nature microbiology

SUPPLEMENTARY INFORMATION

ARTICLE NUMBER: 16058 | DOI: 10.1038/NMICROBIOL.2016.58

Selection of antigenically advanced variants of seasonal influenza viruses

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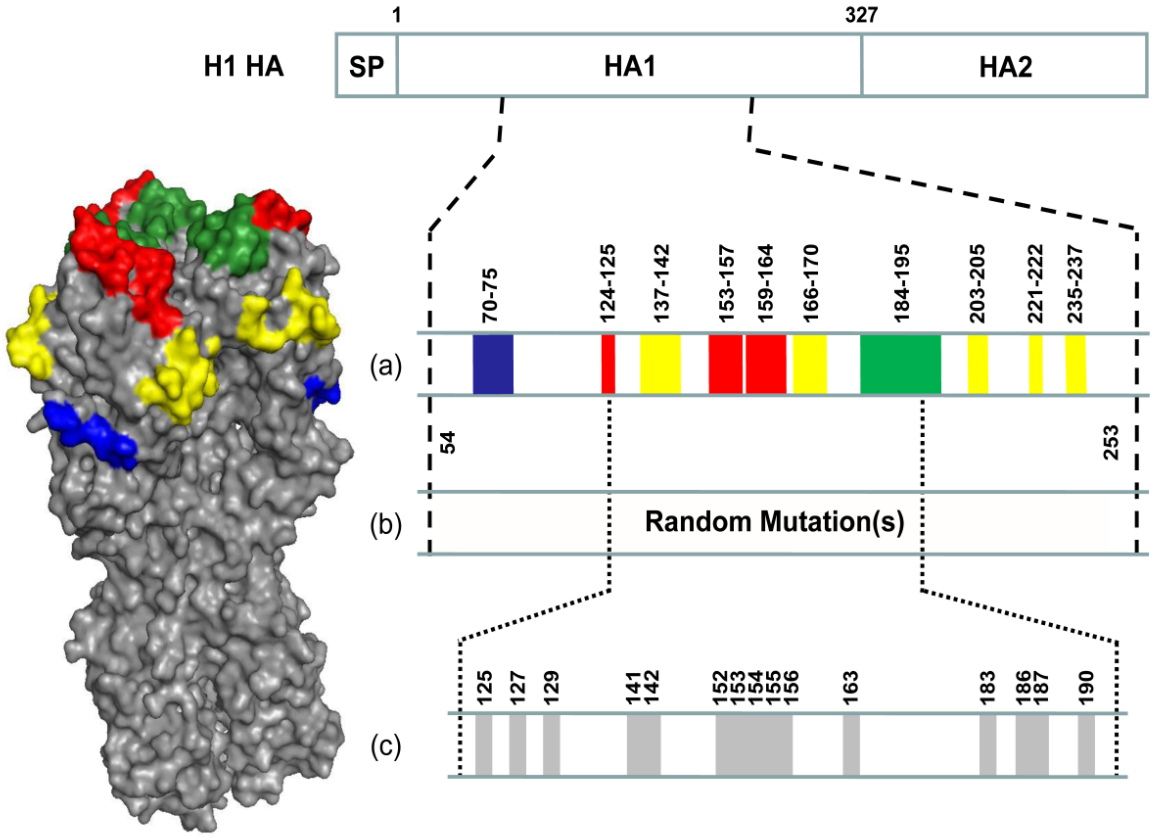
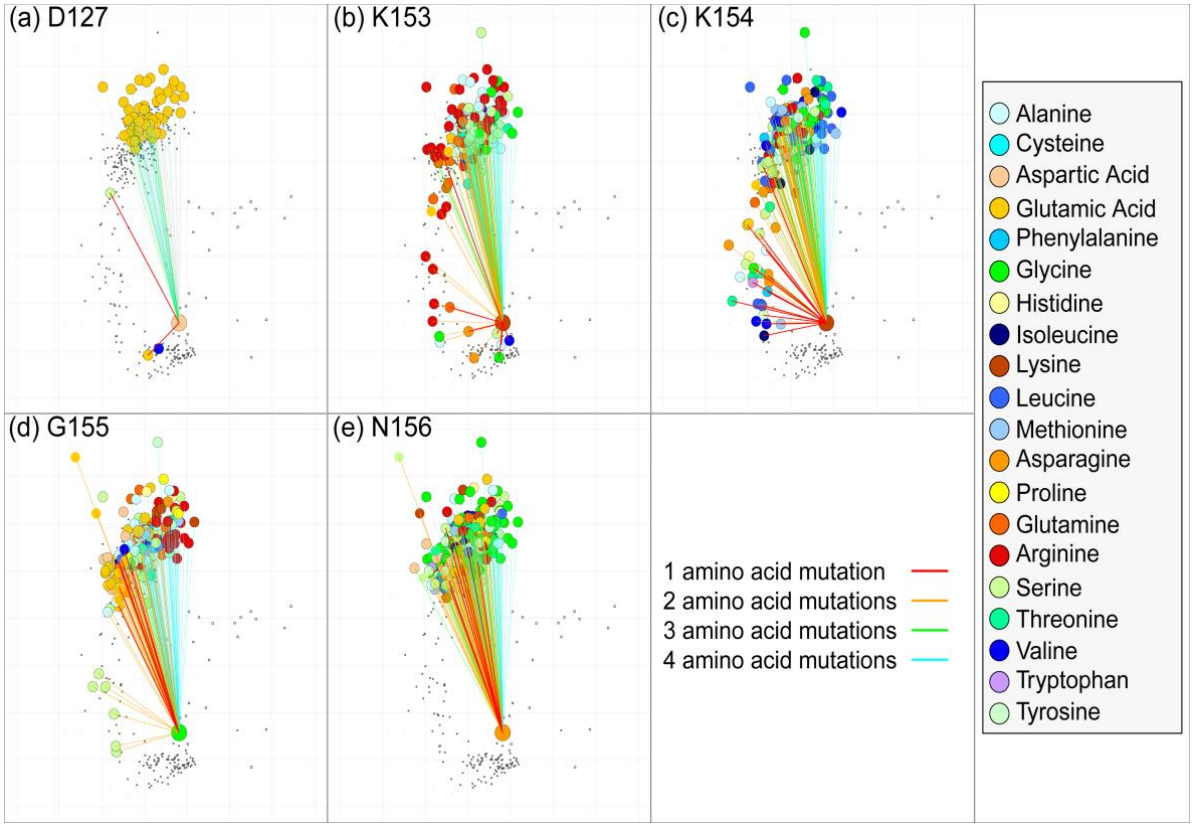
DOI: 10.1038/NMICROBIOL.2016.58



Supplementary Figure 1 | Schematic diagram of pandemic (H1N1) 2009 influenza A viral haemagglutinin (HA). A schematic diagram of the HA gene with the coding regions of the HA1 and HA2 subunits is shown on top; SP indicates the signal peptide sequence. The three dimensional structure of A/California/04/2009 HA (PDBID: 3LZG)[1] is shown on the left. The antigenic epitopes (also shown in (a)) are indicated as follows: Sa site, red; Sb site, green; Ca site, yellow; Cb site, blue. The region targeted for random mutagenesis is shown in (b), and specific sites targeted for mutagenesis are shown in (c).



Supplementary Figure 2 | Color-coded antigenic maps for mutations at positions where large antigenic changes were detected. Large antigenic changes were detected for amino acid mutations at HA positions 127, 153, 154, 155, and 156. Here we show antigenic map where viruses containing amino acid mutations at each of these positions are shown as larger circles. The color of the circle indicates the amino acid of the mutant virus at that position. The lines connecting the mutant strain to wild-type A/Norway/3858/2009 virus are colored according to the total number of amino acid mutations (red=1, orange=2, green=3, cyan=4) between the two strains.

