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ORIGINAL ARTICLE

Responses to the Sb epitope contributed to antigenic drift of the influenza A 2009 H1N1 virus

S. Ketklao[1] · C. Boonarkart[1] · S. Phakaratsakul[1] · P. Auewarakul[1] · Ornpreya Suptawiwat[2]

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Abstract

Immunodominance is recognized as a key factor in the antigenic drift of seasonal influenza viruses. In the immunodominance model, each individual in a population predominantly responds to a single epitope among the five antigenic epitopes of the viral hemagglutinin (HA), driving escape mutations one at a time, and sequential mutations in multiple individuals who respond to different epitopes eventually generate a drifted strain with mutations in epitopes that are targeted by a majority of the population. A focused antibody response to the Sa epitope in people born between 1965 and 1979 was believed to contribute to a mutation at HA residue 163 and the first antigenic drift of the 2009 pandemic influenza A H1N1 virus. A serine-to-threonine mutation at HA residue 185 in the Sb epitope emerged in 2010 even before the 163 mutation. We show here that a large fraction of the population in 2010-2011 had responses to the Sb epitope, as shown by 47% of tested sera having altered titers to the S185T mutant. Responses to the Sb epitope showed an age-specific trend similar to that found for the response to Sa epitope in these subjects. Together, the focused responses to Sa and Sb epitopes may have driven the first antigenic drift of the 2009 pandemic H1N1 virus.

Introduction

Antigenic drift plays a pivotal role in the evolution and persistence of seasonal influenza in the human population. It is a global-scale event that allows new strains to infect people who have been infected and are immune to previously circulating strains. A drifted strain usually carries mutations in several major epitopes of the viral HA surface protein. There are five major B cell epitopes located around the receptor-binding site on the HA head of influenza A H1N1 – Sa, Sb, Ca1, Ca2, and Cb – which are the main targets of hemagglutination-inhibiting and neutralizing antibodies [1–3].

While the mutation rate of influenza virus is high, it is not high enough to allow mutations in all five major epitopes to arise simultaneously in an infected individual or even in the whole world population. Therefore, it is believed that antigenic drift occurs in a stepwise fashion. An individual predominantly responds to one or a few epitopes and drives mutations only in that particular epitope. Mutations accumulate after the virus sequentially infects multiple individuals who respond to different epitopes. At the population level, the number of mutated epitopes required to cause an antigenic drift depends on the coverage of epitope responses in the majority of the population. When the majority of the population have responses focused on the same epitope, it is easier for a new drifted strain to emerge, since there is less mutation required than when diverse epitopes are involved [4]. It was previously reported that people born between 1965 and 1979 had antibody responses to the HA Sa epitope of the pandemic 2009 influenza A H1N1 virus, and this focused response led to the emergence of a lysine-to-glutamine mutation at HA position 163 (K163Q) in the Sa epitope, which was responsible for the first antigenic drift of the 2009 H1N1 virus [5, 6]. While this K163Q mutation was first detected in 2012, a serine-to-threonine mutation at HA position 185 (S185T) in the Sb epitope emerged in 2010, and it is still unknown if this mutation contributed to antigenic

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S. Ketklao and C. Boonarkart contributed equally to this manuscript.

✉ Ornpreya Suptawiwat
ornpreya.sup@pccms.ac.th

[1] Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

[2] Faculty of Medicine and Public Health, HRH Princess Chulabhorn College of Medical Science, Chulabhorn Royal Academy, Bangkok, Thailand

escape and antigenic drift. A previous report using a human monoclonal antibody suggested an emergence of viruses with mutations at the Sb site [7]. We therefore investigated whether responses to the Sb epitope may have contributed to the first antigenic drift of the 2009 pandemic H1N1 virus by testing antibody responses to the Sa and Sb epitopes in a community-based cohort in 2010-2012.

Materials and methods

Serum samples and ethical approval

Annual serum samples spanning the 3-year period of 2010-2012 from the same 98 healthy subjects were randomly selected from a community-based cohort, "Hepatocellular carcinoma screening and surveillance program in Thai patients with chronic hepatitis B infection". They were retrieved from the Biorepository Unit, HRH Princess Chulabhorn College of Medical Science, Chulabhorn Royal Academy. This project was approved by the Human Research Ethics Committee, Chulabhorn Research Institute, project code 021/2561. All subjects who participated in this study provided written informed consent.

Viruses and mutants

Influenza viruses were propagated in Madin-Darby canine kidney (MDCK) cells. The wild-type virus strains were A/Thailand/104/2009 (H1N1) and A/KAN/SC023/2016 (H1N1), which were kindly provided by Asst. Prof. Dr. Kobporn Boonnak, Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Mutant viruses with K163Q and S185T mutations in the HA gene of the A/Thailand/104/2009 (H1N1) HA plasmid were generated by the DpnI mutagenesis technique. The primers for the K163Q mutant were HA-K163Q forward (CATACCCAAAGCTCAGCCAAT CCTACATTAATG) and HA-K163Q reverse (CATTAA TGTAGGATTGGCTGAGCTTTGGGTATG), and the primers for S185T mutant were HA-S185T forward (CACCAT CCATCTACTACTGCTGACCAACAAAG) and HA-S185T reverse (CTTGTTGGTCAGCAGTAGTAGATGGATGGT G). Mutant viruses containing a mutation at amino acid position 163 or 185 in the HA gene were rescued using a reverse genetics technique by which each mutant HA gene was combined with seven other genes from influenza virus strain A/Puerto Rico/8/1934 (H1N1).

