Table 2 | Frequency of multiple mutations in nine amino acids of the H1N1pdm HA in egg-passaged (P1 and P6), and MDCK-passaged samples derived from #1.

		119	125	129	133	183	187	215	222	223	Total (%)
Egg (P1)	Wild type	K	N	N	T	S	D	A	D	Q	4.04
	Mutation									R	91.5
									G		0.2
		N								R	0.4
				S						R	0.4
					S					R	0.4
						P				R	0.2
									G	R	2.42
		N							G	R	0.2
Egg (P6)	Wild type	K	N	N	T	S	D	A	D	Q	0
	Mutation		D								2.73
										R	2.12
			D				E				0.6
			D					V			0.3
			D						G		0.6
			D							R	75.8
							E			R	1.2
			D				E			R	9.09
			D					V		R	0.3
			D						G	R	5.76
			D			P			G	R	0.3
			D				E		G	R	1.2
MDCK	Wild type	K	N	N	T	S	D	A	D	Q	88.57
	Mutation	N									0.21
			D								0.21
				S							0.42
					S						0.21
							E				0.21
								V			5.61
									G		1.45
										R	1.45
							E			R	0.62
								V	G		0.42
			D				E			R	0.62

Critical substitutions such as K119N, N125D, N129S, D187E, A215V, D222G, Q223R, and two additional substitutions T133S and S183P, as controls, are shown.

the #2-P6 viruses (**Table 3**), and K119N, N125D, N129S, A215V, and D222G mutations found in the #3-P5 viruses predominated (**Table 4**). The N125D-Q223R (76%) genotype derived from #1 and K119N-N129S-D222G (45.5%), and N125D-D187E-Q223R (21.8%) genotypes derived from #2 and K119N-A215V-D222G (39%), N125D-D222G (17%), and N125D-N129S-D222G (11%) genotypes derived from #3 were also detected as major populations (**Tables 2–4**). The shift in HA genotype through passaging in eggs is assessed in **Figure 1**. Of particular interest, sequences containing double and/or triple amino acid mutations, which were abundantly detected in egg-passaged viruses, were also found as a minor population in its nasal specimen. For example, HA harboring N125D-D187E-Q223R (**Table 2**; **Figure 1**), K119N-N129S-D222G and N125D-Q223R (**Table 3**; **Figure 1**), or K119N-D222G (**Table 4**) was detected in #1, #2, and #3 clinical samples (Yasugi et al., 2012), respectively. These results suggest that egg-adapted viruses, likely represented by α2,3-linkage-tropic virus, were also present in human upper airways as a minor population and transmitted in humans during the outbreak of H1N1pdm. Because 2,6Gal expression is abundant and 2,3Gal expression is minor in epithelial cells of the upper respiratory tracts (Shinya et al., 2006), it might make sense that α2,3-linkage-tropic viruses exist as a minor population in human upper airways.

In contrast to the first wave, both the G222 and R223 variants had almost disappeared (0.01–0.07 and 0.47–0.63%, respectively) in five nasal swabs (#4 to #8) obtained from individuals with a mild case of H1N1pdm in December 2010 (second wave; Yasugi et al., 2012). We then inoculated the five specimens (#4 to #8) into embryonated chicken eggs and serially passaged them six times in eggs. Hemagglutination was faintly detected in samples in the

Table 3 | Frequency of multiple mutations in nine amino acids of the H1N1pdm HA in egg-passaged (P1 and P6), and MDCK-passaged samples derived from #2.

		119	125	129	133	183	187	215	222	223	Total (%)
Egg (P1)	Wild type	K	N	N	T	S	D	A	D	Q	2.94
	Mutation	N									0.29
			D								0.59
								V			2.35
									G		13.8
										R	65.6
		N								R	0.29
			D						G		9.41
			D							R	2.06
				S						R	0.29
						P				R	0.59
							E			R	0.29
								V		R	0.29
									G	R	1.18
Egg (P6)	Wild type	K	N	N	T	S	D	A	D	Q	0.5
	Mutation	N									1.98
			D								0.5
									G		0.5
										R	0.5
		N		S							3.96
		N							G		6.93
			D				E				0.5
			D						G		0.99
			D							R	7.92
				S					G		3.96
				S						R	0.5
		N		S				V			0.5
		N		S					G		45.5
			D				E		G		0.5
			D				E			R	21.8
		N		S			E		G		0.5
		N		S			E			R	0.99
			D				E		G	R	1.48
MDCK	Wild type	K	N	N	T	S	D	A	D	Q	98.84
	Mutation				S						0.19
						P					0.19
								V			0.39
									G		0.39

Critical substitutions such as K119N, N125D, N129S, D187E, A215V, D222G, Q223R, and two additional substitutions T133S and S183P, as controls, are shown.

first passage (P0) but was detected in the third or fourth passage and showed 2^5–2^7 hemagglutination titers in the fifth or sixth passage (P5 or P6) in the four specimens except #8 (**Table 5**). PCR products amplifying the same region as #1 to #3 (first wave; Yasugi et al., 2012) were prepared and were examined using conventional direct PCR sequencing. The G222 (#4, #6) and R223 (#5 and #7) variants were detected even in P1 passages in the four specimens (**Table 6**), although both variants had very minor populations in nasal specimens. In addition, no other variants, except the above two amino acids, were detected (data not shown); thus, further high-throughput sequencing analysis was not performed in #4 to #8.

DISCUSSION

Previous work demonstrated that D222G and/or Q223R mutation led to the binding of α2,3-SA-resialylated chicken red blood cells (Chen et al., 2010). D222G and/or Q223R mutations might also be crucial for the tropism and/or pathogenesis of H1N1pdm. The function(s) of the other substitutions shown in **Tables 2–4** are largely unknown; however, significant similarities in the HA genotype between the viruses resident in upper airways (Yasugi et al., 2012) and egg-adapted viruses (**Tables 2–4**) suggest that the minor genotype of α2,3-linkage-tropic viruses in upper airways became dominant after passaging through chicken eggs. Viruses derived from #1 and

Table 4 | Frequency of multiple mutations in nine amino acids of the H1N1pdm HA in egg-passaged (P1 and P5) samples derived from #3.

		119	125	129	133	183	187	215	222	223	Total (%)
Egg (P1)	Wild type	K	N	N	T	S	D	A	D	Q	4
	Mutation	N									0.33
						P					35.7
									G		57.3
										R	0.67
		N				P					0.33
				S					G		0.33
						P			G		1.33
Egg (P5)	Wild type	K	N	N	T	S	D	A	D	Q	1.03
	Mutation	N									2.07
			D								2.07
									G		4.66
										R	0.52
		N	D								0.52
		N						V			5.7
		N							G		2.07
			D	S							1.55
			D						G		17.1
				S					G		0.52
								V	G		8.29
		N						V	G		38.9
			D	S				V			1.55
			D	S					G		10.9
			D					V	G		0.52
			D	S				V	G		2.07

Critical substitutions such as K119N, N125D, N129S, D187E, A215V, D222G, Q223R, and two additional substitutions T133S and S183P, as controls, are shown.

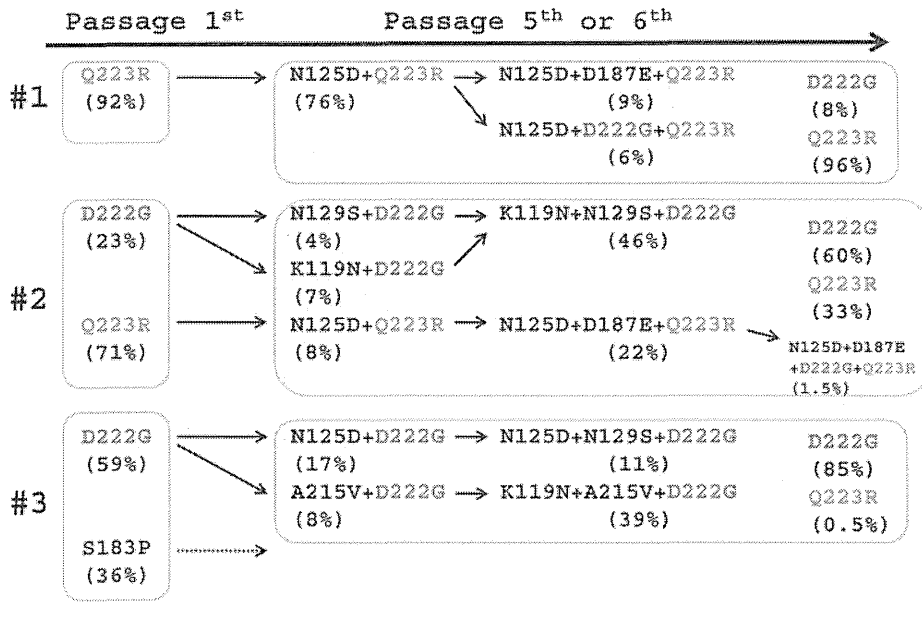

FIGURE 1 | Putative shift in HA genotype through the passage in eggs of H1N1pdm (first wave).

possessing HA containing N125D-Q223R and N125D-D187E-Q223R, viruses derived from #2 containing N125D-Q223R and K119N-N129S-D222G, and viruses derived from #3 containing K119N-D222G and N125D-S207N-D222G, were detected in both

Table 5 | HA titer of virus second wave H1N1pdm samples passaged in eggs.

Sample	HA titer (2 Log 10)						
	P0	P1	P2	P3	P4	P5	P6
#4	1	1	1	5	4	5	4
#5	1	6	7	7	6	7	7
#6	1	1	7	5	5	5	3
#7	1	7	7	5	6	6	5
#8	1	1	1	0	0	0	0

Table 6 | D222G and Q223R mutations on second wave H1N1pdm samples.

