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Egg-adaptive mutations in H3N2v vaccine virus enhance egg-based production without loss of antigenicity or immunogenicity

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Abstract

The recently detected zoonotic H3N2 variant influenza A (H3N2v) viruses have caused 343 documented cases of human infection linked to contact with swine. An effective vaccine is needed for these viruses, which may acquire transmissibility among humans. However, viruses isolated from human cases do not replicate well in embryonated chicken eggs, posing an obstacle to egg-based vaccine production. To address this issue, we sought to identify egg-adaptive mutations in surface proteins that increase the yield of candidate vaccine viruses (CVVs) in eggs while preserving their immunizing effectiveness. After serial passage of a representative H3N2v isolate (A/Indiana/08/2011), we identified several egg-adaptive combinations of HA mutations and assessed the egg-based replication, antigenicity, and immunogenicity of A/Puerto Rico/8/34 (H1N1, PR8)-based 6 + 2 reverse genetics CVVs carrying these mutations. Here we demonstrate that the respective combined HA substitutions G186₁V + N246₁K, N165₁K + G186₁V, T128₁N + N165₁K + R76₂G, and T128₁N + N165₁K + I10₂M, all identified after egg passage, enhanced the replication of the CVVs in eggs without substantially affecting their antigenicity or immunogenicity. The mutations were stable, and the mutant viruses acquired no additional substitutions during six subsequent egg passages. We found two crucial mutations, G186V, which was previously defined, and N246K, which in combination improved virus yield in eggs without significantly impacting antigenicity or immunogenicity. This combination of egg-adaptive mutations appears to most effectively generate high egg-based yields of influenza A/Indiana/08/2011-like CVVs.

Keywords

Influenza A virus; Egg adaptation; Antigenicity; Immunogenicity; Vaccine virus; Hemagglutinin H3 mutations; Zoonosis; Variant virus

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1. Introduction

Swine H3N2 influenza viruses isolated from humans are termed H3N2 variant viruses (H3N2v). All H3N2v detected in the U.S. since 2011 have consisted of North American triple reassortant swine H3N2 influenza virus [1,2] containing the M gene from the 2009 pandemic H1N1 (pH1N1) viruses [3,4]; these H3N2v have caused 343 human cases, which clinically resemble uncomplicated seasonal influenza [5–9]. Almost all cases have been epidemiologically linked to contact with swine [6] but limited human-to-human transmission has been observed within some households and in a child-care setting [5,9]. In the ferret model, 2011 H3N2v isolates [10] and triple-reassortant swine H3N2 viruses [11] are moderately pathogenic, grow efficiently in the upper and lower respiratory tracts, and are readily transmissible via both direct contact and respiratory droplets. Adults possess some immunity to the H3N2v viruses, which are antigenically related to mid-1990s seasonal human influenza viruses. However, young children lack immunity, and recent seasonal influenza vaccines are not expected to be protective [12–16]. Together, these facts indicate a public health threat.

An H3N2v-specific vaccine virus stock should be readily available for immediate vaccine production in case of an outbreak. Optimal vaccine production requires that candidate vaccine viruses (CVVs) grow at high yield in eggs without significant change in antigenicity or immunogenicity. However, most H3N2v and seasonal human H3N2 field isolates replicate poorly in eggs. The G186V substitution in the receptor-binding domain of H3 HA1 is reported to be egg-adaptive and improve virus yield, as are amino acid changes at positions 183, 194–196, and 226 [17–20]. Five antigenic sites (A–E) have been mapped on the H3 HA1 molecule, which largely encase the surface of the HA globular head and include residues around the receptor binding site [21,22]. Therefore, while egg-adaptive mutations may enhance H3N2v vaccine production, the retention of viral antigenicity and immunogenicity must be carefully assessed.

We induced egg-adaptive mutations in the representative H3N2v A/Indiana/08/2011 (Ind08), which grows poorly in eggs. We identified several egg-adaptive mutations in Ind08 HA and evaluated their impact on the replication, antigenicity, and immunogenicity of PR8-based 6 + 2 CVVs. We identified four combinations of HA mutations that are maintained through six passages in eggs and that enhance replication without substantially affecting antigenicity or immunogenicity. Of these four, the combination G186V + N246K (the subscript indicates HA1 [23]) produced the greatest HA yields.