Hemagglutination inhibition (HAI) assay

The HAI assay was performed as described previously [8]. The test sera were pretreated with receptor-destroying enzyme (RDE; Denka Seiken, Japan) at 37 °C for 18 hours and subjected to heat inactivation at 56 °C in a water bath for 30 minutes. To prevent nonspecific agglutination, the treated sera were incubated with 50% goose red blood cells for 60 minutes at 4 °C. The treated sera were serially diluted twofold from 1:40 to 1:2560. Twenty-five microliters of each diluted serum was incubated with 25 µl of the test virus, containing 4 HA units, in a 96-well V-shaped microtiter plate for 30 minutes at room temperature. Thereafter, 50 µl of a 0.5% suspension of goose red blood cells was added to each well, and the plate was kept at 4 °C for 30 minutes. Virus backtitration was performed in parallel. The highest serum dilution that completely inhibited the hemagglutination reaction was defined as the HAI titer. HAI assays were performed in two independent experiments. A serum sample was considered specific for an HA epitope if it exhibited greater than a one-fold reduction in HAI titer against a mutant virus in two independent experiments.

Phylogenetic analysis

One hundred sixty-seven full-length sequences of H1N1 HA1 were randomly selected from the NCBI Influenza Virus Database between 2009 and 2019, and there sequences covered all geographical regions. The HA amino acid positions are numbered using the H1 numbering system. H1N1 HA1 sequences were aligned using ClustalW multiple alignment in Bioedit version 7.1.11. A rooted phylogenetic tree was constructed by the maximum-likelihood method with the JTT+G model in MEGA7 software.

Results

The S185T mutation in the Sb epitope emerged before the K163Q mutation in the Sa epitope: the first mutation of H1N1 2009 virus antigenic drift

To understand the epitopes involved in the antigenic drift of influenza A H1N1, HA amino acid sequences from randomly selected H1N1 viruses obtained since 2009, together with those of vaccine strains, were aligned. A number of mutations in three of the five known antigenic sites that persisted throughout the evolution of the virus were observed (Fig. 1). There were three mutations in the Sa epitope, S162N, K163Q, and S164T, two mutations in the Sb epitope, S183P and S185T, and one mutation in the Cb epitope, S74R. These mutations emerged at different time points along the single-branch phylogenetic tree typical of the pattern of positive selection in seasonal influenza virus evolution (Fig. 2). The first mutation to emerge was the S185T mutation in the Sb epitope, which was detected in 2010, followed by the K163Q mutation in the Sa epitope,

