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H1N1 seasonal influenza virus evolutionary rate changed over time

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ABSTRACT

It was previously shown that the seasonal H1N1 influenza virus antigenic drift occurred at a slower rate than the seasonal H3N2 virus during the first decade of the 21th century. It was hypothesized that the slower antigenic evolution led to a decrease in average ages of infection, which in turn resulted in lower level of global viral circulation. It is unclear what caused the difference between the two viruses, but a plausible explanation may be related to the fact that the H1N1 virus had been in human population for much longer than the H3N2 virus. This would suggest that H1N1 antigenic drift in an earlier period may have been different from a more recent period. To test this hypothesis, we analyzed seasonal H1N1 influenza sequences during various time periods. In comparison to more recent H1N1 virus, the older H1N1 virus during the first half of the 20th century showed evidences of higher nonsynnonymous/synonymous ration (dN/dS) in its hemagglutinin (HA) gene. We compared amino acid sequence changes in the HA epitopes for each outbreak season and found that there were less changes in later years. Amino acid sequence diversity in the epitopes as measured by sequence entropy became smaller for each passing decade. These suggest that there might be some limit to the antigenic drift. The longer an influenza virus has drifted in human population, the less flexibility it may become. With less flexibility to adapt and escape the host immunity, the virus may have to rely more on younger naïve population.

1. Introduction

Seasonal influenza viruses are highly variable. Antigenic drift occurs frequently and makes necessary the annual update of vaccine strains. Hemagglutinin (HA) is the major viral envelope protein and contains major antigenic determinants. Influenza HA evolves under a strong positive selective pressure from the host immune response (Fitch et al., 1991). Because of this strong immune pressure, old influenza strains neutralizable by herd immunity become regularly extinct. This strong immune pressure allows antigenically escape strains to outgrow old strains and cause antigenic drift and new outbreaks (Hay et al., 2001). This antigenic drift is a global event, in which a new variant spreads out globally (Rambaut et al., 2008). Although it is generally accepted that seasonal influenza viruses evolve under strong positive selection exerted by host immunity, the degree of the positive selection can be different. It has been previously shown that during the first decade of the 21st century the H1N1 and influenza B viruses had lower rates of antigenic escape than the H3N2 virus with (Bedford et al., 2015). While the H3N2 virus strictly followed the single lineage phylogeny with periodic emergence of new global strains and extinction of old strains, the H1N1 and influenza B viruses had lower level of global viral circulation and higher level of local persistence resulting in a transient multi-branch phylogeny (Bedford et al., 2015). It was hypothesized that the lower rates of immune escape of influenza B and H1N1 may have led to younger average ages of infection as compared to H3N2. It was also hypothesized that the lower average ages of infection may explain the reduced global viral circulation as children travel long-distances less frequently than adults (Bedford et al., 2015). However, it is unclear why influenza B and the H1N1 virus showed lower rates of antigenic escape. It is possible that this is a difference in intrinsic properties of the viruses. However, as influenza B and H1N1 were older than H3N2, it is also possible that this difference is related to the length of time that the viruses have circulated in human population. The H1N1 virus entered human population in 1918 and influenza B had circulated in human population for much longer, whereas the H3N2 virus came into human population in 1968 (Cox and Subbarao, 2000).

Influenza viruses are believed to be in an optimal balance with their natural hosts. In water fowls, influenza A viruses were shown to be in an evolutionary stasis. This is probably a result of a long-term co-evolution of the viruses and hosts (Webster et al., 1992). Accordingly, the

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Table 1
The values of dN/dS and LRT tests for HA1 sequences by CodeML analysis.

	dN/dS	LRT (M7–M8)	P-value
1918–1957 (72 strains)	0.436	56.21	< 0.00001
1977–2008 (71 strains)	0.271	27.88	< 0.00001
1933–1943 (22 strains)	0.485	14.09	0.00017
1945–1957 (45 strains)	0.387	46.22	< 0.00001
1977–1984 (48 strains)	0.286	21.35	< 0.00001
1985–1997 (83 strains)	0.339	63.66	< 0.00001
1998–2008 (107 strains)	0.256	31.46	< 0.00001
2009–2015 (106 strains)	0.237	8.10	0.00442

longer a virus circulates in a host species, the more it should become closer to this evolutionary stasis. This hypothesis would predict that antigenic drift of a seasonal influenza virus will decrease with time. It is, however, unclear how long it would take to see this effect. The evolutionary stasis of avian influenza A viruses in water fowls may have taken thousands of years. A century is a relatively short time period in comparison. However, if the observed difference in the antigenic drift of H1N1 and H3N2 was a direct result of the difference in time length of the viral circulation, half a century must have been sufficient to cause an observable difference. Comparing old and recent H1N1 evolution may provide some insight into this hypothesis. Since the H1N1 epidemic was interrupted between 1950s to 1977 by H2N2 and H3N2, we decided to compare the evolution of H1N1 before and after 1977.