2. Materials and methods

2.1. Viruses

Wild type (WT) Ind08 virus was kindly provided by the Influenza Division, U.S. Center for Disease Control and Prevention, Atlanta, GA. Virus stocks were propagated in MDCK cells at 37°C or in the allantoic cavities of 10- to 11-day-old specific pathogen-free embryonated chicken eggs at 37°C for 2 days. Viruses were titrated by assaying plaque-forming units (PFU) in MDCK cells and by hemagglutination assay using turkey or chicken red blood cells (TRBC or CRBC).

2.2. Serial passage and cloning

To improve Ind08 virus yield in eggs, we conducted four serial passages in embryonated chicken eggs, obtaining a high hemagglutination titer (TRBC titer 512). The egg-adapted viruses were then cloned by 2-cycle plaque purification (C4/E4/C2) or inoculation of eggs at a 20% egg infectious dose (EID₂₀)(C4/E4/E1). Finally, stocks of the isolated clones were obtained by one additional passage in eggs (C4/E4/C2/E1 or C4/E4/E1/E1). The HA and NA gene segments of WT or egg-adapted Ind08 viruses were amplified from viral RNA by one-step reverse transcription–polymerase chain reaction (RT-PCR) using segment-specific 3' and 5' terminal primers. HAs and NAs from WT or different egg-adapted cloned viruses were sequenced and cloned into virus rescue plasmid pHW2000 [24].

2.3. Generation of variant vaccine viruses by reverse genetics

Reassortant 6 + 2 CVVs were generated by co-transfecting 293 T cells with eight cDNA plasmids encoding the HA and NA genes of WT or egg-adapted Ind08 virus and the remaining six genes of PR8 virus, as described previously [24]. Reverse genetic viruses rescued in 293 T cells were propagated in MDCK cells (passage history: C1, C2, etc.) or in the allantoic cavities of eggs (passage history: E1, E2, etc.).

2.4. Virus purification

Eggs were inoculated with different CVVs (E5 stock, 75 eggs each) and incubated at 37°C for 2 days. Allantoic fluid (E6 virus) was harvested and clarified by centrifugation (Sorval SAL-1500 rotor) at low speed (10,000 rpm) for 15 min at 4°C. Virus was inactivated by treatment with β -propiolactone (0.0005% v/v) at 4°C for 3 days and again clarified at low speed (10,000 rpm) for 15 min at 4°C. Viruses were pelleted by centrifugation (27,000 rpm in a Beckman SW32Ti rotor) for 90 min at 4°C through 25% sucrose cushion (33 ml allantoic fluid over 5 ml sucrose solution). Pellets were suspended in PBS, and suspensions of the respective viruses were combined. The volume was adjusted to 18 ml with PBS, and the suspension was centrifuged at 24,000 rpm through a 20 ml, 25–70% continuous sucrose gradient for 2.5 h at 4°C in a Beckman SW32Ti rotor. The virus band was harvested; the volume was then adjusted to 38 ml with PBS, and virus was pelleted by centrifugation (27,000 rpm) for 60 min at 4°C in a Beckman SW32Ti rotor. The pellet was suspended in PBS to a final volume 1/1000 the original volume of allantoic fluid (e.g., 400 μ l for 400 ml allantoic fluid), thereby producing a 1000 $\times$ virus concentrate. Viral protein concentrations were measured using Quick Start Bradford Protein Assay kit and bovine gamma-globulin standard (Bio-Rad Laboratories Inc., Hercules, CA).