Sample	Passage					
	P0	P1	P3	P4	P5	P6
#4	No	D222G	D222G	No	D222G	No
#5	No	Q223R	Q223R	Q223R	Q223R	Q223R
#6	ND	D222G*	D222G	D222G	D222G	D222G
#7	No	Q223R	Q223R	Q223R	Q223R	Q223R

No, no mutation on 222 and 223 position.

ND, not done.

**Double peak= double peak as in GGT (wild type) and GAT (D to G) on position 222.*

clinical specimens and egg-passaged samples (**Figure 1**; Yasugi et al., 2012). These results suggest a direct linkage of α2,3-tropic viruses between the clinical samples and egg-adapted samples in each case.

However, such a direct linkage could not be observed in the second wave of H1N1pdm, because the G222 and R223 variants were almost undetected (0.01–0.07 and 0.47–0.63%, respectively) in five nasal swabs (#4 to #8; Yasugi et al., 2012). Thus, D222G or Q223R mutation may occur in inoculated eggs. Further investigations of clonal recombinant virus using reverse genetics are required to address this question. We also failed to rescue egg-adapted virus from #8 (**Table 5**). In addition, neither D222G nor Q223R genotype was detected in the sixth passage of #4 (**Table 6**), although egg-adapted viruses well grew (**Table 5**). These results might suggest that factor(s) other than mutation(s) in HA-RBS (Lu et al., 2005) are involved in egg adaptation.

We showed that MDCK-passaged virus isolates contain a similar ratio of α2,3-tropic and α2,6-tropic viruses compared to the original H1N1pdm (first wave) viruses identified in human specimens (**Tables 2** and **3**; Yasugi et al., 2012). Takemae et al. (2010) demonstrated that embryonated chicken egg-isolated classical H1 swine influenza viruses harbored substitutions including D187V/N and D222G in the HA, whereas MDCK isolates retained HA genes identical to those of the viruses present in the swine nasal swab samples. Passaging in MDCK cells may therefore be a better approach to establish genetic diversity and specific HA genotypes *in vivo* in human and swine influenza viruses. While egg adaptation is currently required to prepare vaccine candidates, alternative approaches involving the use of accredited anchorage-dependent and -independent preparations of the African Green monkey kidney (Vero), MDCK, and other cell lines have been pursued by several manufacturers in recent years (Audsley and Tannock, 2008). Our results, in this study, suggest the advantage of the cell-based influenza vaccine approach, which is able to maintain the genetic stability of clinical strains.

This study, together with our previous report (Yasugi et al., 2012), suggest that α2,3-SA-specific viruses, including G222 and R223, existed in humans as a minor population in the early phase (first wave) of the pandemic, and that D222 and Q223 became more dominant through human-to-human transmission (second wave) during the epidemic. Newly emergent influenza A viruses may have been dual specific but not exclusively α2,6-SA-specific during the early phase of the pandemic and adapted during multiple cycles of human-to-human transmission (Yasugi et al., 2012). Further investigation is required to determine the proportion of α2,3-tropic and α2,6-tropic viruses found in tissues and organs infected with other human-, avian-, and swine-derived influenza viruses. Deep sequencing approaches will provide a more comprehensive analysis of genetic diversity in egg- and MDCK-passaged viruses and original virus isolates *in vivo* and will help us to understand quasispecies of influenza viruses more precisely.

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REFERENCES

Audsley, J. M., and Tannock, G. A. (2008). Cell-based influenza vaccines: progress to date. *Drugs* 68, 1483–1491.

Chen, Z., Wang, W., Zhou, H., Suguitan, A. L. Jr., Shambaugh, C., Kim, L., Zhao, J., Kemble, G., and 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.

Cohen, J. (2009). Swine flu outbreak. Out of Mexico? Scientists ponder swine flu's origins. *Science* 324, 700–702.

Daidoji, T., Koma, T., Du, A., Yang, C. S., Ueda, M., Ikuta, K., and Nakaya, T. (2008). H5N1 avian influenza virus induces apoptotic cell death in mammalian airway epithelial cells. *J. Virol.* 82, 11294–11307.

Gambaryan, A. S., Robertson, J. S., and Matrosovich, M. N. (1999). Effects of egg-adaptation on the receptor-binding properties of human influenza A and B viruses. *Virology* 258, 232–239.

Gamblin, S. J., Haire, L. F., Russell, R. J., Stevens, D. J., Xiao, B., Ha, Y., Vasisht, N., Steinhauer, D. A., Daniels, R. S., Elliot, A., Wiley, D. C., and Skehel, J. J. (2004). The structure and receptor binding properties

of the 1918 influenza hemagglutinin. *Science* 303, 1838–1842.

Lu, B., Zhou, H., Ye, D., Kemble, G., and Jin, H. (2005). Improvement of influenza A/Fujian/411/02 (H3N2) virus growth in embryonated chicken eggs by balancing the hemagglutinin and neuraminidase activities, using reverse genetics. *J. Virol.* 79, 6763–6771.

Matrosovich, M., Tuzikov, A., Bovin, N., Gambaryan, A., Klimov, A., Castrucci, M. R., Donatelli, I., and Kawaoka, Y. (2000). Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. *J. Virol.* 74, 8502–8512.

Nakamura, S., Maeda, N., Miron, I. M., Yoh, M., Izutsu, K., Kataoka, C., Honda, T., Yasunaga, T., Nakaya, T., Kawai, J., Hayashizaki, Y., Horii, T., and Iida, T. (2008). Metagenomic diagnosis of bacterial infections. *Emerging Infect. Dis.* 14, 1784–1786.

Nakamura, S., Yang, C. S., Sakon, N., Ueda, M., Tougan, T., Yamashita, A., Goto, N., Takahashi, K., Yasunaga, T., Ikuta, K., Mizutani, T., Okamoto, Y., Tagami, M., Morita, R., Maeda, N., Kawai, J., Hayashizaki, Y., Nagai, Y., Horii, T., Iida, T., and Nakaya, T. (2009). Direct metagenomic detection of viral pathogens in nasal and fecal specimens using an unbiased high-throughput sequencing approach. *PLoS ONE* 4, e4219. doi:10.1371/journal.pone.0004219

Nicholls, J. M., Chan, R. W., Russell, R. J., Air, G. M., and Peiris, J. S. (2008). Evolving complexities of influenza virus and its receptors. *Trends Microbiol.* 16, 149–157.

Rogers, G. N., Paulson, J. C., Daniels, R. S., Skehel, J. J., Wilson, I. A., and Wiley, D. C. (1983). Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. *Nature* 304, 76–78.

Shinya, K., Ebina, M., Yamada, S., Ono, M., Kasai, N., and Kawaoka, Y. (2006). Avian flu: influenza virus receptors in the human airway. *Nature* 440, 435–436.

Stevens, J., Corper, A. L., Basler, C. F., Taubenberger, J. K., Palese, P., and Wilson, I. A. (2004). Structure of the uncleaved human H1 hemagglutinin from the extinct 1918 influenza virus. *Science* 303, 1866–1870.

Takemae, N., Ruttanapumma, R., Parchariyanon, S., Yoneyama, S., Hayashi, T., Hiramatsu, H., Sriwilaijaroen, N., Uchida, Y., Kondo, S., Yagi, H., Kato, K., Suzuki, Y., and Saito, T. (2010). Alterations in receptor-binding properties of swine influenza viruses of the H1 subtype after isolation in embryonated chicken eggs. *J. Gen. Virol.* 91, 938–948.

Ueda, M., Daidoji, T., Du, A., Yang, C. S., Ibrahim, M. S., Ikuta, K., and Nakaya, T. (2010). Highly pathogenic H5N1 avian influenza virus induces extracellular Ca2+ influx, leading to apoptosis in avian cells. *J. Virol.* 84, 3068–3078.

Vines, A., Wells, K., Matrosovich, M., Castrucci, M. R., Ito, T., and Kawaoka, Y. (1998). The role of influenza A virus hemagglutinin residues 226 and 228 in receptor specificity and host range restriction. *J. Virol.* 72, 7626–7631.

WHO Global Alert and Responses. (2009). *Preliminary Review of D222G Amino Acid Substitution in the Haemagglutinin of Pandemic Influenza A (H1N1) 2009 Viruses.*

Yasugi, M., Nakamura, S., Daidoji, T., Kawashita, N., Ramadhany, R., Yang, C. S., Yasunaga, T., Iida, T., Horii, T., Ikuta, K., Takahashi, K., and Nakaya, T. (2012). Frequency of D222G and Q223R hemagglutinin mutants of pandemic (H1N1) 2009 influenza virus in Japan between 2009 and 2010. *PLoS ONE* 7, e30946. doi:10.1371/journal.pone.0030946

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APPENDIX

Table A1 | Primer pairs used to amplify segments of the H1N1pdm genome.