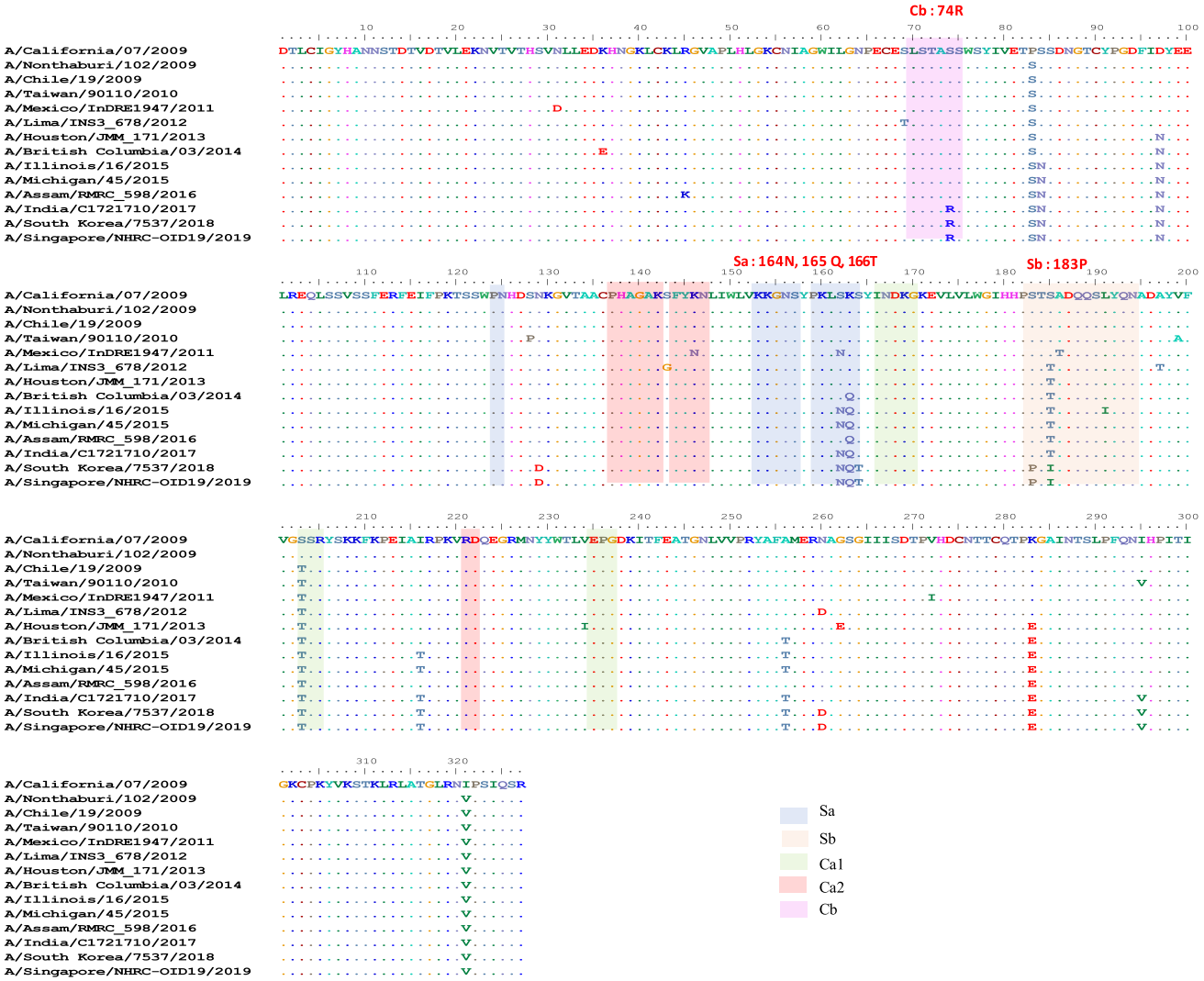

Fig. 1 The antigenic sites in HA1 of H1N1 viruses. An alignment of the amino acid sequences from randomly selected H1N1 viruses obtained since 2009, together with vaccine strains, is shown. The HA1 region is shown using H1 numbering. A number of mutations were observed in three of the five known antigenic sites that persisted throughout the evolution of the virus. There were three mutations in the Sa epitope, S162N, K163Q, and S164T, two mutations in the Sb epitope, S183P and S185T, and one mutation in Cb epitope, S74R

which was first detected in 2012. While the K163Q mutation has been recognized as the key mutation driving the first antigenic drift of the 2009 pandemic H1N1 virus, the contribution of the S185T mutation to antigenic drift had not been analyzed. The S74R mutation in the Cb epitope emerged in 2017, whereas the S162N mutation in the Sa epitope and the S164T and S183P mutations emerged in 2017 and 2018, respectively.

HAI titer against WT-2009, 2009-K163Q, 2009-S185T and WT-2016

To detect antibody responses to the Sa and Sb epitopes, HAI titers against the wild-type A/Thailand/104/2009 (H1N1) virus (WT-2009) were compared to those against the mutant with the K163Q mutation (2009-K163Q), the mutant with the S185T mutation (2009-S185T), and the wild-type A/KAN/SC023/2016 (H1N1) virus (WT-2016). We used serum samples collected from a cohort in a province in northeastern Thailand in 2010-2012. The 2009 H1N1 pandemic reached Thailand in May 2009 and caused three epidemic waves. The first wave occurred between May and November 2009, the second was from December 2009 to April 2010, and the third was from June to October 2010. It has been estimated that approximately 7 percent of the blood donors from whom samples were collected in September 2009 had been infected after the first wave of the 2009 influenza pandemic [9]. In Thailand, the influenza vaccine is offered only to certain risk groups and the elderly, and the vaccine coverage in the general population is extremely low.

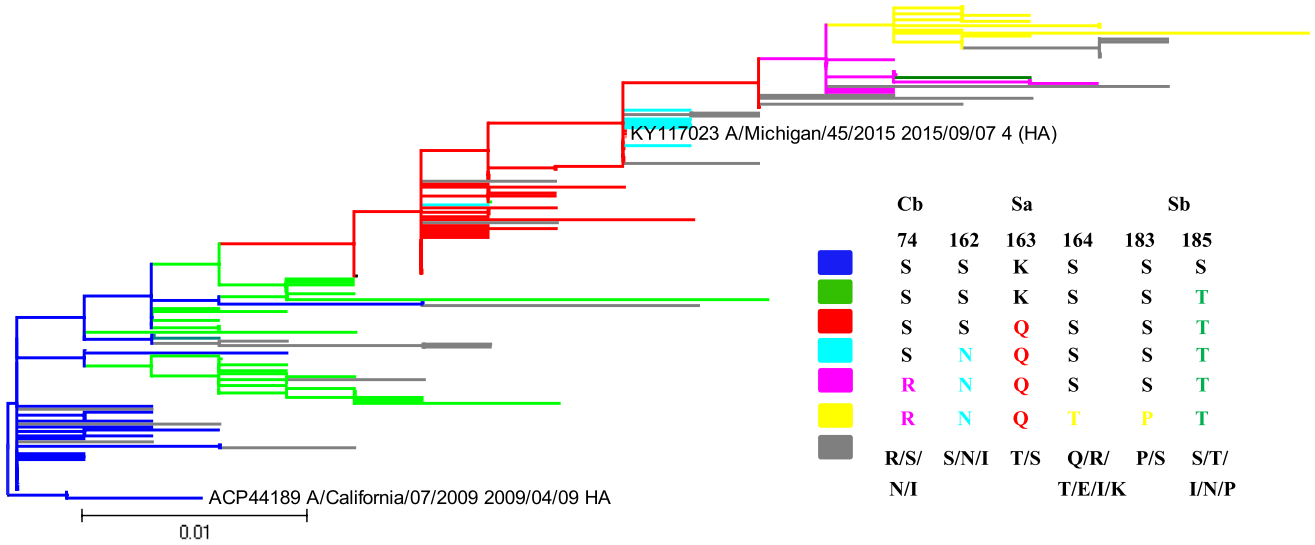

Fig. 2 Maximum-likelihood tree based on H1N1 HA1 amino acid sequences. One hundred sixty-seven full-length sequences of H1N1 HA1 were randomly selected from the NCBI database between 2009 and 2019, and these covered all geographical regions. Branch lengths are drawn to scale. The scale bar represents 0.01 units of amino acid change per site