2.5. Deglycosylation and SDS-PAGE analysis of viral proteins

Ten microliter of each virus concentrate was treated or mock-treated with 2 μ l PNGase F (New England Bio Labs, Ipswich, MA) in a 30- μ l volume (37°C, 4 h) as instructed by the manufacturer, and mixed with 15 μ l of SDS-protein sample buffer containing μ -mercaptoethanol. The sample was heated in boiling water for 5 min and proteins were separated by SDS-PAGE in a 12% polyacrylamide gel (Mini-Protein TGX, Bio-Rad Laboratories Inc.). Protein bands were stained with Coomassie blue dye, scanned, and analyzed by ImageJ software [25].

2.6. Hemagglutination inhibition (HI) assay

The HI assay was based on the WHO-recommended procedure [26]. Briefly, 25 µl of two-fold serial dilutions of RDE-treated ferret antisera were incubated with 25 µl of virus suspended in PBS (hemagglutination titer = 8) at room temperature for 1 h, then incubated with 50 µl of TRBC (0.5% suspension in PBS). Titers were recorded as the reciprocal of the highest serum dilution that inhibited hemagglutination. We considered isolates to be antigenically distinct if they exhibited greater than four-fold decrease in HI titer relative to the wild type virus and post-infection ferret antiserum raised against the wild type virus or conversely if the wild type virus had greater than a four-fold decrease in HI titer relative to the test virus and corresponding homologous ferret antiserum.

2.7. Ferret studies

All animal experiments were conducted in an Animal Biosafety Level 2 facility at St. Jude Children's Research Hospital, in compliance with the policies of the National Institutes of Health and the Animal Welfare Act and with the approval of the St. Jude Animal Care and Use Committee.

Four- to six-month-old male ferrets (Triple F farms, Sayre, PA) serologically negative for A/Perth/16/2009 (H3N2) and A/CA/04/2009 (H1N1) influenza viruses by HI assay (HI titer 10) were lightly anesthetized with isoflurane and inoculated intranasally with 10⁶ PFU of virus in 0.5 ml PBS (250 µl per nostril). Serum samples were collected at day 21 post-inoculation (p.i.).

3. Results

3.1. Identification of egg-adaptive mutations in Ind08 HA

We first determined the baseline sequence of the HA- and NA-specific RT-PCR products of our WT Ind08 stock virus (MDCK passage 4, C4). The sequence of the HA PCR product was identical to the Ind08 GenBank sequence (JN638733 and JQ070800). However, the NA PCR product showed mixed nucleotides Y (T/C) at position(positive sense) 231, causing an Ile(I)/Thr(T) variation at amino acid position 71, as reported previously (JN638732).

Having confirmed that MDCK passage of stock virus had not affected the HA or NA sequence, we conducted four parallel serial passages in eggs, until a high hemagglutination titer (512) was obtained. The HA- and NA-specific RT-PCR products of egg-adapted Ind08 virus were then sequenced. NA sequence was identical to that of WT virus, including mixed nucleotide Y (T/C) at position 231. In contrast, HA exhibited several mixed nucleotides, indicating het-erogeneity of the egg-adapted Ind08 virus stock (C4/E4). To identify the combination(s) of mutation(s), egg-adapted viruses were then cloned and their HA and NA genes were sequenced. Apart from the nucleotides encoding Ile or Thr at amino acid position 71, the NA genes of our cloned viruses were identical to those of the WT virus (Table 1). Several combinations of HA mutations were identified (Table 1).

3.2. Effect of egg-adaptive mutations on CVV replication in eggs

To confirm that the identified HA1 and HA2 amino acid changes (Table 1) would increase CVV yield in eggs, we created a panel of PR8-based 6 + 2 CVVs containing Ind08 WT or egg-adaptive mutant HA and WT NA (I71). As expected, CVV RG265, with WT Ind08 HA and NA (I71), grew well in MDCK cells but poorly in eggs (Table 2). In a hemagglutination assay, MDCK-grown RG265 had a titer of 32 with CRBCs but 256 with TRBCs (Table 2), which is consistent with previous observations that H3N2 viruses in general bind more efficiently to TRBC than to CRBC [27,28]. However, CVVs with egg-adaptive HA mutations showed comparable hemagglutination titers with TRBCs and CRBCs, which differed no more than two-fold even after six egg passages (Table 2). Because of these results, subsequent hemagglutination assays used only TRBCs. CVV RG275, containing HA mutation H183₁L, grew very poorly in MDCK cells and moderately in eggs. RG267, containing HA-G186₁V, grew well in eggs (hemagglutination titer: 512, log₁₀ pfu/ml = 8.6), and replication was enhanced by the additional mutation N246₁K. CVVs RG277 and RG281, containing the HA amino acid substitutions N165₁K + D90₂N (the subscript two indicates HA2) and HA-N165₁K + I10₂M, respectively, yielded titers similar to those of RG267 (Table 2). Together, these results indicate that the identified egg-adaptive mutations were indeed responsible for the enhanced growth of the CVVs in eggs.

