TABLE 3 Amino acid substitutions of escape mutants HAs which affect reactivity with each MAb

MAb	Amino acid substitution(s)[a] in each antigenic site			
	Sa site	Sb site	Ca2 site	Pa site
n3	P141L; N142D; K171E; K171N; K171T; G172E; G172V; K180E; K180N			
n4	P141T; P141L; N142D; H143Y; K171E; K171N; K171T; K171Q; G172E; G172V; S174L; K180E; K180N			
n5	P141L; N142D; H143Y; K171E; K171N; K171T; K171Q; G172E; G172V; S174L; K180E; K180N			
n6	P141L; N142D; H143Y; K171E; K171N; K171T; K171Q; G172E; G172V; S174L; K180E; K180N			
n7	P141L; N142D; K171E; K171N; K171T; K171Q; G172E; G172V; S174L; K180E; K180N			
n8	P141L; N142D; K171E; K171N; K171T; K171Q; G172E; G172V; S174L; K180E; K180N			
n9	P141T; P141L; N142D; K171E; K171N; K171T; K171Q; G172E; G172V; S174L; K177N; K177T	N173I; N173D		
n10	P141T; P141L; N142D; K171E; K171N; K171T; K171Q; G172E; G172V; S174L; K177N; K177T	N173I; N173D		
n11	P141T; P141L; N142D; K171E; K171N; K171T; K171Q; G172E; G172V; K177T; K177E	N173I; N173D		
n12	P141T; P141L; N142D; K171E; K171N; K171T; K171Q; G172E; G172V; K177N; K177T; K177E	N173I		
n13	P141T; P141L; N142D; K171E; K171N; K171T; K171Q; G172E; G172V	N173I		
n15	G172V	K170E; K170Q; K170T; N173I; N173D; S202N; Q206E; Q210L; Q210E; N211D; A212E		
n16		K170E; K170Q; K170T; N173I; N173D; S202N; Q206E; Q210L; N211D; A212E		
n17			A151V; A151G; P154S; A156V; A156T; A156D; G157E; A158E; K159N; S200P	
n18			A151G; P154S; P154T; A156D; G157E; G157R; A158E; A158V; K159N; S200P; R238K	K147N; K147Q
n2	G172E	K170E	K159N	K147N; K147Q

[a] All mutations that decreased the HI titer of each MAb at least 8-fold compared to the titer with the parent virus.

DISCUSSION

The prototypes of the pdm09 virus are A/California/4/2009 (MDCK isolate) and A/California/7/2009 (egg isolate). The primary sequence of A/Narita/1/2009 HA1 is similar to that of the prototype viruses (Fig. 1). HA1 from A/Narita/1/2009 differs from A/California/4/2009 and A/California/7/2009 by two amino acids and one amino acid, respectively. These differences were not observed in the antigenic region, and the reactivity of the ferret antiserum against A/California/7/2009 is similar among those viruses (data not shown). Therefore, we have analyzed the antigenic structure of HA of A/Narita/1/2009, as a representative pdm09 virus, using 16 anti-HA MAbs and their escape mutants. Most epitopes of the MAbs were located in the antigenic sites Sa, Sb, and Ca2. In addition, epitope n2 was defined in a novel antigenic site (Pa) and was proximal to the receptor-binding site (Fig. 2).

Based on the antigenic analysis of PR8-Mt. Sinai HA, sites Sa and Sb are defined as operationally distinct areas that are separated by residues 170/173 of the Sb site and 172/174 of the Sa site, which lie in the same region of the polypeptide chain (10). Although sites Sa and Sb are contiguous and have been suggested to share a close linkage, simultaneous binding of antibodies to each site has not been identified (10, 11, 20). However, a study of the crystal structure of human MAb 2DI in complex with the 1918 pandemic HA demonstrated that the epitope of MAb 2DI, which is derived from survivors of the 1918 pandemic, contained the residues at positions 142 (in the Sa site) and 171 to 183 (in the Sa

