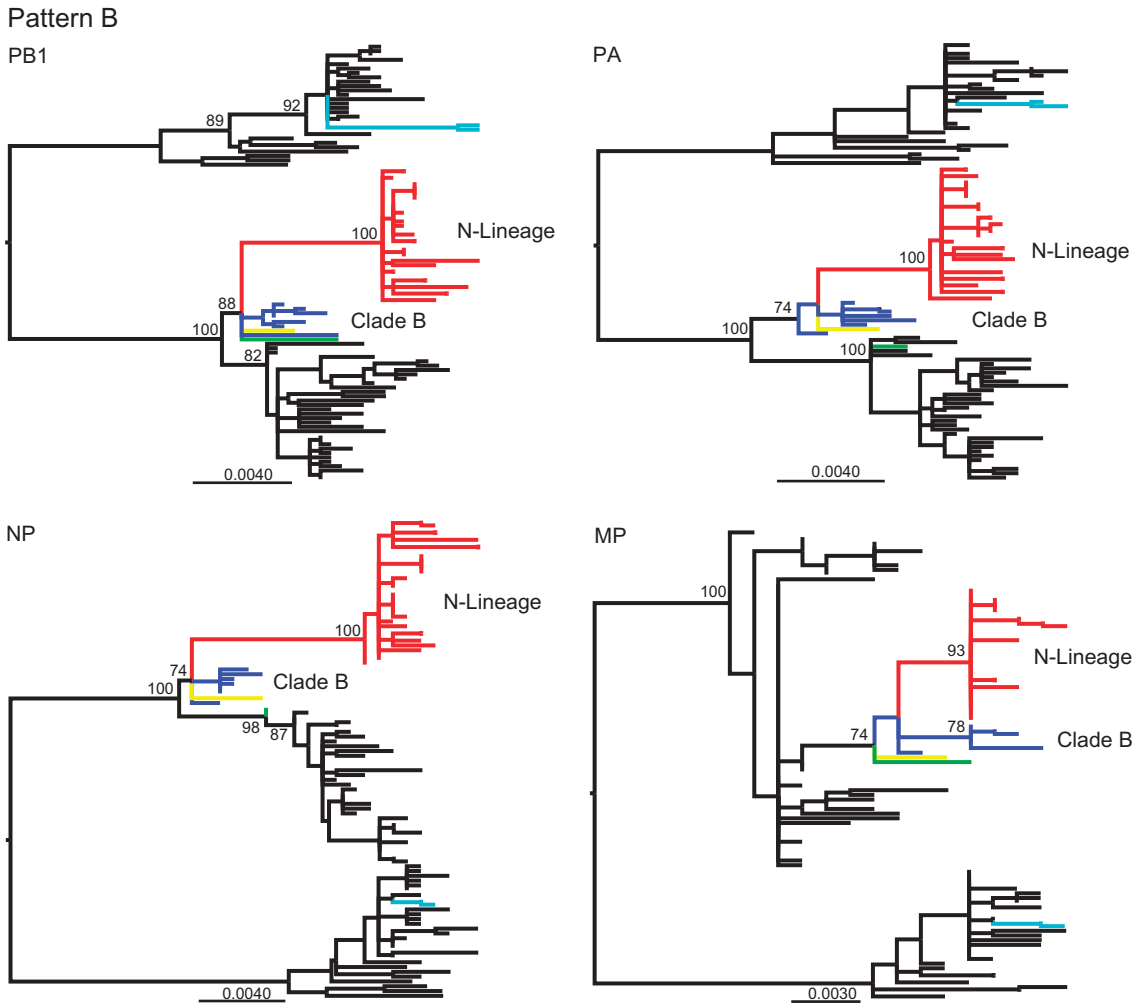

FIG. 3. (Continued)

Health Inc., PA, USA), showed a constant level of adamantane sales around 1.5 million doses annually during 2000-2005, while drug resistance increased dramatically (fig. 5). Notably, a high level of resistance to adamantanes (>90%) continued among A/H3N2 specimens tested through May 2006, even though in January 2006 the CDC recommended against the use of adamantanes for the treatment and prophylaxis of influenza (Anonymous 2006). Similar tracking of drug use internationally reveals zero sales of adamantanes between 2000 and 2006 in Japan and New Zealand (personal communication regarding data from the Midas® tool, by Pamela L. Sauerwald, IMS Health Inc., PA, USA). Taking into account the CDC recommendation against adamantane use, we project that the prevalence of adamantane resistance will approach 100% in the US in the near future, even as sales of adamantanes continue to decline (fig. 5).

