1 Identification of amino acid substitutions supporting antigenic change of

2 A(H1N1)pdm09 viruses.

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14 Running head: H1N1 pdm09 virus: possibilities for antigenic change

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22 **Abstract**

23 The majority of currently circulating influenza A(H1N1) viruses are antigenically similar to the
24 virus that caused the 2009 influenza pandemic. However, antigenic variants are expected to
25 emerge as population immunity increases. Amino acid substitutions in the hemagglutinin
26 protein can result in escape from neutralizing antibodies, affect viral fitness, and change
27 receptor preference. Here we constructed mutants with substitutions in the hemagglutinin of
28 A/Netherlands/602/09 in an attenuated backbone to explore amino acid changes that may
29 contribute to emergence of antigenic variants in the human population. Our analysis revealed
30 that single substitutions affecting the 151 – 159 loop located adjacent to the receptor binding
31 site caused escape from ferret and human antibodies elicited after primary A(H1N1)pdm09
32 virus infection. The majority of these substitutions resulted in similar or increased replication
33 efficiency *in vitro* compared to the virus carrying the wildtype hemagglutinin, and did not result
34 in a change of receptor preference. However, none of the substitutions was sufficient to
35 escape from the antibodies in sera from individuals that experienced both seasonal and
36 pandemic A(H1N1) virus infections. These results suggest that antibodies directed against
37 epitopes on seasonal A(H1N1) viruses contribute to neutralization of A(H1N1)pdm09
38 antigenic variants, thereby limiting the number of possible substitutions that could lead to
39 escape from population immunity.

40 **Importance**

41 Influenza A viruses can cause significant morbidity and mortality in humans. Amino acid
42 substitutions in the hemagglutinin protein can result in escape from antibody-mediated
43 neutralization. This allows the virus to re-infect individuals that have acquired immunity to
44 previously circulating strains through infection or vaccination. To date, the vast majority of
45 A(H1N1)pdm09 strains remain antigenically similar to the virus that caused the 2009 influenza
46 pandemic. However, antigenic variants are expected to emerge as a result of increasing
47 population immunity. We show that single amino acid substitutions near the receptor binding
48 site were sufficient to escape from antibodies specific for A(H1N1)pdm09 viruses, but not
49 from antibodies elicited in response to infections with seasonal A(H1N1) and A(H1N1)pdm09
50 viruses. This study identifies substitutions in A(H1N1)pdm09 viruses that support escape from
51 population immunity, but also suggests that the number of potential escape variants is limited
52 by previous exposure to seasonal A(H1N1) viruses.

53 **Introduction**

54 Influenza pandemics occur when a novel influenza A virus is introduced in the human
55 population and spreads around the globe. Since existing antibody responses are typically not
56 cross-reactive with the antigenically novel virus, the virus encounters little pre-existing
57 humoral immunity and can cause severe outbreaks. Three influenza pandemics occurred
58 during the 20th century: A(H1N1) virus in 1918, A(H2N2) virus in 1957, and A(H3N2) virus in
59 1968 (1). In each case the newly introduced subtype replaced the previous subtype. In 1977
60 an A(H1N1) virus that caused epidemics in the early 1950s was reintroduced in the human
61 population (2), and continued to co-circulate with A(H3N2) until 2009. In April 2009 a swine-
62 origin A(H1N1) virus (A(H1N1)pdm09) caused the first influenza A virus pandemic of the 21th
63 century (3). It replaced the previously circulating seasonal A(H1N1) virus, but continues to co-
64 circulate with seasonal A(H3N2) virus (4).

65

66 A prerequisite for the influenza virus to infect the host cell is the binding of the hemagglutinin
67 (HA) surface protein to sialylated glycan receptors on the host cell through its receptor-
68 binding site (RBS). HA is the main target of neutralizing antibodies and is therefore a critical
69 component of influenza vaccines (5). Influenza viruses continually escape antibody-mediated
70 neutralization by variation of the amino acids in the HA protein. This process is referred to as
71 antigenic drift, and allows the virus to infect individuals that are immune to contemporary or
72 previously circulating antigenic variants.

73

74 Studies from the 1980s identified four immunodominant antigenic regions within the HA of
75 A(H1N1) virus (6, 7). Similar antigenic regions were identified for A(H3N2) (8) and A(H5N1)
76 viruses (9, 10). Amino acid substitutions in these so called antigenic sites, which cover much
77 of the HA globular head, can result in escape from antibody recognition. More recently it was
78 shown that major antigenic change during evolution of A(H3N2) and A(H5N1) viruses and
79 recent antigenic change of seasonal A(H1N1) and influenza B viruses were predominantly
80 caused by single substitutions that occurred near the RBS (11, 12).

81

Antigenic change may also be a secondary effect of substitutions in HA that facilitate more efficient replication in the human host. HA is pivotal in adaptation of zoonotic influenza A viruses to a new host because of its function in receptor binding (13). Human influenza viruses bind to sialic acids (SAs) linked to the galactose in an α2,6-linkage, avian influenza viruses have a preference for α2,3-linked SAs, while swine viruses bind either α2,6 or both α2,3 and α2,6 linked SAs (14). We hypothesized that substitutions that modify or fine-tune receptor specificity, thereby altering host range and tissue tropism, may result in escape from antibodies directed at the RBS area. Finally, addition or removal of carbohydrate side chains on HA has been associated with changes in the antigenic properties of influenza viruses (15, 16).

The HA of A(H1N1)pdm09 viruses is antigenically most similar to that of recent classical and triple reassortant swine A(H1N1) viruses (17, 18). The HA of these swine viruses descended from the 1918 pandemic influenza virus but in contrast to the human lineage did not undergo extensive antigenic drift, as was reported for A(H3N2) swine viruses (19). Structural analyses suggested high antigenic similarity between the A(H1N1)pdm09 virus and A(H1N1) viruses that circulated in the first decades after the 1918 pandemic (20, 21). Accordingly, age groups that experienced A(H1N1) virus infection before 1950 were partially immune to the A(H1N1)pdm09 virus (22).

During the five years after its emergence multiple antigenic variants of A(H1N1)pdm09 virus have been detected, but the vast majority of recently isolated viruses remain antigenically similar to the A/California/7/2009 vaccine virus (4). However, as population immunity to A(H1N1)pdm09 virus builds up it becomes beneficial for the virus to be antigenically different from the pandemic strain. Therefore, the goal of the present study was to explore molecular changes that contribute to antibody escape of A(H1N1)pdm09 virus.

We selected 25 single and five double substitutions based on substitutions that were shown to be important for antigenic change in other subtypes, changes in receptor specificity, or genetic differences between A(H1N1)pdm09, swine A(H1N1) and seasonal A(H1N1) viruses.

112 The substitutions were introduced into the HA gene of influenza virus A/Netherlands/602/09 in
113 an attenuated virus backbone, and their antigenic effect was tested in hemagglutination
114 inhibition (HI) assays using a ferret antiserum prepared to A/Netherlands/602/09. Mutants that
115 displayed altered antigenic properties were further tested to a larger panel of ferret antisera
116 and human sera. In addition, the impact of these substitutions on replication kinetics and
117 receptor specificity was evaluated.
118
119 **Materials and Methods**
120 Cells
121 293T cells were cultured in DMEM (Lonza, Breda, The Netherlands) supplemented with 10%
122 FCS (Sigma-Aldrich, Zwijndrecht, The Netherlands), 100 IU/ml penicillin, 100 µg/ml
123 streptomycin, 2 mM glutamine, 1mM sodiumpyruvate, non-essential amino acids (Lonza,
124 Breda, The Netherlands), and 500 µg/mL geneticin (Life Technologies, Bleiswijk, The
125 Netherlands). Madin-Darby Canine Kidney (MDCK) cells were cultured in EMEM (Lonza)
126 supplemented with 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2mM glutamine,
127 1.5 mg/ml sodiumbicarbonate (Lonza), 10mM Hepes (Lonza), and non-essential amino acids.
128
129 Plasmid construction
130 A/Netherlands/602/09 was isolated from a patient in the Netherlands during the 2009
131 influenza pandemic (23), and was used in this study to represent the antigenic properties of
132 A(H1N1)pdm09 viruses. The full HA gene was cloned in the modified pHW2000 expression
133 plasmid as previously described (24-26). Mutations were introduced with the minimal number
134 of nucleotide substitutions necessary to change the amino acid. When more than one single
135 nucleotide change could lead to the desired substitution, the codon change observed in
136 naturally occurring amino acid substitutions was selected (e.g. genetic differences between
137 human A(H1N1) viruses). If the desired amino acid substitution did not occur previously in
138 A(H1N1) viruses, the mutation was introduced using a codon observed in A(H3N2) viruses.
139 Mutations were introduced in the HA gene using the QuickChange multi-site directed
140 mutagenesis kit (Agilent Technologies, Amstelveen, The Netherlands) according to the
141 manufacturers instructions. The presence of introduced mutations and absence of undesired

142 additional mutations was confirmed by sequence analysis of the modified HA gene.