PB2-5′	F	TGTAAAACGACGGCCAGTATGGAGAGAATAAAAGAACTGAGAG
	R	GCTTGTCCTTTTGAAAGTGAACCCA
PB2-3′	F	TGCAAAAGTGCTTTTCCAGAACTGG
	R	CAGGAAACAGCTATGACCCTAATTGATGGCCATCCGAATTC
PB1-5′	F	TGTAAAACGACGGCCAGTATGGATGTCAATCCGACTCTAC
	R	ATTTTCATTCCACTTAGTGTTGTCC
PB1-middle	F	CAAAGATGCAGAGAGAGGCAAG
	R	CAGGTCCTGTAGAATCTGTCCAC
PB1-3′	F	GATTTTGCTCTCATAGTGAATGCAC
	R	CAGGAAACAGCTATGACCTTATTTTTGCCGTCTGAGTTCTTCAATGGTGG
PA-5′	F	TGTAAAACGACGGCCAGTATGGAAGACTTTGTGCGACAATG
	R	GTCCTCAAGAATGGTTCAATTTTGG
PA-middle	F	ATGGATTCGAGCCGAACGGCTGCATTG
	R	TATGTACACTCCCTTCATTATGTATTCAG
PA-3′	F	TTGATGAAATAGGAGAAGATGTTGC
	R	CAGGAAACAGCTATGACCCTACTTCAGTGCATGTGTGAG
HA-5′	F	TGTAAAACGACGGCCAGTATGAAGGCAATACTAGTA
	R	CGGGATATTCCTTAATCCTGTRGC
HA-3′	F	GTGCTATAAACACCAGCCTYCCA
	R	CAGGAAACAGCTATGACCTTAAATACATATTCTACACTGTAGAG
NP	F	TGTAAAACGACGGCCAGTATGGCGTCTCAAGGCACCAAACG
	R	CAGGAAACAGCTATGACCTCAACTGTCATACTCCTCTG
NA-5′	F	TGTAAAACGACGGCCAGTATGAATCCAAACCAAAAGATAATAACCATTG
	R	AGAATCAGGATAACAGGAGC
NA-3′	F	GAATGTGCATGTGTAAATGG
	R	CAGGAAACAGCTATGACCTTACTTGTCAATGGTAAATGGCAACTCAG
M	F	TGTAAAACGACGGCCAGTAGCAAAAGCAGGTAGAT
	R	CAGGAAACAGCTATGACCAGTAGAAACAAGGTAGTTT
NS	F	TATAAAACGACGGCCAGTAGCAAAAGCAGGGTGACAA
	R	CAGGAAACAGCTATGACCAGTAGAAACAAGGGTGTTT

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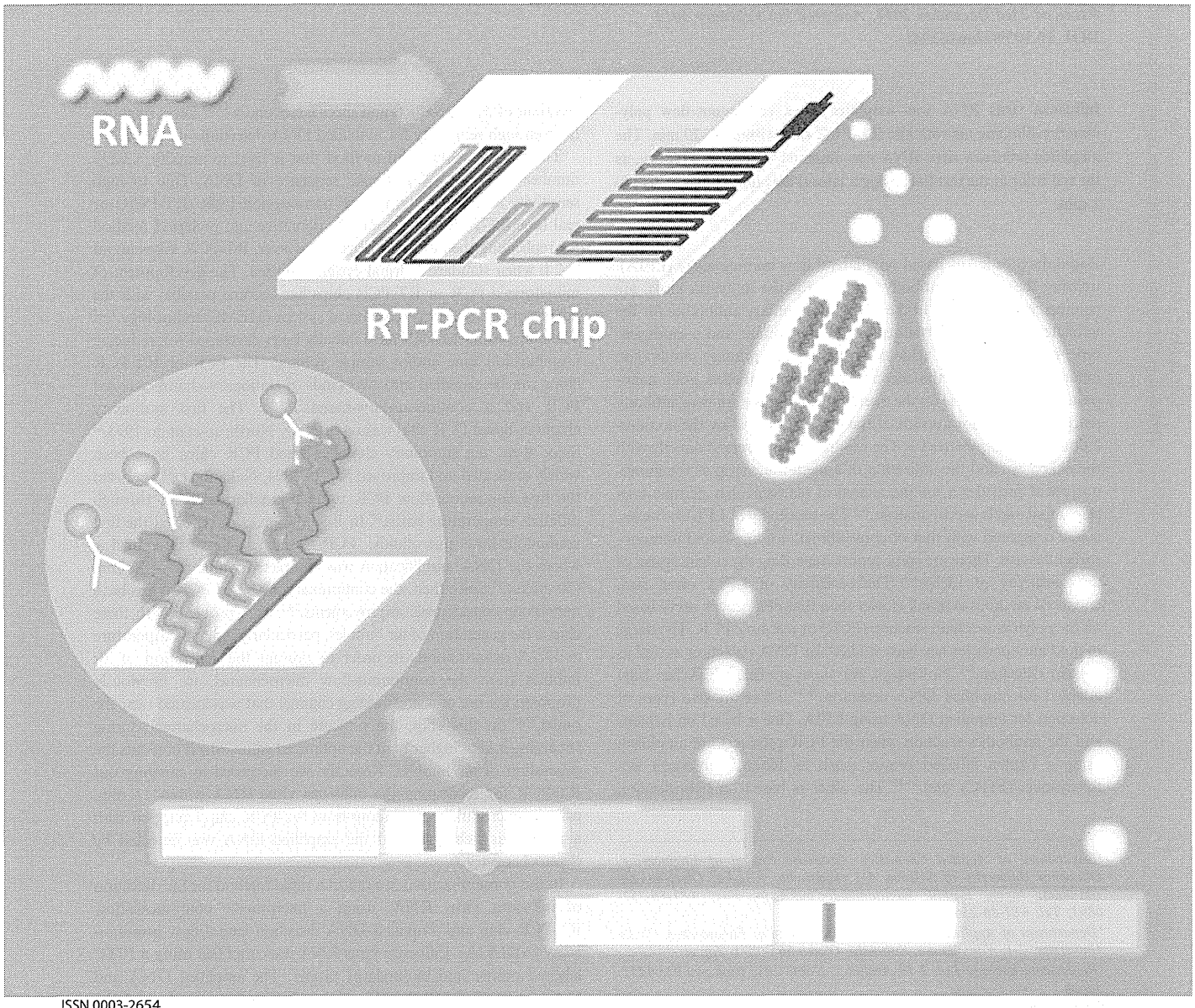

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COMMUNICATION

Detection of influenza virus using a lateral flow immunoassay for amplified DNA by a microfluidic RT-PCR chip

Naoki Nagatani,*[a] Keiichiro Yamanaka,[b] Hiromi Ushijima,[c] Ritsuko Koketsu,[d] Tadahiro Sasaki,[d] Kazuyoshi Ikuta,[d] Masato Saito,[b] Toshiro Miyahara[a] and Eiichi Tamiya[b]

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Influenza virus RNA was amplified by a continuous-flow polydimethylsiloxane microfluidic RT-PCR chip within 15–20 min. The amplified influenza virus RNA was observed with the naked eye, as the red color at the test line, using a lateral flow immunoassay within 1 min.

Rapid diagnostic kits based on a lateral flow immunoassay (LFIA), utilizing the antigen–antibody reaction for the influenza virus, are commercially available. In the LFIA, the primary antibodies for the influenza virus are immobilized on the membrane, and a sandwich-type immunoassay is achieved between the primary antibodies, influenza virus, and the secondary antibody-labelled gold nanoparticles at the test line on the membrane. After the antigen–antibody reaction, a red color appears at the test line, caused by the accumulation of gold nanoparticles. The results can be observed directly with the naked eye and the utilization of a membrane strip as the immunosorbent provides a unique analytical platform that permits one-step, rapid and low-cost analysis.[1–3] The sensitivity of LFIA however, is low compared with that of conventional enzyme-linked immunosorbent assays. There are some reports regarding the enhancement of the sensitivity of LFIA.[4–6] The sensitivity of LFIA using such enhanced methods is usually lower than that of a genetic assay based on the polymerase chain reaction (PCR) or real-time PCR. The usage of gold nanoparticles has been studied for DNA detection as well as protein detection.[7–9] Nowadays, for these studies, LFIA has been adapted for amplified DNA detection.[10–19] There are two types of detection for amplified DNA using LFIA. One is based on haptens and the antibodies reaction when the PCR products are amplified, using a hapten labelled primer (such as biotin, fluorescein isothiocyanate (FITC), *etc*).[10–15] The other is based on hybridization reactions of PCR products and specific sequences.[16–20] These methods have enough sensitivity for amplified DNA detection.

The PCR is a powerful method that is used to amplify a large number of copies of a specific sequence of DNA. Due to high sensitivity and specificity, reverse transcription PCR (RT-PCR) or real time RT-PCR has become the definitive and preferred method for replicating the influenza virus.[21] However, RT-PCR takes about 1–2 h when standard thermal cyclers are used. The development of miniaturized PCR or RT-PCR chips has become possible with the advent of micro electro-mechanical system (MEMS) technology.[22,23] The chips using MEMS have initially been developed for reducing amplification time and/or sample volume. The PCR or RT-PCR chips can be classified into two kinds: a stationary chamber-based PCR and a continuous-flow-based PCR. The first stationary chamber-based PCR chip was reported by Northrup *et al.* in 1993.[24] Since then, the stationary chamber-based PCR chips have been widely replicated and improved.[25–28] In 1994, Nakano *et al.* reported the first continuous-flow PCR using a capillary passing through different temperature baths.[29] In 1998, Kopp *et al.* reported the first continuous-flow microfluidic PCR, using a glass-based device, where the DNA amplification was completed within 1.5–18.7 min (20 cycles).[30] Since then, the continuous-flow-based PCR chips have undergone considerable improvements.[20,31–35] A problem with these chip is the generation of air bubbles, particularly at high temperature in DNA denaturation. In order to prevent the generation of air bubbles from the continuous-flow microchannel, we previously proposed the use of a pressurizing channel that was located near the outlet.[33,35] In this chip, the pressure in the microchannel during streaming is kept higher than the optimized value, which prevents the generation of air bubbles. Recently, we succeeded in carrying out RT-PCR for replicating the influenza virus RNA within 15 min, using a microfluidic continuous-flow RT-PCR chip equipped with a pressurizing channel, and the amplified DNA was detected by electrochemistry.[36]

In this communication, we report a rapid method for the detection of influenza virus RNA, using a microfluidic continuous-flow RT-PCR chip and amplified DNA detection lateral flow immunoassay (ADLFIA). Influenza virus RNA was amplified using a FITC labelled primer and biotinylated primer. The amplified DNA and biotin antibody-labelled gold nanoparticles were mixed, and the mixture was absorbed by capillary action for ADLFIA, immobilizing FITC antibodies at the test line. As a result, a red color, caused by the

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accumulation of gold nanoparticles, was observed at the test line within 1 min (Scheme 1).