2. Materials and methods

2.1. Sequences

We focused our analyses on the HA1 gene because it codes for the main antigenic protein of the virus that showed antigenic drift and its sequences are the most abundantly available. Four hundred and eleven full length sequences of H1N1 HA1 were retrieved from NCBI Influenza virus database of the full length sequences were selected and aligned with Bioedit program. After alignment, sequences with 100% similarity were excluded from the analyses. All available sequences from 1918 to 1987 were included, and sequences were randomly selected from the more the recent years (1988–2015) to cover all geographical regions. The sequences were arbitrarily divided into 6 groups by decade (Table 1).

2.2. Phylogenetic analyses

Phylogenetic trees based on HA1 nucleotide sequences were constructed by maximum likelihood method implemented in PAUP version 4.0. The resulted trees were further used as guide trees for estimating selection pressure in CodeML application program in Phylogenetic Analysis by Maximum Likelihood (PAML) package (Yang, 2007). We used models M7 and M8, where M7 contains 10 ω categories to describe ω amongst sites, all constrained to be < 1; M8 differs from M7 only in that it estimates ω for an extra class of sites (p10) at which ω can be > 1 (Yang, 1997). Models were compared using a likelihood ratio test and the Bayes Empirical Bayes (BEB) method was used for a posteriori estimation of individual codons under positive selection (Yang et al., 2005). Phylogenetic tree based on HA1 amino acid sequences were constructed by maximum likelihood method in MEGA program version 6.0.

2.3. Hamming distance and $P_{epitope}$

Hamming distance of HA1 epitopes was calculated by comparing consensus sequence of each year to that of the previous year. $P_{epitope}$, which has been previously shown to correlate with antigenic distance (Deem and Pan, 2009), was also calculated by comparing consensus sequences of the epitope residues of two consecutive years. For each epitope, the P-value is defined as the proportion of different amino

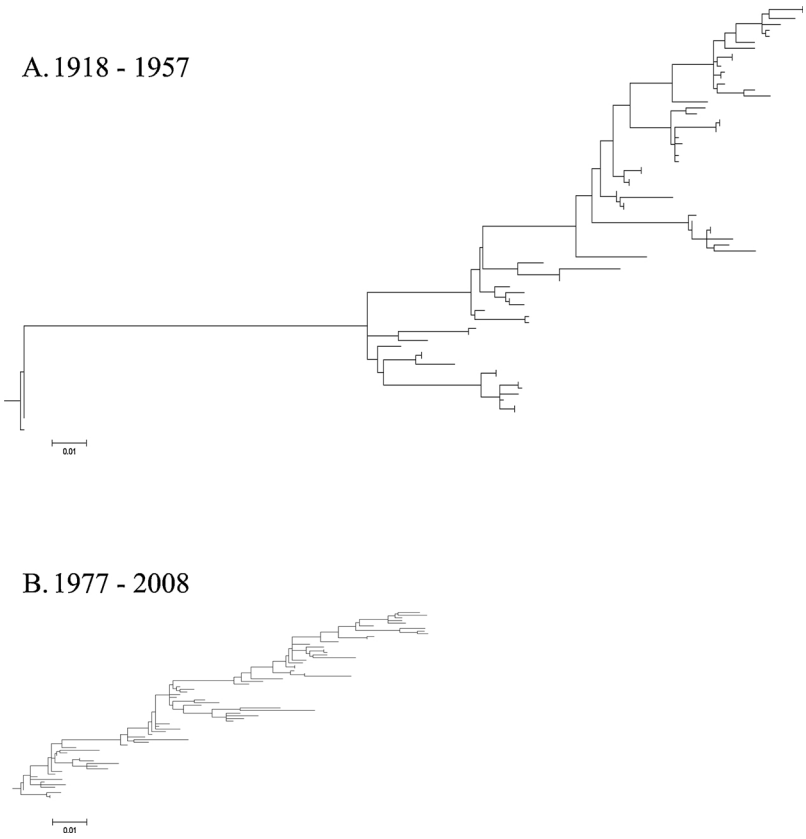

Fig. 1. Phylogenetic analysis of H1N1 HA1 nucleotide sequences. The phylogeny trees were generated by maximum likelihood from HA1 nucleotide sequences of human influenza A viruses isolated from 1918 to 1957 (a) and 1977 to 2008 (b). Branch lengths are drawn to scale. The scale bar represents 0.01 units of nucleotide change per site.