3.3. Antigenicity of vaccine viruses

A fundamental criterion for CVVs is that their antigenicity must remain consistent with that of their parental strain. We therefore performed a one-way HI assay of all vaccine viruses, using ferret antisera to MDCK-grown WT CVV (RG265-C1, Table 2). This reference antiserum inhibited hemagglutination for the egg-passaged (E1) CVVs RG271 (HA L194₁I/Y233₁H) and RG275 (HA H183₁L) more weakly than did homologous virus, RG265-C1, indicating that antigenicity varied in the HAs of RG271-E1 and RG275-E1. Conversely, all other egg-passaged (E1) CVVs (RG267, RG269, RG273, RG277, RG279, and RG281) had HI titers essentially similar (differing <two-fold) to that of RG265-C1, indicating no substantial variation in antigenicity, and these viruses were selected for further study. We found that the NA amino acid change I71 T (with WT or mutant HA) did not affect virus growth (in MDCK cells or eggs) or antigenicity (data not shown); therefore, I71 NA was selected for further study.

3.4. Growth, antigenicity, and genetic stability of vaccine viruses after six passages in eggs

CVVs must undergo several passages in eggs between generation and final vaccine production. Therefore, we serially passaged the selected CVVs six times in eggs. Virus yield was not substantially different after six passages in eggs (E6) (Table 2). CVV RG269, which carried the G186₁V + N246₁K HA substitutions, grew best (TRBC hemagglutination titer = 2048, log₁₀ PFU/ml = 9.4); RG277, containing the N165₁K + D90₂N HA substitutions, grew least readily (TRBC hemagglutination titer = 256, log₁₀ PFU/ml = 7.9) and was excluded from further study.

To compare virus yield, as measured by hemagglutination titer and PFU/ml, to viral protein yield at E6, an equal volume of each CVVs E6 allantoic fluid was purified, concentrated

(1000×), and viral protein concentrations were measured using Quick Start Bradford Protein Assay kit and bovine gamma-globulin standard. To estimate further relative HA protein yield, viral proteins were deglycosylated, and analyzed by SDS-PAGE. Total viral protein yield and protein yield (relative to RG267-E6 yield) as measured by the deglycosylated HA1 and NA combined band intensity, grossly paralleled the hemagglutination titers, and RG269 had the highest yield (Fig. 1 and Table 3).

The HA and NA RT-PCR products of the CVVs were sequenced after six passages in eggs. We observed no change in the NA sequence. The WT HA of RG265 acquired mutations similar to those of RG271 (L194_I + Y233_H) and displayed significant variation in antigenicity (Table 2). We detected no additional HA mutations in viruses RG269 and RG273, indicating high genetic stability. The HA changes acquired by other CVVs are shown in Table 2. To understand the effect of additional mutations acquired by RG279 and RG281 during six passages in eggs, we rescued two additional CVVs, designated RG314 (HA-T128_N + N165_K + R76_G) and RG316 (HA-T128_N + N165_K + I10_M). After six passages in eggs, RG314 and RG316 exhibited growth and antigenicity similar to that of RG279 (E6) and RG281 (E6), respectively. Sequencing of their RT-PCR products revealed no additional mutations in their HAs and NAs.