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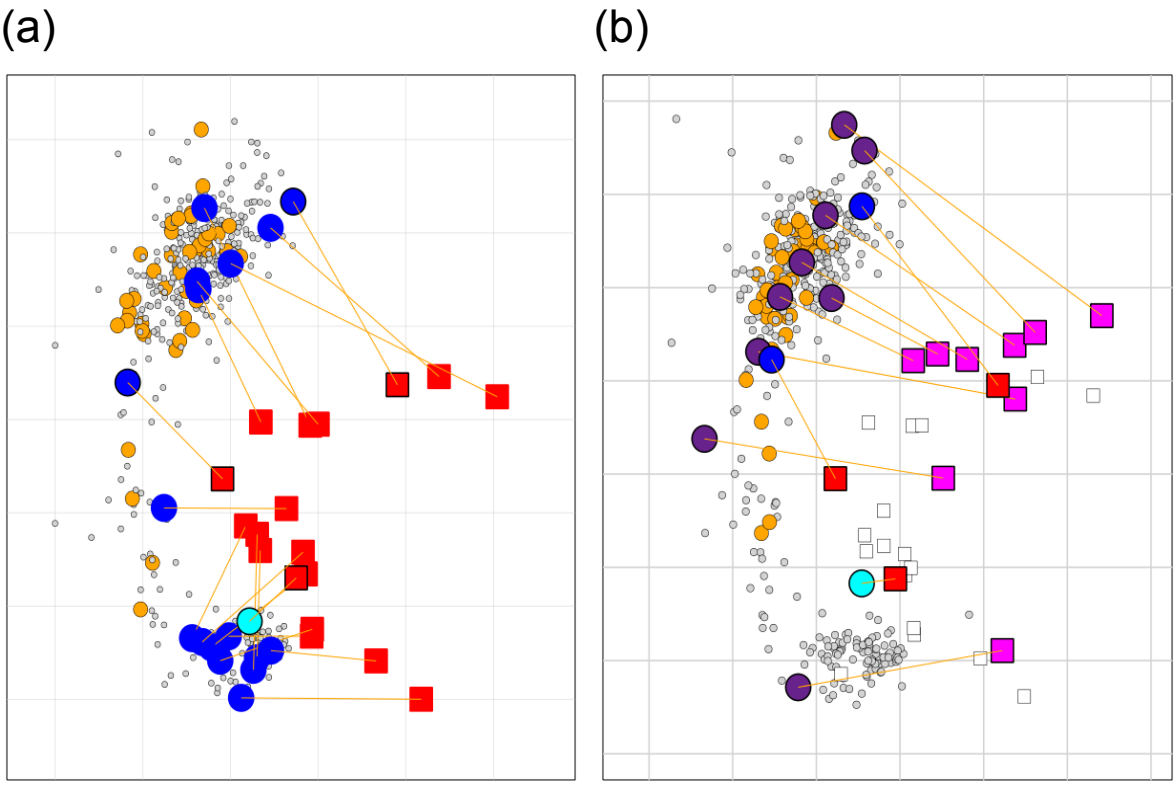
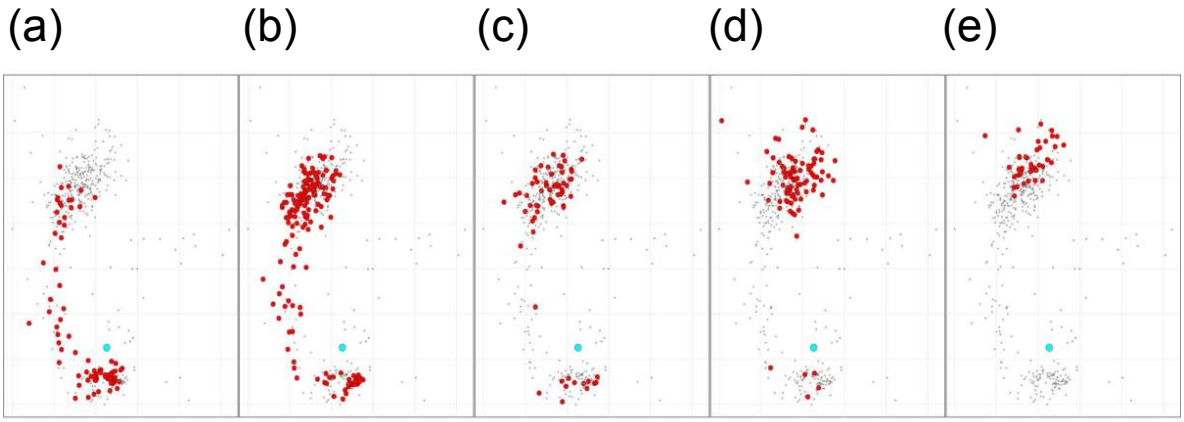


Supplementary Figure 3 | Antigenic maps comparing the initial and additional sets of antisera. Shown in (**a**) are the positions of the initial set of antisera (red boxes) with lines connecting their homologous antigens (blue circles = mutant viruses; cyan circle = A/Norway/3858/2009). The positions of the viruses selected for further testing with new antisera (approximately 13% of the total number of mutant viruses) are shown as orange circles. These viruses were selected based upon their original position in the map as well as the biophysical properties of the amino acids at positions 153-156. Shown in (**b**) an antigenic map based on data with nine additional ferret antisera (pink squares) raised against nine mutant viruses (purple circles). HI titres were determined for selected viruses (represented by orange, purple, blue and cyan circles) against these new antisera together with three antisera (red squares) shown in panel **3a** that were used to generate the original antigenic map. The faint antisera squares in panel **3b** represent the original sera that were not retested in this experiment. The map in **3b** was generated by analyzing the HI of the viruses (depicted by orange, purple, blue and cyan circles) tested with the nine new (pink squares) plus three original (red squares) antisera, along with the original HI data used to make the map in **3a**. In both panels, the remaining antigenic escape variants examined in this study are shown as small grey circles. No significant changes in the positions of viruses or antisera were seen between the two maps.



Supplementary Figure 4 | Positions of strains by numbers of amino acid mutations. In each panel, mutant viruses (red circles) possessing a given number of amino acid mutations (**a** = one, **b** = two, **c** = three, **d** = four, or **e** = five mutations) are shown on an antigenic map in comparison to wild-type A/Norway/3858/2009 virus (cyan circle).