143

144 Construction of recombinant virus stocks

145 Plasmids containing wildtype or modified A/Netherlands/602/09 HA genes were used to
146 generate recombinant viruses consisting of the (modified) HA gene and seven remaining
147 gene segments of A/Puerto Rico/8/34, as described previously (25). Briefly, 293T cells were
148 seeded in 100mm dishes one day prior to transfection. Cells were transfected overnight with
149 40 µg of plasmid DNA. Transfection medium was subsequently replaced with medium
150 containing 2% FCS. Cells were incubated for 72 hours at 37°C and 5% CO_2 before harvesting
151 the supernatant. The virus stocks were propagated by inoculation of MDCK cells with 2 mL of
152 supernatant of transfected cells. After 2 hours the inoculum was replaced by MDCK infection
153 medium consisting of EMEM, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2mM glutamine,
154 1.5 mg/ml sodium bicarbonate, 10mM Hepes, non-essential amino acids, and 25 µg/ml
155 trypsin. Viruses were harvested after incubation for 72 hours at 37°C and 5% CO_2. The
156 culture supernatants were subjected to ultracentrifugation to increase the viral particle
157 concentration if hemagglutination titers were below 12 hemagglutinating units (HAU). The
158 presence of introduced mutations and absence of unwanted additional mutations was
159 confirmed by sequencing of the HA gene. Work with recombinant viruses was performed
160 under biosafety level 2 conditions under a permit from the Ministry of Infrastructure and the
161 Environment.

162

163 Antisera

164 Ferret antisera were prepared by intranasal inoculation with 500 µL virus stock. Antisera were
165 collected fourteen days after inoculation. Ferrets were housed and experiments were
166 conducted in strict compliance with European guidelines (EU directive on animal testing
167 86/609/EEC) and Dutch legislation (Experiments on Animal Act, 1997). The protocol was
168 approved by an independent animal experimentation ethical review committee 'stichting DEC
169 consult'. Animal welfare was monitored daily and all animal handling was performed under
170 light anesthesia (ketamine) to minimize animal discomfort.

171

172 Human serum samples were selected from the serum bank of the Viroscience department.
173 Sera from patients in whom the possibility of non-naturally obtained antibody responses to
174 A(H1N1)pdm09 virus (e.g. vaccination, intravenous immunoglobulin administration) existed or
175 sera from patients with immune deficiencies (e.g. auto-immune disease, HIV-positive status,
176 use of immunosuppressive medication) were excluded from use in this study. In addition, only
177 sera of patients who, or of whom the caregivers, did not object to scientific use of leftover
178 materials were included in this study. The study protocol was reviewed and approved by the
179 medical ethics board of the Erasmus University Medical Center (study number MEC-2012-
180 181). Informed consent was waived because patient inclusion was performed retrospectively
181 and anonymously.
182
183 HI assay
184 HI assays were performed using standard procedures (27). Briefly, ferret antisera or human
185 sera were pre-treated overnight with the receptor destroying enzyme *Vibrio cholerae*
186 neuraminidase (VCNA) at 37°C followed by inactivation for 1 hour at 56°C. 2-fold serial
187 dilutions of the pre-treated sera, starting at a 1:20 dilution, were mixed with 25 µL virus stock
188 containing 4 HAU and the mixture was incubated at 37°C for 30 minutes. Subsequently, 25
189 µL 1% turkey erythrocytes was added and hemagglutination patterns were read after a 1 hour
190 incubation at 4°C. The HI titer is expressed as the reciprocal value of the highest serum
191 dilution that completely inhibited agglutination of turkey erythrocytes.
192
193 Plaque assay
194 The assay was performed as described (28). In brief, MDCK cells were seeded in a 6 well
195 plate to reach 90% confluency the following day. One hour after inoculation with virus, the
196 inoculum was replaced with a 1:1 mixture of 2.4% Avicel (FMC biopolymers, Brussels,
197 Belgium) with 2xEMEM infection medium. After 36 hours, cells were washed with PBS and
198 incubated with 80% Aceton for at least 30 minutes at -20°C. Fixed and permeabilized cells
199 were washed 3 times with PBS, and incubated for 1 hour at 37°C with mouse-anti-NP
200 monoclonal antibody (1 mg/ml HB65, ATCC). Following three washes with PBS, cells were
201 incubated for 1 hour at 37°C with rabbit-anti-mouse-FITC (Life Technologies, Bleiswijk, The

202 Netherlands). Cells were washed with PBS and allowed to air dry. Plaques were scanned on
203 a Typhoon 9410 variable mode imager (GE Healthcare, Diegem, Belgium). This data was
204 analysed with ImageQuant TL Colony counter & Image feature measurement (Amersham
205 Biosciences, Freiburg, Germany). Plaque size was plotted as the radius of the plaques.
206
207 Modified turkey red blood cell hemagglutination assay
208 The modified turkey red blood cell (TRBC) assay was performed as described previously (29)
209 with modifications. Briefly, SAs were removed from the turkey erythrocyte surface by
210 incubating 20% turkey erythrocytes in a total volume of 62,5 μL phosphate-buffered saline
211 supplemented with 50mU of VCNA (Roche, Almere, The Netherlands) in 8 mM calcium
212 chloride at 37°C for 1 hour. The removal of SAs was confirmed by absence of
213 hemagglutination of the treated turkey erythrocytes by control viruses. Turkey erythrocytes
214 stripped of SAs were resialylated to contain only α2,3- or α2,6-linked SAs using either 0.5 mU
215 α2,3-(N)-sialyltransferase (Calbiochem, CA) or 2 mU of α2,6-(N)- sialyltransferase (Japan
216 Tobacco, Inc., Shizuoka, Japan), and 1.5 mM cytidine monophospho-N-acetylneuraminic
217 (CMP) sialic acid (Sigma-Aldrich, Zwijndrecht, Netherlands) at 37°C for 2 hours in a total
218 volume of 75 μl. Subsequently, resialylated turkey erythrocytes were washed with PBS and
219 were resuspended to a final concentration of 0.5% in PBS containing 1% bovine serum
220 albumin. Standard hemagglutination assays were performed to confirm correct resialylation
221 using control viruses with known receptor specificities for α2,3- and α2,6-linked SAs. The
222 receptor specificity of mutant viruses was tested by conventional hemagglutination assay with
223 the modified TRBCs using cell culture supernatants.
224
225 Structural analysis
226 Amino acid positions were plotted on the HA crystal structure of the A/California/04/09 virus
227 [PDB accession code 3LZG (42)] using MacPyMOL (The PyMOL Molecular Graphics System,
228 Version 1.3, Schrödinger, LLC) to visualize the trimer.

229 **Results**

230 *Selection of substitutions*

231 The antigenic regions of seasonal A(H1N1) virus have previously been mapped (6, 7), but the
232 substitutions that caused antigenic change during A(H1N1) virus evolution remain almost
233 entirely unknown. Additionally, fine-grain differences in receptor specificity beyond the coarse
234 distinction for α2,3-SA or α2,6-SA preference that allow more efficient replication in the
235 human host and that may change the virus antigenically are currently incompletely
236 understood (13, 30). Because of the limited insight in the molecular basis for previous
237 antigenic change of A(H1N1) viruses we selected substitutions to introduce into the
238 A/Netherlands/602/09 representative virus HA gene based on the following three approaches.

239

240 First, we aligned the HA sequences of 28 viruses representing pre-1957 and post-1977
241 seasonal A(H1N1) viruses, swine A(H1N1) viruses and A(H1N1)pdm09 viruses, and selected
242 substitutions based on genetic differences between these viruses (Table 1). We hypothesized
243 that part of the substitutions that became fixed during A(H1N1) virus evolution had a selective
244 advantage because of their role in immune escape. In addition, amino acid differences
245 between swine or A(H1N1)pdm09 viruses and pre-2009 human A(H1N1) viruses may
246 contribute to adaptation to the human host and potentially change the virus antigenically as a
247 secondary effect. Previous work indicated a critical role for antibodies targeting the RBS area
248 in virus neutralization (11, 12) and we therefore focused primarily on amino acid differences in
249 this region of the HA. Substitution 84SN is located away from the RBS, but was consistently
250 different between A(H1N1)pdm09 viruses and all swine and human A(H1N1) viruses (H1
251 numbering is used throughout, unless indicated otherwise).