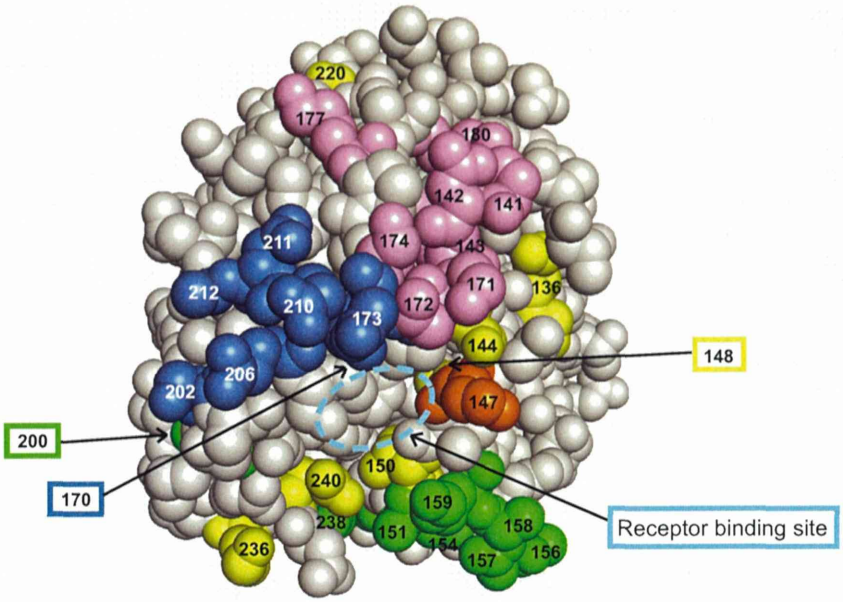

FIG 2 Antigenic structure of HA of A/Narita/1/2009. Positions of amino acid changes in the escape mutants are located on the globular head of HA of A/Narita/1/2009, shown with numbering. The three-dimensional structure of A/Narita/1/2009 HA was generated by the protein structure homology-modeling server "SWISS MODEL" using the coordinates of A/California/4/2009 (PDB ID 3al4A). The image was created using the software program PyMOL. The residues at each antigenic site are colored as pink for the Sa site, sky blue for the Sb site, green for the Ca2 site, and orange for residue 147. The residues discussed in the text are colored yellow.

and Sb sites). Similarly, in this study, epitopes of mouse MAbs n9 to n13 and n15 were composed of residues in the Sa and Sb sites (16).

Based on the residues of epitopes n2 and EM4C04 (13), we have determined that the novel antigenic site (Pa) of pdm09 HA is between sites Sa, Sb, and Ca2 and residue 147. In a previous study using mouse MAbs, this epitope in the Pa site was not identified in PR8-Mt. Sinai HA (20). We therefore considered that the Pa antigenic site may be specific to pdm09 HA. Recently, epitopes of broadly neutralizing human MAbs, i.e., 5J8 and CH65, were found around the receptor-binding site (15, 16). MAb 5J8 was derived from a healthy, middle-aged woman and had HI activity against A/South Carolina/1/1918 and A/California/4/2009. Epitope 5J8 is composed of residues 147, 151, and 236, which are near epitope n2. Because residue 151 is near residue 159, epitope 5J8 may partially overlap epitope n2 and comprise the Pa site. In contrast, MAb CH65 was obtained by rearranging the heavy- and light-chain genes derived from a subject immunized with the 2007

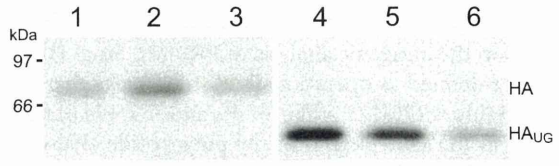

FIG 3 Immunoprecipitation analysis of the HA molecules of escape mutants carrying amino acid substitutions at position 177. MDCK cells infected with parental virus (lanes 1 and 4) and mutants with the K177T (lanes 2 and 5) and K177N (lanes 3 and 6) substitutions were labeled with [^{35}S]methionine for 30 min in the absence of tunicamycin (lanes 1 to 3) or for 60 min in the presence of tunicamycin (lanes 4 to 6) at 7 h postinfection. Cells were immunoprecipitated with MAb n17 to site Ca2, and the resulting precipitates were analyzed by SDS-PAGE.