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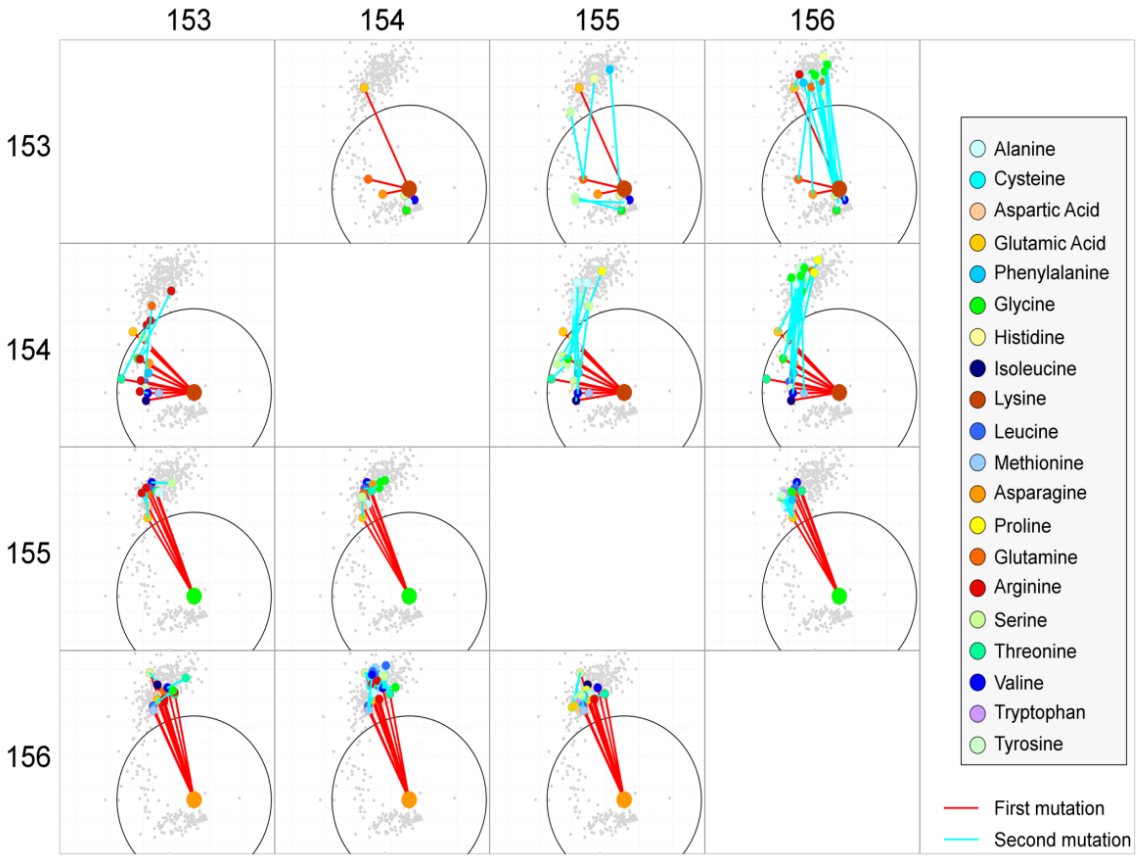
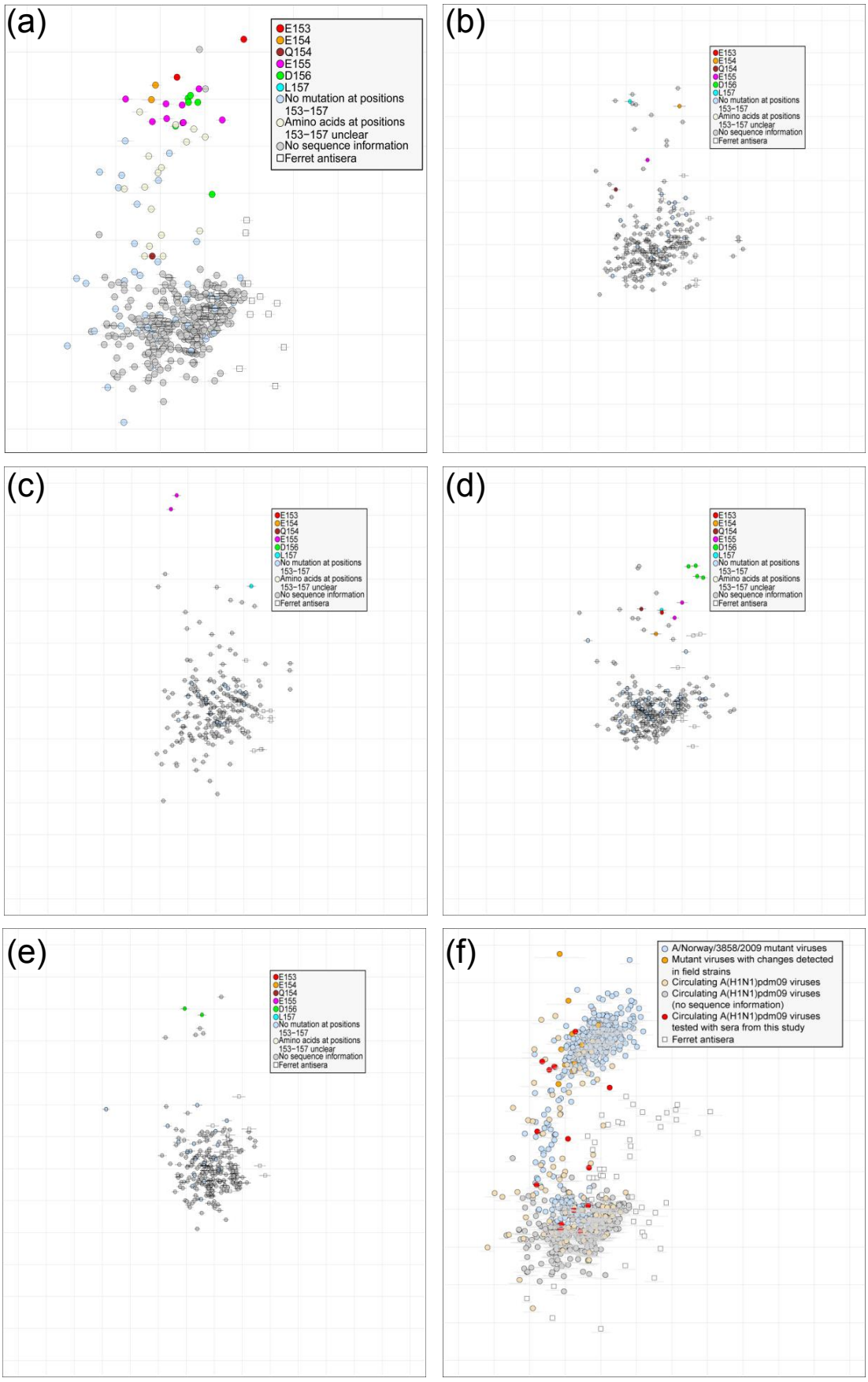


Supplementary Figure 5 | Antigenic maps showing the effects of double amino acid mutations at positions shown to cause a large antigenic change (i.e., positions 153, 154, 155, and 156). In each row, red lines connect the wild-type A/Norway/3858/2009 strain to viruses with a single amino acid mutation at the particular position (labels on left). Cyan lines connect those mutants which have an additional amino acid mutation in a second position (labels on top of each column). The color of the circle indicates the amino acid (see key in figure). Circles indicate a distance of 4 HI units from the reference virus; i.e., mutants outside the circle would necessitate a vaccine update.


(a)
E153
E154
Q154
E155
D156
L157
No mutation at positions 153–157
Amino acids at positions 153–157 unclear
No sequence information
Ferret antisera
(b)
E153
E154
Q154
E155
D156
L157
No mutation at positions 153–157
Amino acids at positions 153–157 unclear
No sequence information
Ferret antisera
(c)
E153
E154
Q154
E155
D156
L157
No mutation at positions 153–157
Amino acids at positions 153–157 unclear
No sequence information
Ferret antisera
(d)
E153
E154
Q154
E155
D156
L157
No mutation at positions 153–157
Amino acids at positions 153–157 unclear
No sequence information
Ferret antisera
(e)
E153
E154
Q154
E155
D156
L157
No mutation at positions 153–157
Amino acids at positions 153–157 unclear
No sequence information
Ferret antisera
(f)
A/Norway/3858/2009 mutant viruses
Mutant viruses with changes detected in field strains
Circulating A(H1N1)pdm09 viruses
Circulating A(H1N1)pdm09 viruses (no sequence information)
Circulating A(H1N1)pdm09 viruses tested with sera from this study
Ferret antisera

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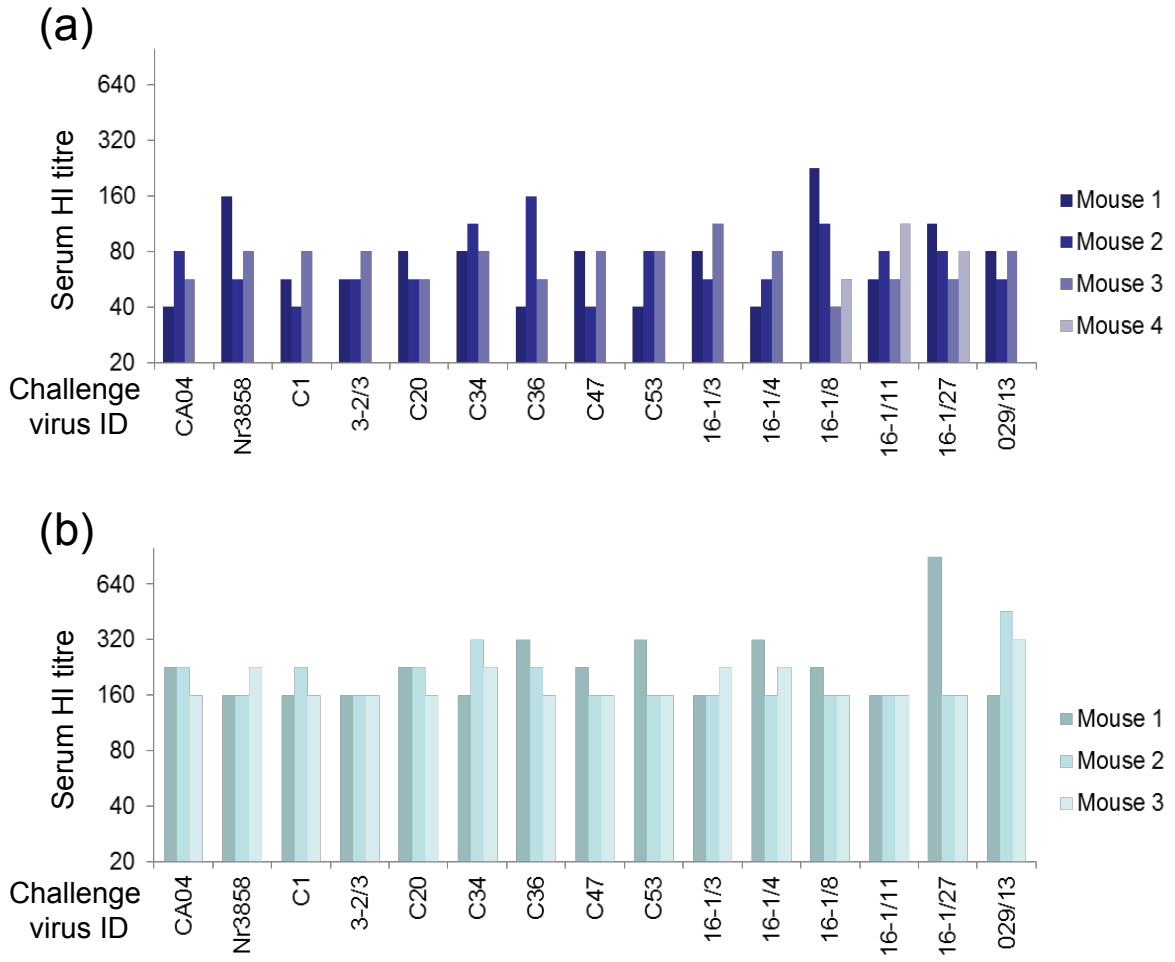
Supplementary Figure 6 | Antigenic maps from WHO Collaborating Centre data from 2009 – 2013. (**a**) Zoomable and searchable labeled antigenic map of 862 A(H1N1)pdm09 circulating strains from WHO surveillance in 2009 and 2010 (pale blue circles). Where an amino acid sequence is available, viruses with mutations at positions 153-157 are coloured by amino acid type; viruses for which the HA sequence has not been determined are shown as light grey circles. For some viruses, the identity of one or more amino acids at positions 153-157 was unclear in sequence data (pale yellow circles). (**b**) – (**e**) Zoomable and searchable labeled antigenic maps of 415, 345, 424, and 512 A(H1N1)pdm09 viruses isolated from December 2010 – March 2011 (**b**), October 2011 – March 2012 (**c**), November 2012 – March 2013 (**d**) and October 2013 – December 2013 (**e**). Where an amino acid sequence is available, viruses with mutations at positions 153–157 are coloured by amino acid type; viruses for which the HA sequence has not been determined are shown as light grey circles. (**f**) Zoomable and searchable labeled antigenic map of merged datasets includes A(H1N1)pdm09 viruses from the mutant screens in this study (light blue circles); A(H1N1)pdm09-based reassortant viruses with changes detected in field strains (orange circles); and circulating viruses from WHO surveillance studies with HA sequence data (pale orange circles) or without HA sequence data (light grey circles). Circulating A(H1N1)pdm09 viruses tested with a subset of sera from this study are shown as red circles.



