

Original Article

Isolation of an Egg-Adapted Influenza A(H3N2) Virus without Amino Acid Substitutions at the Antigenic Sites of Its Hemagglutinin

Tomoko Kuwahara¹, Emi Takashita¹, Seiichiro Fujisaki¹, Masayuki Shirakura¹, Kazuya Nakamura¹, Noriko Kishida¹, Hitoshi Takahashi¹, Noriko Suzuki², Yoshihiro Kawaoka^{3,4,5}, Shinji Watanabe¹, and Takato Odagiri^{1*}

¹*Influenza Virus Research Center, National Institute of Infectious Diseases, Tokyo;*

²*Saitama Institute of Public Health, Saitama;* ³*Division of Virology, Department of Microbiology and Immunology;*

⁴*Department of Special Pathogens, International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo, Japan;* and ⁵*Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, WI, USA*

SUMMARY: Antigenic changes in the hemagglutinin protein of recent A(H3N2) viruses often arise when these viruses adapt to their egg host. By serial egg passages of a cell-propagated virus, we successfully isolated an egg-adapted influenza A(H3N2) virus, A/Saitama/103/2014, without amino acid substitutions at the antigenic sites of its hemagglutinin protein but with multiple substitutions in its neuraminidase protein. Antigenic analysis of this egg-adapted A/Saitama/103/2014 virus indicated that its antigenicity did not differ from that of the World Health Organization prototype cell-propagated vaccine virus: A/Hong Kong/4801/2014. Our results suggest that this strategy may facilitate egg-based vaccine production without antigenic alterations in hemagglutinin by egg adaptation.

INTRODUCTION

Vaccination plays a key role in preventing infection and severe outcomes caused by influenza viruses. Most influenza vaccines are produced in embryonated chicken eggs; however, during egg adaptation of viruses, amino acid substitutions arise at the antigenic sites of the hemagglutinin (HA) protein (1–4), resulting in antigenic changes to the virus and decreased vaccine effectiveness. Recent studies indicate that the T160K or L194P substitution at an antigenic site of the HA of recent H3N2 viruses during egg passaging significantly affects antigenicity and the induction of appropriate protective antibodies, thus pointing to an association with low vaccine effectiveness (5,6). Here, we successfully isolated an egg-adapted influenza A/Saitama/103/2014 virus with amino acid substitutions in its neuraminidase (NA) but no antigenic changes in its HA by inoculating the cell-grown virus into eggs. Because no significant antigenic changes occurred in the HA of this virus even after extensive passages (> 21), the World Health Organization (WHO) has listed this extensively egg-passaged virus as a candidate vaccine virus for the development and production of vaccines for use in the 2017–2018 northern hemisphere influenza season (7).

MATERIALS AND METHODS

Cells: MDCK cells were maintained in minimum essential medium supplemented with 10% heat-inactivated

fetal calf serum. MDCK-SIAT1 cells, i.e., engineered MDCK cells with over-expressed α 2,6-linked sialic acid residues (8), were kindly provided by Dr. Matrosovich (Philipps Univ., Marburg, Germany) and were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5% heat-inactivated fetal calf serum, a penicillin-streptomycin solution (Sigma-Aldrich), and 1 mg/mL geneticin (Gibco, Grand Island, NY, USA). NIID-MDCK cells are established and authorized MDCK cells for cell-based vaccine production (9). NIID-MDCK cells were maintained in OptiPRO SFM (Gibco) supplemented with 2 mM L-glutamine. All the cell lines were cultured at 37°C and 5% CO₂.

Viruses: All Japanese virus isolates used in this study were collected through the national influenza surveillance system of Japan without a formal ethical review. The cell-grown viruses were amplified in NIID-MDCK or MDCK-SIAT1 cells at 34°C for 48 h. The egg-grown viruses were propagated in the amniotic or allantoic cavities of 10-day-old embryonated chicken eggs at 35°C for 48 h.