Fig. 2. Maximum likelihood trees of H1N1 HA1 nucleotide sequences (a) and amino acid sequences (b). The H1N1 HA1 sequences were arbitrarily divided into 6 groups by decade. All available sequences from 1918 to 1987 were included, and sequences were randomly selected from the more the recent years (1988–2015) to cover all geographical regions. Branch lengths are drawn to scale. The scale bar represents 0.01 units of nucleotide or amino acid change per site. Tree lengths are provided in parentheses under the time periods.

acids between these two sequences. The largest of the five P-values is defined as $P_{epitope}$, and the corresponding epitope is defined as the dominant epitope (Deem and Pan, 2009). We used previously described 62 amino acid residues in 5 epitopes (A–E) for these analyses (Huang et al., 2012).

2.4. Sequence entropy

Shannon entropy is a measure of uncertainty or unpredictability of information content. It has been used to describe diversity of amino acid sequences and identify antigenic sites of influenza HA (Huang et al., 2012; Pan and Deem, 2011). To measure entropy of the HA

Table 2
The values of dN/dS and LRT tests for HA1 sequences, which were deleted the egg-adapted positions, by CodeML analysis.

	dN/dS	LRT (M7–M8)	P-value
1918–1957 (72 strains)	0.404	42.54	< 0.00001
1977–2008 (71 strains)	0.259	29.16	< 0.00001
1933–1943 (22 strains)	0.456	8.65	0.00327
1977–1984(48 strains)	0.300	27.59	< 0.00001
1985–1977 (83 strains)	0.333	66.89	< 0.00001
1988–1997 (72 strains)	0.308	39.63	< 0.00001
1998–2008 (107 strains)	0.228	15.85	< 0.00001
2009–2015 (106 strains)	0.232	6.23	0.01256

antigenic sites, alignment was compiled for each time period containing all amino acid positions that were previously mapped in antigenic sites (Caton et al., 1982; Deem and Pan, 2009; Huang et al., 2012). Shannon entropy of each amino acid position in the alignment, which is defined as $H = \sum_{I=1}^{20} Pil$, where *Pi* is proportion or probability of an amino acid, was then calculated by a web-based entropy calculator at http://imed.med.ucm.es/PVS/.

3. Results

3.1. Evolution of H1N1 HA during difference periods

It was previously shown that seasonal H1N1 virus in 2000–2008 had a slow rate of antigenic drift than H3N2 virus in the same period (Bedford et al., 2015). We suspected that this might be because H1N1 had been in human population for much longer. This would mean that earlier H1N1 would have had faster antigenic drift than the more recent H1N1 virus. To test this hypothesis, HA1 gene sequences of seasonal H1N1 viruses from the period before and after the interruption by H2N2 and H3N2 viruses during 1957–1977 were compiled and analyzed. We chose to analyze only the HA1 part of the gene because it is more variable than the HA2 and contains all the variable antigenic epitopes of the HA globular head. Although both the phylogenetic trees of HA1 before 1957 and after 1977 showed the typical monophyletic pattern indicating positive selection, the tree before 1957 has a much longer main trunk (Fig. 1). The longer trunk suggests that the HA1 gene before 1957 had more mutations accumulating in the length of the main trunk. Shorter main trunk, on the other hand, suggests that more mutations were lost in the dead-end side branches and did not contribute to the main trunk length. We next generated both nucleotide and amino acid phylogenetic trees of the H1 HA1 by decade (Fig. 2). Only the main trunk length of amino acid tree significantly shrank down for each passing decade (one-tail Spearman correlation of amino acid tree; *P*-value = 0.0292, nucleotide tree *P*-value = 0.0681), except for the period of 1998–2008. Nevertheless, there was a clear trend toward shorter main trunk in the more recent history of the H1N1 virus. This includes that 2009 pandemic virus, which surprisingly had little changes during the last 7 years since its emergence.