HA position															
127	D	D	E	E	E	E	E	E	E	E	E	E	E	E	D
153	K	K	S	A	A	Q	S	G	R	R	A	A	G	A	S
154	K	K	L	L	L	N	L	I	L	R	L	K	L	N	G
155	G	G	K	R	N	S	R	N	S	E	Q	H	R	R	Y
156	N	N	G	G	K	G	G	R	G	H	K	G	G	G	G

Supplementary Figure 7 | Serum HI titres of immunized mice subsequently challenged with selected escape variants. Three or four mice per group were immunized by intranasal infection with $10^{1.5}$ PFU of A/California/04/2009 virus (**a**) or by vaccinating twice (with a two-week interval) with an A/California/07/2009 HA split vaccine (**b**). Four weeks later, serum HI titres were determined using standard HI assays with 0.5% turkey red blood cells and the amount of A/California/04/2009 virus equivalent to 8 haemagglutination units. For better data comparison, we list on the X-axis the viruses with which the respective groups of mice were subsequently challenged (for results of the challenge experiment, see Supplementary Fig. 9).

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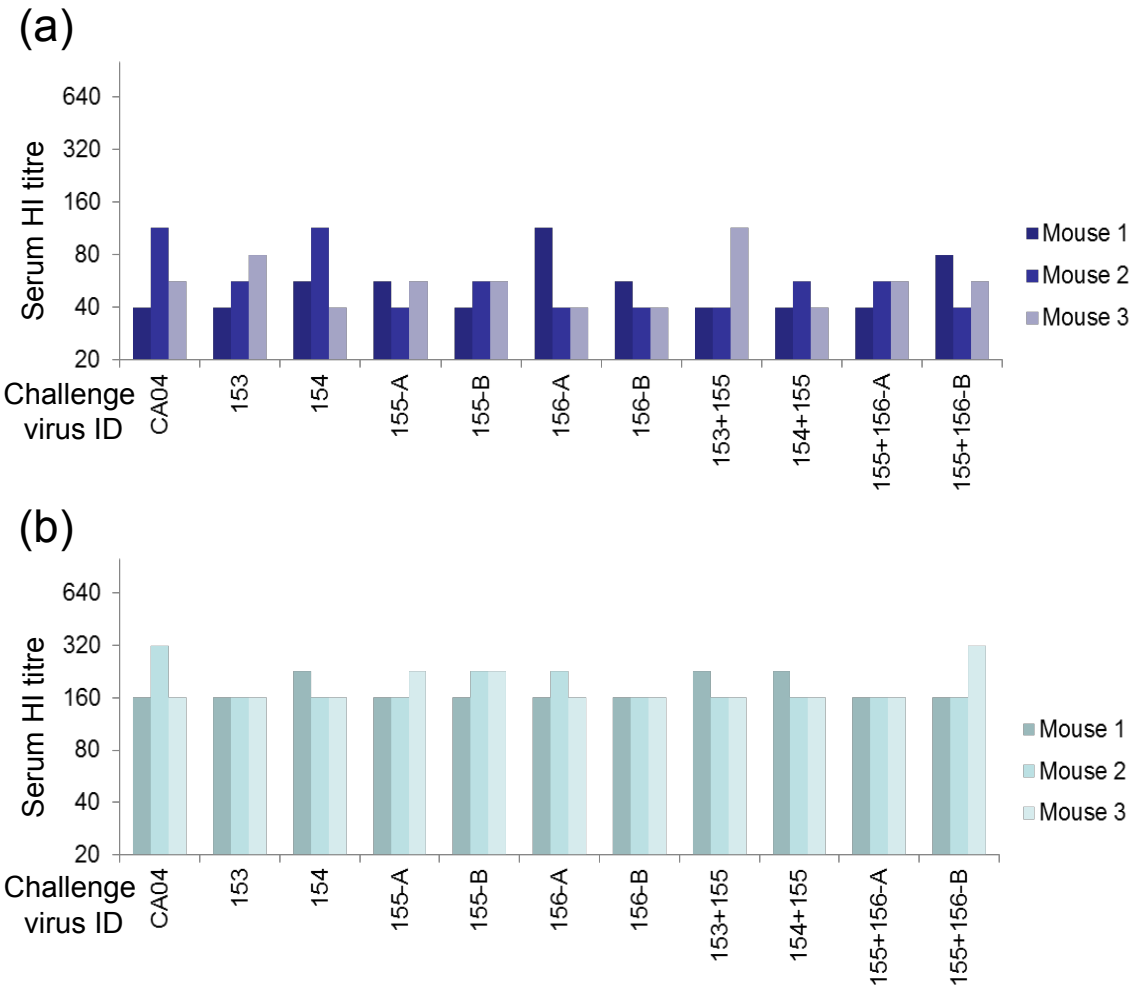
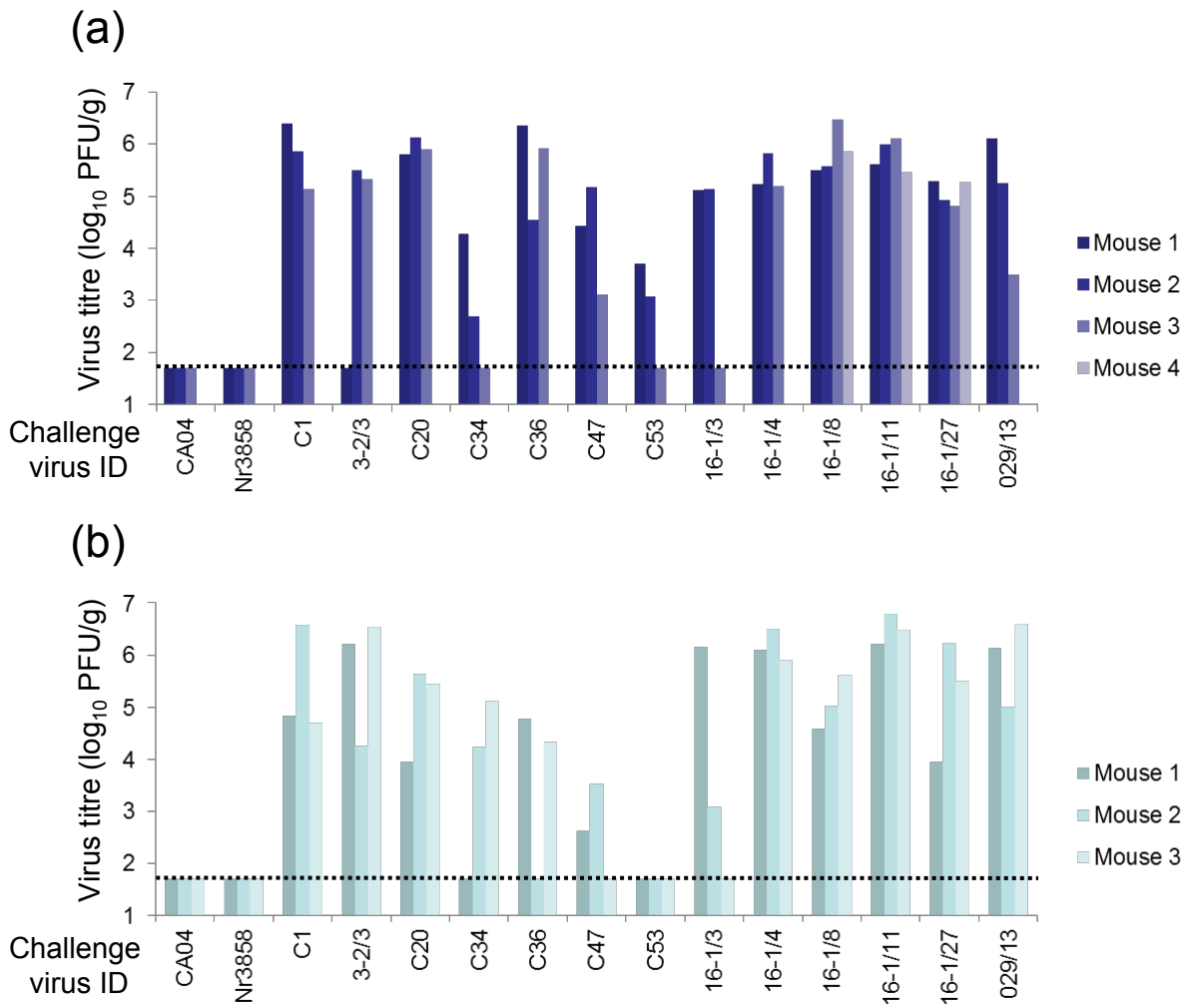