Firstly, ADLFIA was evaluated using amplified DNA from influenza virus RNA with a standard thermal cycler. RT-PCR for influenza virus RNA (A/H1N1) was performed using a one-step RT-PCR method (PrimeScript® One Step RT-PCR Kit Ver.2, Takara Bio Inc.). The reaction mixture for the RT-PCR contained 25 μL of 2 × 1 step buffer, 2 μL of PrimeScript® 1 step Enzyme Mix, 5 μL of 4 μM 5′-FITC labelled primer (M30: 5′-TTCTAACCGAGGTC GAAACG-3′), 5 μL of 4 μM 5′-biotinylated primer (M264R2: 5′-ACAAAGCGTCTACGCTGCAG-3′),[18] 2 μL of various concentrations of isolated influenza RNA and 11 μL of RNase free distilled water. The RT-PCR was carried out using a standardized RT-PCR thermal cycling protocol that consisted of RT at 50 °C for 30 min, followed by denaturation at 95 °C for 2 min and PCR for 30 cycles at 95 °C for 30 s of denaturation, 60 °C for 30 s of annealing and 72 °C for 1 min of primer extension. After RT-PCR, the reaction solution (5 μL) was electrophoreted on 3% (w/v) agarose gel containing ethidium bromide in running buffer and the amplified DNA was visualized by UV illumination (Fig. 1A). The specific amplification of 232 bp was clearly observed at an initial concentration 40 pg μL^{-1} of template RNA in the RT-PCR mixture. However, no amplification was evident at lower initial concentrations. The amplifications after RT-PCR were also confirmed by ADLFIA. The ADLFIA was prepared by a similar method to that described in a previous report.[5] The FITC antibody solution (BETHYL laboratories Inc.) was prepared to 1 mg mL^{-1} by diluting with 50 mM phosphate-buffered saline (pH 7.2). For the immobilization of the FITC antibody at the test line on the nitrocellulose membrane, the FITC antibody solution of 650 μL was mixed with 20% (w/v) sucrose solution, diluted with 50 μL of 50 mM KH_2PO_4 buffer (pH 7.5) and 50 μL of 2-propanol. For the preparation of the control line, 40 μL of the mouse IgG antibody (DakoCytomation) was mixed with both 60 μL of 2-propanol and 1100 μL of 50 mM KH_2PO_4 buffer (pH 7.5). The FITC antibody solution and the mouse IgG antibody solution were applied to the nitrocellulose membrane (Hi-Flow Plus HF135, Merck Millipore) by a dispensing system (Biojet Quanti 300, BioDot Inc.). After drying the membrane for 1 h at room temperature, the non-specific protein adsorption was blocked by immersing it in a 50 mM boric acid solution containing 0.5% (w/v) casein (pH 8.5) and incubating it for 30 min at room temperature. Then, the blocked membrane was washed with a 5.0 mM phosphate buffer (pH 7.5) containing 0.01% (w/v) sodium dodecyl sulfate for 30 min at room temperature. After drying the membrane overnight, it was prepared on a backing sheet and the absorbent pad was pasted. The membrane was cut into individual strips (4.0 mm $strip^{-1}$), using a guillotine cutting system (BioDot Inc.) and these strips were used for ADLFIA. Biotin antibody-labelled gold nanaparticles were also prepared by a similar method to that described in a previous report.[5] The biotin antibody solution at 50 μg mL^{-1} was prepared with 50 mM KH_2PO_4 buffer (pH 7.5) and the final volume was 250 μL. 200 μL of the diluted biotin antibody solution was added to a 1.8 mL colloidal solution (Tanaka Kikinzoku Kogyo) of gold nanoparticles (40 nm in mean diameter, 0.0065% (w/v)) and mixed immediately. The mixture was kept at room temperature for 10 min, for the immobilization of antibodies onto the surface of the gold nanoparticles by physical adsorption. After the immobilization, 100 μL of 1% (w/v)

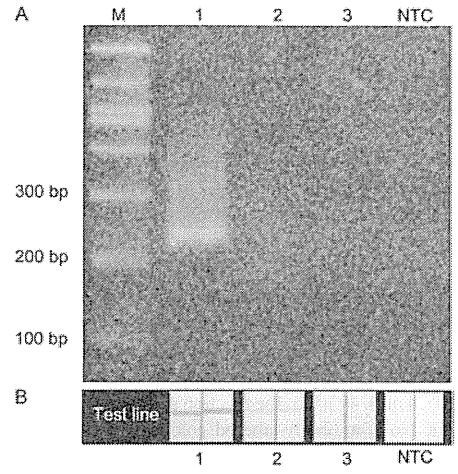
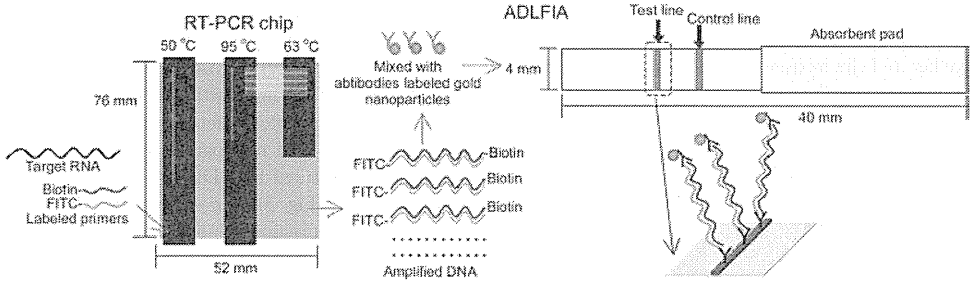


Fig. 1 Detection of amplified DNA from the influenza virus RNA, with a standard thermal cycler, by agarose gel electrophorsis (A) and ADLFIA (B). For both A and B, lane 1, 2 and 3 show the initial concentrations of template RNA (40, 4, 1.6 pg μL^{-1}), respectively. Lane M contains a 100 bp DNA ladder as a molecular weight standard. Lane NTC means non-template control.



Scheme 1 Schematic representation of the detection of the influenza virus using the continuous-flow RT-PCR chip and ADLFIA. Influenza virus RNA (target RNA) is amplified by the continuous-flow RT-PCR chip (52 × 76 mm) using an FITC labelled and biotinylated primer set. The amplified DNA is mixed with biotin antibodies labelled with gold nanoparticles. The size of the ADLFIA strip is 4 × 40 mm. FITC antibody and mouse IgG antibody are immobilized on the test and control line, respectively. The mixture is absorbed for ADLFIA, the amplified DNA complex with biotin antibody-labelled gold nanoparticles is captured by the FITC antibody at the test line. Finally, a red color appears as the result of accumulation of gold nanoparticles.

polyethylene glycol (PEG), which was dissolved in a 50 mM KH_2PO_4 buffer (pH 7.5), and 200 μL of 10% (w/v) bovine serum albumin (BSA), which was also dissolved in a 50 mM KH_2PO_4 buffer (pH 9.0), were added to block the surfaces of non-coated gold nanoparticles. After the immobilization and the blocking procedure, biotin antibody-labelled gold nanoparticles were separated by a centrifugal operation (12 000 × *g* for 15 min at 4 °C). The obtained pellet was pulse-sonicated for a few seconds and was added to 2 mL of preserving solution (1% (w/v) BSA, 0.05% (w/v) PEG 20000 and 150 mM NaCl in a 20 mM Tris–HCl buffer, pH 8.2). Once mixed, biotin antibody-labelled gold nanoparticles were collected by the same procedure as described above. After the pulse sonication, biotin antibody-labelled gold nanoparticles were suspended in a preserving solution and the absorbance was adjusted to O.D. 520 = 6 (optical density at 520 nm). 40 μL of the reaction solution after RT-PCR and 4 μL of the biotin antibody-labelled gold nanoparticles were mixed together in a microtiter 96-well plate. Each of the mixed solutions was then absorbed for ADLFIA by the capillary force (Fig. 1B). At the initial concentration of 40 pg μL^{-1}, the red color at the test line was clear for the template RNA in RT-PCR mixture. The red color at the initial concentration 4 pg μL^{-1} was observed within 1 min. Although the photograph was not clear, we could observe a very pale red color with the naked eye at the test line at the initial concentration of 1.6 pg μL^{-1} after 5 min. The red color at the test line was not observed in the non-template control. As a result of agarose gel electrophoresis, the specific DNA band was not observed except for an initial concentration of 40 pg μL^{-1}. This means that, although there is the amplified DNA from the influenza virus RNA in the reaction solution at the initial concentrations of 4 pg μL^{-1} and 1.6 pg μL^{-1}, the amount of amplified DNA by RT-PCR in 5 μL of the reaction solution is not enough to be observed on agarose gel electrophoresis. The amplification by RT-PCR was confirmed with ADLFIA using 40 μL of the reaction solution. The result indicates that ADLFIA can be used to detect the amplified DNA from the influenza virus RNA and the sensitivity of ADLFIA is comparable or superior to that of agarose gel electrophoresis.

When RT-PCR or PCR is carried out by a continuous-flow microfluidic chip using MEMS technology, a long time is required to collect 40 μL of the reaction solution, due to the small channel. To overcome the problem of sample collection, the sensitivity of ADLFIA was evaluated. The reaction solution after RT-PCR, at an initial concentration of 40 pg μL^{-1} of the template RNA mixture, was diluted (10, 100, 1000 times) with 10 mM phosphate buffer (pH 7.4) and measured using ADLFIA (Fig. 2). 40 μL of the diluted RT-PCR solution and 4 μL of the biotin antibody-labelled gold nanoparticles were mixed together and the mixture was measured by ADLFIA, using the same procedure described above. A red color at the test line for the reaction solutions diluted 10 and 100 times was observed. Although the red color for the reaction solution diluted 100 times was paler than that diluted 10 times, the red color was detected clearly with the naked eye. A red color at the test line was not observed for the reaction solution diluted 1000 times. These results mean that the sensitivity of ADLFIA is enough to detect amplified DNA and the volume required for detection is 0.04 μL when the amplified DNA is detected on agarose gel electrophoresis. The high sensitivity and low volume for detection of amplified DNA using ADLFIA are very attractive for the continuous-flow RT-PCR chip.