3.5. Immunogenicity of the vaccine viruses after 6 passages in eggs

For two-way antigenic analysis, two ferrets were inoculated with 10⁶ PFU of selected CVVs (E6). Antisera collected on day 21 p.i. were titrated by HI against homologous virus and the reference virus RG265-C1 (WT HA). All CVVs induced robust antibody responses, with homologous HI titers ranging from 320 to 1280. HI titers against WT RG265-C1 virus were not substantially (more than two-fold) lower than those against homologous virus (Table 4). Therefore, the egg-adaptive HA mutations G186_V + N246_K, N165_K + G186_V, T128_N + N165_K + R76_G, and T128_N + N165_K + I10_M did not significantly alter viral immunogenicity.

4. Discussion

Although several egg-adaptive HA mutations have previously been identified in H3N2 viruses [15–18], their implications for H3N2v vaccine virus production have not been examined. Here, we identified egg-adaptive HA mutations in the representative H3N2v Ind08 and examined their effect on production of PR8-based 6 + 2 CVVs. Ind08 virus with the combined substitutions G186_V and N246_K produced the greatest HA protein yield and offer the best means of egg-based production of Ind08-like vaccine.

Ind08 grows very poorly in eggs, posing a crucial barrier to vaccine production. We serially passaged Ind08 in eggs until high titers were obtained. Subsequent sequence analysis of the hemagglutinin RT-PCR products revealed a heterogeneous population of viruses with mixed nucleotides encoding different amino acid at single HA residues. We then cloned the egg-adapted viruses to directly identify the combinations of HA mutations. Previous attempts to overcome poor growth in eggs involved introducing and testing a list of multiple mutations in different combinations [16,17]. Our more straightforward approach of cloning egg-

adapted viruses reduced the number of HA clones and PR8-based reverse genetics viruses to be tested.

We observed four groups of egg-adaptive HA mutations or combinations of mutations in Ind08: (1) H183₁L and L194₁I + Y233₁H, the only group that significantly altered the antigenicity of CVVs; (2) G186₁V, the most common egg-adaptive mutation identified in H3N2 viruses [17–20]; (3) mutations in the N-linked glycosylation sites 126-NWT-128, 165-NVT-167, and 246-NWT-248, either alone or accompanied by other mutation(s); and (4) I102₁M, R762₁G, and D902₁N in HA2, together with mutations in HA1.

The egg-adaptive mutation of the highly conserved basic polaramino acid His183₁ to the nonpolar Leu (H183₁L) dramatically reduced virus growth in MDCK cells and yielded poor to moderate growth in eggs, consistent with the previous observation that His183₁ interacts directly via hydrogen bond with bound sialic acid in human erythrocytes and that the H183₁F mutation severely impairs sialic acid binding [29]. The egg-adaptive mutation H183₁F was previously reported to improve the egg growth of PR8-based A/Fujian/411/2002 (H3N2) CVV [16]. We found that the double mutation L194₁I + Y233₁H improved the growth of CVV RG271 in eggs. However, HA-H183₁L and HA-L194₁I + Y233₁H exhibited significantly altered antigenicity (Table 2). Although amino acids at positions 183, 194, and 233 were not predicted to reside in any of the five antigenic sites [19,20], the close proximity of positions 183 and 194 to the dominant antigenic site (B) may have altered the antigenicity of CVVs RG271 (L194₁I/Y233₁H) and RG275 (H183₁F). It is noteworthy that although vaccine virus RG265 (WT HA) grew moderately during six passages in eggs, it underwent a substantial change in antigenicity (HI titer to RG265 = 640/400 for E6 virus vs. 2560/2000 for homologous C1 virus, Table 2). Sequence analysis showed that the virus had acquired the HA mutations L194₁I + Y233₁H, similar to those in RG271, which underwent the greatest change in antigenicity (Table 2). This result indicates that it is preferable to use selected egg-adaptive HA mutations rather than generate stock vaccine virus indiscriminately by passaging CVV in eggs until high titers are obtained. In such cases, reverse genetics will provide a clear advantage over conventional methods of reassortment to generate CVVs.