Sequence analysis: Viral RNA was extracted from virus isolates by the QIAamp Viral RNA Kit (Qiagen, Hilden, Germany). Nucleotide sequences were determined by Sanger sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit and 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) or by deep sequencing with MiSeq Reagent Kits v2 on the MiSeq next-generation sequencer platform (Illumina, San Diego, CA, USA) (10). DNA sequences were assembled and analyzed with Sequencher ver. 4.9 software (Gene Codes, Ann Arbor, MI, USA) or were aligned in CLC Genomics Workbench 8 (CLC bio, Aarhus, Denmark). Primer sequences for the Sanger sequencing are available upon request. Sequences of the HA and NA genes that were obtained in this study were deposited in the EpiFlu database of the Global Initiative on Sharing Avian Influenza Data (GISAID). Accession numbers are shown in Table 1.

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*Corresponding author: Mailing address: Influenza Virus Research Center, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan. Tel: +81-42-848-7161, Fax: +81-42-561-6149, E-mail: todagiri@nih.go.jp

An enzyme-linked immunosorbent assay (ELISA)-based microneutralization assay: This assay was performed as described in the WHO manual (11) with a minor modification. Briefly, 100 TCID₅₀ (50% tissue culture infectious dose) of each virus was mixed with serially diluted post-infection ferret serum (2-fold dilution), which was pretreated with a receptor-destroying enzyme (RED II; Denka Seiken, Tokyo, Japan), and the mixture was incubated for 1 h at 37°C. After that, a suspension of MIDCK-SIAT1 cells (2×10^4 cells) was added into each well, and the plates were incubated at 35°C and 5% CO₂ for 18 to 20 h. Cells were then fixed with 80% acetone in phosphate buffered saline (PBS), and an ELISA was carried out to identify the wells positive for virus growth by means of a mixture of mouse anti-influenza A nucleoprotein monoclonal antibody clones A1 and A3 (1:15,000 dilution; Millipore, Bedford, MA, USA) as primary antibodies and a goat anti-mouse immunoglobulin G (IgG) antibody conjugated with horseradish peroxidase (1:2,000 dilution; Kirkegaard & Perry Laboratories [KPL], Gaithersburg, MD, USA) as a secondary antibody. The neutralization titer was determined as the reciprocal of the highest serum dilution as described in the WHO manual (11).

Cloning of viruses by limiting dilution: Viruses were serially diluted (dilution range: 10^{-4} – 10^{-7}), and each dilution was inoculated into the allantoic cavities of 10-day-old embryonated chicken eggs. Eggs inoculated with the virus were incubated at 35°C for 48 h. Viruses from the highest dilution showing a hemagglutination with 0.5% chicken red blood cells were passaged and analyzed.

Table 1. Accession numbers of sequences of the HA and NA genes deposited in EpiFlu database

Virus name	Passage history	HA_ID	NA_ID
A/Saitama/103/2014	Clinical specimen	EPI1022843	EPI1022844
A/Saitama/103/2014	C3	EPI1105790	EPI1105791
A/Saitama/103/2014	C3E4	EPI1105792	EPI1105793
A/Saitama/103/2014	C3E8	EPI1105796	EPI1105797
A/Saitama/103/2014	CEXP002 (C3E20E8/E1)	EPI1105794	EPI1105795

RESULTS

Isolation of the egg-adapted influenza A(H3N2) virus: The influenza A/Saitama/103/2014 (Saitama) virus was initially isolated from a clinical sample, which was collected through the national influenza surveillance system of Japan and was passaged twice in NIID-MDCK cells (and was designated as strain C3). Because we did not have a sufficient amount of the clinical sample to inoculate both NIID-MDCK cells and eggs for virus isolation, we prioritized inoculation of the sample into NIID-MDCK cells to secure the A/Saitama/103/2014 virus. To obtain egg-adapted viruses, the NIID-MDCK cell-propagated C3 virus was serially passaged in the amniotic cavities and then in the allantoic cavities of 10-day-old embryonated chicken eggs. The virus grew well in the amniotic cavities even at the first passage, with a high hemagglutination titer when 1% guinea pig red blood cells were used ($> 1:256$). However, for full adaptation in eggs, which means that hemagglutination activity was detected in all eggs that we used for virus passaging, the virus needed 4 further passages in the amniotic cavities (designated as C3E4) and 4 passages in the allantoic cavities (designated as C3E8).