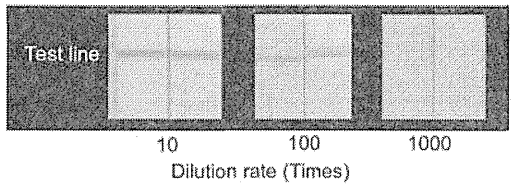


Fig. 2 Sensitivity of detection of ADLFIA. RT-PCR mixture at an initial concentration of 40 pg μL^{-1} of the influenza virus RNA was diluted (10, 100, 1000 times) and tested by ADLFIA.

The continuous-flow RT-PCR chip for this study, fabricated using polydimethylsiloxane (PDMS) (Dow Corning) by standard soft lithography, was the same design as that described in our pervious report.[36] The RT-PCR chip consisted of four zones: the RT reaction zone, the initial denaturation zone, the thermal cycle zone (30 cycles) and the pressurizing-channel zone. The size of the channel was 50 × 50 μm and the pressurizing-channel was 20 × 50 μm. The total length of the flow channel was 1280 mm and the total volume was 3.1 μL. Access holes were drilled above the inlet and outlet locations and fluorinated ethylene propylene tubes (BSA Inc.) were inserted into these holes and sealed with PDMS. The PDMS chip was bonded irreversibly to the glass substrate *via* oxygen plasma-treatment. The experimental set up for continuous-flow RT-PCR on the chip consisted of a microsyringe pump (KD Scientific Inc.) and a 100 μL syringe (Hamilton). A cartridge heater and a temperature sensor were packaged in a 10 × 10 × 70 mm aluminium block, and the temperature was controlled by PID regulation to maintain the optimized constant temperature (Kyushu-Nisso Co.). The chip was placed on the three individual heaters set at 50, 95 and 63 °C, for reverse transcription to synthesize cDNA from RNA, for denaturation and for annealing and extension, respectively. The RT-PCR mixture for the continuous-flow RT-PCR chip contained 2 × PrimeSTAR Max Premix (PrimeScript® High Fidelity RT-PCR kit; Takara Bio Inc.), 0.4 U μL^{-1} of RNase Inhibitor (Takara Bio Inc.), 0.15 U μL^{-1} of SpeedSTAR® HS DNA polymerase (Takara Bio Inc.), 8 U μL^{-1} of PrimeScript RTase (Takara Bio Inc.), 1 μg μL^{-1} of BSA to prevent the adsorption of RT-PCR regents on PDMS, 0.5 μM FITC labelled and biotin biotinylated primer set for the influenza virus, as described above, and 0.16 pg μL^{-1} of influenza virus RNA. The RT-PCR mixture was streamed using a syringe pump at the fixed flow rate 0.5 μL min^{-1} and the total flow-through time from inlet to outlet was 15 min. The RT-PCR mixture from the outlet was collected at different periods of 15 to 20, 20 to 25 and 25 to 30 min. The total volume collected from the outlet during each period was approximately 2 μL. The reaction solutions (1 μL) were electrophoreted on 3% (w/v) agarose gel. The collected reaction solutions were also diluted to 40 times with a 10 mM phosphate buffer (pH 7.4) and 40 μL of the diluted reaction solution and 4 μL of the biotin antibody-labelled gold nanoparticles were mixed together and the mixture was measured by ADLFIA, using the same procedure as described above (Fig. 3). The specific amplification of 232 bp was observed for the reaction solution collected from the outlet during 15–20 min but it was not clear. The amount of amplified DNA increased with the collection period from 25–30 to 30–35 min on the agarose gel. Meanwhile, the amplification was not observed in the non-template control at any collection period (Fig. 3A). A pale red color at the test line was observed for the reaction solution collected from the outlet during 15–20 min, within 1 min (Fig. 3B). A red color at the test line was clear for the collection period of 25–30 and 30–30

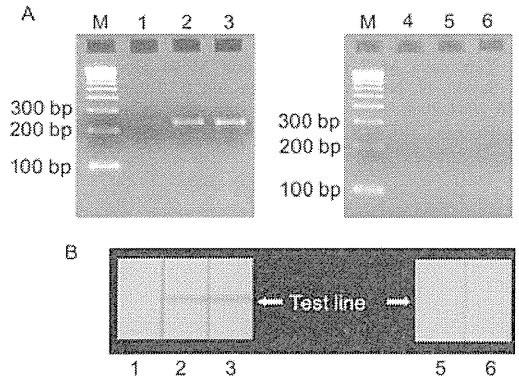


Fig. 3 Detection of amplified DNA from influenza virus RNA, with the continuous-flow RT-PCR chip, by agarose gel electrophoresis (A) and ADLFIA (B). In both A and B, lanes 1, 2 and 3 show the RT-PCR solution collected from the continuous-flow RT-PCR chip at 15–20, 20–25 and 25–30 min (total flow-through time from inlet to outlet), respectively. Lanes 4, 5, 6 show the non-template RT-PCR solution collected from the continuous-flow RT-PCR chip at 15–20, 20–25, 25–30 min (total flow-through time from inlet to outlet), respectively. Lane M contains a 100 bp DNA ladder as a molecular weight standard.

min as well, for agarose gel electrophoresis. The red color at the test line was not observed in the non-template control. The depth of the red color at the test line was in proportion to the amount of amplified DNA on the agarose gel when 1 μL of the reaction solution was measured using ADLFIA. In this study, 40 μL of the diluted reaction solution was needed to detect amplified DNA with a 40 mm wide ADLFIA strip. The volume of diluted solution however, can be reduced when a narrow width ADLFIA is applied.

Conclusions

The amplified DNA from influenza virus RNA, using a FITC labelled primer and biotinylated primer, by the microfluidic continuous-flow RT-PCR chip was detected by ADLFIA. The sensitivity of ADLFIA is comparable to that of agarose gel electrophoresis. The detection of amplified DNA by staining agarose gel electrophoresis with fluorescent dyes needs additional equipment such a UV illuminator. The detection by ADLIFA, however, needs only the naked eye. ADLFIA will be appended to a conjugate pad containing a dried excess volume of biotin antibody-labelled gold nanoparticles for a one-step assay. The conjugate pad appending ADLFIA can integrate easily into the solution exuding from the outlet of the chip. The integration enables the easy detection by ADLFIA without a mixing step. We are continuing our effort towards a battery powered portable PCR chip, and a battery powered portable PCR chip with ADLFIA has the potential to give the portable system a diagnostic test.

Acknowledgements

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Notes and references

1 Y. Tsuda, Y. Sakoda, S. Sakabe, T. Mochizuki, Y. Namba and H. Kida, *Microbiol. Immunol.*, 2007, **51**, 903–907.
2 K. H. Chan, S. Y. Lam, P. Puthavathana, T. D. Nguyen, H. T. Long, C. M. Pang, K. M. Chan, C. Y. Cheung, W. H. Seto and J. S. M. Peiris, *J. Clin. Virol.*, 2007, **38**, 169–171.
3 T. Miyoshi-Akiyama, K. Narahara, S. Mori, H. Kitajima, T. Kase, S. Morikawa and T. Kirikae, *J. Clin. Microbiol.*, 2010, **48**, 703–708.
4 T. Ono, M. Kawamura, S. Arao and H. Nariuchi, *J. Immnuol. Meth.*, 2003, **272**, 221–218.
5 N. Nagatani, R. Tanaka, T. Yuhi, T. Endo, K. Kerman, Y. Takamura and E. Tamiya, *Sci. Technol. Adv. Mater.*, 2006, **7**, 270–275.
6 R. Tanaka, T. Yuhi, N. Nagatani, T. Endo, K. Kerman, Y. Takamura and E. Tamiya, *Anal. Bioanal. Chem.*, 2006, **385**, 1414–1420.
7 C. A. Mirkin, R. L. Letsinger, R. C. Mucic and J. J. Storhoff, *Nature*, 1996, **382**, 607–609.
8 Y. Weizmann, F. Patolsky and I. Willner, *Analyst*, 2001, **126**, 1502–1504.
9 K. Sato, M. Onoguchi, Y. Sato, K. Hosokawa and M. Maeda, *Anal. Biochem.*, 2006, **350**, 162–164.
10 D. Kozwich, K. A. Johansen, K. Landau, C. A. Roehl, S. Woronoff and P. A. Roehl, *Appl. Environ. Microbiol.*, 2000, **66**, 2711–2717.
11 T. Suzuki, M. Tanaka, S. Otani, S. Matsuura, Y. Sakaguchi, T. Nishimura, A. Ishizaka and N. Hasegawa, *Diagn. Microbiol. Infect. Dis.*, 2006, **56**, 275–280.
12 T. Nishimura and S. Kure, *Hum. Mutat.*, 2003, **22**, 166–172.
13 K. Takada, Y. Sakaguchi, C. Oka and M. Hirasawa, *J. Periodontol.*, 2005, **76**, 508–512.
14 P. Corstjens, M. Zuiderwijk, A. Brink, S. Li, H. Feindt, R. S. Niedbala and H. Tanke, *Clin. Chem.*, 2001, **47**, 1885–1893.
15 M. Blažxková, M. Koets, P. Rauch and A. van Amerogen, *Eur. Food Res. Technol.*, 2009, **229**, 867–894.
16 S. Knoll, R. F. Vogel and L. Niessen, *Lett. Appl. Microbiol.*, 2002, **34**, 144–148.
17 S. Pischke, D. W. Büttner, E. Liebau and P. Fischer, *Trop. Med. Int. Health*, 2002, **7**, 526–531.
18 H. Helmy, P. Fischer, H. A. Farid, M. H. Bradley and R. Ramzy, *Trop. Med. Int. Health*, 2004, **9**, 158–163.
19 J. Aveyard, M. Mehrabi, A. Cossins, H. Braven and R. Wilson, *Chem. Commun.*, 2007, 4251–4253.
20 S. H. Lee, S. W. Kim, J. Y. Kang and C. H. Ahn, *Lab on a Chip*, 2008, **8**, 2121–02127.
21 World Health Organization, Recommendations and laboratory procedures for detection of avian influenza A(H5N1) virus in specimens from human case; http://www.who.int/influenza/resources/documents/RecAIlabtestsAug07.pdf (last accessed 10 November 2011).
22 C. Zhang and D. Xing, *Nucleic Acids Res.*, 2007, **35**, 4223–4237.
23 P. A. Auroux, Y. Koc, A. deMello, A. Manz and P. J. R. Day, *Lab Chip*, 2004, **4**, 534–546.
24 M. A. Northrup, M. T. Ching, R. M. White and R. T. Watson, 1993, *Proceedings of the 7th Internayional Conference on Solid-State Sensors and Actuators (Transducers '93). Yokohama, Japan, 7–10 June*, 924–926.
25 Y. Hataoka, L. H. Zhang, T. Yukimasa and Y. Baba, *Anal. Sci.*, 2005, **21**, 53–56.
26 C. Ke, A. M. Kelleher, H. Berney, M. Sheehan and A. Mathewson, *Sens. Actuators, B*, 2007, **120**, 538–544.
27 Y. Matsubara, K. Kerman, M. Kobayashi, S. Yamamura, Y. Morita and E. Tamiya, *Biosens. Bioelectron.*, 2005, **20**, 1482–1490.
28 C. Consolandi, M. Severgnin, A. Frosini, G. Caramenti, M. De Fazio, F. Ferrara, A. Zocco, A. Fischetti, M. Palmieri and G. De Bellis, *Anal. Biochem.*, 2006, **353**, 191–197.
29 H. Nakano, K. Matsuda, M. Yohda, T. Nagamune, I. Endo and T. Yamane, *Biosci., Biotechnol., Biochem.*, 1994, **58**, 349–352.
30 M. U. Kopp, A. J. de Mello and A. Manz, *Science*, 1998, **280**, 1046–1048.
31 C. S. Zhang, J. L. Xu, W. L. Ma and W. L. Zheng, *Biotechnol. Adv.*, 2006, **24**, 243–284.
32 J. A. Kim, J. Y. Lee, S. Seong, S. H. Cha, S. H. Lee, J. J. Kim and T. H. Park, *Biochem. Eng. J.*, 2006, **29**, 91–97.
33 T. Nakayam, Y. Kurosawa, S. Furui, K. Kerman, M. Kobayashi, S. R. Rao, Y. Yonezawa, K. Nakano, A. Hino, S. Yamamura,