252

253 A second approach was based on information regarding the molecular basis for antigenic
254 change during evolution of other influenza virus subtypes. Substitutions at seven positions
255 (145, 155, 156, 158, 159, 189, and 193, H3 numbering) were entirely responsible for the
256 major antigenic changes during evolution of A(H3N2) virus (11), three of which (151, 185, and
257 189, H5 numbering) were also identified as key positions for antigenic change of A(H5N1)
258 clade 2.1 virus (12). The corresponding A(H1N1)pdm09 virus positions were identified and

259 where possible the substitution responsible for the observed antigenic change was introduced
260 (Table 1). Multiple mutants were made if it was not possible to introduce the desired
261 substitution. For example, 156QH was responsible for a major antigenic change during
262 A(H3N2) virus evolution and we therefore generated mutants with 153KQ and 153KH.
263
264 Third, we selected substitutions shown or predicted to affect receptor binding specificity of
265 influenza viruses of the A(H1N1), A(H3N2) or A(H5N1) subtypes (Table 1). A(H1N1)pdm09
266 viruses containing the substitutions 222DE and 222DG were previously tested for their effects
267 on receptor binding and antigenic properties by Chutinimitkul *et al.* (24). That work
268 additionally suggested that amino acid differences at positions 142, 187, 222, and 224 were
269 responsible for differences in receptor binding between a 1918 A(H1N1) virus and
270 A/Netherlands/602/09 (24, 31). Mutants with these substitutions, or combinations thereof,
271 were included in the current more extensive analyses. Substitutions 222QL and 224GS
272 (223QL and 225GS in H1 numbering) were previously shown to affect the receptor specificity
273 of A(H5N1) virus (32).
274
275 Recombinant viruses with the modified A/Netherlands/602/09 HA gene and the seven
276 remaining gene segments of A/Puerto Rico/8/34 were rescued by reverse genetics. We were
277 unable to rescue the 127DN and 186AK mutants. The mutant with 187DE did not agglutinate
278 turkey erythrocytes even upon concentration of the virus and was therefore omitted from the
279 antigenic analyses. After MDCK passaging, we found addition of substitution 153KE to mutant
280 222DG224EA and this mutant was therefore excluded from this study. Figure 1A indicates the
281 positions of introduced substitutions on an A/California/04/09 HA crystal structure.
282
283 *Analysis of antigenic properties using ferret antisera*
284 HI assays with a ferret antiserum prepared to A/Netherlands/602/09 were performed to test if
285 the mutant viruses could escape recognition by antibodies against the wildtype virus. When
286 comparing HI titers obtained to an individual antiserum, viruses were considered substantially
287 antigenically different from the reference virus if the HI titer was at least 4-fold (2 $\log_2$) lower.
288 Ten of the 27 mutants were substantially antigenically different from A/Netherlands/602/09

289 (Fig. 2): 224EA, 127DT, 155GE, 156ND, 156NG, 153KE, 156NY, 156NS, 152VT156NS, and
290 155GE224EA.
291
292 Two groups of mutants were further tested in HI assays to a panel of ferret A(H1N1)pdm09
293 antisera. One group contained the ten viruses that were substantially antigenically different
294 from A/Netherlands/602/09, the second group contained seven mutants; 84SN, 152VT,
295 190SN, 222DE, 222DG, 223QL, and 223QR that were antigenically similar to
296 A/Netherlands/602/09 (Fig. 1B and 2). These groups are hereafter referred to as escape
297 mutants and non-escape mutants, respectively. The panel of 14 ferret antisera was prepared
298 to seven A(H1N1)pdm09 viruses isolated between 2009 and 2011. Of these, six wildtype
299 viruses were antigenically similar to A/Netherlands/602/09, in agreement with the fact that
300 these viruses did not have any of the substitutions present in the mutants. However,
301 A/Netherlands/219/11 contains the 155GE substitution and had a more than 8-fold (3 $\log_2$)
302 lower mean HI titer (data not shown).
303
304 The mean HI titers of the non-escape mutants and 224EA were less than 2-fold lower than
305 that of A/Netherlands/602/09 to the panel of ferret antisera (Fig. 3). Escape mutants 156ND,
306 127DT, 155GE, and 156NG had 3 to 4-fold lower mean HI titers than A/Netherlands/602/09
307 (4-fold for 155GE when antisera prepared to A/Netherlands/219/11 were not included). The
308 mean HI titers of 156NS, 152VT156NS, 156NY, 155GE224EA, and 153KE were up to 14-fold
309 (3.8 $\log_2$) lower than A/Netherlands/602/09 HI titers. These results indicate that the amino
310 acid substitutions in all escape mutants except 224EA caused evasion of recognition by
311 antisera raised to this panel of A(H1N1)pdm09 viruses. Mutants 156NS and 152VT156NS
312 were antigenically similar, as were 152VT and A/Netherlands/602/09, thus indicating that
313 156NS was solely responsible for the antigenic change of this double mutant. Introduction of
314 224EA as single substitution had only minor effects on HI titers to most antisera. The
315 155GE224EA double mutant displayed a mean HI titer decrease of 12-fold (3.6 $\log_2$),
316 indicating that these substitutions had a cumulative antigenic effect.
317
318 *Analysis of antigenic properties using human infant sera*

319 Antibodies in sera obtained from infants that experienced a primary A(H1N1)pdm09 virus
320 infection were elicited in response to a single antigenic variant of influenza virus, as is the
321 case for the antibody repertoire of inoculated ferrets. We next examined if the substitutions
322 that led to antigenic variation as tested by ferret antisera were sufficient to escape recognition
323 by human antibodies. 49 surplus sera from unvaccinated infants were tested in HI assays for
324 the presence of antibodies to A(H1N1)pdm09 virus. Six sera from infants born in 2009 or
325 2010 that were between 6 and eleven months of age at the time of sampling had detectable
326 HI titers to A/Netherlands/602/09. HI titers to A/Brisbane/59/07 (seasonal A(H1N1)) were
327 below the detection limit of the HI assay at a starting dilution of 1/40. This suggested that the
328 infants experienced a primary infection and that maternal antibodies potentially present in the
329 sera did not influence our results. Escape and non-escape mutants were tested in HI assays
330 to sera 6, 11, 14 and 16. Non-escape mutants were not included in HI assays with sera 7 and
331 25 because of insufficient material available for these sera.
332
333 Escape and non-escape mutants had 3.5 – 14-fold lower mean HI titers than
334 A/Netherlands/602/09 (Fig. 4A). The variation in HI titers between the different infant sera was
335 larger than for ferret antisera and contradicting results were obtained with different sera. For
336 example, mutant 222DE was antigenically similar to A/Netherlands/602/09 using ferret sera
337 and infant serum 14, yet the HI titer to serum 16 was more than 10-fold lower. Sera 6, 7 and
338 16 poorly discriminated between the mutants. Serum 7 had a low HI titer of 160 to
339 A/Netherlands/602/09 and HI titers of the mutants tested to this serum were similarly low or
340 up to 4-fold higher. The HI titers to sera 6 and 16 were at least 8-fold lower than that of
341 A/Netherlands/602/09 for all mutants.
342
343 To test if escape mutants had an antigenic advantage over non-escape mutants in evasion of
344 the infant sera that discriminated between the mutants, the results from sera 7 (that had
345 similarly high titers to all viruses), and 6 and 16 (that had low titers to all mutants) were
346 omitted and the analysis was repeated. Non-escape mutants and 156ND had mean HI titers
347 that ranged from similar to 2-fold lower than A/Netherlands/602/09, individual HI titers to the
348 different sera were up to 3-fold lower (Fig. 4B, 4C). The remaining mutants, which were all