The nature of selection was determined by the dN/dS ratio of the HA1 nucleotide sequence alignment separately constructed from the sequences before 1957 and after 1977. Although the log-likelihood ratios indicate significant positive selection for both periods, the HA1 gene of early 20th century before 1957 showed a higher dN/dS ratio of 0.436, whereas the more recent HA1 after 1977 had dN/dS ratio of only 0.271 (Table 1). Because the dN/dS ratio of the sequences before 1957 includes the 1918 pandemic virus and because a pandemic virus may evolve rapidly in order to adapt to the new transmission condition in human population, the ratio might be biased toward higher positive selection. In order to clearly see changes in the selective pressure over time, the dN/dS ratio was determined for HA1 sequences of each decade (Table 1). Excluding the 1918 pandemic virus, the evolution of early HA1 during 1933–1943 showed a dN/dS ratio of 0.485, clearly higher than those of the following decades. The period of 1945–1957 also showed a moderately higher dN/dS ratio than the more recent periods. These indicate that the HA1 of seasonal H1N1 virus may have evolved with a gradually increasing negative selective pressure over a period of about half a century and then continued to evolve with a steadily level of selection over the subsequent period (one-tail Spearman correlation; *P*-value = 0.0083). Because virus isolation in cell culture was only available after 1950s, earlier sequences were therefore derived solely from egg-cultured viruses. To provide a supporting evidence that the observed difference in the dN/dS ratio among the time periods was not due to a bias of having egg-derived sequences in the earlier period, we deleted amino acid position 129, 163, 187, 190, 225 and 226, which were shown to contain egg-adapted mutations (Gambaryan et al., 1999; Robertson et al., 1991), from the alignments and reanalyzed for dN/dS ratio. Similar decrease in dN/dS ratio with time was observed after deletion of the egg-adapted positions (one-tail Spearman correlation; *P*-value = 0.0514) (Table 2).

3.2. Changes in antigenic residues

Amino acid difference in 5 epitopes of HA1 was shown to correlate with antigenic distance and reduced vaccine efficacy against mismatched strains (Deem and Pan, 2009; Huang et al., 2012). The difference in epitope amino acid sequence can be quantified either by Hamming distance or $P_{epitope}$ (Deem and Pan, 2009; Huang et al., 2012). To understand the trend in antigenic evolution, Hamming distance and $P_{epitope}$ were calculated by comparing consensus epitope sequences between two consecutive years. The values were used to represent levels of antigenic change in each outbreak season. Both Hamming distance and $P_{epitope}$ showed a clear decreasing trend with time (Fig. 3). For each individual epitope, the P values of most epitopes except for epitope C showed the decreasing trend (Fig. 3).

3.3. Diversity in the antigenic sites

The decreasing dN/dS in the more recent H1N1 HA1 suggested that the more recent virus had slow antigenic drift. This would mean less diversity in the antigenic sites of the HA1. In order to determine the amino acid diversity in the HA1 antigenic sites, amino acid alignment at positions of previously described antigenic sites was constructed for HA1 sequences of each decade. Sequence entropy, which is a quantitative measure of uncertainty of amino acid at each position, was then calculated (Pan and Deem, 2011). In accordance to the decreasing dN/dS, the entropy of the amino acid positions in the HA1 antigenic sites showed a clear decreasing trend over the decades (Fig. 4). This indicates lower diversity in the antigenic sites, which may link to slower antigenic drift, in the more recent viruses. Because the number of available old strains used in the analysis was much lower than the more recent strains, the different sample sizes might cause a bias in the analysis. To ensure the validity of the entropy results, recent strains were randomly selected for 22 strains per decade and reanalyzed for entropy. The random resampling was performed for 10 times. The mean and maximum entropy of the resampling sets of recent strains still showed similarly lower levels than those of older strains (Fig. 5).