Our results indicate that G186₁V is a key HA mutation for the adaptation of Ind08 to eggs without significant impairment of antigenicity or immunogenicity. This amino acid substitution in the receptor-binding domain of H3 HA is reported to increase the yield of A/Singapore/21/2004 (H3N2), A/Fujian/411/2002 (H3N2), and A/Wisconsin/67/05 (H3N2) viruses in eggs [17–20]. In fact, sequence database searching indicated that the passage history of all human H3N2 isolates with the HA 186₁V either included eggs or was unknown (with the exception of CY113477, with a passage history in tMK1/MDCK1). Therefore, it is possible that the 186₁V in the HA of human H3N2 influenza viruses reported in Gene Bank originated from laboratory egg passage.

The N-linked glycosylation sites 126-NWT-128, 165-NVT-167, and 246-NWT-248 are highly conserved in recent human and swine H3N2 isolates, and the latter two motifs occur in avian H3N2 isolates [30]. Although removal of an N-glycosylation site may unmask antigenic sites and may potentially alter antigenicity [28], we observed no significant change

in antigenicity after mutation of any single glycosylation site (N126₁K, T128₁N, N165₁K, and N246₁K) or even after the double mutation T128₁N + N165₁K. CVV RG269, carrying the combined mutations G186₁V + N246₁K, produced the greatest virus yield (significantly greater than the RG267 [G186₁V] yield) with no detectable change in HA properties after six egg passages (Table 2). Analysis of viral proteins supported our yield data (hemagglutination and PFU assays), showing an E6-RG269 (G186₁V + N246₁K) yield almost double that of E6-RG267 (G186₁V) (Fig. 1). Notably, although sequence analysis of the E6-RG267 RT-PCR product indicated acquisition of the N246₁K HA mutation (creating an RG269-type HA), the yield of RG269 remained substantially greater than that of RG267, possibly reflecting undetected heterogeneity of RG267. Upon 6 passages in egg, RG269 (G186₁V + N246₁K) and RG273 (N165₁K + G186₁V) HA did not acquire any other detectable mutation, whereas additional mutation N246₁K was detected in RG267 (G186₁V) HA. This led us to hypothesize that HA mutation G186₁V though play important role in egg adaptation, but may not be sufficient. Therefore, it is possible that RG267 HA along with N246₁K, acquired some other undetected (lower population) mutation which rendering it growth to reach to the same level of RG269. Increased H3N2 CVV growth due to the N246₁K HA mutation has not been reported previously, and it will be important to examine the effect of this mutation on H3N2 CVVs that carry the HA-186₁V mutation (e.g., influenza A/Victoria/361/2011[H3N2]).

The I102M, R762G, and D902N HA2 mutations we observed in this study were all accompanied by the N165₁K mutation. While their immediate impact on viral growth is uncertain, mutations surrounding these areas have been reported to influence HA acid stability [23]. Protein analysis showed reduced migration of the HA2 band of RG314-E6 in SDS-PAGE. It is possible that this altered migration pattern of HA2 could be a reflection of differential post-translational modification due to substitution of a charged Arg with neutral Gly at position 76 (R762G). This mutation may increase activation pH favoring improved virus replication in eggs. Supporting this possibility is prior work showing a Glu to Gly mutation at position 81 (E812G) of HA2 in an H3N2 virus (X31), which increased activation pH by 0.3, likely destabilized a hydrogen-bonded salt link with Arg 76, which is important for stabilizing the trimeric arrangement of α -helices [31].

Taken together, our results show that the egg-adapted H3N2v vaccine virus E6-RG269 (G186V + N246K), which acquired no other detectable HA mutations, retained its original antigenicity and immunogenicity while producing the greatest yield of HA protein. This vaccine virus may therefore be the best candidate for egg-based H3N2v vaccine production.

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Abbreviations

H3N2v	H3N2 variant
CVV	candidate vaccine virus
MDCK	Madin-Darby canine kidney cells
TRBC	turkey red bold cells
CRBC	chicken red blood cells
PFU	plaque forming unit.