349 escape mutants, had mean HI titers 2 to 10-fold lower than A/Netherlands/602/09 HI titers,
350 and had one or more individual HI titers that was at least 4-fold lower than HI titers to
351 A/Netherlands/602/09. Therefore, substitutions responsible for antigenic change as measured
352 by ferret antisera can also mediate escape from recognition by human antibodies elicited in
353 response to a primary infection with an A(H1N1)pdm09-like virus. However, in contrast to the
354 ferret sera, the infant sera were sometimes non-discriminative (serum 7) or reacted low
355 across the board with all mutants (sera 6 and 16). Thus, mutants 127DT, 153KE, 155GE,
356 156ND, 156NG, 156NS, 156NY, 152VT156NS, and 155GE224EA escaped from recognition
357 by antibodies in ferret sera and human infant sera (Figs. 3 and 4).
358
359 *Analysis of virus replication*
360 Next, plaque assays were performed to test the effect of the introduced substitutions on
361 replicative fitness. Plaque sizes were determined at a fixed time-point after inoculation and
362 were used as a proxy for replication efficiency, i.e. larger plaques indicate more efficient
363 replication.
364
365 The majority of the mutants displayed plaque sizes similar to A/Netherlands/602/09 (Fig. 5).
366 Single mutants 187DE, 156NY, 224EA, 84SN, and 222DG displayed reduced plaque sizes.
367 Mutants 127DT, 153KH, 153KE, 155GE, 153KQ, and 156ND had predominantly larger
368 plaques. Of the ten mutants that had an antigenic effect in HI assays with ferret or infant sera,
369 only 156NY and 224EA had substantially smaller plaques. The 155GE224EA double mutant
370 displayed plaque sizes similar to A/Netherlands/602/09, indicating that 155GE compensated
371 for the adverse effect of 224EA. Thus, eight mutants (127DT, 155GE, 156ND, 156NG,
372 153KE, 156NS, 152VT156NS, and 155GE224EA) were antigenically different from
373 A/Netherlands/602/09 with no apparent loss of replication efficiency (Figs. 3, 4, and 5).
374
375 *Effect of substitutions on receptor binding specificity*
376 To test if substitutions that caused antigenic change in the HI assays had altered receptor
377 binding specificity, the escape mutants were tested in hemagglutination assays using normal
378 TRBCs or TRBCs resialylated to contain either α2,3- or α2,6-linked SAs. Removal of SAs

379 from the TRBC surface and correct resialylation was confirmed in hemagglutination assays
380 using the avian A/Vietnam/1194/2004 (H5N1) and A/Netherlands/213/03 (H3N2) viruses.
381 A/Netherlands/602/09 and all mutants yielded hemagglutination titers to TRBCs reconstituted
382 with α2,6-linked SA (Table 2). Only mutants 153KE, 155GE and 156ND showed a very weak
383 binding to TRBCs with α2,3-linked SAs. Mutants 156NG and 156NS showed markedly
384 reduced binding to α2,6-reconstituted TRBCs as compared to unmodified TRBCs. Although
385 some mutations that affect antigenic properties appeared to influence receptor specificity to
386 some extent, a clear tendency towards reduced affinity for α2,6-linked SAs was not
387 demonstrated.
388
389 *Frequency of substitutions in natural isolates*
390 We analyzed the frequencies of the tested substitutions in 10,422 A(H1N1)pdm09 sequences
391 from GenBank submitted between April 2009 and February 2014 (data not shown). Four
392 substitutions were detected in more than 1% of the sequences: three non-escape mutants;
393 222DE, 222DG, and 223QR (4.80, 1.94, and 1.60%, respectively), and escape mutant 155GE
394 (1.46%). Of the mutants with substantially increased plaque sizes, substitutions 155GE,
395 156ND, 153KE were found in 1.46, 0.36 and 0.17%, of the sequences, respectively.
396 Substitutions 127DT and 153KH were not detected. Substitution 156NS, which had a large
397 antigenic effect but did not affect plaque size was detected in 0.18% of the sequences. Other
398 substitutions in escape mutants that did not affect plaque size or resulted in substantially
399 smaller plaques were not detected.
400
401 *Analysis of antigenic properties using human sera*
402 The majority of the human population has a more broadly reactive antibody repertoire than
403 influenza-virus inoculated ferrets or infants after primary infection due to previous infections or
404 vaccination with seasonal A(H1N1) viruses (33, 34). Therefore we also tested the mutants in
405 HI assays to human sera obtained from individuals anticipated to have experienced both
406 seasonal A(H1N1) virus and A(H1N1)pdm09 virus infections. We first tested the reactivity of a
407 panel of pre-2009 swine (-origin) viruses (A/swine/Shope/56 and A/New Jersey/8/76) and
408 human vaccine strains (A/USSR/92/77, A/Chile/1/83, A/Taiwan/1/86, A/New Caledonia/20/99,

409 and A/Brisbane/59/07) to a ferret antiserum prepared to A/Netherlands/602/09. HI titers were
410 below the detection limit of the HI assay at a starting dilution of 1/20 (data not shown),
411 indicating that these viruses were antigenically distinct from A(H1N1)pdm09 viruses. 21 sera
412 obtained from individuals aged 7 to 85 at the time of serum collection (October 2009 – May
413 2011) were selected based on their ability to inhibit A/Netherlands/602/09 in HI assays. The
414 sera were tested in HI assays to ten seasonal A(H1N1) viruses covering the period from 1977
415 to 2009 (Fig. 6A). All sera had detectable HI titers to five or more seasonal A(H1N1) viruses,
416 demonstrating a broadly reactive antibody repertoire. Subsequently, the escape and non-
417 escape mutants and A/Netherlands/602/09 were tested in HI assays using the selected sera
418 (Figs. 6B and C). The mean HI titers of escape as well as non-escape mutants were less than
419 1.5-fold (0.54 $\log_2$) lower than A/Netherlands/602/09 HI titers, suggesting that none of the
420 mutants escaped recognition by this panel of sera. The majority of individual HI titers to the
421 different sera were also less than 2-fold lower than A/Netherlands/602/09 HI titers. Six
422 mutants had a single titer that was more than 2-fold lower than the A/Netherlands/602/09 HI
423 titer: 155GE, 155GE224EA, 223QL, 222DG displayed a 4-fold lower HI titer; 224EA and
424 127DT had 8 and 12-fold lower HI titers, respectively. The antigenic effect of the substitutions
425 in neither non-escape nor escape mutants was therefore sufficient to escape recognition by
426 this panel of human sera.
427
428 **Discussion**
429 In this study we attempted to identify amino acid substitutions that contribute to antibody
430 escape of A(H1N1)pdm09 viruses. Substitutions introduced into the HA of
431 A/Netherlands/602/09 were selected based on genetic and antigenic changes of influenza A
432 viruses that circulated in the past and on substitutions associated with changes in receptor
433 binding specificity. We show that at least nine mutants were antigenically distinct from
434 A/Netherlands/602/09 in HI assays using ferret antisera and found that the substitutions that
435 caused escape from ferret antibodies also evaded antibodies in some human sera collected
436 after primary infection.
437

438 Substitution 127DT introduces a glycosylation pattern (N-X-S/T-X) that potentially adds a
439 carbohydrate side chain at position 125. Carbohydrate side chains can mask antibody
440 epitopes and have been shown to change the antigenic properties of influenza viruses (15,
441 16). Seasonal A(H1N1) viruses that circulated since the 1930s until 1986 had a glycosylation
442 site starting at position 127, viruses that circulated from 1986 onwards had a glycosylation
443 site starting at position 125. Both glycosylation sites were absent from the 1918 A(H1N1) and
444 A(H1N1)pdm09 viruses. Absence of glycosylation at these positions has been suggested to
445 contribute to the antigenic difference between seasonal A(H1N1) viruses and A(H1N1)pdm09
446 viruses (35). Our results indicate that re-introduction of a potential glycosylation site in this
447 region was sufficient to escape from antibodies generated in response to A(H1N1)pdm09
448 virus.

449

450 Substitutions 153KE and 155GE in A(H1N1)pdm09 virus are analogous to substitutions
451 156KE and 158GE that were responsible for major antigenic changes during evolution of
452 A(H3N2) viruses (11). The observation that these substitutions changed the antigenic
453 properties of an A(H1N1)pdm09 virus is also in agreement with previous studies that reported
454 escape of A(H1N1)pdm09 virus from monoclonal antibodies (36, 37), and from polyclonal
455 antibody responses in ferrets (35, 38, 39).

456

457 The four different substitutions introduced at position 156 (ND, NG, NY and NS) all had a
458 large antigenic effect. Two additional substitutions on this position, 156NK and 156NE, were
459 previously reported to escape antibody neutralization of A(H1N1)pdm09 virus (37, 38).
460 Interestingly, the large changes in biophysical properties of the substituted amino acids
461 introduced by 156ND or 156NY (charge and volume differences, respectively) had a similar
462 antigenic effect to the small difference in biophysical properties introduced by 156NS. This
463 finding emphasizes the potential importance of this position for antigenic change of
464 A(H1N1)pdm09 viruses.