4. Discussion

Our data are in agreement with previously published data showing that amino acid substitution rate and positive selective pressure of seasonal influenza virus H1N1 during 1918–1957 were higher than during 1977–2009 (Furuse et al., 2010), suggesting that evolutionary rate of the H1N1 seasonal influenza virus has slowed down with time. However, the analyses included viruses from the beginning of the pandemic in 1918, and it was shown that for many emerging viruses a rapid increase of viral diversity was a phenomenon usually observed at the beginning of an epidemic when expansion of viral population was

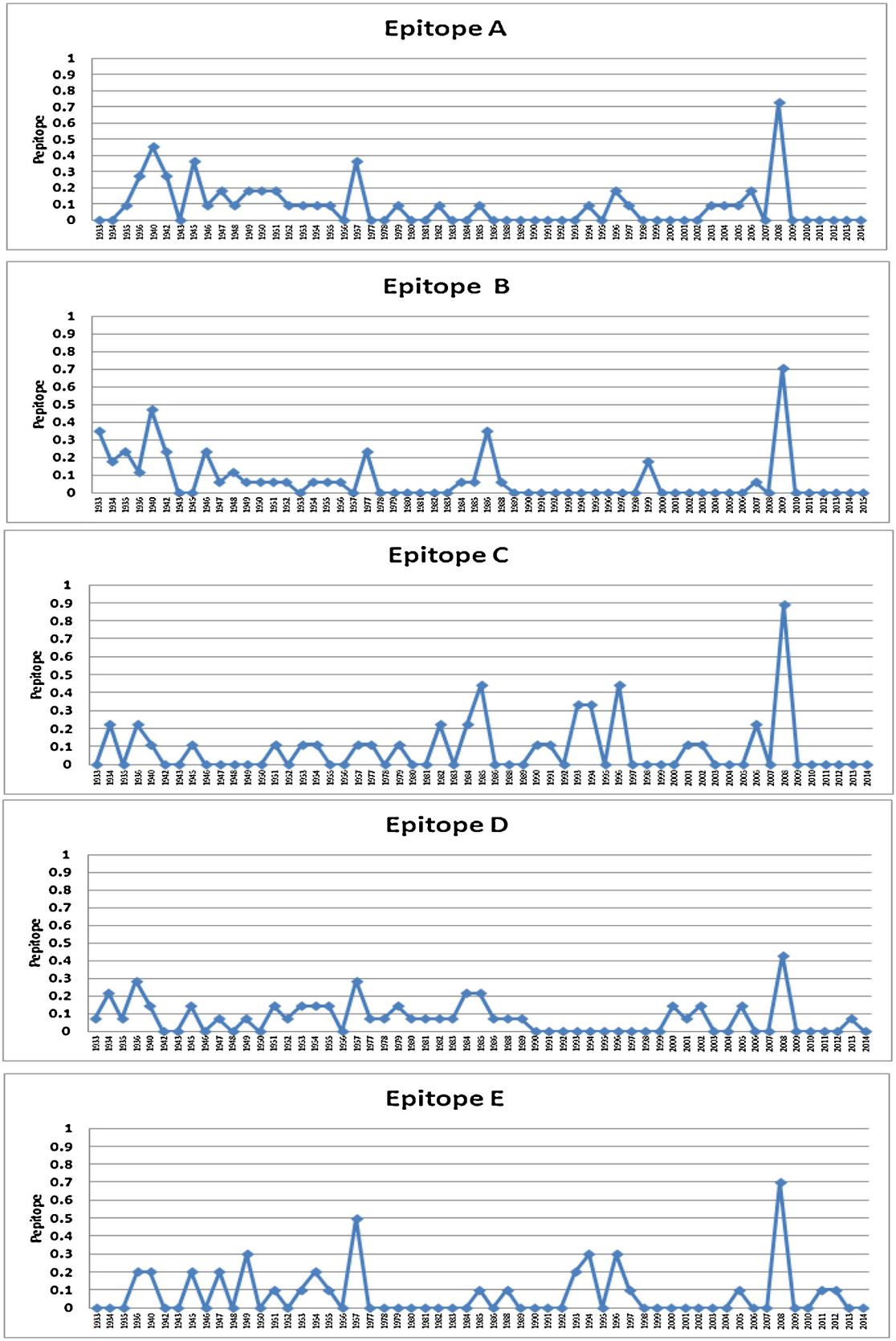

Fig. 3. P epitope values of the HA antigenic sites represent antigenic changes by year. The values were calculated by comparing consensus sequences of the epitope residues of two consecutive years. The P epitope is defined as the proportion of different amino acids between these two sequences.