HA position											
153	K	E	K	K	K	K	K	E	K	K	K
154	K	K	E	K	K	K	K	K	Q	K	K
155	G	G	G	E	E	G	G	E	E	E	E
156	N	N	N	N	N	S	T	N	N	S	K

Supplementary Figure 8 | Serum HI titres of immunized mice subsequently challenged with A(H1N1)pdm09-based reassortant viruses possessing HA changes detected in field strains. Three mice per group were immunized by intranasal infection with $10^{1.5}$ PFU of A/California/04/2009 (**a**) or by vaccinating twice (with a two-week interval) with an A/California/07/2009 HA split vaccine (**b**). Four weeks later, serum HI titres were determined using standard HI assays with 0.5% turkey red blood cells and the amount of A/California/04/2009 virus equivalent to 8 haemagglutination units. For better data comparison, we list on the X-axis the viruses with which the respective groups of mice were subsequently challenged (for results of the challenge experiment, see Supplementary Fig. 10).



HA position															
127	D	D	E	E	E	E	E	E	E	E	E	E	E	E	D
153	K	K	S	A	A	Q	S	G	R	R	A	A	G	A	S
154	K	K	L	L	L	N	L	I	L	R	L	K	L	N	G
155	G	G	K	R	N	S	R	N	S	E	Q	H	R	R	Y
156	N	N	G	G	K	G	G	R	G	H	K	G	G	G	G

Supplementary Figure 9 | Immune evasion in mice by HA mutant viruses identified in this study. Three or four mice per group were immunized by intranasal infection with $10^{1.5}$ PFU of A/California/04/2009 (**a**), or by vaccination with an A/California/07/2009 HA split vaccine (**b**). Four weeks later, serum HI titres against A/California/04/2009 were determined (Supplementary Fig. 7) and mice were challenged with 10^6 PFU of A/California/04/2009, A/Norway/3858/2009, or the representative antigenic escape mutants shown on the X-axis (see also Supplementary Table 18). Shown are lung virus titers on day 4 post-challenge. Dashed lines, detection limit of virus (1.7 log_{10} PFU/g).

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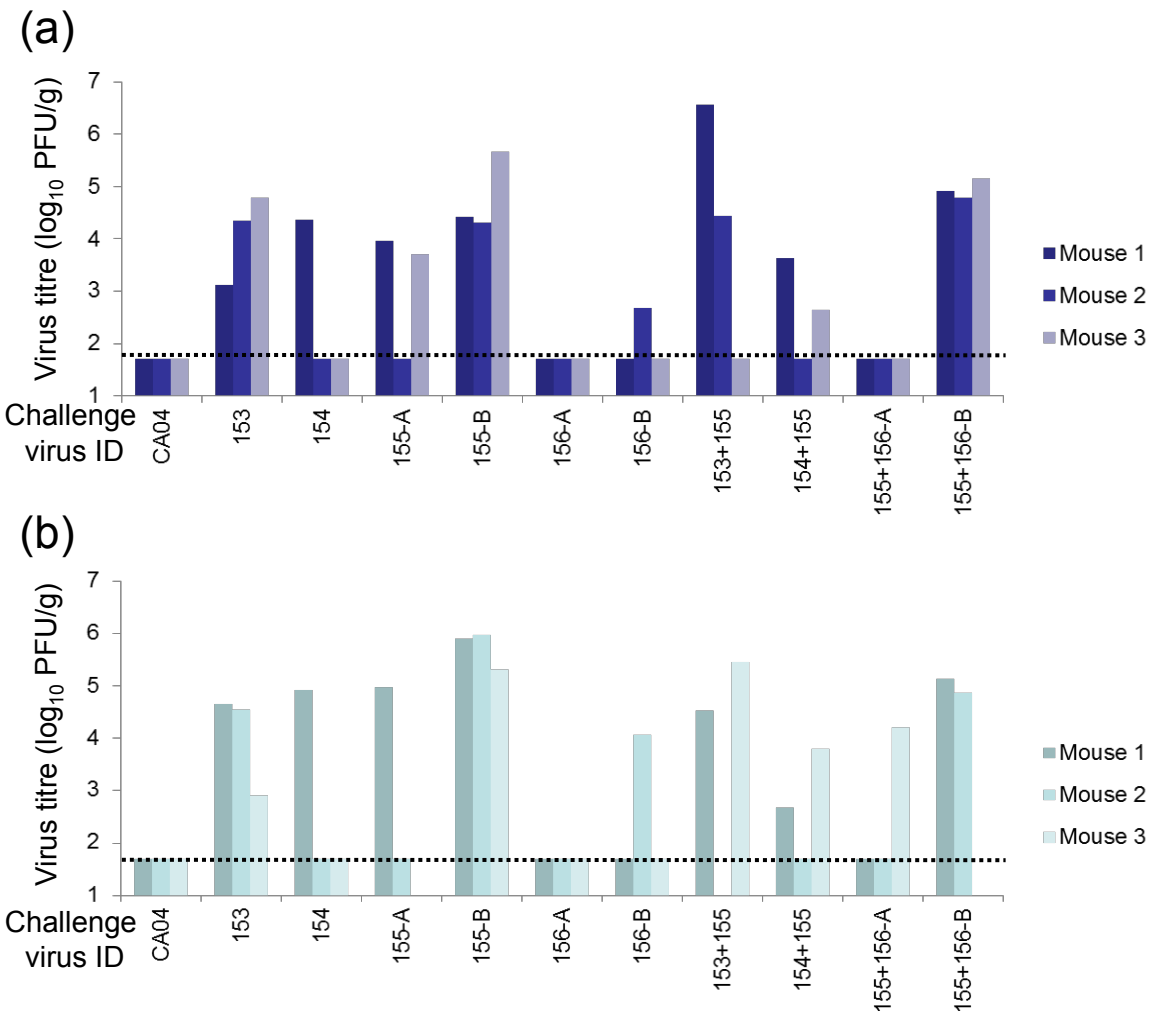
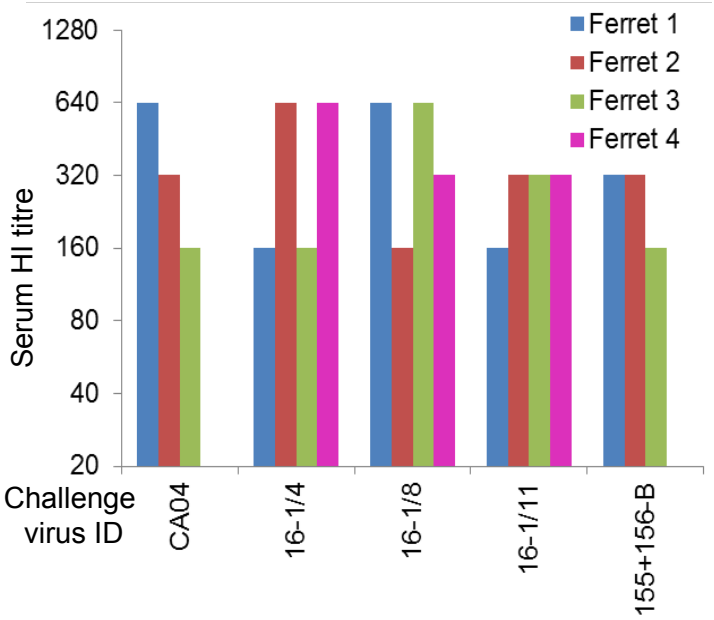