trivalent vaccine, including the seasonal H1N1 virus lacking residue 147 of HA. The crystal structure of the complex between CH65 and A/Solomon Islands/3/2006 HA, which had a deletion of residue 147, suggested that residues 150, 151, and 240, which were located on the right edge of the receptor-binding site, were involved in the interaction. A neutralization assay showed that among the historical H1N1 viruses, the viruses with an insertion at position 147 were resistant to CH65. Taking these results into consideration, the region around position 147 is presumably a component of epitopes for human antibodies against H1HA. Considering the location of residue 147, a mutation at this position may significantly affect the receptor-binding ability. However, in this study, two escape mutants of Narita/1/2009, carrying the mutations K147N and K147Q, were isolated using MAb n2. In another study, a similar escape mutant of A/California/4/2009, with K147Q, was selected by human MAb 5J8 (15). During the circulation of the H1N1 seasonal influenza viruses in 1947 to 1957 and 1977 to 1995, only mutations K to R and R to K were recognized, and residue 147 was deleted after 1995. Similarly, residue 144, which is located near position 147 of older H1N1 viruses, was also deleted immediately before the disappearance of these viruses in 1957 (21). Deletions of residues in close proximity to the receptor-binding site of HA seem preferable for the escape of H1N1 viruses from immune selection in the human population. It is well known that the glycosylation of HA prevents its neutralization by antibodies. A previous study implied that pdm09 HA modified with additional glycosylation sites at positions 142 and 177 was resistant to neutralization by antibodies to wild-type HA (22). Therefore, glycosylation at these positions has implications for the antigenic drift of pdm09 viruses. However, as indicated in this study, the asparagine at position 177 was not glycosylated, even though the mutation affected reactivity with the MAb.

TABLE 4 Antigenic analysis of pdm09 natural isolates in Yamagata Prefecture and seasonal H1N1 viruses using representative MAbs

Virus strain	Amino acid change(s)[a]	HI titer[b]							
		MAbs of respective antigenic site							
		Sa			Sb	Ca2		Pa	Postinfection ferret antiserum against A/California/7/2009
		n3	n7	n10	n16	n17	n18	n2	
pdm09 natural isolates									
A/Narita/1/2009		12,800	640	6,400	25,600	25,600	25,600	640	1,280
2009-2010 season									
A/Yamagata/232/2009	S220T	6,400	320	3,200	25,600	12,800	12,800	640	1,280
A/Yamagata/752/2009	G172E, K188R, S220T, A214T	<[c]	<	<	6,400	6,400	3,200	80	640
A/Yamagata/143/2010	V36I, D52G, A203T, S220T	12,800	320	3,200	12,800	12,800	25,600	320	640
2010-2011 season									
A/Yamagata/203/2011	V64I, A151T, A158S, S200P, S220T, I312V	6,400	640	6,400	12,800	<	160	640	1,280
A/Yamagata/206/2011	K136N, S160G, S202T, S220T, A214T	6,400	640	6,400	1,600	2,560	2,560	640	1,280
2012-2013 season									
A/Yamagata/264/2012	D114N, S202T, S220T, F227S, V251I, K300E	6,400	320	1,600	320	12,800	12,800	320	640
A/Yamagata/87/2013	S101G, S160G, K180I, S202T, A214T, S220T	20	80	1,600	320	12,800	12,800	320	640
Seasonal H1N1 viruses[d]									
A/Solomon Islands/3/2006		<	<	<	<	40	20	<	NT[e]
A/Brisbane/59/2007		<	<	<	<	<	<	<	NT

[a] Amino acid differences in HA1 between pdm09 natural isolates and A/Narita/1/2009 are shown.
[b] The HI test was performed with 0.5% turkey erythrocytes. The HI titer is expressed as the reciprocal of the highest antibody dilution which completely inhibited hemagglutination.
[c] <, less than 20.
[d] Amino acid sequences of seasonal H1N1 viruses are shown in Fig. 1.
[e] NT, not tested.

Amino acid changes at positions 170 to 174 have been identified after the cell culture adaptation of pdm09 viruses (9, 23, 24). In this study, the variants with G172E comprised one-third of all of the mutants. However, each variant lost reactivity with the MAbs that were used for the selection of the variant, suggesting that variants with G172E were not selected during adaptation to MDCK cells.