Among the 98 serum samples collected in 2010, 56 (57%) showed an HAI titer ≥ 40 against WT-2009. Of these, 44 (79%) and 23 (41%) showed greater than a one-fold reduction in HAI titer against 2009-K163Q and 2009-S185T, indicating responses targeting the Sa and Sb epitope, respectively. These were counted as having significant reduction when both of the duplicate titers were concordant with the reduction. One sample (2%) showed a higher HAI titer against 2009-S185T than against WT-2009, and 45 (80%) showed a reduction in HAI titer against WT-2016, while eight (14%) maintained a similar titer to WT-2016 and three (5%) gave indeterminate results (Table 1). None of the 56 individuals with an HAI titer ≥ 40 against WT-2009 in 2010 showed a significant change in their titers against any of the four viruses in sera collected in 2011. Among the 42 subjects who were negative for HAI antibody in 2010, nine (21%) showed seroconversion, with an HAI titer ≥ 40 against WT-2009 in sera collected in 2011. Of these, one sample (11%) maintained a similar titer against WT-2016. Eight samples (89%) showed more than a one-fold reduction in the HAI titer against WT-2016 in 2011, and six (67%) and two (22%) showed a reduction in their HAI titer against 2009-K163Q and 2009-S185T, respectively. Only two samples (22%) showed higher HAI titer against 2009-S185T than against WT-2009, indicating a response to the Sb epitope induced by infection by viruses with the S185T mutation (Table 2). This indicated that the majority of the circulating viruses in 2011 in the cohort site still had the wild-type sequence at position 185. In 2012, five more samples (12%) showed seroconversion (Table 3). None of these showed a significant change in HAI titers against 2009-K163Q, while four (80%) of them had a drop in their HAI titer against 2009-S185T (Fig. 3A), and four (80%) of them showed more than a one-fold reduction in the HAI titer against WT-2016. Together, in this 3-year period, 70 sera showed a positive antibody response to the 2009 H1N1 virus. Of these, 33 (47%) had altered HAI titers against the S185T mutant as compared to WT-2009, indicating responses to the Sb epitope. The titer of each serum collected during 2010-2012 against WT-2009, 2009-K163Q, 2009-S185T and WT-2016 is shown in Fig. 3B.

Responses to the Sa and Sb epitope varied among age groups

The response to the 2009 pandemic H1N1 virus (WT-2009) in the subjects tested in 2010, as determined by an HAI titer ≥ 40, was 57%. The average age of those who had an HAI titer ≥ 40 was 41 ± 11 years old. Among these subjects with antibody response to the pandemic virus WT-2009, only 14% maintained similar titers to WT-2016. This indicated the expected antigenic drift of WT-2016 from the responses in 2010-2011. This 14% of the sera that could still recognize WT-2016 well without a drop in titer might have had antibodies to epitopes that were not mutated in the 2016 virus, i.e. Ca1 and Ca2. Sa-specific antibodies in 2010 sera were found in 44 subjects (79%) in all age groups (1946-1988) whose titers against 2009-K163Q showed a significant drop. In accordance with the previously published data [5], people born between 1965 and 1979 had the most frequent responses to the Sa epitope. It was found that 91% of people (30 out of 33) born between 1965 and 1984 had

Table 1 Comparison of HAI titers against WT-2009, 2009-K163Q, 2009-S185T, and WT-2016 in individuals with an HAI titer > 40 against WT-2009 in 2010. Each serum sample was tested in two independent HAI assays (n1 and n2) for each virus. The ratio WT-2009/2009-K163Q n1 is the ratio of the HAI titer against WT-2009 to the HAI titer against 2009-K163Q from the n1 experiment, and so on. Sera indicated to have a reduction in HAI titer had a greater than one-fold reduction in both experiments, and sera showing similar HAI titers are those with a ratio of 1 in both experiments. One sample, with values shown in bold, showed a higher HAI titer against 2009-S185T than against WT-2009