Y. Takamura and E. Tamiya, *Anal. Bioanal. Chem.*, 2006, **386**, 1327–1333.
34 T. H. Fang, N. Ramalingman, D. Xian-Dui, T. S. Ngin, Z. Xianting, A. T. L. Kuan, E. Y. P. Huat and G. Hai-Qing, *Biosens. Bioelectron.*, 2009, **24**, 2131–2136.
35 T. Nakayama, H. M. Hiep, S. Furui, Y. Yonezawa, M. Saito, Y. Takamura and E. Tamiya, *Anal. Bioanal. Chem.*, 2009, **396**, 457–464.
36 K. Yamanaka, M. Saito, K. Kondoh, M. M. Hossain, R. Koketsu, T. Sasaki, N. Nagatani, K. Ikuta and E. Tamiya, *Analyst*, 2011, **136**, 2064–2068.

NEUTRALIZATION TITERS AGAINST INFLUENZA A (H3N2) AND INFLUENZA B VIRUSES AMONG A NON-VACCINATED POPULATION FROM THAILAND

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Abstract. Influenza A and B viruses are viral respiratory pathogens that can cause severe infections among birds and mammals. Neutralization assays using human sera are useful to evaluate the risk of circulating viruses to humans. In this study, 359 serum samples from healthy Thai volunteers, who had not been vaccinated against influenza for at least five years, were investigated by microneutralization (MN) assays against influenza A H3N2 and influenza B viruses in 2009. There was no significant difference in neutralization activities against 2006 and 2008 isolates of influenza A H3N2 viruses. However, neutralization titers to influenza B viruses among 2008 isolates were quite low. The results indicate the non-vaccinated study population had some neutralizing antibodies against influenza A H3N2 but not against influenza B viruses.

Keywords: influenza A virus, influenza B virus, neutralization titer, healthy Thais

INTRODUCTION

Influenza A and B viruses are the viral respiratory pathogens that can cause severe infections among birds and mammals. Since the first influenza A (H3N2) virus pandemic in 1968 which killed an estimated 750,000 people worldwide, the virus has had the tendency to predominate in prevalence over influenza A H1N1 and influenza B, especially over the past ten years (Finkelman *et al*, 2007; Colin *et al*, 2008). In Thailand, influenza (H3N2) virus predominated during 2005 and 2007 and type B viruses predominated in 2008 (Simmerman *et al*, 2009). Influenza viruses have both dynamic antigenic drifts

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and antigenic shifts. Antigenic drift is an accumulation of point mutations in the hemagglutinin (HA) gene, responsible for inhibiting receptor binding and preventing reinfection with the same strain, which allows the viruses to escape the immunologic pressure of the host (Hampson, 2002), whereas antigenic shift is the process by which two or more different strains of a virus combine to form a new subtype having a mixture of the surface antigens of the original strains (Carrat and Flahault, 2007). Molecular epidemiological studies have suggested antigenic changes occur in H3N2 virus more frequently than H1N1 virus (Rambaut *et al*, 2008). The World Health Organization (WHO) has been using antigenic, genetic and epidemiologic data from the current epidemic virus strains to identify antigenic variants with the potential to cause future epidemics and to develop vaccines (WHO, 2007). During 2007-2008 influenza season in the northern hemisphere there were changes in all vaccine strains: H1N1, H3N2 and influenza B viruses (WHO, 2008). This change in candidate vaccine strains suggests potential antigenic drift in 3 subtypes of influenza viruses. We reported significant antigenic drift in H1N1 viruses between 2006 and 2008 among healthy Thai volunteers in 2009, with remarkably lower neutralization activities against H1N1 isolates in 2008 than 2006 isolates (Kanai *et al*, 2010). Influenza A (H3N2) and influenza B viruses have been co-circulating worldwide with influenza A (H1N1) viruses. H1N1 and H3N2 viruses have been co-circulating worldwide since 1977 and have continued to do so even during the H1N1 2009 virus pandemic (WHO, 2011).

To better understand the epidemiology of influenza A (H3N2) and influenza B viruses in Thailand, we examined influenza virus neutralizing activities among non-vaccinated, healthy Thais against recent virus isolates to carry out risk analysis among Thais to current circulating viruses and the virus evolution driven by human antibodies.

MATERIALS AND METHODS

Healthy volunteer sera

In this study, we collected serum samples from 359 healthy volunteers in Prachuap Khiri Khan (n=119), Phetchabun (n=120) and Nakhon Sawan (n=120) Provinces, Thailand, during May to October 2009. None of the volunteers had vaccinations during the previous 5 years and signs of influenza infection during sampling. This study was approved by the ethics committee of the Department of Medical Sciences, Ministry of Public Health, Thailand. Written informed consent was obtained from each participant before inclusion in this study. Serum samples were treated with receptor destroying enzyme (RDE: Denka Seiken, Tokyo, Japan) at 37°C for 18 hours and followed by incubation at 56°C for 1 hour to remove non-specific neutralizing factors to influenza viruses.

Influenza viruses

The influenza A (H3N2) isolates in 2006 (TH/45/06; TH/46/06; TH/47/06; TH/49/06) and in 2008 (TH/743/08; TH/592/08) and influenza B isolates in 2008 (B/TAK/725/08; B/TRAT/433/08; TH/749/08, TH/533/08), which were clinical isolates in Thailand, were kindly provided by the Thai National Influenza Center (Thai NIC), National Institute of Health, Thailand. Viruses were cultured in Madin-Darby canine kidney (MDCK) cell culture and detected by the virus working dilution by focus-forming units (FFU) before use as previously described (Okuno *et al*, 1990).

Microneutralization assay

The microneutralization (MN) assay was carried out as described previously (Kanai *et al*, 2010). Pretreated sera were serially diluted (1:10 to 1:1,280) with Dulbecco's modified essential medium (DMEM) in a 96 well round-bottom microplate. Then, each serum dilution and then DMEM without serum as a control were each combined with an equal volume (25 µl) of virus fluid adjusted to give a final control count of about 50-100 focus-forming units (FFU) per well and then incubated at 37°C for 60 minutes in a 5% CO_2 incubator. After 16 hours, the cells were fixed with absolute ethanol and reacted with mouse monoclonal antibodies against influenza A virus NP protein (clone A3, 1:1,000 dilution) or influenza B virus NP protein (clone B2, 1:1,000 dilution), followed by horseradish peroxidase-conjugate goat anti-mouse IgG antibody (A2304, Sigma Aldrich, Steinheim, Germany. 1:400 dilution). Mouse monoclonal antibodies were kindly provided by Dr Yoshinobu Okuno (Kanonji Institute, the Research Foundation for Microbial Diseases of Osaka University, Kanonji, Kagawa, Japan). Virus-infected cells were visualized by 3,3-diaminobenzidine tetrahydrochloride hydrate (D5637; Sigma Aldrich, Steinheim, Germany). Human serum taken from vaccinated adults who had a positive antibody titer to influenza A H3N2 or influenza B virus was used as a positive control. The MN titers were expressed as a reciprocal of the maximum dilution giving a 50% reduction compared with the control.

Statistical analysis

The average MN titer among the virus isolates were compared by two-tailed Students *t*-test. The prevalences of positive samples were compared by chi-square test. A $p<0.05$ was considered statistically significant.