Since the emergence of the pdm09 virus as a pandemic virus in 2009, stringent surveillance of pdm09 viruses has been applied to detect antigenic variants. From 2009 to 2013, amino acid changes at positions 220 (Ca1 site) and 202 (Sb site) were found in the mainstream of the evolutionary pathway of pdm09 HA (Fig. 4). However, as shown in Table 4, hemagglutination abilities of natural isolates with the mutations S202T and S220T were efficiently inhibited by postinfection ferret sera against the A(H1N1)pdm09 virus. No antigenic differences from the vaccine strain, A/California/7/2009, were reported for the natural isolates in the various region of the world in 2013 (25, 26). In the case of the reappearance of H1N1 viruses in 1977, several antigenic variants, A/Lackland/3/78 and A/Brazil/11/78, showed 4- to 8-fold decreases in reactivity with the postinfection ferret sera against A/USSR/90/77. These variants also failed to react with MAb 264 against A/USSR/90/77 HA (27, 28). Using binding assays of MAb 264 with mutant HA, we have identified E233K as a mutation responsible for the

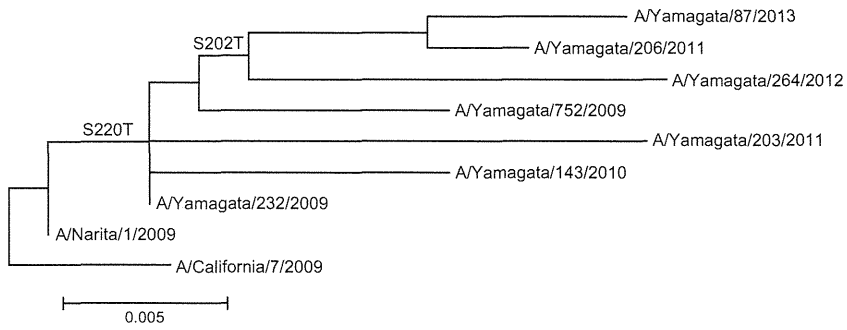

FIG 4 Phylogenetic tree of the HA1 polypeptide of the natural isolates from 2009 to 2013 that were used in this study. A phylogenetic tree was constructed using the neighbor-joining method in the CLUSTAL W software program, version 2.1. Numbers refer to the mainstream amino acid changes that were fixed in most of the subsequent strains.

antigenic drift of A/USSR/90/77 (29). In fact, this mutation was found in the mainstream of the evolutionary pathway (30). It is difficult to identify such a crucial mutation in antigenic variants, which usually possess extra mutations unrelated to antigenicity. In the present study, we identified the residues that composed the epitopes of 16 MAbs against A/Narita/1/2009 HA. The use of such MAbs should be helpful in determining critical amino acid substitutions for the antigenic drift of A(H1N1)pdm09 viruses, once such drift occurs. Consequently, these findings may help the WHO to make recommendations for the next vaccine virus among pdm09 H1N1 viruses.

ACKNOWLEDGMENTS

This study was supported in part by a Health Labor Sciences Research Grant, Research on Emerging and Re-emerging Infectious Diseases (to E.N.; no. H22-//Shinko-Ippan-001//) from the Ministry of Health, Labor and Welfare of Japan.

We are greatly indebted to Katsuhisa Nakajima for helpful suggestions and valuable discussions. We thank Yasushi Suzuki for preparing the image of 3-D structure and Yoko Kadowaki for her assistance with sequence analysis.

We declare that we have no potential conflicts of interests related to this article.