We then compared the amino acid sequences of the HA and NA proteins of this series of Saitama viruses with those of the virus in a clinical sample from which the Saitama virus was originally isolated (Table 2). To this end, nucleotide sequence analyses were conducted by Sanger sequencing or by deep sequencing. After nucleotide sequence analyses, we found that the cell-propagated C3 virus had no amino acid substitutions in its HA but had acquired the T148I substitution in its NA (Table 2). The NA T148I substitution is associated with virus adaptation to cell culture and may confer a receptor-binding property to NA (12,13). The C3E4 virus, which replicated well in the amniotic cavities, acquired an additional substitution, T153N, in its NA. The egg-adapted C3E8 virus acquired the HA H435L substitution, which is located in the stem region of HA, and 8 substitutions (R150H, N329T, S331G, S335G, H347R, N358G, N367D, and T369K) in addition to substitutions T148I and T153N in NA (Table 2).

Virus neutralization by antiserum against the egg-

Table 2. Amino acid substitutions in egg-adapted influenza A/Saitama/103/2014 (H3N2)

Passage history ¹⁾	amino acid residue														
	HA					NA									
	160	225	435	143	148	150	153	329	331	335	347	358	367	369	437
clinical specimen	T	D	H	V	T	R	T	N	S	S	H	N	N	T	L
C3					I ²⁾										
C3E4					I ²⁾		N								
C3E8			L		I ²⁾	R/H	N	N/T ²⁾	S/G ²⁾	S/G	H/R	N/G	N/D ²⁾	T/K ²⁾	
C3E8-m2			L		I ²⁾		N	T ²⁾						K ²⁾	
C3E8-m7			L	E	I ²⁾	H	N		G ²⁾	G	R	G	D ²⁾		
CEXP002 ³⁾	K ²⁾	G	L		I ²⁾		N	T ²⁾			N			K ²⁾	S

¹⁾: NIID-MDCK cell-grown (C3) virus was passaged 4 times in the amniotic cavities (C3E4) and then 4 times in the allantoic cavities (C3E8) of eggs. Cloned viruses (C3E8-m2 and C3E8-m7) were obtained by limiting dilution in the allantoic cavities.

²⁾: May result in the loss of a glycosylation site.

³⁾: Virus that had been listed as a candidate vaccine virus for the development and production of vaccines for use in the 2017–2018 northern hemisphere influenza season.

adapted influenza A(H3N2) virus: Given that the egg-adapted Saitama (C3E8) virus possessed a single substitution, H435L, in the stem region of HA, we performed an ELISA-based microneutralization (MN) assay to assess the antigenicity of the egg-adapted Saitama virus using post-infection ferret antisera against the Saitama and A/Hong Kong/4801/2014 (Hong Kong) viruses, the latter being an A(H3N2) prototype vaccine strain for the 2017–2018 northern hemisphere influenza season. As shown in Table 3, ferret antisera against the cell-propagated Saitama and Hong Kong viruses effectively inhibited the Saitama (C3E8) virus, suggesting that the antigenicity of the egg-adapted Saitama virus was retained even after egg adaptation and that the Saitama (C3E8) virus was antigenically a Hong Kong-like virus. We also found that the HA H435L substitution did not affect the antigenicity of the Saitama (C3E8) virus.

We then examined the virus-neutralizing activity of the antiserum, raised against the egg-adapted Saitama (C3E8) virus toward recently circulating H3N2 viruses (Table 3) by the MN assay. Replication of all the circulating viruses tested was effectively inhibited by the antisera against the Saitama (C3E8) virus as well as the cell-propagated Saitama and Hong Kong viruses with a ≤ 4 -fold reduction in MN titers as compared with the homologous virus titers. Nevertheless, most of the viruses tested were poorly inhibited by the antiserum against the egg-propagated Hong Kong virus with a ≥ 8 -fold reduction in MN titers as compared with the homologous virus titer. This is because the egg-propagated Hong Kong virus possesses egg-adaptive mutations in the head region of its HA (N96S, T160K, and L194P). Our findings thus suggest that the antiserum against the egg-adapted Saitama (C3E8) virus will show better coverage against currently circulating vi-

ruses than will the antiserum against the egg-propagated Hong Kong virus.