HA position											
153	K	E	K	K	K	K	K	E	K	K	K
154	K	K	E	K	K	K	K	K	Q	K	K
155	G	G	G	E	E	G	G	E	E	E	E
156	N	N	N	N	N	S	T	N	N	S	K

Supplementary Figure 10 | Immune evasion in mice by HA mutations found in field strains. Three mice per group were immunized by intranasal infection with $10^{1.5}$ PFU of A/California/04/2009 (**a**), or by vaccination with an A/California/07/2009 HA split vaccine (**b**). Four weeks later, serum HI titres against A/California/04/2009 were determined (Supplementary Fig. 8) and mice were challenged with 10^6 PFU of A/California/04/2009 or with A/Norway/3568/2009-based reassortant viruses possessing changes in HA at positions 153-156 detected in field strains as shown on the X-axis (see also Supplementary Table 19). On day 4 post-challenge, virus titres in mouse lungs were determined. Dashed lines, detection limit of virus (1.7 $\log_{10}$ PFU/g).



HA position					
127	D	E	E	E	D
153	K	A	A	G	K
154	K	L	K	L	K
155	G	Q	H	R	E
156	N	K	G	G	K

Supplementary Figure 11 | Serum HI titres of immunized ferrets subsequently challenged with escape variants identified in this study or found in a field strain. Three or four ferrets per group were infected by intranasal inoculation with 500 PFU of A/California/04/2009 virus. Twelve months later, serum HI titres were determined by using standard HI assays with 0.5% turkey red blood cells. For better data comparison, on the x-axis we list the viruses with which the respective groups of ferrets were subsequently challenged (for results of the challenge experiment, see Fig. 4).

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Supplementary Table 1. Human A(H1N1)pdm09 sera used in this study.

Serum ID	MN titre[c]	Donor age (years)	Date of collection	Screens of wild-type viruses[d]	Screens of virus libraries[e]
C26[a]	512	11	November 21, 2009	+	−
C42[a]	512	8	November 21, 2009	+	−
C61[a]	512	9	November 21, 2009	+	−
C32[a]	256	11	November 21, 2009	−	+
C53[a]	256	12	November 21, 2009	−	+
C59[a]	256	9	November 21, 2009	+	+
C82[a]	512	10	January 30, 2010	+	+
C83[a]	512	11	January 30, 2010	+	+
C84[a]	256	9	January 30, 2010	+	+
C86[a]	512	8	January 30, 2010	−	+
C87[a]	256	14	January 30, 2010	+	+
C89[a]	1024	9	January 30, 2010	−	+
C91[a]	512	9	January 30, 2010	−	+
C96[a]	256	10	January 30, 2010	−	+
C101[a]	256	13	January 30, 2010	−	+
C103[a]	256	8	January 30, 2010	−	+
A114[a]	256	46	January 30, 2010	−	+
A163[b]	1024	39	March 25, 2010	−	+

[a]Sera from clinical patients infected with an A(H1N1)pdm09 virus, based on a positive result with a rapid diagnosis kit. Sera were selected from a larger panel of sera based on high microneutralization (MN) titres (>256).

[b]Serum from an individual with no known respiratory infection during the 2009–2010 season who was vaccinated in 2009.

[c]Microneutralization (MN) titres were determined using standard MN assays[2,3]. Briefly, 100 $TCID_{50}$ of A/Osaka/364/2009 (H1N1) (isolated August, 2009) were incubated with 2-fold serial dilutions of human sera treated with receptor-destroying enzyme (RDE; Denka Seiken Co., Ltd., Tokyo, Japan) for 30 min at 35°C. The mixtures were then added to confluent MDCK cells in 96-well plates to determine the neutralizing activity.

[d]Sera used in antigenic screens of wild-type viruses (see Supplementary Table 2).

[e]Sera used in antigenic screens of virus libraries (see Supplementary Tables 5, 10, and 11).

Supplementary Table 2. HA mutations identified in antigenic escape mutants selected from clinical A(H1N1)pdm09 isolates with human convalescent sera[a].

Serum ID	HA mutation(s) found in plaque-purified viruses (number of clones with mutations/number of clones examined) using:	
	A/Norway/3206-3/2009	A/Yokohama/UT-K1205T/2009
C26	ND (0/12 clones)[b]	-[c]
C42	K153E+N239K (12/12 clones)	-
C61	K153E+N239K (12/12 clones)	-
C59	K153E+N239K (12/12 clones)	D127E (12/12 clones)
C82	ND (0/12 clones)	D127E (10/12 clones)
C83	K153E+N239K (10/12 clones)	D127E (6/12 clones)
C84	ND (0/12 clones)	ND (0/12 clones)
C87	K153E+N239K (6/12 clones)	D127E (4/12 clones) K163E (8/12 clones)

[a]4.0×10^6 PFU of virus were mixed with each of the convalescent human sera and then used to infect MDCK cells. Virus-containing culture supernatants were collected from cells incubated with the highest concentration of antiserum at which a cytopathic effect was observed. Viruses were plaque-purified in MDCK cells. The HA genes of 12 plaque-purified viruses per group were sequenced.

[b]ND, not detected, i.e., no mutations in HA were found.

[c]-, not tested.

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Supplementary Table 3. Antigenic characterization of escape mutants from A(H1N1)pdm09 viruses by HI assay[a].

Virus	HI titre with antisera raised against:		
	WT Nr3206	Nr3206-K153E+N239K	WT Yk1205
WT Nr3206[b]	**1280**[g]	320	-
Nr3206-K153E+N239K[c]	160	**320/640**	-
WT Yk1205[d]	-[h]	-	**1280**
Yk1205-D127E[e]	-	-	320/640
Yk1205-K163E[f]	-	-	640

[a]HI titres were determined by standard HI assays with 0.5% turkey red blood cells, the amount of virus equivalent to 8 haemagglutination units, and the indicated ferret antiserum.
[b]WT Nr3206, wild-type A/Norway/3206-3/2009.
[c]Nr3206-K153E+N239K, A/Norway/3206-3/2909 possessing K153E and N239K mutations.
[d]WT Yk1205, wild-type A/Yokohama/UT-K1205T/2009.
[e]Yk1205-D127E, A/Yokohama/UT-K1205T/2009 possessing a D127E mutation.
[f]Yk1205-K163E, A/Yokohama/UT-K1205T/2009 possessing a K163E mutation.
[g]HI titres to homologous viruses are indicated in boldface.
[h]-, not tested.

Supplementary Table 4. Diversity of a mutant A/Norway/3858/2009 HA cDNA plasmid library[a].

		Clone number (%)
Total clones sequenced		24
Wild-type clones		2 (8%)
Non-functional mutant clones[b]		6 (25%)
Functional mutant clones	1 amino acid change	5 (31%)
	2 amino acid changes	6 (38%)
	3 amino acid changes	3 (19%)
	4 amino acid changes	1 (6%)
	5 amino acid changes	1 (6%)
	Total number of clones with amino acid change(s)	16 (67%)

[a]Random mutations were introduced by error-prone PCR into the globular head region (amino acid positions 54–253) of A/Norway/3858/2009 HA. Twenty-four cDNA clones were sequenced to evaluate the diversity of the library.