Sample ID	Age	Year of birth	HAI titers								Ratio					
			WT-2009		2009-K163Q		2009-S185T		WT-2016		WT-2009/2009-K163Q		WT-2009/2009-S185T		WT-2009/WT-2016	
			n1	n2	n1	n2	n1	n2	n1	n2	n1	n2	n1	n2	n1	n2
No. 81	22	1988	160	160	160	160	160	160	160	160	1	1	1	1	1	1
No. 2	23	1987	80	80	40	40	80	40	< 40	< 40	2	2	1	2	> 2	> 2
No. 50	23	1987	640	320	160	160	160	160	40	40	4	2	4	2	16	8
No. 88	24	1986	40	40	< 40	< 40	< 40	< 40	< 40	< 40	> 1	> 1	> 1	> 1	> 1	> 1
No. 62	26	1984	80	80	40	40	40	40	40	40	2	2	2	2	2	2
No. 30	27	1983	40	80	40	40	40	40	< 40	< 40	1	2	1	2	> 1	> 2
No. 82	28	1982	320	320	160	160	320	160	160	160	2	2	1	2	2	2
No. 44	29	1981	40	40	< 40	< 40	40	40	< 40	< 40	> 1	> 1	1	1	> 1	>1
No. 59	30	1980	40	40	< 40	< 40	40	40	< 40	< 40	> 1	> 1	1	1	> 1	> 1
No. 72	30	1980	40	40	< 40	< 40	40	40	< 40	< 40	> 1	> 1	1	1	> 1	> 1
No. 65	31	1979	320	160	160	80	160	80	80	80	2	2	2	2	4	2
No. 23	32	1978	40	40	< 40	< 40	40	< 40	< 40	< 40	> 1	> 1	1	> 1	> 1	> 1
No. 57	34	1976	80	40	< 40	< 40	80	40	< 40	< 40	> 2	> 1	1	1	> 2	> 1
No. 51	35	1975	40	40	< 40	< 40	< 40	< 40	< 40	< 40	> 1	> 1	> 1	> 1	> 1	> 1
No. 92	35	1975	40	40	< 40	< 40	< 40	< 40	< 40	< 40	> 1	> 1	> 1	> 1	> 1	> 1
No. 94	36	1974	640	320	320	160	640	320	160	160	2	2	1	1	4	2
No. 46	37	1973	160	80	80	40	80	40	40	40	2	2	2	2	4	2
No. 29	38	1972	40	80	< 40	< 40	40	< 40	< 40	< 40	> 1	> 2	1	> 2	> 1	> 2
No. 70	38	1972	40	80	< 40	< 40	40	40	< 40	< 40	> 1	> 2	1	2	> 1	> 2
No. 71	38	1972	80	40	40	40	40	40	< 40	< 40	2	1	2	1	> 1	> 2
No. 84	38	1972	40	40	< 40	< 40	< 40	< 40	< 40	< 40	> 1	> 1	> 1	> 1	> 1	> 1
No. 79	39	1971	40	40	< 40	< 40	40	40	< 40	< 40	> 1	> 1	1	1	> 1	> 1
No. 6	39	1971	40	40	< 40	< 40	< 40	< 40	< 40	< 40	> 1	> 1	> 1	> 1	> 1	> 1
No. 20	39	1971	40	40	< 40	< 40	< 40	< 40	< 40	< 40	> 1	> 1	> 1	> 1	> 1	> 1
No. 35	39	1971	80	80	40	40	80	40	< 40	40	2	2	1	2	> 2	2
No. 60	40	1970	320	320	160	160	160	160	160	160	2	2	2	2	2	2
No. 96	40	1970	40	40	< 40	< 40	40	40	40	40	> 1	> 1	1	1	1	1
No. 97	40	1970	40	40	< 40	< 40	40	< 40	< 40	< 40	> 1	> 1	1	> 1	> 1	> 1
No. 3	41	1969	40	40	< 40	< 40	< 40	< 40	< 40	< 40	> 1	> 1	> 1	> 1	> 1	> 1
No. 21	41	1969	80	40	40	40	80	40	< 40	< 40	2	1	1	1	> 2	> 1
No. 54	41	1969	80	80	40	40	40	40	< 40	40	2	2	2	2	> 2	2
No. 63	41	1969	40	40	< 40	< 40	< 40	< 40	40	40	> 1	> 1	> 1	> 1	1	1
No. 67	42	1968	40	40	< 40	< 40	40	40	< 40	< 40	> 1	> 1	1	1	> 1	> 1
No. 53	43	1967	40	40	< 40	< 40	40	< 40	40	40	> 1	> 1	1	> 1	1	1
No. 95	43	1967	40	40	< 40	< 40	< 40	< 40	< 40	< 40	> 1	> 1	> 1	> 1	> 1	> 1
No. 12	44	1966	40	40	< 40	< 40	< 40	< 40	40	40	> 1	> 1	> 1	> 1	1	1
No. 93	44	1966	40	40	< 40	< 40	< 40	< 40	< 40	< 40	> 1	> 1	> 1	> 1	> 1	> 1
No. 36	46	1964	40	40	< 40	< 40	40	40	40	40	> 1	> 1	1	1	1	1
No. 17	**48**	**1962**	**40**	**40**	**40**	**< 40**	**80**	**80**	**< 40**	**< 40**	**1**	**> 1**	**0.5**	**0.5**	**> 1**	**> 1**
No. 25	49	1961	40	40	40	40	40	40	< 40	< 40	1	1	1	1	> 1	> 1
No. 7	49	1961	40	40	40	40	40	40	40	40	1	1	1	1	1	1
No. 41	49	1961	40	40	40	40	40	40	< 40	40	1	1	1	1	> 1	1

Table 1 (continued)

Sample ID	Age	Year of birth	HAI titers								Ratio					
			WT-2009		2009-K163Q		2009-S185T		WT-2016		WT-2009/2009-K163Q		WT-2009/2009-S185T		WT-2009/WT-2016	
			n1	n2	n1	n2	n1	n2	n1	n2	n1	n2	n1	n2	n1	n2
No. 90	49	1961	40	80	< 40	< 40	40	40	40	40	> 1	>2	1	2	1	2
No. 43	49	1961	80	80	80	80	80	80	40	40	1	1	1	1	2	2
No. 34	50	1960	160	160	160	160	160	80	160	80	1	1	1	2	1	2
No. 61	50	1960	320	320	160	160	320	160	80	80	2	2	1	2	4	4
No. 86	50	1960	40	40	< 40	< 40	< 40	< 40	< 40	< 40	> 1	> 1	> 1	> 1	> 1	> 1
No. 39	52	1958	80	80	80	40	80	40	40	40	1	2	1	2	2	2
No. 68	55	1955	80	80	< 40	< 40	80	80	< 40	< 40	> 2	> 2	1	1	> 2	> 2
No. 33	56	1954	80	80	< 40	< 40	40	40	< 40	< 40	> 2	> 2	2	2	> 2	> 2
No. 8	57	1953	40	40	< 40	< 40	40	40	< 40	< 40	> 1	> 1	1	1	> 1	> 1
No. 28	58	1952	40	40	40	40	< 40	< 40	< 40	< 40	1	1	> 1	> 1	> 1	> 1
No. 89	58	1952	40	40	< 40	< 40	< 40	< 40	< 40	< 40	> 1	> 1	> 1	> 1	> 1	> 1
No. 69	62	1948	40	40	< 40	< 40	40	40	< 40	< 40	> 1	> 1	1	1	> 1	> 1
No. 91	62	1948	40	40	< 40	< 40	< 40	< 40	< 40	< 40	> 1	> 1	> 1	> 1	> 1	> 1
No. 11	64	1946	40	40	< 40	< 40	< 40	< 40	40	40	> 1	> 1	> 1	> 1	1	1