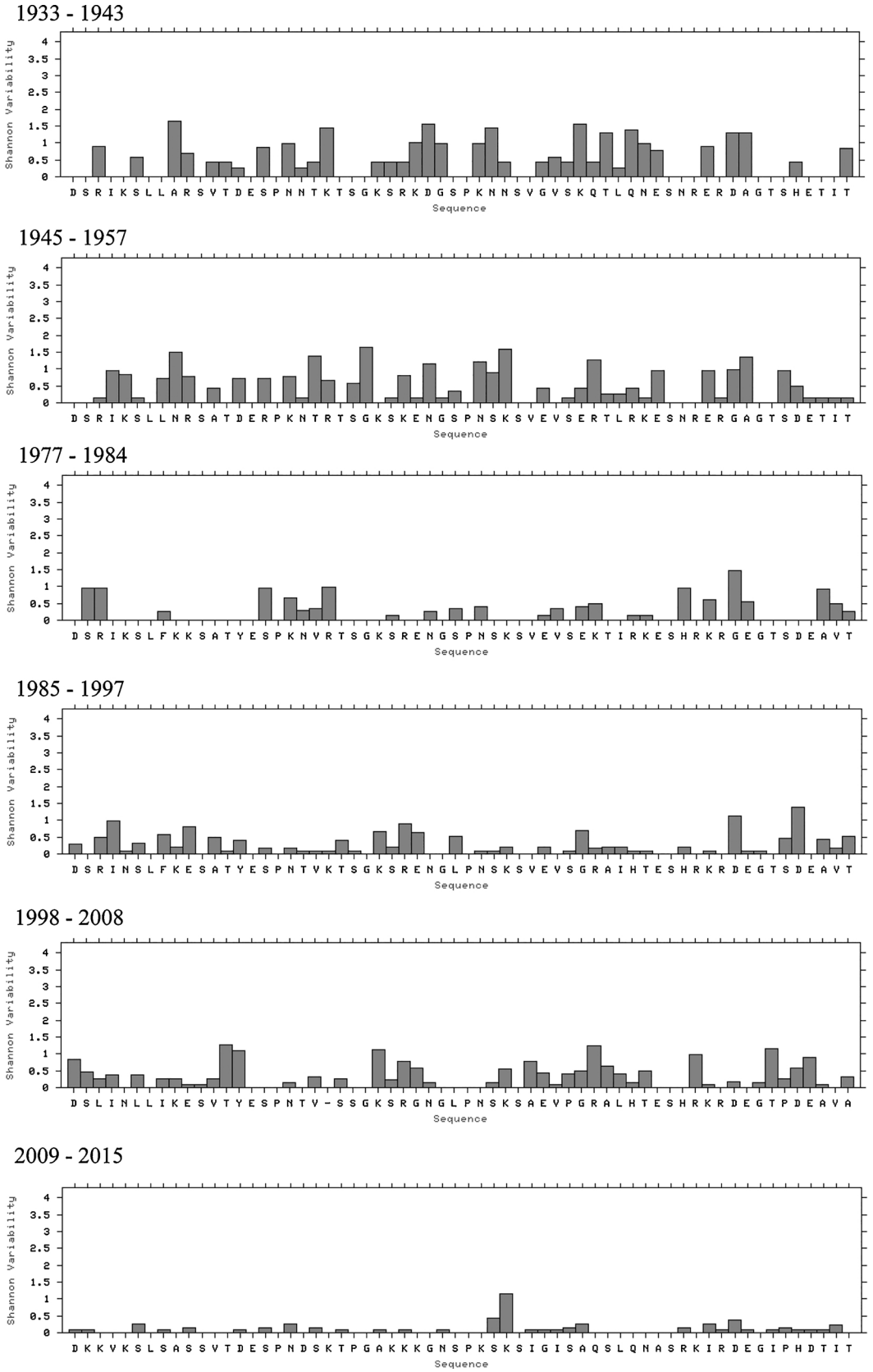

Fig. 4. Shannon entropy of 62 amino acid residues in the HA antigenic sites by decade. Amino acid residues previously identified as antigenic sites were obtained from HA1 sequences arbitrarily divided into 6 groups by decade. Shannon entropy was then calculated by a web-based entropy calculator at http://imed.med.ucm.es/PVS/.