[b]"Non-functional" clones acquired a stop codon or a nucleotide deletion so that functional HA protein should no longer be expressed.

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Supplementary Table 6. Libraries possessing random mutations at one, two, or four amino acid positions of A/Norway/3858/2009 HA[a].

Mutation target (HA amino acid position)	*E. coli* transformation efficiency (CFU[b])	Transfectant virus titre (PFU/ml)	Stock virus titre (PFU/ml)
125	2.7×10^{6}	1.1×10^{7}	2.0×10^{8}
127	2.9×10^{5}	5.0×10^{4}	1.4×10^{8}
129	7.9×10^{5}	9.0×10^{6}	5.0×10^{7}
141	4.0×10^{5}	1.0×10^{6}	1.3×10^{8}
142	1.5×10^{5}	4.4×10^{6}	7.0×10^{7}
152	2.6×10^{6}	2.5×10^{5}	1.1×10^{8}
153	8.8×10^{5}	3.3×10^{7}	5.5×10^{7}
154	3.4×10^{5}	7.0×10^{7}	1.0×10^{8}
155	1.4×10^{6}	1.2×10^{7}	1.8×10^{8}
156	9.3×10^{5}	1.1×10^{7}	1.5×10^{8}
163	1.9×10^{5}	1.3×10^{7}	3.0×10^{7}
183	6.8×10^{4}	5.1×10^{5}	6.0×10^{7}
186	7.4×10^{5}	1.7×10^{6}	1.1×10^{8}
187	3.4×10^{5}	3.4×10^{4}	2.0×10^{8}
190	6.2×10^{5}	1.5×10^{6}	1.4×10^{8}
153+154	7.8×10^{5}	5.0×10^{6}	9.0×10^{7}
153+155	7.5×10^{5}	2.1×10^{7}	1.2×10^{8}
153+156	6.6×10^{5}	1.7×10^{7}	4.3×10^{8}
154+155	7.2×10^{5}	2.4×10^{7}	2.2×10^{8}
154+156	5.6×10^{5}	2.7×10^{7}	7.5×10^{7}
155+156	1.1×10^{6}	1.0×10^{7}	2.7×10^{8}
153+154+155+156	1.6×10^{6}	6.8×10^{7}	3.1×10^{8}
D127E 153+154+155+156	7.5×10^{6}	4.9×10^{7}	4.3×10^{8}

[a]Mutagenesis was carried out for the indicated amino acid position(s) by performing PCR with mixtures of primers encoding all possible nucleotide combinations at the targeted codon(s). To represent all possible amino acid combinations at one, two or four amino acid positions, at least 20, 400, or 1.6×10^{5} variants need to be tested.
[b]Colony-forming units.

Supplementary Table 7. Antisera used to screen A/Norway/3858/2009 HA libraries possessing random mutations at one, two, or four amino acid positions.

Targeted site in HA (amino acid position)	Antisera used for screening (raised in ferrets unless noted otherwise)
125	Nr3858-N125D[a]
127	Nr3858-D127E
129	Nr3858-N129D
153	Nr3858-K153E
154	Nr3858-K154N
155	Nr3858-G155E
156	Nr3858-N156D
163	Nr3858-K163E
183	Nr3858-S183P
141	WT Nr3858[b]
142	WT Nr3858
152	WT Nr3858
186	WT Nr3858
187	WT Nr3858
190	WT Nr3858
153+154	Nr3858-K153E and Nr3858-K154N
153+155	Nr3858-K153E and Nr3858-G155E
153+156	Nr3858-K153E and Nr3858-N156D
154+155	Nr3858-K154N and Nr3858-G155E
154+156	Nr3858-K154N and Nr3858-N156D
155+156	Nr3858-G155E and Nr3858-N156D
153+154+155+156	Human antiserum A163
D127E 153+154+155+156	Human antiserum A163

[a]Nomenclature example: Nr3858-N125D, A/Norway/3858/2009 possessing a N125D mutation.
[b]WT Nr3858, wild-type A/Norway/3858/2009.

DOI: 10.1038/NMICROBIOL.2016.58

Supplementary Table 12. Diversity of an A/Norway/3858/2009 HA plasmid library possessing random mutations at amino acid positions 153–156[a].

Codon for amino acid at position:			
153	154	155	156
AAA[b]	**AAA**	**GGA**	**AAT**
GCT	ATG	AAC	CTG
AAC	TAG*	GAT	TGC
CGA	TTT	GGA	AGA
ACA	GGG	AAA	AGT
GGT	TCC	ATC	AGG
TTC	TGT	TTT	AGG
TCC	TCC	TCC	CTA
TGT	GCT	GTA	GAC
AAA	CAC	TCG	AAT
TGT	CAT	CGT	TCC
TGA*[c]	AAA	TGA*	GAT
GGT	AGG	GTG	ATA
TCC	GGT	ATT	GAA
TTG	CCT	TTT	TTT
AAA	ATT	ATT	CGC
ACG	ATC	TCG	GCA
TCT	TCG	GGG	AGT
GTT	TGA*	TGT	GAC
TCA	TCG	GAT	TGT
GGT	TCA	TAA*	GAC
GCT	AGG	CAA	TTC
TTT	GCA	AGA	TAA*
TTC	GTT	TAC	GGG
CCC	TGG	GCG	GCG
AAG	GAC	TAA*	GAG
TTT	GTA	CGA	ACC
CTG	GGA	TTT	TGA*
ACG	GGT	CCA	AAA
AAC	AGC	GTA	CTA
TTT	GAC	CCT	ACT
GGC	TCC	TGG	TCG
AAA	AGA	GAG	TCG
AGC	AAA	GTG	TGC
GAA	AGG	CCA	GCC

[a]Mutagenesis targeting amino acid positions 153–156 of A/Norway/3858/2009 HA was carried out as described in the Methods section. Thirty-four cDNA clones were sequenced to confirm the diversity of the library.

[b]Nucleotide sequences of wild-type A/Norway/3858/2009 HA gene are shown in boldface.

[c]Asterisk (*), stop codon.

Supplementary Table 13. Diversity of an A/Norway/3858/2009 HA plasmid library possessing the D127E mutation and random mutations at amino acid positions 153–156[a].

Codon for amino acid at position:			
153	154	155	156
AAA[b]	**AAA**	**GGA**	**AAT**
ACA	AGC	AGC	TTC
GGA	CTG	GAT	ATC
AGG	GTC	TAG*	GTA
ATT	ATA	GGT	TTA
CAC	ACG	TTA	CAG
TGA*[c]	CTA	GGC	CCG
AAA	GGT	TCG	CCC
GGT	AGT	GAG	GGT
AAA	AAA	GGA	AAT
GTA	GAA	TGA*	TGC
GGA	ACC	AAG	TTT
AAT	GTG	TGT	GGC
GCT	TGC	TTT	CCA
GGT	CAC	AGT	AGT
TTG	CCA	GTC	AGC
TAA*	CGG	ATG	AGC
TTC	TTA	GGG	TGT
GAA	GGG	CTG	CAT
TTC	TAT	CAG	TCC
CAG	GAC	AAT	GCT
AGT	GCG	ACC	GGA
TCG	TCA	ATA	ACC
GTA	TCC	TTG	GAG
TCA	GTT	ATC	CGG
CAA	CGT	CTG	CGC
GTG	TGT	TCG	TGG
TGG	TCT	GAA	AAA

[a]Mutagenesis targeting amino acid positions 153–156 of A/Norway/3858/2009 HA possessing the D127E mutation was carried out as described in the Methods section. Twenty-seven cDNA clones were sequenced to confirm the diversity of the library.
[b]Nucleotide sequences of wild-type A/Norway/3858/2009 HA gene are shown in boldface.
[c]Asterisk (*), stop codon.

DOI: 10.1038/NMICROBIOL.2016.58

Supplementary Table 14. Selection of antigenic escape mutants in mice.

Mouse ID	Mouse strain	HI titre of mouse serum[a]	Challenge virus	Virus titre in lungs[c] (PFU/g)
1	BALB/c	80	WT CA04[b]	<50
2		80		<50
3		<10		7.39×10^{6}
4		<10	Nr3858 with 5 mutations[d]	1.18×10^{7}
5		80		2.42×10^{2}
6		<10		8.57×10^{6}
7	C57BL6/J	40	WT CA04	<50
8		80/160		<50
9		80		<50
10		80	Nr3858 with 5 mutations	1.05×10^{3}
11		80/160		<50
12		80/160		<50

[a]HI titres were determined by standard HI assays with 0.5% turkey red blood cells, the amount of wild-type A/California/04/2009 virus equivalent to 8 haemagglutination units, and the sera collected from the indicated mice four weeks post-infection.
[b]WT CA04, wild-type A/California/04/2009 virus.
[c] The virus detection limit was 50 PFU/g.
[d]Nr3858 with 5 mutations, A/Norway/3858/2009 possessing the prefixed D127E mutation and random mutations at positions 153–156 of HA.