Discussion

Our phylogenetic analysis of influenza A/H3N2 virus genome sequence data reveals the existence of a distinct lineage (N-lineage) of predominantly adamantane resistant viruses at high frequency in many locations globally. As adamantane drugs have rarely been used in countries like New Zealand and Japan in recent years, and used in the US for decades (at constant or recently declining rates) with extremely low levels of drug resistance, it is unlikely that local drug selection pressure is directly responsible for the recent spread and maintenance of the N-lineage in these countries. Indeed, the N-lineage also contains a small subset of viruses that do not possess the S31N mutation and, importantly, none of the other mutations associated with adamantane resistance were observed in our data set. We therefore propose that the rapid spread of adamantane resistance is due to its interaction with other genomic mutations, most likely through hitch-hiking with advantageous mutations located elsewhere in the viral genome, although the possibility of selectively mediated epistatic interactions cannot be excluded. This hypothesis is further supported by the observation that adamantane resistant mutants have similar fitness to wild-type viruses in animal passage experiments, so that resistant viruses may revert slowly, if at all, in the absence of adamantane selection pressure (Bean et al. 1989). Also, we note that there is no correlation

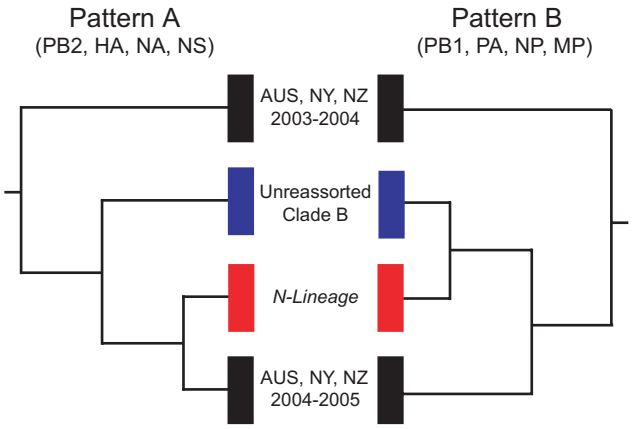

FIG. 4.—Schematic representation of the hybrid origin of the N-lineage, derived from the phylogenetic patterns observed in figure 2. The occurrence of reassortment is strongly suggested by the mismatch in tree topologies. Branch lengths are not drawn to scale. AUS = Australia, NY = New York State, NZ = New Zealand.

between the geographical distribution of adamantane resistance in influenza A/H5N1 viruses infecting poultry in the Far East and local drug-use prevalence (Cheung et al. 2006), again countering the drug selection theory. However, it will be necessary to undertake experimental tests of the phenotypic consequences of the 17 amino acid substitutions that characterize the N-lineage.

Our genome-wide phylogenetic analysis also reveals that a 4 + 4 reassortment event involving 2 phylogenetically distinct lineages of A/H3N2 influenza virus was responsible for the genesis of the N-lineage. Although multiple reassortment events have previously been observed in influenza A virus evolution (Lindstrom et al. 2004), we believe that this is the first time that such a 4 + 4 segment reassortment event has been reported. Hence, our study further demonstrates the potential for reassortment to profoundly effect patterns of genetic diversity, and hence the fitness, of influenza viruses. However, whether the patterns of segment evolution observed reflect functional compatibilities, or merely chance associations, is unknown and clearly merits additional study. Further, that viruses of phylogenetic pattern B were evidently at low frequency during 2003–2005, yet continuing to accumulate nucleotide substitutions, suggests that a more intensive global survey of viral genetic diversity may uncover additional distinct lineages of A/H3N2, including those that exhibit important phenotypic differences such as drug resistance.