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Fig. 1. SDS-PAGE analysis of deglycosylated viral proteins. Purified virus (E6) concentrate was treated or mock-treated with PNGase F and proteins were separated on a 12% polyacrylamide gel. Protein bands were stained with Coomassie blue and analyzed by densitometry. Protein intensity was estimated by combining the deglycosylated HA1 and NA bands; the sum is presented as a percentage of the RG267-E6 band intensity.

Table 1
Amino acid changes in the HA and NA of egg-adapted influenza A/Indiana/08/2011 (H3N2v).

Virus passage history	Isolate number	HA titer (TRBC/CRBC)	HA1 residues					HA2 residues					NA residues	
			165	183	186	194	233	246	10	90	71			
C4	WT	128/16	N	H	G	L	Y	N	I	D	I/T			
C4/E4/E1/E1	5	4096/4096			V						T			
	24	4096/4096			V			K			T			
	40	128/256				I	H				I			
	46	128/256				I	H				I			
C4/E4/C2/E1	55	512/2028		L							T			
	58	512/2028		L							I			
	71	4096/4096	K		V						T			
	89	2048/2048	K						I/M		I			
	91	512/2046		L							T			
	92	512/2046		L							T			
	93	512/2046		L							T			
	103	2048/4096	K							N	I			

TRBC, turkey red blood cells; CRBC, chicken red blood cells.

^aMDCK cell (C4)-grown A/Indiana/08/2011 (H3N2v) virus was passaged four times in eggs (C4/E4). Single infectious units were isolated by inoculating eggs with virus EID₅₀ (C4/E4/E1) or by two cycles of plaque purification in MDCK cells (C4/E4/C2) followed by one passage in eggs (C4/E4/E1/E1 or C4/E4/C2/E1) to obtain stock viruses. vRNA was isolated from WT (C4) or egg-adapted cloned virus isolates. HA and NA were sequenced as described in the Methods.

Table 2

Growth and antigenicity of candidate vaccine viruses with different passage histories.

CVV	Mutations in HA ^a (cloned from virus isolate #)	MDCK passage 1 (C1)			Egg passage 1 (E1)			Egg passage 6 (E6)			HA mutations ^c after E6		
		HA titer (TRBC/CRBC)	PFU/ml log ₁₀	HI titer ^b to ref. antisera	HA titer (TRBC/CRBC)	PFU/ml log ₁₀	HI titer ^b to ref. antisera	HA titer (TRBC/CRBC)	PFU/ml log ₁₀	HI titer ^b to ref. antisera			
		#1	#2	#1	#2	#1	#2	#1	#2	#1	#2		
RG265	WT	256/32	8.7	2560	2000	<2/<2	5.7	ND	ND	512/1024	640	400	L194 ₁ I (>80%)Y233 ₁ Y (≈40%)H (≈60%)
RG267	G186 ₁ V(5)	256/128	8.5	1600	1280	512/512	8.6	1280	1280	1024/2048	1280	1280	N246 ₁ K (>80%)
RG269	G186 ₁ V/N246 ₁ K (24)	64/64	7.4	1920	1440	1024/2048	9.3	1920	1440	2048/4096	1280	1280	None
RG271	L194 ₁ I/Y233 ₁ H (40)	128/128	8.1	1280	960	512/1024	8.7	640	720	ND	ND	ND	N/A
RG273	N165 ₁ K/G186 ₁ V (71)	128/64	7.9	2560	2560	1024/1024	8.8	2560	2560	1024/2048	1280	1280	None
RG275	H183 ₁ L (93)	<2/<2	4.8	ND	ND	128/256	7.7	960	800	ND	ND	ND	N/A
RG277	N165 ₁ K/D90 ₂ N (103)	128/64	8.0	2560	2560	512/1024	8.8	2560	2560	256/512	1440	1600	N126 ₁ K (>80%)
RG279	N165 ₁ K (89)	512/256	8.6	2560	2560	256/256	8.1	2560	2560	1024/2048	1280	1280	K190 ₁ V(2/8) or T128 ₁ N/R76 ₂ G(6/8) ^d
RG314	T128 ₁ N/N165 ₁ K/R76 ₂ G	ND	ND	ND	ND	1024/1024	8.4	ND	ND	1024/1024	1280	1280	None
RG281	N165 ₁ K/I10 ₂ M (89)	128/128	7.8	2560	2560	512/1024	8.8	2560	2560	1024/1024	2560	2560	T128 ₁ N (>80%)
RG316	T128 ₁ N/N165 ₁ K/I10 ₂ M	ND	ND	ND	ND	512/1024	8.4	ND	ND	512/1024	1280	1280	None