Supplementary Table 16. Virus strains and HA mutants used to raise ferret antisera for antigenic cartography.

Virus type	Virus strain or HA mutant
A(H1N1)pdm09	A/California/04/2009
	A/Norway/3568/2009
	A/Norway/3206-3/2009
	A/Norway/3206-3/2009-K153E+N239K[a]
	A/Yokohama/UT-K1205T/2009
	A/Yokohama/UT-K1205T/2009-K163E
	A/Norway/3858/2009
	A/Norway/3858/2009-N125D
	A/Norway/3858/2009-D127E
	A/Norway/3858/2009-N129D
	A/Norway/3858/2009-K153E
	A/Norway/3858/2009-K154N
	A/Norway/3858/2009-G155E
	A/Norway/3858/2009-N156D
	A/Norway/3858/2009-K163E
	A/Norway/3858/2009-S183P
	A/Norway/3858/2009-K154G+N156V
	A/Norway/3858/2009-K153S+K154L+G155R+N156S
	A/Norway/3858/2009-D127E+K153A+K154L+G155R+N156G
Seasonal human and swine H1N1 viruses	A/swine/Iowa/15/1930
	A/New Jersey/8/1976
	A/Wisconsin/10/1998
	A/Wisconsin/87/2005
	A/Ohio/01/2007
	A/Texas/14/2008

[a]A/Norway/3206-3/2009-K153E+N239K, A/Norway/3206-3/2009 possessing K153E and N239K mutations.

DOI: 10.1038/NMICROBIOL.2016.58

Supplementary Table 17. A(H1N1)pdm09 HA mutants used to raise additional ferret antisera to potentially improve mutant virus resolution by antigenic cartography.

A(H1N1)pdm09 HA mutant	Property
A/Norway/3858/2009-N125G	Antigenically close to wild-type viruses
A/Norway/3858/2009-D127S	Amino acid close to 153/156 loop in structure of HA
A/Norway/3858/2009-K153H+N156H	All large aromatic amino acids
A/Norway/3858/2009-K154N+S183P	Antigenically in intermediate cluster
A/Norway/3858/2009-D127E+K153S+N156T	All negative or polar amino acids
A/Norway/3858/2009-K153A+K154A+N156G	All small, hydrophobic amino acids
A/Norway/3858/2009-K153Q+K154Q+G155R	All positive amino acids
A/Norway/3858/2009-K153S+K154G+G155Y+N156G*	Virus most distant from others antigenically
A/Norway/3858/2009-D127E+K153R+K154G+G155H+N156S	Mixed size and charge of amino acids
A/Norway/3858/2009-D127E+K153R+K154R+G155E+N156H	All large amino acids

*The position of this antiserum in the antigenic map was not well-coordinated and the antiserum was excluded from the analysis.

Supplementary Table 18. Virus strains and HA mutants used to challenge immunized mice[a].

Virus ID	Virus strain or HA mutant	Method by which the HA mutant was selected
CA04	A/California/04/2009	NA[b]
Nr3858	A/Norway/3858/2009	NA
C1	Nr3858-D127E+K153S+K154L+G155K+N156G[a]	*In vitro*[c] and *In vivo*[d]
3-2/3	Nr3858-D127E+K153A+K154L+G155R+N156G	*In vivo*
C20	Nr3858-D127E+K153A+K154L+G155N+N156K	*In vitro* and *In vivo*
C34	Nr3858-D127E+K153Q+K154N+G155S+N156G	*In vitro* and *In vivo*
C36	Nr3858-D127E+K153S+K154L+G155R+N156G	*In vitro* and *In vivo*
C47	Nr3858-D127E+K153G+K154I+G155N+N156R	*In vitro*
C53	Nr3858-D127E+K153R+K154L+G155S+N156G	*In vitro*
16-1/3	Nr3858-D127E+K153R+K154R+G155E+N156H	*In vivo*
16-1/4	Nr3858-D127E+K153A+K154L+G155Q+N156K	*In vivo*
16-1/8	Nr3858-D127E+K153A+G155H+N156G	*In vivo*
16-1/11	Nr3858-D127E+K153G+K154L+G155R+N156G	*In vivo*
16-1/27	Nr3858-D127E+K153A+K154N+G155R+N156G	*In vivo*
029/13	Nr3858-K153S+K154G+G155Y+N156G	*In vitro*

[a]Nr3858-D127E+K153S+K154L+G155K+N156G, A/Norway/3858/2009 possessing the D127E, K153S, K154L, G155K, and N156G mutations in HA.
[b]NA, not applicable.
[c]*In vitro* selection with human antiserum A163.
[d]*In vivo* selection in immunized mice.

DOI: 10.1038/NMICROBIOL.2016.58

Supplementary Table 19. Reassortant viruses used to challenge immunized mice[a].

Virus ID	HA mutation compared to A/Norway/3858/2009	Origin of HA and NA genes[b]
153	K153E	A/New York/4557/2009
154	K154E	A/New York/4459/2009
155-A	G155E	A/Bangkok/INS425/2010
155-B	G155E	A/Jiangsu/1/2009
156-A	N156S	A/Qingdao/1610/2009[c]
156-B	N156T	A/Wisconsin/629/D00223/2009
153+155	K153E+G155E	A/Miyazaki/40/2011[d,e]
154+155	K154Q+G155E	A/Okinawa/41/2010[d,e]
155+156-A	G155E+N156S	A/Cameroon/LEID/01/11/1398/2010[e]
155+156-B	G155E+N156K	A/Townsville/56/2010

[a]HA and NA genes of reassortant viruses were derived from A(H1N1)pdm09 field strains, while the remaining six genes were from A/Norway/3568/2009.

[b]A(H1N1)pdm09 field strains were identified from the influenza viral genome sequence database Global Initiative on Sharing All Influenza Data (GISAID) or from the National Center for Biotechnology Information .

[c]Since the A/Qingdao/1610/2009 NA gene sequence is not publicly available, we used the NA gene of A/Norway/3568/2009 virus.

[d]The HA2 portions of the A/Miyazaki/40/20111 and A/Okinawa/41/2010 HA gene segments are not publicly available; we, therefore, constructed chimera between the A/Miyazaki/40/2011 and A/Okinawa/41/2010 HA1 sequences and the A/Norway/3568/2009 HA2 sequence.

[e]Mixed mutations are present in the targeted positions of the original HA sequences [153(K/E)+155(G/E) for A/Miyazaki/40/2011; 154(K/Q)+155(G/E) for A/Okinawa/41/2010; 155(G/E)+156S for A/Cameroon/LEID/01/11/1398/2010]. Based on this information, the K153E+G155E mutations were introduced into virus 153+155, K154Q+G155E mutations were introduced into virus 154+155, and G155E+N156S mutations were introduced into virus 155+156-A.

Supplementary Table 22. Human sera used in A/Texas/50/2012 (H3N2) escape mutant selection

Serum ID[a]	Group No.[b]	HI titre[c]	Donor age (years)	Date of collection
LS 2361308A	2	40	31	December 1, 2013
LS 2361081A	2	40	33	December 4, 2013
LS 2361252A	3	80	46	December 10, 2013
LS 5543568A	2	160	37	December 30, 2013
LS 2362317A	3	160	53	January 21, 2014
LS 8812408A	1	160	25	January 23, 2014
LS 8812407A	1	160	29	January 23, 2014
LS 8812404A	2	160	30	January 23, 2014
LS 8812400A	1	80	23	January 23, 2014
LS 5543871A	3	80	59	January 23, 2014

[a]Sera from anonymized healthy donors (Lampire Biological Products, Pipersville, PA).
[b]For some antigenic screens, human sera were combined based on the donor's age: Group 1 (G1): Age 23-29; Group 2 (G2): Age 30-37; Group 3 (G3): Age 46-59.
[c]Haemagglutination inhibition (HI) titres were determined using standard HI assays with 0.75% guinea pig red blood cells and the amount of A/Texas/50/2012 (H3N2) virus equivalent to 8 haemagglutinating units.

DOI: 10.1038/NMICROBIOL.2016.58

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