REFERENCES

1. Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, Ma SK, Cheung CL, Raghwani J, Bhatt S, Peiris JS, Guan Y, Rambaut A. 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 459:1122–1125. http://dx.doi.org/10.1038/nature08182.
2. World Health Organization. 2010. Recommended viruses for influenza vaccines for use in the 2010-2011 northern hemisphere influenza season. Wkly. Epidemiol. Rec. 85:81–92.
3. World Health Organization. 2011. Influenza A(H1N1) 2009 virus: current situation and post-pandemic recommendations. Wkly. Epidemiol. Rec. 86:61–72.
4. Nakajima K, Desselberger U, Palese P. 1978. Recent human influenza A (H1N1) viruses are closely related genetically to strains isolated in 1950. Nature 274:334–339. http://dx.doi.org/10.1038/274334a0.
5. Kendal AP, Joseph JM, Kobayashi G, Nelson D, Reyes CR, Ross MR, Sarandria JL, White R, Woodall DF, Noble GR, Dowdle WR. 1979. Laboratory-based surveillance of influenza virus in the United States during the winter of 1977-1978. I. Periods of prevalence of H1N1 and H3N2 influenza A strains, their relative rates of isolation in different age groups, and detection of antigenic variants. Am. J. Epidemiol. 110:449–461.
6. Dapat IC, Dapat C, Baranovich T, Suzuki Y, Kondo H, Shobugawa Y, Saito R, Suzuki H. 2012. Genetic characterization of human influenza viruses in the pandemic (2009-2010) and post-pandemic (2010-2011) periods in Japan. PLoS One 7:e36455. http://dx.doi.org/10.1371/journal.pone.0036455.
7. Barr IG, Cui L, Komadina N, Lee RT, Lin RT, Deng Y, Caldwell N, Shaw R, Maurer-Stroh S. 2010. A new pandemic influenza A(H1N1) genetic variant predominated in the winter 2010 influenza season in Australia, New Zealand and Singapore. Euro Surveill. 15:19692.
8. Strengell M, Ikonen N, Ziegler T, Julkunen I. 2011. Minor changes in the hemagglutinin of influenza A(H1N1)2009 virus alter its antigenic properties. PLoS One 6:e25848. http://dx.doi.org/10.1371/journal.pone.0025848.
9. Guarnaccia T, Carolan LA, Maurer-Stroh S, Lee RT, Job E, Reading PC, Petrie S, McCaw JM, McVernon J, Hurt AC, Kelso A, Mosse J, Barr IG, Laurie KL. 2013. Antigenic drift of the pandemic 2009 A(H1N1) influenza virus in A ferret model. PLoS Pathog. 9:e1003354. http://dx.doi.org/10.1371/journal.ppat.1003354.
10. Caton AJ, Brownlee GG, Yewdell JW, Gerhard W. 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell 31:417–427. http://dx.doi.org/10.1016/0092-8674(82)90135-0.
11. Gerhard W, Yewdell J, Frankel ME, Webster R. 1981. Antigenic structure of influenza virus haemagglutinin defined by hybridoma antibodies. Nature 290:713–717. http://dx.doi.org/10.1038/290713a0.
12. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, Sessions WM, Xu X, Skepner E, Deyde V, Okomo-Adhiambo M, Gubareva L, Barnes J, Smith CB, Emery SL, Hillman MJ, Rivailler P, Smagala J, de Graaf M, Burke DF, Fouchier RA, Pappas C, Alpuche-Aranda CM, Lopez-Gatell H, Olivera H, Lopez I, Myers CA, Faix D, Blair PJ, Yu C, Keene KM, Dotson PD, Jr, Boxrud D, Sambol AR, Abid SH, St George K, Bannerman T, Moore AL, Stringer DJ, Blevins P, Demmler-Harrison GJ, Ginsberg M, Kriner P, Waterman S, Smole S, Guevara HF, Belongia EA, Clark PA, Beatrice ST, Donis R, Katz J, Finelli L, Bridges CB, Shaw M, Jernigan DB, Uyeki TM, Smith DJ, Klimov AI, Cox NJ. 2009. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science 325:197–201. http://dx.doi.org/10.1126/science.1176225.
13. O'Donnell CD, Vogel L, Wright A, Das SR, Wrammert J, Li GM, McCausland M, Zheng NY, Yewdell JW, Ahmed R, Wilson PC, Subbarao K. 2012. Antibody pressure by a human monoclonal antibody targeting the 2009 pandemic H1N1 virus hemagglutinin drives the emergence of a virus with increased virulence in mice. mBio 3(3):e00120–00112. http://dx.doi.org/10.1128/mBio.00120-12.