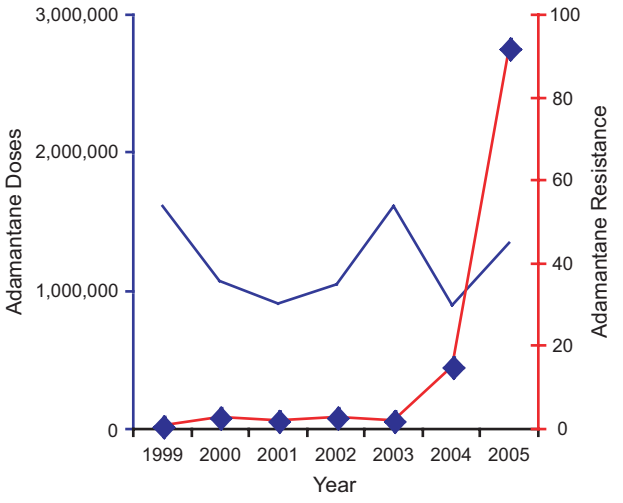

FIG. 5.—Contrasting adamantane drug use and resistance prevalence in the US. The annual numbers of doses used (blue line) were taken from a national prescription audit data for 12-month (July–July periods). The prevalence of adamantane resistance in A/H3N2 viruses for the USA 1999–2005 (red line) were taken from recent CDC studies (Anonymous 2006; Bright et al. 2005; Bright et al. 2006; Hayden 2006). Projections of drug use for seasons past 2005–2006 were performed assuming the CDC guidelines recommending against adamantane usage would reduce drug use rapidly to a base level consistent with current adamantane use for Parkinsons disease. Projections of future resistance were based on the persistence of the S31N mutation in future A/H3N2 lineages.

Although the global spread of S31N is unlikely to be directly linked to drug pressure, it is possible that local adamantane use in countries such as China could have played a key role in elevating the prevalence of multiple lineages carrying the S31N replacement since 2003. This local advantage could then have set the stage for global resistance by enhancing the possibility of linking the S31N mutation with advantageous mutations in other viral lineages. More light will be shed on the role of local drug selection once more full-genome sequences are made available from a wider range of localities, including those from tropical regions where influenza viruses circulate year-round.

Our findings also have important implications for the clinical usefulness of adamantanes for the future treatment of A/H3N2 influenza, for our understanding of the evolution and seasonal spread of influenza viruses, and for the possibility of using this class of antiviral drugs in a future influenza pandemic. In particular, our data suggest that the regional spread of adamantane resistance is in part a stochastic process, relying on the chance association between the S31N mutation and fitness-enhancing mutations located elsewhere in the viral genome. However, after linkage to beneficial mutations has been established, reversion to A/H3N2 sensitivity would require increasingly rare adamantane sensitive viruses to be fortuitously associated with unrelated beneficial mutations. Adamantanes may therefore not be a useful option for prophylaxis and treatment of seasonal influenza A/H3N2 infections in the long term, regardless of the efforts to reduce drug use. The observation of the recent fixation of S31N in swine influenza viruses in Europe supports this likely scenario of future persistence of adamantane resistance in human influenza A/H3N2 viruses.

It is of major public health significance that adamantanes, the only class of inexpensive antiviral drugs for influenza, may now be obsolete for the prophylaxis and treatment of A/H3N2 influenza. Similarly, adamantanes may also be ineffective should a pandemic virus emerge by reassortment between avian influenza A (such as H5N1) and circulating human H3N2 viruses if the MP gene of the reassortant virus has a human origin (as was the case in the 1957 and 1968 pandemics). To date, influenza viruses resistant to the second-generation neuraminidase inhibitors (Tamiflu and Relenza) appear to have reduced fitness, and are transmitted infrequently, if at all (Hayden 2006), suggesting that the conditions necessary for hitch-hiking are

absent. However, this possibility should not be discounted, especially since partially Tamiflu resistant A/H5N1 viruses have been recently reported in treated patients in Vietnam (Le et al. 2005) and intense use of neuraminidase inhibitors for aggressive prophylaxis is suggested in current plans for containment and mitigation of pandemic influenza (Regoes and Bonhoeffer 2006; Ferguson et al. 2006; Germann et al. 2006; Longini and Halloran 2005).

Supplementary Material

Supplementary materials are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

We thank Derek Smith, Gus Rimmelzwaan, Alison Deckhut, Suzanne Epstein, David Woodland, Jack Bennick and Andrew Kress for valuable discussions, and all those contributing to the Influenza Genomics Project, including Maria Giovanni, Virginia Wells, Sylvia Mai, Elodie Ghedin, David Spiro, Kirsten St George, Jill Taylor, Andrew Kress, Laurel Edelman, David Lipman and Yiming Bao. Finally, we thank Masako Tsuzuki, Kenji Sakae and Hiroko Minagawa for laboratory contributions in Japan and Pamela Sauerwald, IMS Health Inc, for contributing unpublished surveillance data on adamantane drug use. JD was supported by the Princeton Center for Quantitative Biology (NIH-P50-GM071508).

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Peter Lockhart, Associate Editor

Accepted May 15, 2007