Abbreviations: TRBC, turkey red blood cells; CRBC, chicken red blood cells; CVV, candidate vaccine virus; HI, hemagglutination inhibition; HA, hemagglutinin; ND, not done; N/A, not applicable.

^aSubscripts 1 and 2 after amino acid position represent HA1 and HA2, respectively.

^bHI titer (using TRBC) to reference ferret antisera against WT vaccine virus RG265-C1; #1 and #2 represent two ferrets per virus. Values are the mean of 3–4 independent assays with each ferret’s serum.

^cMutations were identified by sequencing PCR products.

^dMutations in RG279-E6 (showing mixed nucleotides) were identified by sequencing eight independent HA clones. Fractions indicate the frequency of each type.

Table 3

Relative viral protein concentration of variant vaccine viruses.

CVVs (egg passage 6)	Protein concentration		Overall protein concentration relative to RG267	
	Measured by Bradford method ^a		As a measure of HA and NA combined protein band intensity ^b relative to RG267	
	Concentration (µg/µl)	Concentration relative to RG267		
RG267	4.2	100	100	100
RG269	7.4	176	185	180
RG273	4.0	95	102	98
RG279	3.2	76	70	73
RG281	3.6	86	78	82
RG314	3.0	71	67	69
RG316	1.9	45	32	38

^aViral protein concentrations were measured using Quick Start Bradford Protein Assay kit and bovine gamma-globulin standard.

^bProtein intensities were estimated by combining the deglycosylated HA1 and NA bands separated in SDS-PAGE and stained with Coomassie blue.

Table 4

Immunogenicity and cross-reactivity (with WT vaccine virus) of variant vaccine viruses.

Ferret serum against CVVs	Ferret #	Serum HI titers of against vaccine virus	
		Homologous	WT (RG265-C1)
RG265-C1	Ferret#1	N/A	2560, 2560, 2560, 2560
	Ferret #2	N/A	2560, 2560, 2560, 2560
RG267-E6	Ferret#1	1280, 1280, 1280, 1280	1280, 1280, 1280, 1280
	Ferret #2	1280, 1280, 1280, 1280	1280, 1280, 1280, 1280
RG269-E6	Ferret#1	640, 640, 1280, 640	640, 640, 1280, 640
	Ferret #2	640, 640, 1280, 640	640, 640, 1280, 640
RG273-E6	Ferret#1	640, 640, 640, 640	320, 320, 320, 320
	Ferret #2	640, 640, 640, 640	320, 320, 320, 320
RG279-E6	Ferret#1	640, 640, 640, 640	320, 320, 640, 320
	Ferret #2	640, 640, 1280, 640	640, 640, 640, 640
RG314-E6	Ferret#1	640, 640, 640, 640	640, 320, 640, 320
	Ferret #2	1280, 1280, 1280, 1280	640, 640, 1280, 640
RG281-E6	Ferret#1	1280, 1280, 1280, 1280	640, 640, 640, 640
	Ferret #2	640, 640, 640, 640	320, 320, 640, 320
RG316-E6	Ferret#1	640, 640, 1280, 1280	640, 640, 1280, 640
	Ferret #2	640, 640, 640, 640	640, 640, 640, 640

Ferrets ($n = 2$) were inoculated intranasally with 10^6 PFU of MDCK (C1)-grown WT virus (RG265-C1, reference) or with the indicated variant virus after 6 passages in eggs. Serum antibody at 21 days p.i. was titrated by HI assay against homologous virus and reference virus. N/A, not applicable.