Antigenicity of the cloned egg-adapted influenza A(H3N2) virus: Because 8 NA substitutions in the Saitama (C3E8) virus were represented in the mixed population (Table 2), we attempted to isolate clones by limiting dilution. After 2 limiting-dilution passages in allantoic cavities, we obtained 2 clones: C3E8-m2 and C3E8-m7. These clones possessed 2 (N329T and T369K) or 7 (V143E, R150H, S331G, S335G, H347R, N358G, and N367D) substitutions, respectively, in addition to the T148I and T153N substitutions in their NA (Table 2). The NA V143E substitution in C3E8-m7 was not originally detected in the C3E8 virus. Both clones possessed only the H435L substitution in their HA.

We then examined the antigenicity of these clones in the MN assay (Table 4). No significant antigenic differences were found between the cell-propagated C3 virus, cell- or egg-propagated Hong Kong virus, and the egg-adapted C3E8-m2 clone carrying the 2 NA substitutions (plus substitutions T148I and T153N) with a ≤ 4 -fold reduction in MN titers compared with the homologous virus titers; however, the C3E8-m7 clone carrying the 7 NA substitutions (plus substitutions T148I and T153N) showed reduced reactivity with antisera against cell- and egg-propagated Hong Kong viruses with a ≥ 8 -fold reduction in MN titers as compared with the homologous viruses (Table 4), suggesting that the 7 NA amino acid changes found in C3E8-m7 may affect C3E8-m7 neutralization by antisera against Hong Kong viruses.

The C3E8 virus was further passaged in eggs (> 13) in our laboratory and then passaged (8 times) at the Center for Biologics Evaluation and Research in the USA, and ultimately yielded the A/Saitama/103/2014 (CEXP002)

Table 3. Virus neutralization by antiserum against egg-adapted influenza A/Saitama/103/2014 (H3N2)

Virus	Passage history ¹⁾	Microneutralization titer using post-infection ferret antisera against:			
		A/Saitama/103/2014		A/Hong Kong/4801/2014	
		Cell ²⁾	C3E8	Cell	Egg ³⁾
A/Saitama/103/2014	C2	<u>1,280</u> ⁴⁾	1,280	1,280	640
	C3E8	1,280	<u>1,280</u>	1,280	160
A/Hong Kong/4801/2014	C6	1,280	1,280	<u>1,280</u>	640
	E8	1,280	1,280	1,280	<u>2,560</u>
A/Aomori/13/2016	C2	1,280	2,560	1,280	640
A/Kawasaki/109/2016	C2	1,280	1,280	640	320
A/Gifu/31/2016	C2	640	1,280	640	640
A/Shimane/50/2016	C2	640	1,280	1,280	640
A/Kanagawa/AC1602/2016	C1	320	1,280	320	80
A/Mie/27/2016	C3	320	1,280	320	20
A/Yokohama/124/2016	C4	640	640	320	160
A/Sapporo/89/2016	C3	320	640	320	320
A/Niigata-C/53/2016	C3	320	320	320	80
A/Tokyo/16161/2016	C3	320	320	80	320

¹⁾ A/Saitama/103/2014 (C3E8) virus is described in footnote of Table 2. Regarding the rest of viruses, C indicates MDCK and/or MDCK-SIAT1 cell-propagated virus and E indicates egg-propagated virus. Number indicates the passage number.

²⁾ Cell-propagated virus.

³⁾ Egg-propagated virus.

⁴⁾ Homologous titers are underlined.

A(H3N2) Virus without Antigenic Changes in Eggs

Table 4. Antigenicity of egg-adapted influenza A/Saitama/103/2014 (H3N2)

Virus	Passage history ¹⁾	Microneutralization titer using post-infection ferret antisera against:		
		A/Saitama/103/2014		A/Hong Kong/4801/2014
		Cell ²⁾	Cell	Egg ³⁾
A/Saitama/103/2014	C3	<u>1,280</u> ⁴⁾	640	1,280
	C3E8-m2	640	640	320
	C3E8-m7	640	80	160
A/Hong Kong/4801/2014	C7	2,560	<u>2,560</u>	2,560
	E9	640	<u>1,280</u>	<u>1,280</u>

¹⁾: NIID-MDCK cell-grown (C3) A/Saitama/103/2014 virus was passaged 4 times in the allantoic cavities and then 4 times in the allantoic cavities (C3E8) of eggs. Cloned viruses (C3E8-m2 and C3E8-m7) were obtained by limiting dilution in the allantoic cavities. A/Hong Kong/4801/2014 virus was propagated in MDCK cells (C7) or eggs (E9).