465

466 The antigenic effect of 155GE224EA in HI assays with ferret antisera was larger than the sum
467 of the effects of the single substitutions. A possible explanation for how substitutions

468 bordering opposite sides of the RBS can amplify each other's effect on antibody escape is
469 given by the greater surface area of an antibody footprint in relation to the size of the RBS
470 (40), which allows more efficient escape from antibodies that cover the RBS in addition to the
471 proportion of antibodies evaded by the individual substitutions. Interestingly, substitution
472 224EA had a small antigenic effect in HI assays with ferret antisera but was substantially
473 different from A/Netherlands/602/09 when tested with infant sera. In tests with infant sera
474 155GE224EA and 224EA were equally different from A/Netherlands/602/09, indicating that
475 155GE did not add to the antigenic effect of 224EA. These results suggest that position 224
476 plays a more prominent role in escape from human antibodies than in escape from ferret
477 antibodies. Antigenic variants with a substitution on this position may therefore not be readily
478 detected in conventional HI assays using ferret antisera.
479
480 The high mutation rate of influenza A viruses and the observation that single substitutions
481 caused substantial antigenic change during evolution of A(H3N2), A(H1N1), and influenza B
482 viruses contradicts the relatively slow rate at which influenza viruses have changed
483 antigenically (11). One possible explanation for this paradox is that substitutions responsible
484 for escape from antibodies targeting the RBS have an adverse effect on HA function,
485 necessitating the co-occurrence of compensatory substitutions which slows down the
486 emergence of new antigenic variants. Substitutions in and surrounding the A(H1N1)pdm09
487 RBS may affect receptor binding and consequently change replication efficiency (41).
488 Surprisingly, we found that most of the substitutions that caused antigenic change in HI
489 assays with ferret and human sera did not result in less efficient virus replication.
490 Substitutions at positions 153 and 156 often had large effects on replication efficiency.
491 Interestingly, change of the lysine at position 153 for three biophysically diverse amino acids
492 all resulted in increased replication efficiency. Replacing the asparagine at position 156 with
493 either an aspartic acid or tyrosine had opposite effects on replication. These results suggest
494 that positions 153 and 156 are key determinants of replication efficiency. It should be noted
495 that replication assays are a surrogate measure of viral replication *in vivo*, and that results
496 may vary with the model system used. In addition, differences in replication efficiency may in
497 part be caused by the use of an A/Puerto Rico/8/34 backbone. However, other studies have

498 also demonstrated that A(H1N1)pdm09 viruses with 153KE, 155GE, and 156ND had
499 increased replication efficiency in eggs or MDCK cells (21, 35), and they were often
500 associated with cell culture adaptation (21, 35, 38).

501

502 The substitutions that caused antigenic change in HI assays with ferret and human infant sera
503 were, with the exception of 224EA, located in or near the 151 - 159 loop. Substitutions in this
504 region have previously been shown to affect the antigenic properties of A(H3N2) and A(H5N1)
505 viruses (11, 12). In addition, these positions often affected replication efficiency of the mutants
506 constructed in this study. Amino acid substitutions at other positions tested in this study that
507 caused similarly large changes in biophysical properties did not substantially change the
508 antigenic properties or replication efficiency. However, it should be noted that it is possible
509 that other amino acid changes outside the 151- 159 loop, that were not included in this study,
510 can result in phenotypic change (42). Our results are in agreement with previous studies that
511 reported mice or human monoclonal antibodies (36, 37) or antibodies in ferret antisera (38)
512 that target the 151 - 159 loop. We here show that several substitutions in or affecting the 151
513 - 159 loop caused antigenic escape while retaining replicative fitness. These results
514 substantiate the importance of this region for antigenic evolution of A(H1N1) viruses.

515

516 We found large differences in the reactivity of infant antisera. Surprisingly, three sera (from
517 the youngest infants) had almost uniform HI titers to all mutants that were either the same as
518 that of A/Netherlands/602/09, or much lower. Infection in the presence of maternally derived
519 antibodies or mechanisms associated with the transformation of the neonatal immune system
520 to a more mature immunological phenotype may explain the difference from the other infant
521 sera (43, 44). Nevertheless, the different reactivities of these three sera warrant further
522 investigation. However, the remaining three sera had reactivities similar to that observed with
523 ferret antisera. This indicates that substitutions that caused escape from ferret antibodies also
524 promote escape from human antibodies elicited in response to infection with an
525 A(H1N1)pdm09-like virus. In contrast, the substitutions responsible for escape from ferret and
526 infant sera were not sufficient to escape recognition by antisera from individuals that
527 experienced infection with seasonal and pandemic A(H1N1) viruses. Thus, the antibody

528 repertoire of the individuals that had a more experienced immune system included antibodies
529 that were absent in the sera of infants. These results suggest that antibodies directed to
530 epitopes on seasonal A(H1N1) viruses are cross-reactive with epitopes on the mutants tested
531 in this study, and thereby complement the antibody repertoire elicited in response to
532 A(H1N1)pdm09 infection alone. The presence of such antibodies in much of the population
533 may also explain why mutations that gave an *in vitro* replication advantage have not been
534 detected at higher frequencies. Carter *et al.* showed that sera from ferrets sequentially
535 infected with antigenically diverse seasonal A(H1N1) viruses can efficiently neutralize
536 A(H1N1)pdm09 virus, whereas sera from ferrets infected with a single seasonal A(H1N1)
537 virus were not cross-reactive (45). Additionally, a recent study by Miller *et al.* indicated that
538 antibodies against previously encountered influenza A viruses are periodically boosted upon
539 natural exposure to drift variants of the same subtype (33), possibly resulting in the more
540 broadly reactive antibody repertoire observed here. This study and other studies (39, 42)
541 suggest that the complexity of the antibody repertoire to A(H1N1) viruses in much of the
542 human population cannot be represented by antigenic analyses based on single-infection
543 ferret antisera. This limits the use of ferret antisera to test the ability of an antigenic variant to
544 escape from population immunity.
545
546 In conclusion, substitutions in or near the RBS can influence the antigenic properties of
547 A(H1N1)pdm09 viruses. Based on the current and previous studies into antigenic change of
548 influenza A viruses (11, 12), it is probable that emerging antigenic variants of A(H1N1)pdm09
549 viruses will escape from population immunity because of substitutions in or near the RBS.
550 However, our results also suggest that the presence of antibodies directed to epitopes on
551 seasonal A(H1N1) and A(H1N1)pdm09 viruses in much of the population limits the number of
552 antigenic variants that can emerge to cause new epidemics.

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564

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732
733
734
735

736 **TABLE 1** Rationale of selected substitutions.