RESULTS

Seroprevalence and MN titer to subtype H3N2 viruses

Serum samples from 359 healthy volunteers collected during May to October 2009 were examined for their MN titer against influenza A (H3N2) (4 virus isolates in 2006 and 2 virus isolates in 2008). Mean MN titers against influenza A H3N2 and influenza B viruses are shown in Fig 1. The overall neutralization antibody levels against the H3N2 viruses in 2006 and 2008 isolates were comparable. Among H3N2 isolates during 2006 and 2008, a higher titer was detected in the 16-20 year old age group (average titer 272.8) and the 31-40 year old age group (average titer 239.3). The lowest titer was found among subjects in the older age (>41 years old) for virus isolates from 2006 and 2008 (average titer 134.0). Most individuals had similar MN titers against to each of the 4 isolates in 2006; only 8 of 119 subjects (6.7%) had a greater than 16-fold difference in MN titers among the 2006 isolates. Twenty-three of 119 subjects (19.3%) had a greater than 16-fold difference in the MN titer for 2 isolates in 2008; all 23 subjects had higher titers against TH/592/08 than TH/743/08 (Table 1).

A HI titer >40 is considered the minimum effective antibody level (Hancock *et al*, 2009). We examined the correlation between HI and MN titers against H1N1 viruses and found a MN titer >80-160 consistently corresponded to a HI titer >40 (Kanai *et al*, 2010). In this study, a MN titer >80 was considered as having an effective antibody titer. The prevalence of effective antibodies against H3N2 2006 isolates (63.7%) was significantly lower

Table 1
Distinct MN titers against 2 isolates of H3N2 viruses (TH/743/08 and TH/592/08) in 2008.

Sample ID	Age	MN titers to H3N2 viruses	
		TH/743/08	TH/592/08
PJ 46	16	40	640
PJ 48	16	20	320
PJ 44	18	10	160
PJ 49	18	40	>1,280
PJ 34	19	20	320
PJ 37	20	<10	160
PJ 39	21	40	640
PJ 3	21	10	160
PJ 19	22	10	160
PJ 9	28	20	320
PJ 33	28	40	>1,280
PJ 7	29	20	320
PJ 43	34	40	640
PJ 30	36	40	640
PJ 42	37	10	160
PJ 25	39	<10	160
PJ 11	41	20	320
PJ 2	42	<10	160
PJ 12	46	20	320
PJ 3	48	20	320
PJ 15	48	20	640
PJ 35	50	<10	80
PJ 6	52	<10	80

Subjects showing more than a 16-fold difference in MN titers are shown.

than against the H3N2 TH/592/08 isolate (94.1%) (p=0.004), but comparable to the H3N2 TH/743/08 isolate (69.4%) (p=0.435).

MN titers against influenza B virus

The MN titers against 4 isolates of influenza B virus in 2008 were examined. The MN titers against influenza B viruses in 2008 were lower than those against the influenza A H3N2 2008 isolates (p<0.001) (Fig 1). The MN titers in each age group varied by the virus isolate. There were no clear patterns among MN titers by age group found against H3N2 viruses. Low level of neutralizing antibodies were found among people of all age groups against influenza B isolates in 2008 with a prevalence of neutralizing antibodies of less than 50%. MN titers against influenza B viruses varied more than titers against H3N2 isolates. B/TRAT/433/08 has a remarkable ability to escape human antibodies, as was seen by the low prevalence of effective antibodies (MN titer >80). Interestingly, sero-prevalence rates of antibodies against B/TRAT/433/08 among 16-20 and 21-30 year olds were 2% (5/25) and 0% (0/35), respectively.

DISCUSSION

In this study, 359 samples collected from non-vaccinated, healthy volunteers were examined for neutralizing activity against influenza A (H3N2) and influenza B viruses. The study demonstrated:1) no antigenic changes between 2006 and 2008 in H3N2 viruses and 2) low neutralizing activity against influenza B viruses in 2008. The WHO changed recommendations for the influenza A strains of H3N2 used in the vaccine for the 2007-2008 influenza season. Significant differences in neutralizing activity against H3N2 isolates would be expected between 2006 and 2008 and would be expected against H1N1 viruses (Kanai *et al*, 2010). The samples in this study were tested against H1N1 virus. The MN titer among the younger population against H1N1 2008 isolates was found by another study (Kanai *et al*, 2010) to be nearly 900.

Since normal human serum is known to have non-specific neutralizing factors against influenza viruses (Ananthanarayan and Paniker, 1960), the samples were

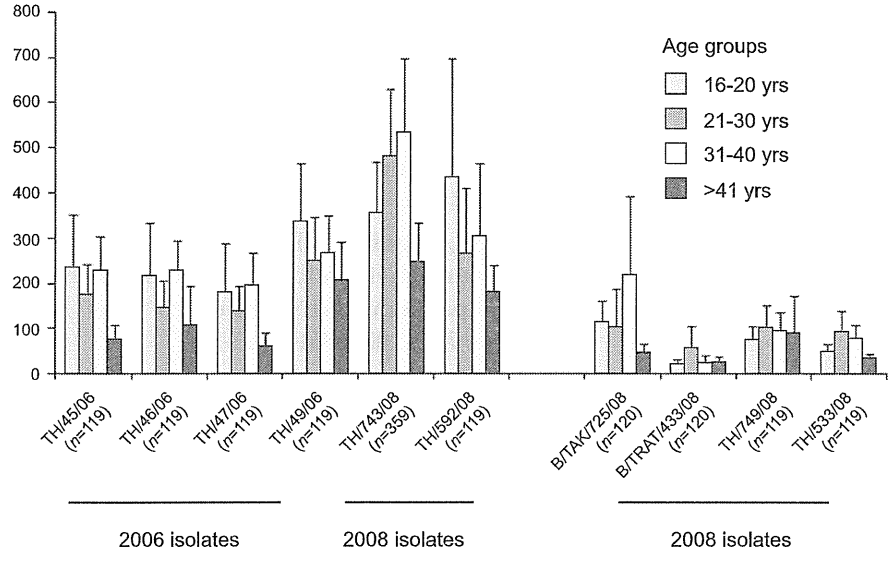

Fig 1–MN antibody titers among 359 healthy volunteers from Thailand against A (H3N2) and influenza B viruses.

treated with respector destroying emzyme (RDE) before testing to remove non-specific inhibitors. Although a negative control using human serum, which could assure the specificity of neutralizing reaction, was not used in this study, a positive reaction can be considered specific for neutralizing antibodies, since many samples were negative against influenza A (H3N2) and influenza B viruses. This study found the population had neutralizing antibodies against H3N2 isolates in 2006 and 2008 with more than a 50% prevalence of effective antibodies. Interestingly, the MN titer against H3N2 virus isolates in 2008 was slightly higher than against H3N2 isolates in 2006, in contrast to the general theory that virus antigenicity changes toward escape from natural antibodies. The effect of antigenic change due to human herd immunity was low, even if changes had occurred in circulating H3N2 viruses in 2008. Neutralization titers against H3N2 viruses in 2006 and 2008 were characterized by distinct age patterns, with higher activity among 16-20 and 31-40-year olds. However, there were no significant differences in neutralization activities between the 2006 and 2008 isolates. Subjects showed distinct MN titers for the 2 isolates of H3N2 2008 viruses. The MN titer against TH/743/08, highest among the 31-40 year old age group and lower among the 21-30 year old age group was distinct for each of the 5 H3H5 isolates examined. Although the sequence data are not available for the isolates used in this study, the current data suggest TH/743/08 is an antigenically evolved virus.

There were low effective neutralization antibodies against influenza B virus isolates in 2008. The lowest titer was seen against B/TRAT/433/08 (mean MN titer of 33) among all age groups. This indicates the human herd immunity was vulnerable to influenza B viruses in 2008. Due to the limited availability of virus isolates, it was not possible to examine influenza B viruses before 2008. The 2008 influenza B virus isolates might be escaped mutations from the herd immunity. During 2008, MN titers against influenza A (H3N2) viruses were higher than those against influenza B viruses. The remarkably low titers against influenza B viruses suggests influenza B virus will predominate during the following season. However, in 2009 after collecting serum samples, pandemic H1N1 2009 predominated in Thailand and only sporadic cases of influenza B virus were reported in Southeast Asian countries (WHO, 2009). This fact indicates

neutralization activity among humans is not the only factor that determines circulation of influenza viruses. Routine investigations for neutralization activity is still important to understand the epidemiology of influenza virus and to predict the epidemiological status during the following season. This type of study should be performed periodically.

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REFERENCES

Ananthanarayan R, Paniker CK, Non-specific inhibitors of influenza viruses in normal sera. *Bull World Health Organ* 1960; 22: 409-19.

Carrat F, Flahault A. Influenza vaccine: the challenge of antigenic drift. *Vaccine* 2007; 25: 6852-62.

Colin AR, Terry CJ, Ian GB, *et al.* The global circulation of seasonal influenza A (H3N2) viruses. *Science* 2008; 320: 340-6.

Finkelman BS, Viboud C, Koelle K, *et al.* Global patterns in seasonal activity of influenza A/H3N2, A/H1N1, and B from 1997 to 2005: viral coexistence and latitudinal gradients. *PLoS ONE* 2007; 2: e1296-96.

Hampson AW. Influenza virus antigens and 'antigenic drift'. *Trends Ecol Evolut* 2002; 17: 334-40.

Hancock K, Veguilla V, Lu X, Zhong W, *et al.* Cross-reactive antibody response to the 2009 pandemic H1N1 influenza virus. *N Engl J Med* 2009; 361: 1945-52.

Kanai Y, Boonsathorn N, Chittaganpitch M, *et al.* The impact of antigenic drift of influenza A virus on human herd immunity: Sero-epidemiological study of H1N1 in healthy Thai population in 2009. *Vaccine* 2010; 28: 5437-44.

Okuno Y, Tanaka K, Baba K, *et al.* Rapid focus reduction neutralization test of influenza A and B viruses in microtiter system. *J Clin Microbiol* 1990; 28: 1308-13.