Table 2 Comparison of HAI titers against WT-2009, 2009-K163Q, 2009-S185T, and WT-2016 in individuals who seroconverted with an HAI titer > 40 against WT-2009 in 2011. Shown here are data for sera in which seroconversion was observed (sera with an HAI titer < 40 in one experiment that had at least a one-fold increase in both experiments). Each serum sample was tested in two independent HAI assays (n1 and n2) for each virus. The ratio WT-2009/2009-K163Q n1 is the ratio between HAI titers against WT-2009 and HAI titers against 2009-K163Q from experiment n1, and so on. Sera indicated to have a reduction in HAI titer had a greater than one-fold reduction in both experiments, and sera showing similar HAI titers are those with a ratio of 1 in both experiments. Two samples, with data shown in bold, showed a higher HAI titer against 2009-S185T than against WT-2009

Sample ID	Age	Year of birth	HAI titer								Ratio					
			WT-2009		2009-K163Q		2009-S185T		WT-2016		WT-2009/2009-K163Q		WT-2009/2009-S185T		WT-2009/WT-2016	
			n1	n2	n1	n2	n1	n2	n1	n2	n1	n2	n1	n2	n1	n2
No. 80	36	1974	160	320	< 40	40	160	160	< 40	< 40	> 4	8	1	2	> 4	> 8
No. 1	**37**	**1973**	**80**	**80**	**40**	**40**	**160**	**160**	**< 40**	**< 40**	**2**	**2**	**0.5**	**0.5**	**> 2**	**> 2**
No. 85	**38**	**1972**	**40**	**80**	**< 40**	**40**	**80**	**160**	**< 40**	**40**	**> 1**	**2**	**0.5**	**0.5**	**> 1**	**2**
No. 52	42	1968	640	640	40	80	640	640	40	80	16	8	1	1	16	8
No. 24	43	1967	80	160	80	160	80	40	40	80	1	1	1	4	2	2
No. 42	46	1964	40	40	< 40	< 40	< 40	< 40	< 40	< 40	> 1	> 1	> 1	> 1	> 1	> 1
No. 10	47	1963	320	320	80	80	160	160	< 40	< 40	4	4	2	2	> 8	> 8
No. 16	50	1960	80	160	80	< 40	80	80	< 40	< 40	1	> 4	1	2	> 2	> 4
No. 38	60	1950	40	80	40	80	40	< 40	40	80	1	1	1	> 2	1	1

antibody responses to K163 in the Sa epitope (Table 4 and Fig. 4). The Sa epitope response rate dropped to 42% (five out of 12) in people born between 1955 and 1964, and six of the seven people (86%) born between 1946 and 1954 were found to respond to this epitope. The responses to the Sb epitope showed a similar trend with 45% (15 out of 33) response rate in people born between 1965 and 1984. There was a similar drop in the response rate (one out of 12; 8%) in people born between 1955 and 1964 and a high response rate (five out of seven; 71%) in people born between 1946

Table 3 Comparison of HAI titers in against WT-2009, 2009-K163Q, 2009-S185T, and WT-2016 in individuals who seroconverted with an HAI titer > 40 against WT-2009 in 2012. Shown here are data for sera in which seroconversion was observed (sera with an HAI titer < 40 in one experiment that had at least a one-fold increase in both experiments). Each serum sample was tested in two independent HAI assays (n1 and n2) for each virus. The ratio WT-2009/2009-K163Q n1 is the ratio between HAI titers against WT-2009 and HAI titers against 2009-K163Q from experiment n1, and so on. Sera indicated to have a reduction in HAI titer had a greater than one-fold reduction in both experiments

Sample ID	Age	Year of birth	HAI titer								Ratio					
			WT-2009		2009-K163Q		2009-S185T		WT-2016		WT-2009/2009-K163Q		WT-2009/2009-S185T		WT-2009/WT-2016	
			n1	n2	n1	n2	n1	n2	n1	n2	n1	n2	n1	n2	n1	n2
No. 58	45	1965	160	160	40	160	80	160	40	40	4	1	2	1	4	4
No. 45	48	1962	40	80	< 40	320	< 40	< 40	< 40	80	> 1	0.25	> 1	> 2	> 1	1
No. 26	51	1959	40	80	< 40	80	< 40	< 40	< 40	80	> 1	1	> 1	> 2	> 1	2
No. 73	58	1952	160	160	80	320	80	80	40	80	2	0.5	2	2	4	2
No. 22	58	1952	640	1280	320	1280	320	320	160	320	2	1	2	4	4	4

and 1954. The responses to Sa and Sb epitopes together accounted for 87% (39 out of 45) of those with an HAI titer drop to WT-2016. This suggests that the other 13% might have had responses to other epitopes. This was more common in people born between 1955 and 1964, as shown by the drop in the response rates to both the Sa and Sb epitopes in this age group (Fig. 4). These suggest an age difference among people with preferential responses to the Sa, Sb, and Cb or other epitopes.