737 **TABLE 2** Agglutination of TRBCs by viruses with wildtype or mutant HAs.
738
739 **FIG 1** Position of amino acid substitutions indicated on an A/California/04/09 HA crystal
740 structure. (A) The three HA monomers are indicated in white, grey and black, the RBS in
741 yellow. Amino acid positions that were mutated in this study are indicated in orange. (B)
742 Zoomed in on the globular head of HA. Amino acid substitutions in mutants that were
743 substantially antigenically different from A/Netherlands/602/09 (escape mutants) are indicated
744 in red; substitutions in mutants that where antigenically similar to A/Netherlands/602/09 and
745 that were included in further antigenic analyses (non-escape mutants) are indicated in blue.
746
747 **FIG 2** HI titer differences between viruses with wildtype or mutant HAs against an
748 A/Netherlands/602/09 ferret antiserum. Viruses with either wildtype or mutant
749 A/Netherlands/602/09 HAs were tested in HI assays to a ferret antiserum prepared to the
750 A/Netherlands/602/09 wildtype virus. Each point represents the $\log_2$ HI titer difference
751 between a mutant and A/Netherlands/602/09. Mutants with HI titers at least 4-fold (2 $\log_2$)
752 lower than A/Netherlands/602/09 (dashed line) were considered substantially antigenically
753 different. The viruses are ordered by the $\log_2$ HI titer difference from A/Netherlands/602/09.
754
755 **FIG 3** HI titer differences between viruses with wildtype or mutant HAs against ferret antisera.
756 Each point in panel (A) represents the $\log_2$ HI titer difference between a mutant and
757 A/Netherlands/602/09 for an individual ferret antiserum. The viruses are ordered by the mean
758 $\log_2$ HI titer difference from A/Netherlands/602/09, which is indicated as red horizontal lines.
759 Names of escape and non-escape mutants are shown in black and grey, respectively. Ferret
760 antisera are indicated in the left most column panel (B), and are ordered top to bottom by a
761 decreasing ability of the serum to inhibit the test viruses in the HI assay. Two antisera
762 (labeled A and B) were prepared to each virus. HI titers are color coded for the difference
763 from A/Netherlands/602/09 (NL602): orange; equal to or higher than A/Netherlands/602/09,
764 yellow; up to 2-fold lower, green; 2 to 4-fold lower, cyan; 4 to 8-fold lower, blue; 8 to 16-fold
765 lower, purple; 16 to 32-fold lower, magenta; at least 32-fold lower.
766
767 **FIG 4** HI titer differences between viruses with wildtype or mutant HAs against human infant
768 sera. **(**A) Symbols, order and nomenclature as in figure 3. (B) HI titers are color coded for the
769 difference from A/Netherlands/602/09: orange; equal to or higher than A/Netherlands/602/09,
770 yellow; up to 2-fold lower, green; 2 to 4-fold lower, cyan; 4 to 8-fold lower, blue; 8 to 16-fold
771 lower, purple; 16 to 32-fold lower, magenta; at least 32-fold lower. NT: The virus serum
772 combination was not tested. (C) The analysis was repeated with inclusion of only the sera that
773 differentiated between the mutants (sera 6, 7, and 16 were omitted). Non-escape mutants
774 were not tested to serum 25 because of insufficient material available for this serum. Grey
775 horizontal lines indicate the mean $\log_2$ HI titer difference from A/Netherlands/602/09 when this
776 serum is also omitted for the other mutants.
777
778 **FIG 5** Effect of substitutions on virus replication. MDCK cells were inoculated with viruses
779 containing wildtype or mutant A/Netherlands/602/09 HAs. After 36 hours the plaque sizes
780 were determined as a measure of replication efficiency. Each point indicates the size of a
781 single plaque. The mutants are ordered by increasing median plaque size, which is indicated
782 as red horizontal lines. Escape mutants are indicated in bold.
783
784 **FIG 6** HI titer differences between viruses with wildtype or mutant HAs against human sera.
785 (A) HI titers of human sera against antigenic variants of seasonal A(H1N1) viruses isolated
786 between 1977 and 2009 (USSR77, A/USSR/90/77; NL78, A/Netherlands/3075/78, TA89,
787 A/Taiwan/1/89; NL87, A/Netherlands/414/87; NC99, A/New Caledonia/20/99; NL99,
788 A/Netherlands/271/99; NL03, A/Netherlands/02/03; NL06, A/Netherlands/364/06; SS06,
789 A/Solomon Islands/03/06; NL09, A/Netherlands/1005/09). (B) HI titer differences between
790 viruses with wildtype or mutant NL602 HAs against human sera. Symbols, order and
791 nomenclature as in figure 3. (C) HI titers are color coded for the difference from
792 A/Netherlands/602/09: orange; equal to or higher than A/Netherlands/602/09, yellow; up to 2-
793 fold lower, green; 2 to 4-fold lower, cyan; 4 to 8-fold lower, blue; 8 to 16-fold lower than

794 A/Netherlands/602/09. The first two digits of the serum number indicate the age of the
795 individual at the time of sampling.
796

TABLE 1 Rationale of selected substitutions.

	Subtype number conversion	Genetic difference between[a]			Position involved in antigenic change of other influenza viruses			
		A(H1N1)pdm09 viruses – swine A(H1N1) viruses	A(H1N1)pdm09 viruses – seasonal A(H1N1) viruses	Swine A(H1N1) viruses – seasonal A(H1N1) viruses	A(H3N2) virus	A(H5N1) virus	Influenza B virus	Substitutions associated with changes in receptor binding specificity
84SN		✓	✓					
127DE	126[b]	✓				✓[e]		
127DN	126[b]	✓				✓[e]		
127DT	126[b]		✓			✓[e]		
142KN	145[c]				(23)			
142KS	145[c]		✓		(23)			
152VT	151[b], 155[c]			✓	(23)	(24)		
153KE	156[c]			✓	(23)			
153KH	156[c]				(23)			
153KQ	156[c]				(23)			
155GE	158[c], 165[d]				(23)		(23)	
156ND	159[c]				(23)			
156NG	159[c]		✓	✓	(23)			
156NS	159[c]			✓	(23)			
156NY	159[c]				(23)			
186AK	185[b], 189[c]				(23)	(24)		
186AQ	185[b], 189[c]				(23)	(24)		
190SD	189[b], 193[c]				(23)	(24)		
190SN	189[b], 193[c]				(23)	(24)		
222DE								(9)
222DG								(9)
223QL	222[b]							(10)
223QR	222[b]							(8)
224EA		✓	✓	✓				(9)
225GS	224[b]							(10)
152VT156NS				✓				
187DE222DG								(9)
155GE224EA								SP
222DG224EA								(9)
223QL225GS								(10)

[a]Genetic differences were identified from an amino acid alignment of A(H1N1) viruses that circulated between 1918 and 2009, including 11 pre-1957 human and five swine A(H1N1) viruses (data not shown). Subtype number conversion is indicated where appropriate; [b]H5, [c]H3, [d]influenza B. [e]Unpublished results. SP Substitution predicted to change receptor binding specificity based on structural modeling (data not shown). Numbers between brackets refer to previous studies listed in the references.

797

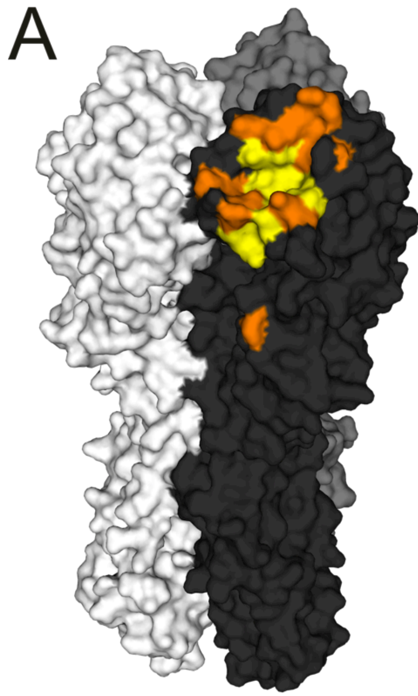
TABLE 2 Agglutination of TRBCs by viruses with wildtype or mutant HAs [a].

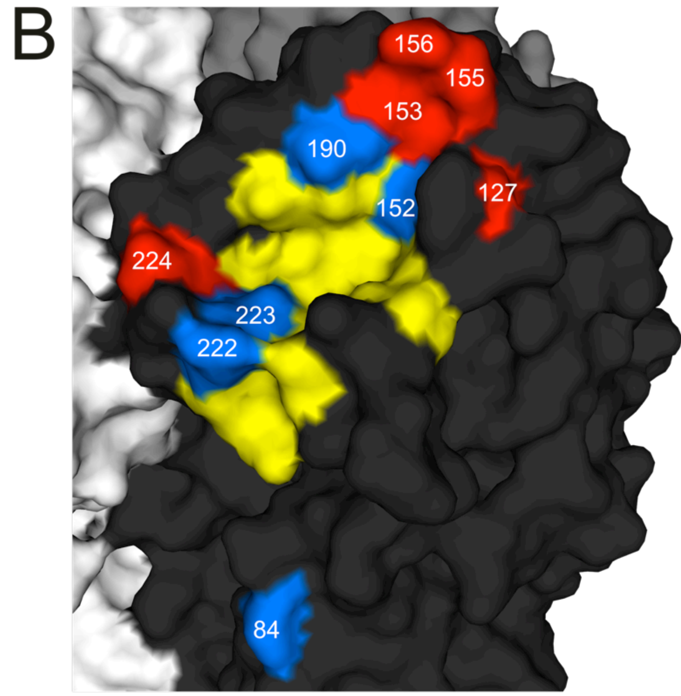
Antigen	HA titer			
	TRBC	VCNA	α2,3-TRBC	α2,6-TRBC
A/Netherlands/602/09	512	0	0	32
127DT	128	0	0	32
153KE	128	0	1	64
155GE	512	0	1	16
156ND	512	0	2	128
156NG	256	0	0	4
156NS	256	0	0	2
156NY	64	0	0	32
224EA	128	0	0	64
152VT156NS	128	0	0	8
155GE224EA	1024	0	0	1024
A/Vietnam/1194/2004	128	0	256	0
A/Netherlands/213/2003	256	0	0	256

[a]Hemagglutination titers are expressed as the HAU with unmodified TRBCs, TRBCs stripped from SAs using VCNA, or TRBC re-sialylated to contain either α2,3 or α2,6- SA. A/Vietnam/1194/2004 and A/Netherlands/213/2003 served as typical avian or human viruses with α2,3 or α2,6- SA preference, respectively.