Rambaut A, Pybus OG, Nelson MI, *et al.* The genomic and epidemiological dynamics of human influenza A virus. *Nature* 2008; 453(7195): 615-19.

Simmerman JM, Chittaganpitch M, Levy J, *et al.* Seasonality and mortality associated with influenza pneumonia in Thailand: 2005–2008. *PLoS ONE* 2009; 4: e7776.

World Health Organization (WHO). Recommended composition of influenza virus vaccines for use in the 2007-2008 influenza season. *Wkly Epidemiol Rec* 2007; 82: 69-76.

World Health Organization (WHO). Recommended composition of influenza virus vaccine for use in the 2008-2009 influenza season. *Wkly Epidemiol Rec* 2008; 83: 81-7.

World Health Organization (WHO). Recommended composition of influenza virus vaccines for use in the 2010 influenza season (southern hemisphere winter). *Wkly Epidemiol Rec* 2009; 84: 421-31.

World Health Organization (WHO). Recommended composition of influenza vaccines for use in the 2012 southern hemisphere influenza season. *Wkly Epidemiol Rec* 2011; 86: 457-68.

Frequency of D222G and Q223R Hemagglutinin Mutants of Pandemic (H1N1) 2009 Influenza Virus in Japan between 2009 and 2010

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Abstract

Background: In April 2009, a novel swine-derived influenza A virus (H1N1pdm) emerged and rapidly spread around the world, including Japan. It has been suggested that the virus can bind to both 2,3- and 2,6-linked sialic acid receptors in infected mammals, in contrast to contemporary seasonal H1N1 viruses, which have a predilection for 2,6-linked sialic acid.

Methods/Results: To elucidate the existence and transmissibility of α2,3 sialic acid-specific viruses in H1N1pdm, amino acid substitutions within viral hemagglutinin molecules were investigated, especially D187E, D222G, and Q223R, which are related to a shift from human to avian receptor specificity. Samples from individuals infected during the first and second waves of the outbreak in Japan were examined using a high-throughput sequencing approach. In May 2009, three specimens from mild cases showed D222G and/or Q223R substitutions in a minor subpopulation of viruses infecting these individuals. However, the substitutions almost disappeared in the samples from five mild cases in December 2010. The D187E substitution was not widespread in specimens, even in May 2009.

Conclusions: These results suggest that α2,3 sialic acid-specific viruses, including G222 and R223, existed in humans as a minor population in the early phase of the pandemic, and that D222 and Q223 became more dominant through human-to-human transmission during the first and second waves of the epidemic. These results are consistent with the low substitution rates identified in seasonal H1N1 viruses in 2008.

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Introduction

In April 2009, a novel swine-derived influenza A virus (H1N1pdm) emerged and spread rapidly around the world [1,2], causing the World Health Organization to declare a pandemic in June. To date, most countries have confirmed infections by the new virus.

Since the first appearance of H1N1pdm, one particular amino acid substitution {aspartic acid to glycine at position 222 (D222G)} within the hemagglutinin (HA) molecule has appeared sporadically [1,3,4,5]. The D222G substitution is known to cause a shift from α2,6-sialic acid receptor specificity to mixed α2,3/α2,6-sialic acid receptor specificity [6,7]. It is noteworthy that G222 is highly conserved among avian viruses [8]. Previously, α2,3-specific avian viruses have been isolated from patients during the initial phases of the pandemics of 1957 and 1968, and avian HA in humans has been shown to be selected for increased affinity for the α2,6 receptor [8]. Also, the G222 substitution was present in the Spanish Flu outbreak of 1918 [9]; however, the existence and transmissibility of H1N1pdm α2,3 sialic acid-specific viruses remain unclear.

To identify whether α2,3 sialic acid-specific viruses, which replicate well in swine, were spread during the early phase of the H1N1pdm pandemic and whether α2,3 sialic acid-specific viruses are easily transmitted, the nucleotide sequences of the HA receptor binding site of H1N1pdm in clinical specimens were determined in this study.

Several specimens obtained from mild H1N1pdm cases during the first (May 2009) and second (Dec 2010) waves of the epidemic in Japan were analyzed. In addition to the D222G substitution, we focused on two other substitutions {aspartic acid187glutamic acid (D187E) and glutamine223argine (Q223R)}, known to be critical

for receptor binding, which cause a shift from α2,6 to α2,3 sialic acid receptor specificity [8,10]. Changes in HA amino acid diversity were identified in individual cases using a high-throughput sequencer.

Results

Sequencing analysis of H1N1pdm HA receptor binding site (RBS) from nasal specimens in May 2009

Three nasal swabs (#1–#3) from mild H1N1pdm cases were obtained in May 2009 in Osaka, Japan. Total RNA was extracted from each sample and the receptor binding site (RBS) within HA was successfully amplified using One Step RT-PCR. The PCR products (369 bp) were cloned into pGEM-T easy vector and analyzed by conventional Sanger technology. More than 100 clones in each specimen were sequenced, and the deduced amino acid sequences were analyzed (Figures 1, S1, S2, and S3). The substitution rate of the deduced amino acids was 0.29–0.41% (average: 0.34%) in the three samples, and mutations commonly observed in more than two patients are listed in Table 1. Four substitutions, K119N, N125D, D222G, and Q223R, were detected in more than 2.0% of the samples (Table 1). Among these substitutions, D222G and Q223R had already been shown to be critical for receptor binding and to cause a shift from α2,6 to α2,3 sialic acid receptor specificity [10]. In addition, the D187E substitution could be critical for the human-to-avian–type receptor switch [9]. A homology modeling and docking study also showed that D222G and Q223R mutants had reduced affinity for the α2,6 sialic acid-linked receptor (Figure S4). Thus, we focused on these five mutations and tried to measure the mutation ratio of unisolated viruses in upper respiratory airways. For a larger scale analysis, the PCR products were examined via a high-throughput sequencing approach using a 454/Roche GS-Junior sequencer.

Deep sequencing of more than 3,000 clones (reads) per specimen showed high rates of amino acid diversity, with the K119N, N125D, D222G, and Q223R substitutions in specimens #1–#3 (Table 2). The results obtained by deep sequencing were consistent with those obtained using Sanger sequencing (Table 1). The D187E substitution was also identified in two specimens through deep sequencing analysis; however, the substitution rate was only slightly increased in one of these (by 0.72% in specimen #2) (Table 2). These results suggest that α2,3 sialic acid-specific viruses, including G222 and/or R223, were present as a minor population in the upper respiratory tract even in mild cases.

Table 1. Deduced amino acid mutations commonly observed in more than two patients in May 2009.

HA position	Wild type	Mutant	Mutant Number[a]	Ratio (%)
119	K	N	8	2.6
125	N	D	9	2.9
129	N	S	4	1.3
145	Y	H	2	0.6
147	N	D	2	0.6
154	K	E	4	1.3
157	S	P	3	1.0
160	K	E	2	0.6
179	I	T	3	1.0
182	P	Q	5	1.6
187	D	E	5	1.6
191	L	P	4	1.3
204	S	P	3	1.0
210	F	L	2	0.6
214	I	V	2	0.6
222	D	G	8	2.6
223	Q	R	10	3.2

[a]Number of each mutant clone out of a total of 313 sequenced clones (#1–#3 specimens).
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Multiple passages of H1N1pdm viruses in embryonated chicken eggs

To elucidate how the genomic diversity (and population) of the RBS, including the D222G and/or Q223R substitutions, might change, we inoculated these specimens into embryonated chicken eggs and serially passaged them five or six times in eggs. Hemagglutination was not detected in any samples of the first passage (P1) but was detected in the third or fourth passage and showed 2^5 to 2^6 hemagglutination titers on the fifth or sixth passage (P5 or P6) (Figure S5). PCR products amplifying the same region as in Figure 1 were prepared from early- and late-passaged viruses (P1 and P5/P6), as well as MDCK-passaged viruses (except #3). The amplicons, approximately 200–500 reads, were examined using deep sequencing.

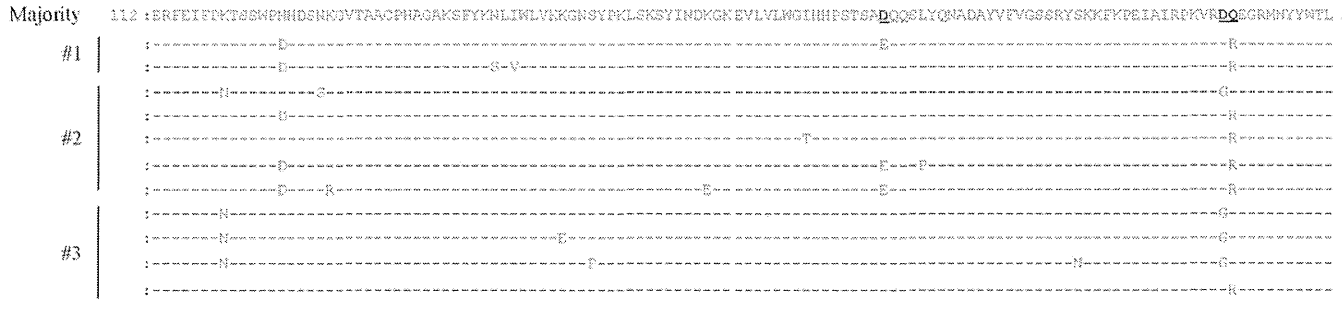

Figure 1. Alignment of the amino acid sequences including D187E, D222G, and Q223R mutants within the receptor binding site of H1N1pdm hemagglutinin. E187, G222, and R223 variants obtained from three clinical specimens (#1, #2, and #3) from the first wave of the outbreak. Three clinical nasal swabs were each subjected to RNA extraction, RT-PCR, and TA cloning. More than one hundred clones per specimen were sequenced using conventional Sanger technology. Positions 187, 222, and 223 are shown in bold and are underlined.
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