Discussion

Previous data [5] indicated that antibody responses to the 2009 pandemic H1N1 influenza virus in people born between 1965 and 1979 were focused on the Sa epitope. Our data now also show that a large fraction of the population in 2010-2012 responded to the Sb epitope. This focused response to the Sb epitope might have contributed to the selective pressure for the first antigenic drift of the 2009 pandemic H1N1 virus. The S185T mutation in the Sb epitope appeared in 2010 and became dominant in 2011, and all strains after 2012 had threonine at this position. The K163Q mutation in the Sa epitope appeared in 2012 and almost completely replaced the wild-type strain in 2014. H1N1 influenza outbreaks and vaccine escape suggesting antigenic drift were first observed in the 2013-2014 influenza season of the northern hemisphere. According to the US CDC influenza reports, the H1N1 pandemic subsided after 2010, and influenza A H3N2 virus dominated the influenza seasons for three years until the 2013-2014 season, when H1N1 virus again became the dominant virus and influenza incidence exceeded the epidemic threshold, indicating a more severe influenza season. The 2010-2011 and the 2011-2012 seasons were within the seasonal baseline, whereas the 2012-2013 season exceeded the epidemic threshold because of the antigenic drift of the H3N2 virus, resulting in a change of the vaccine strain from A/Perth/16/2009 (H3N2)-like virus to A/Victoria/361/2011 (H3N2)-like virus. Although the S185T mutant emerged and became dominant before 2012, it did not cause a major outbreak until the K163Q mutation came along in 2013. It is likely that the response to the Sa epitope could suppress the Sb epitope mutant because the majority of the population had responses to both epitopes in the same individuals. It is also possible that the antigenic drift of the H3N2 virus in 2012 contributed to the suppression of H1N1 by competition. Nevertheless, the fact that the H1N1 virus causing antigenic drift and outbreaks in 2013-2014 harbored no mutations in the antigenic epitopes other than the two mutations in the Sa and Sb epitopes indicates that these two epitopes were the targets of the first antigenic drift of the pandemic H1N1 virus and that the focused responses to these two epitopes were the main driving force of the drift.

Although our data and those of Linderman, et al. [5] showed age-specific differences in the response to the Sa epitope, the levels of differences were not similar between the two studies. While the previous data showed that the response to K163 dropped sharply in people born in 1980-1984 and was undetected in people born between 1985 and 1997, our data showed that individuals born in 1985-1988 still had antibodies to K163 to some extent. Whether this difference was due to differences in previous experiences with seasonal H1N1 viruses in different geographical regions or possibly to racial differences is unclear. The small sample