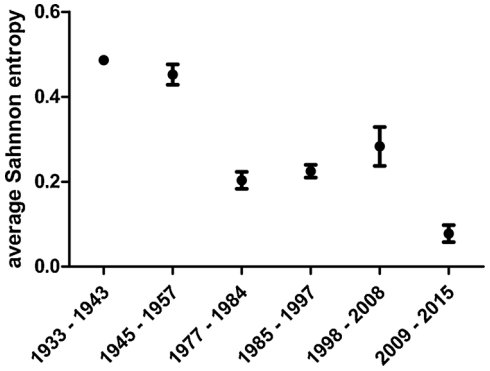

Fig. 5. Mean Shannon entropy of the HA antigenic sites by decade. Ten sets of randomly selected 22 strains per decade were analyzed for Shannon entropy of the HA antigenic sites, except for the 1933–1943 decade where only one set of sequences was available. Mean ± SD of the entropy values are shown for each decade.

unhindered by competition among viral variants, some of which may be later selected out by purifying selection resulting in reduced diversity when the epidemic became more mature (Faria et al., 2016; Gire et al., 2014; Meyer et al., 2015). Our decade-wise analyses, on the other hand, included only viruses starting more than a decade after the pandemic, and should be much less affected by the initial phase of epidemic.

The antigenic drift as a global event is understandable in the view of current global community when any corner of the world can be reached within a couple of days and a large number of people travel across continents and countries in airliners every day. This is in sharp contrast to the first few decades of the history of influenza in humans. In the early 20th century, traveling around the world in 80 days was still a scientific fiction. International traveling had not become a part of everyday life until the 1950s when commercial airliners began to operate. The rapid expansion of international travel thereafter has transformed human society in many ways including the risk of rapid spread of new pathogens. Massive international travel is probably a pivotal factor in the thorough mixing and global circulation of seasonal influenza strains, which play an important role in the antigenic drift and evolution of seasonal influenza (Lemey et al., 2014). It was previously proposed that slower antigenic drift of H1N1 seasonal influenza virus as compared to H3N2 virus was associated with lower ages of infection that lead to reduced global circulation and increased local persistence of viral strains (Bedford et al., 2015). Given constant viral and host factors, massive increase in international travel should have resulted in a more rapid antigenic drift. However, while international travel was dramatically increasing through commercial air travel after 1970s, the antigenic drift of H1N1 seasonal influenza virus was getting slower. This suggests that the decreasing antigenic drift of H1N1 HA was an intrinsic property of the virus rather than a result of changing transmission environment.

The reason for the decrease in the antigenic evolutionary over time is unclear. It is possible that structural constraint may restrict changes for preservation of the HA function and viral fitness. Although it has been previously shown that the HA was highly flexible to changes and could accommodate more mutations than other viral proteins (Heaton et al., 2013), it was also shown that there was preference of certain amino acids in some positions in HA antigenic sites (Kryazhimskiy et al., 2008). This implies that there may be some restriction in the changes of HA antigenic sites. In addition, it was proposed that in addition to immune escape the evolution of HA may also direct toward optimization of receptor binding avidity (Hensley et al., 2009).

The current H1N1 virus seemed to follow the trend of the old seasonal H1N1 virus with slow antigenic drift. There were little changes in antigenic property of the virus since its emergence as a pandemic virus (Koel et al., 2015). Previous data showed that the HA of this virus was mutating toward stability and optimal receptor binding rather than immune evasion (Castelan-Vega et al., 2014; de Vries et al., 2013). Complex interaction with host immunity targeting highly conserved epitopes has been proposed to be responsible for the extinction of the old seasonal H1N1 virus and may play a role in limiting antigenic escape of the current H1N1 virus (Pica et al., 2012).

Antigenic drift of influenza B virus was shown to be even lower than H1N1 virus (Bedford et al., 2015). In addition to antigenic drift rate, nucleotide mutation and amino acid substitution rates in influenza B was observed to be lower than those of influenza A H3N2 and H1N1 viruses (Bedford et al., 2015). There was also observation that mutation rate in HA1 of H1N1 was lower than that of H3N2 during 1980–2000 (Ferguson et al., 2003). These indicate that influenza B and H1N1 viruses are evolving at lower rates than H3N2 virus. Because influenza B had been in human population much longer than H1N1, it suggests that the decrease of evolution rate over time applies to both influenza B and H1N1 viruses. This implies that H3N2 may follow the same pattern. Understanding the changes in evolution rate of seasonal influenza viruses may help us foresee the future trend of seasonal influenza viruses.

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