14. Rudneva I, Ignatieva A, Timofeeva T, Shilov A, Kushch A, Masalova O, Klimova R, Bovin N, Mochalova L, Kaverin N. 2012. Escape mutants of pandemic influenza A/H1N1 2009 virus: variations in antigenic specificity and receptor affinity of the hemagglutinin. Virus Res. 166:61–67. http://dx.doi.org/10.1016/j.virusres.2012.03.003.
15. Krause JC, Tsibane T, Tumpey TM, Huffman CJ, Basler CF, Crowe JE, Jr. 2011. A broadly neutralizing human monoclonal antibody that recognizes a conserved, novel epitope on the globular head of the influenza H1N1 virus hemagglutinin. J. Virol. 85:10905–10908. http://dx.doi.org/10.1128/JVI.00700-11.
16. Whittle JR, Zhang R, Khurana S, King LR, Manischewitz J, Golding H, Dormitzer PR, Haynes BF, Walter EB, Moody MA, Kepler TB, Liao HX, Harrison SC. 2011. Broadly neutralizing human antibody that recognizes the receptor-binding pocket of influenza virus hemagglutinin. Proc. Natl. Acad. Sci. U. S. A. 108:14216–14221. http://dx.doi.org/10.1073/pnas.1111497108.
17. Nakajima S, Nakajima K, Nobusawa E, Zhao J, Tanaka S, Fukuzawa K. 2007. Comparison of epitope structures of H3HAs through protein modeling of influenza A virus hemagglutinin: mechanism for selection of antigenic variants in the presence of a monoclonal antibody. Microbiol. Immunol. 51:1179–1187. http://dx.doi.org/10.1111/j.1348-0421.2007.tb04013.x.
18. Nobusawa E, Sato K. 2006. Comparison of the mutation rates of human influenza A and B viruses. J. Virol. 80:3675–3678. http://dx.doi.org/10.1128/JVI.80.7.3675-3678.2006.
19. Tsuchiya E, Sugawara K, Hongo S, Matsuzaki Y, Muraki Y, Li ZN, Nakamura K. 2001. Antigenic structure of the haemagglutinin of human influenza A/H2N2 virus. J. Gen. Virol. 82:2475–2484.
20. Lubeck MD, Gerhard W. 1981. Topological mapping antigenic sites on the influenza A/PR/8/34 virus hemagglutinin using monoclonal antibodies. Virology 113:64–72. http://dx.doi.org/10.1016/0042-6822(81)90136-7.
21. Nakajima S, Nishikawa F, Nakajima K. 2000. Comparison of the evolution of recent and late phase of old influenza A (H1N1) viruses. Microbiol. Immunol. 44:841–847. http://dx.doi.org/10.1111/j.1348-0421.2000.tb02572.x.
22. Wei CJ, Boyington JC, Dai K, Houser KV, Pearce MB, Kong WP, Yang ZY, Tumpey TM, Nabel GJ. 2010. Cross-neutralization of 1918 and 2009 influenza viruses: role of glycans in viral evolution and vaccine design. Sci. Transl. Med. 2:24ra21. http://dx.doi.org/10.1126/scitranslmed.3000799.
23. Yang H, Carney P, Stevens J. 2010. Structure and receptor binding properties of a pandemic H1N1 virus hemagglutinin. PLoS Curr. 2:RRN1152. http://dx.doi.org/10.1371/currents.RRN1152.
24. Chen Z, Wang W, Zhou H, Suguitan AL, Jr, Shambaugh C, Kim L, Zhao J, Kemble G, Jin H. 2010. Generation of live attenuated novel influenza virus A/California/7/09 (H1N1) vaccines with high yield in embryonated chicken eggs. J. Virol. 84:44–51. http://dx.doi.org/10.1128/JVI.02106-09.
25. World Health Organization. 2013. Review of the 2012-2013 winter influenza season, northern hemisphere. Wkly. Epidemiol. Rec. 88:225–232.
26. World Health Organization. 2013. Review of the 2013 influenza season in the southern hemisphere. Wkly. Epidemiol. Rec. 88:509–520.
27. Webster RG, Kendal AP, Gerhard W. 1979. Analysis of antigenic drift

in recently isolated influenza A (H1N1) viruses using monoclonal antibody preparations. Virology **96**:258–264. http://dx.doi.org/10.1016/0042-6822(79)90189-2.

28. **Nakajima S, Kendal AP.** 1981. Antigenic drift in influenza A/USSR/90/77(H1N1) variants selected in vitro with monoclonal antibodies. Virology **113**:656–662. http://dx.doi.org/10.1016/0042-6822(81)90194-X.
29. **Nobusawa E, Nakajima K, Nakajima S.** 1987. Determination of the epitope 264 on the hemagglutinin molecule of influenza H1N1 virus by site-specific mutagenesis. Virology **159**:10–19. http://dx.doi.org/10.1016/0042-6822(87)90342-4.
30. **Raymond FL, Caton AJ, Cox NJ, Kendal AP, Brownlee GG.** 1983. Antigenicity and evolution amongst recent influenza viruses of H1N1 subtype. Nucleic Acids Res. **11**:7191–7203. http://dx.doi.org/10.1093/nar/11.20.7191.