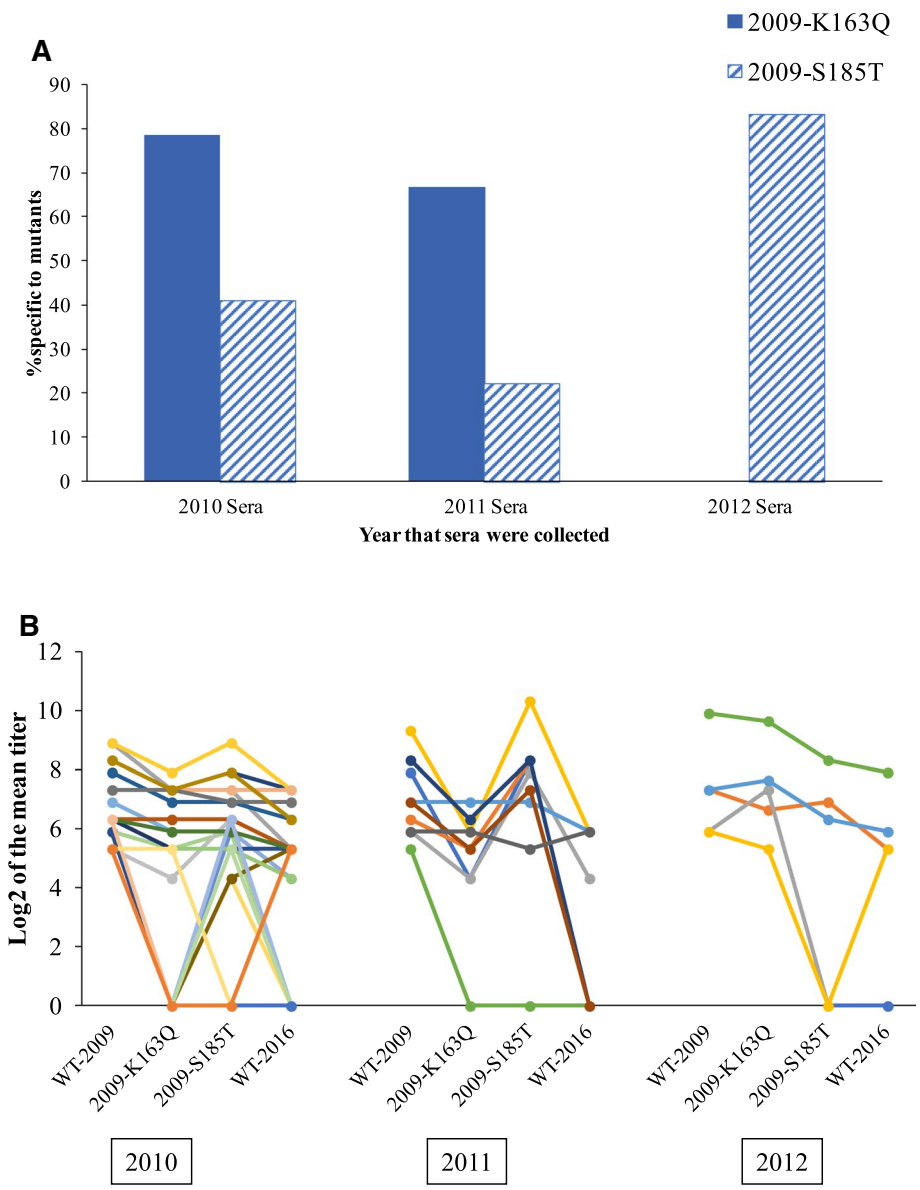

Fig. 3 HAI titer against WT-2009, 2009-K163Q, 2009-S185T and WT-2016. (**A**) Specific response rate to the Sa and Sb epitopes among sera with an HAI titer ≥ 40 against WT-2009 during 2010-2012, as determined by the percentage of sera with a reduction in HAI titer of at least one-fold to the K163Q and S185T mutant, respectively, as compared to WT-2009. (**B**) A graph showing log2 of the mean titer of each serum against WT-2009, 2009-K163Q, 2009-S185T and WT-2016 during 2010-2012. The mean titer was calculated from the titers obtained in two independent experiments. A titer < 40 is assumed to be 0

Table 4 The fraction of 2010 sera with a drop in HAI titer as compared to WT-2009 among the total seropositive sera in each decade of birth for 2009-K163Q, 2009-S185T, and WT-2016. Sera were counted as having a drop in titer when at least a one-fold reduction in HAI titer was observed in both experiments

Year of birth	2009-K163Q	2009-S185T	WT-2016
1946-1954	6/7 (86%)	5/7 (71%)	6/7 (86%)
1955-1964	5/12 (42%)	1/12 (8%)	7/12 (58%)
1965-1974	20/22 (91%)	11/22 (50%)	18/22 (81%)
1975-1984	10/11 (91%)	4/11 (36%)	11/11 (100%)
1985-1988	3/4 (75%)	2/4 (50%)	3/4 (75%)

size of the younger age group in our study makes accurate estimation of the actual response rate in this age group difficult.

In the United States, during the seasonal influenza season of 2010-2013 [10, 11], children aged 0-4 years and adults aged ≥ 65 years were the age groups with the highest rates of influenza-associated hospitalization. However, from September 2013 to February 2014 [12], the largest groups that were affected by influenza were young and middle-aged adults (18-64 years old). This implies that, in this particular influenza season, the antigenic drift had occurred in a way that disproportionately affected middle-aged adults. This suggests that the antigenic drift in 2013 might not have affected children who had not responded to the drifted Sa and Sb epitopes.

The glycosylation site at position 129, which appeared after 1985, is predicted to shield the antigenic site involving K163 [5]. It has been proposed that people born before 1980 responded to the unshielded epitope, with this site

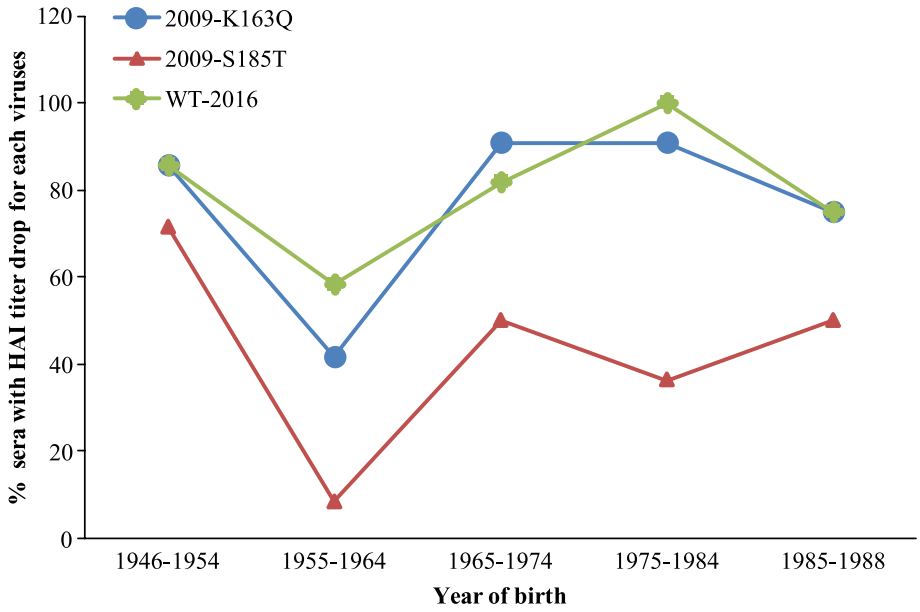

Fig. 4 Percentages of 2010 sera with a drop in titer to the K163Q and S185T mutants and WT-2016 in comparison to WT-2009. The sera were counted as having a drop in titer when they showed at least a one-fold reduction in the HAI titer in two independent experiments

representing the "original antigenic sin". They therefore developed responses targeting this epitope, which shared similarity in the pandemic H1N1 virus. Our data also showed some age-specific differences in the responses to the S185 epitope, which suggest that the Sb epitope shared some similarity to previous seasonal H1N1 viruses causing an original antigenic sin. These data contribute to our understanding of the role of previous exposures and antigenic sins on the anti-influenza immune response and antigenic drift, which might help in predicting future antigenic drift.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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