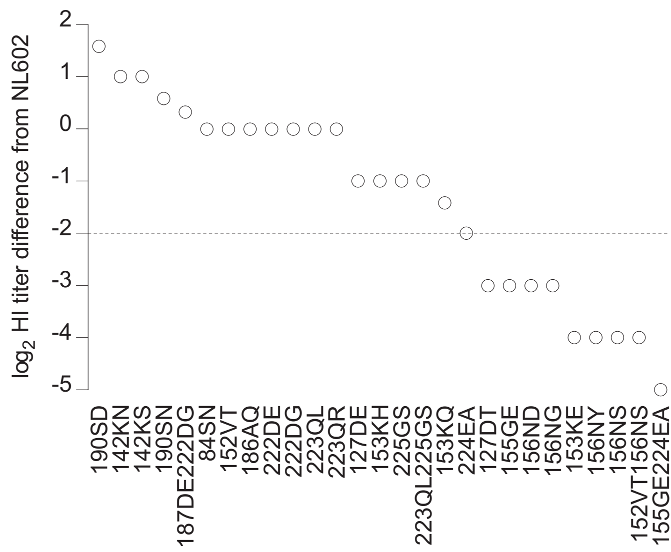
798

799





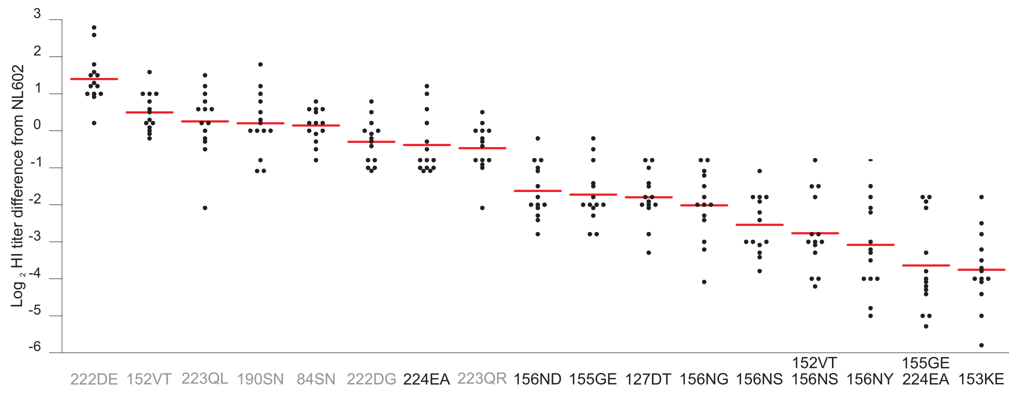
800 **FIG 1**



801

802 **FIG 2**

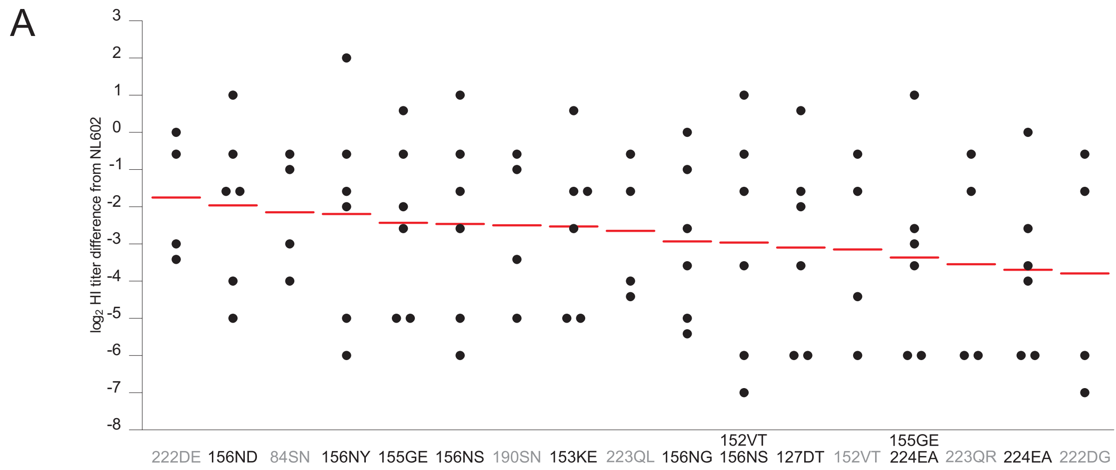
A



B

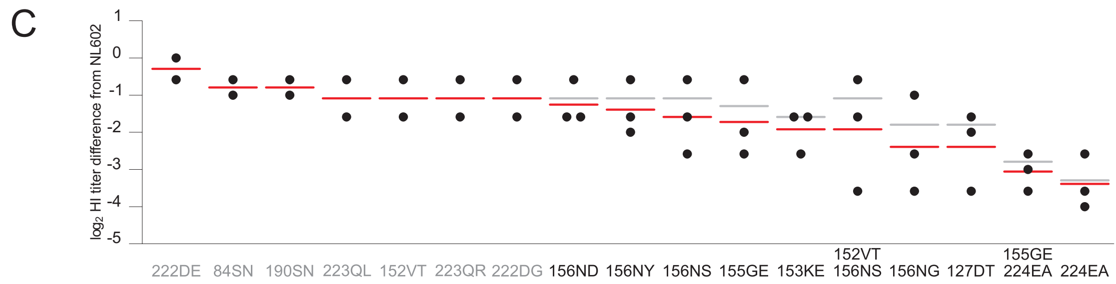
	NL602	222DE	152VT	223QL	190SN	84SN	222DG	224EA	223QR	156ND	155GE	127DT	156NG	156NS	152VT 156NS	156NY	155GE 224EA	153KE
NL/151B/11	2217	7680	2715	2560	5120	1280	3840	2560	1920	1920	1280	640	1280	640	1280	1280	640	320
NL/219A/11	2217	2560	1920	1920	2560	2560	1920	1280	1280	1280	1920	1280	1280	640	640	640	640	640
NL/2001B/09	1109	7680	2217	2560	3840	1280	1280	2560	1280	640	320	640	480	320	160	240	80	120
NL/151A/11	1810	5120	1920	1280	2560	1280	2560	1280	960	640	640	480	640	480	640	640	480	160
NL602A	2217	5120	3840	3840	3840	2560	1280	1280	1280	640	320	640	640	480	320	240	113	160
NL/219B/11	1358	2560	1280	320	640	1280	1280	640	320	640	960	480	640	640	480	320	320	240
NL/007A/10	2560	5120	2560	5120	2560	2560	2560	3840	1280	640	960	640	640	480	320	320	160	160
NL/2001A/09	1280	7680	3840	1280	2560	1280	1280	2560	960	320	320	640	320	160	80	80	60	80
NL/007B/10	1568	3840	1810	1280	1920	1280	1280	1280	1280	320	320	160	320	160	160	160	160	120
NL602B	2560	5120	5120	3840	2560	3840	1280	1280	2560	640	640	640	640	320	320	160	139	160
CAL/004A/09	2560	5120	3840	3840	2560	3840	1280	1280	2560	1280	640	960	320	320	320	160	80	120
CAL/004B/09	2560	7680	5120	3840	2560	3840	1920	1280	2560	480	640	640	480	240	160	80	80	80
CAL/007A/09	2715	7680	3840	7680	1280	3840	1280	1280	3840	640	640	640	160	320	320	240	160	160
CAL/007B/09	2217	5120	2560	2560	1280	3840	1280	1280	1280	320	320	320	240	160	120	80	57	40