²⁾: Cell-propagated virus.

³⁾: Egg-propagated virus.

⁴⁾: Homologous titers are underlined.

virus. The titer of the CEXP002 virus was approximately $10^{8.5}$ 50% egg infectious dose/mL, which was comparable to that of high-growth reassortant virus A/Hong Kong/4801/2014 (X-263): the virus strain used for vaccine production in Japan (data not shown). This CEXP002 virus possessed 2 substitutions (T160K and D225G) in its HA in addition to H435L and 3 substitutions (N329T, H347N, and T369K) in NA with one more substitution (L437S) in addition to T148I and T153N compared with the C3E8 virus (Table 2). Although the CEXP002 virus acquired 2 amino acid substitutions (T160K and D225G) at an antigenic site and at the receptor-binding site of HA, respectively, this virus remained antigenically similar to the vaccine virus, A/Hong Kong/4801/2014 (data not shown). A possible reason is that the whole structure of HA of the Saitama strain may not be strongly affected even after a loss of the glycosylation site at position 160 at an antigenic site. Accordingly, the WHO has listed the A/Saitama/103/2014 (CEXP002) virus as a candidate vaccine virus for the development and production of vaccines for the 2017–2018 northern hemisphere influenza season (7).

DISCUSSION

Several amino acid substitutions associated with egg adaptation of A(H3N2) viruses have been reported (14–17); however, almost all of these substitutions are located in the head region of HA, including receptor-binding and antigenic sites, and this situation resulted in antigenic changes. Here, we obtained an egg-adapted Saitama virus possessing a single substitution (H435L; in the stem region of HA), that does not affect HA antigenicity and multiple substitutions in NA (Tables 2 and 3).

Egg-adapted clones C3E8-m2 and C3E8-m7 possess 2 (N329T and T369K) and 7 (V143E, R150H, S331G, S335G, H347R, N358G, and N367D) substitutions, respectively, in addition to T148I and T153N in their NA. Because three of these positions (146–148, 329–331, and 367–369) in NA are a part of potential N-glycosylation sites, and both clones have substitutions at these 3 sites, these substitutions may result in the loss of glycosylation on NA for both viruses. This finding thus suggests that a loss of NA glycosylation may be important for viral adap-

tation in eggs.

Recent A(H3N2) viruses with the NA D151G substitution have been shown to bind to avian α 2-3-linked receptors, and this binding by NA may contribute to virus entry (18,19). Similarly, the NA T148I substitution has been suggested to confer receptor-binding activity to NA (10), perhaps via α 2-3-linked receptors. Thus, it is possible that the T148I substitution in NA plays the role of a trigger inducing other substitutions in NA in eggs, where many α 2-3-linked receptors are expressed, rather than inducing substitutions in HA. Given that the egg-adapted C3E8-m2 virus replicated well in eggs without any substitutions in the head of HA, the 4 substitutions (T148I, T153N, N329T, and T369K) in NA may be sufficient to confer adequate receptor-binding activity to NA for efficient replication in eggs. Studies designed to test this concept are now underway. It has been reported that the NA T148I substitution arises during virus amplification in cell culture (11). Our results also suggest that it is possible to obtain a virus that does not acquire substitutions at an antigenic site and/or the receptor-binding site of its HA by our procedure, that is, passages in cells followed by passages in eggs, and that this approach may become a solution to the problem of egg-adapted antigenic changes in H3N2 viruses.

The NA substitutions in the egg-adapted C3E8-m2 virus did not strongly affect viral antigenicity when tested with ferret antisera raised against the Saitama and Hong Kong viruses (Table 4), even though the amino acid residues at positions 329 and 369 are known NA antigenic sites (20,21). Although the roles of these 2 NA substitutions in egg adaptation of the A(H3N2) virus remain unclear, our observations suggest that the egg-adapted Saitama virus may be a useful tool for improving our understanding of the egg adaptation mechanisms of recent A(H3N2) viruses.

In summary, we isolated an egg-adapted influenza A(H3N2) strain, A/Saitama/103/2014, which has no amino acid substitutions at the antigenic sites of its HA, resulting in the absence of important antigenic changes even after egg passages. Our results suggest that our strategy may facilitate the production of egg-based vaccine viruses without antigenic alterations.

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Conflict of interest None to declare.

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