FIG 3



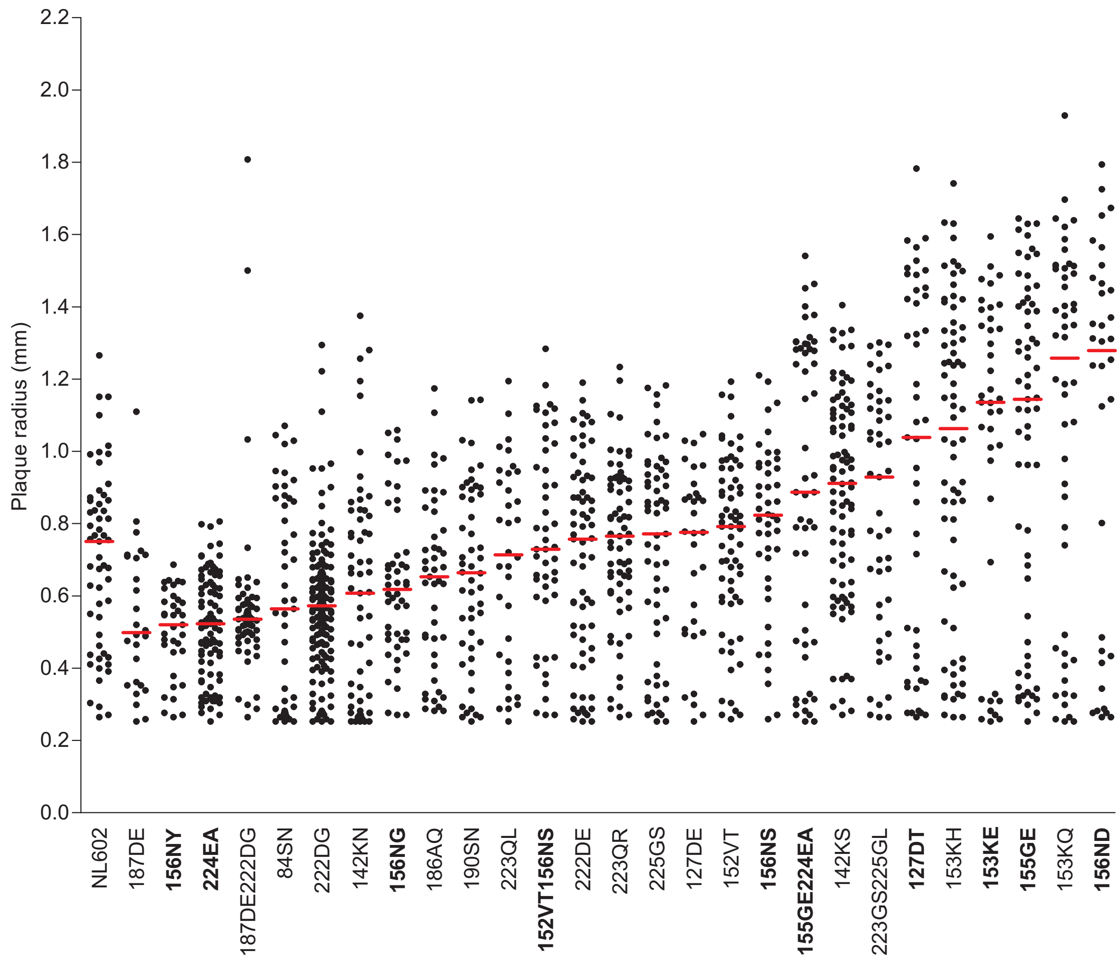
B

	NL602	222DE	156ND	84SN	156NY	155GE	156NS	190SN	153KE	223QL	156NG	152VT 156NS	127DT	152VT	155GE 224EA	223QR	224EA	222DG
serum 7	160	NT	320	NT	640	240	320	NT	240	NT	160	320	240	NT	320	NT	160	NT
serum 14	480	480	320	320	320	320	320	320	160	320	240	320	160	320	80	320	80	320
serum 25	1920	NT	640	NT	480	320	320	NT	320	NT	160	160	160	NT	160	NT	160	NT
serum 11	1920	1280	640	960	640	480	640	960	640	640	320	640	480	640	240	640	120	640
serum 6	1280	160	80	160	40	40	40	120	40	80	30	10	20	60	20	20	20	<10
serum 16	640	60	20	40	10	20	10	20	20	30	20	<10	<10	10	<10	<10	<10	<10



805

806 **FIG 4**



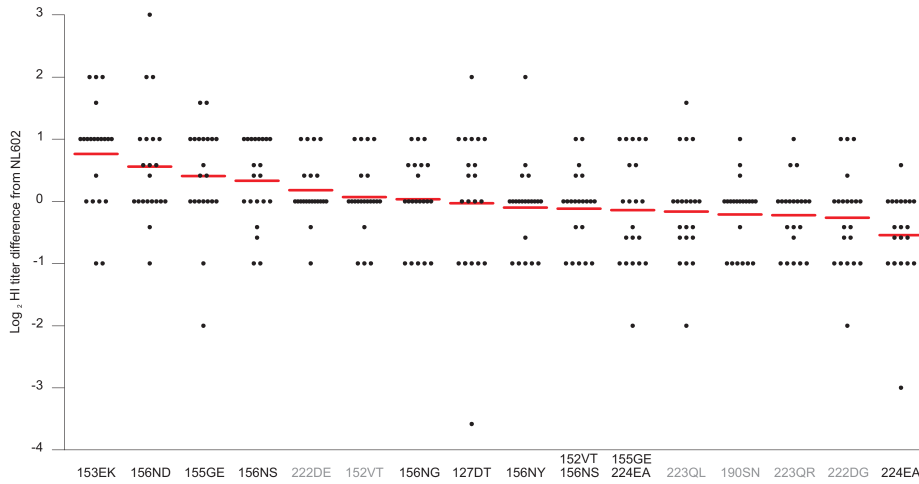
807

808 **FIG 5**

A

	USSR77	NL78	TA89	NL87	NC99	NL99	NL03	NL06	SS06	NL09
55 2273	960	960	640	1280	160	1280	160	320	160	640
34 25621	640	1280	640	1280	<10	640	<10	160	10	160
18 12105	20	40	80	160	320	160	320	640	160	160
19 13519	<10	<10	320	640	80	640	160	160	160	160
24 4717	240	160	640	5120	1280	1920	640	1280	1280	480
40 23692	160	640	160	320	80	160	80	80	80	80
50 24601	40	40	160	160	80	80	80	160	40	40
07 10613	80	160	320	640	320	640	320	640	320	160
29 4404	40	80	640	1280	320	640	160	480	320	120
37 1493	1280	1280	2560	5120	<60	5120	160	2560	640	640
46 26710	640	640	80	1280	<10	320	<10	320	40	160
32 26442	<10	20	640	1280	160	1280	160	320	160	40
43 1077	2560	1920	1280	5120	30	5120	<10	1280	160	1280
43 1849	960	640	1280	2560	<10	2560	<10	640	80	640
50 2824	320	480	320	1280	<10	640	20	160	<10	240
37 25032	640	1280	<30	40	<30	80	<30	<30	<30	640
07 2965	<10	10	<10	60	120	40	80	160	40	40
41 2453	160	160	10	40	<10	20	<10	<10	<10	10
85 3763	<10	10	10	40	40	<10	20	40	<10	<10
18 21877	40	60	320	640	320	640	160	640	320	80
52 24131	80	320	10	160	640	160	240	80	<10	320

B



C

	NL602	153EK	156ND	155GE	156NS	222DE	152VT	156NG	127DT	156NY	152VT 156NS	155GE 224EA	223QL	190SN	223QR	222DG	224EA
55 2273	1280	3840	1920	3840	2560	2560	2560	1920	1280	1920	2560	1920	3840	2560	1920	1280	1920
34 25621	640	1280	1280	1280	960	640	1280	640	640	640	640	640	640	640	640	1280	640
18 12105	80	80	80	160	160	160	80	160	160	320	80	160	160	80	120	160	80
19 13519	40	160	320	80	80	80	80	80	80	40	80	80	80	40	80	80	40
24 4717	160	640	640	320	320	160	80	320	640	160	160	320	160	160	160	160	160
40 23692	160	320	320	160	240	160	320	160	80	160	240	160	160	160	160	160	160
50 24601	80	160	80	80	80	80	80	80	40	80	80	120	80	80	80	80	80
07 10613	160	320	240	160	160	160	80	160	320	160	160	320	160	160	160	160	120
29 4404	80	160	160	160	160	160	80	120	160	80	80	160	160	40	80	40	80
37 1493	3840	7680	3840	5120	5120	5120	5120	5120	5120	5120	3840	2560	2560	3840	3840	2560	2560
46 26710	480	960	640	640	640	640	640	480	640	640	640	320	320	640	320	640	320
32 26442	160	640	640	320	320	160	160	160	320	160	160	80	160	80	160	80	80
43 1077	2560	5120	2560	5120	5120	2560	2560	3840	3840	2560	1920	2560	1280	3840	1920	1920	1920
43 1849	1280	2560	2560	3840	2560	1280	1280	1920	1280	1280	960	1280	640	1280	960	960	960
50 2824	640	1280	960	960	640	640	640	640	960	640	640	160	160	320	320	640	80
37 25032	3840	5120	3840	3840	2560	5120	3840	3840	320	2560	5120	2560	2560	3840	3840	2560	2560
07 2965	640	640	640	640	640	640	640	320	320	320	320	480	480	480	480	640	320
41 2453	320	320	320	320	320	320	320	160	160	160	160	160	240	160	160	160	160
85 3763	80	40	40	20	40	80	80	40	80	40	40	40	80	80	40	40	40
18 21877	640	640	640	640	480	320	480	320	320	320	320	320	480	320	320	320	320
52 24131	2560	1280	1920	1280	1280	1920	1280	1280	1280	1280	1280	1280	1280	1280	1280	640	1280

809

810 **FIG 6**