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Bioengineering a highly productive vaccine strain in embryonated chicken eggs and mammals from a non-pathogenic clade 2·3·4·4 H5N8 strain

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ABSTRACT

The clade 2·3·4·4 H5Nx is a highly pathogenic avian influenza (HPAI) virus, which first appeared in China and has spread worldwide since then, including Korea. It is divided into subclades a - d, but the PR8-derived recombinant clade 2·3·4·4 a viruses replicate inefficiently in embryonated chicken eggs (ECEs). High virus titer in ECEs and no mammalian pathogenicity are the most important prerequisites of efficacious and safer vaccine strains against HPAI. In this study, we have synthesized hemagglutinin (HA) and neuraminidase (NA) genes based on the consensus amino acid sequences of the clade 2·3·4·4a and b H5N8 HPAIVs, using the GISAID database. We generated PR8-derived H5N8 recombinant viruses with single point mutations in HA and NA, which are related to efficient replication in ECEs. The H103Y mutation in HA increased mammalian pathogenicity as well as virus titer in ECEs, by 10-fold. We also successfully eradicated mammalian pathogenicity in H103Y-bearing H5N8 recombinant virus by exchanging PB2 genes of PR8 and 01310 (Korean H9N2 vaccine strain). The final optimized H5N8 vaccine strain completely protected against a heterologous clade 2·3·4·4c H5N6 HPAIV in chickens, and induced hemagglutination inhibition (HI) antibody in ducks. However, the antibody titer of ducks showed age-dependent results. Thus, H103Y and 01310PB2 gene have been successfully applied to generate a highly productive, safe, and efficacious clade 2·3·4·4 H5N8 vaccine strain in ECEs.

1. Introduction

Clade 2·3·4·4 H5Nx highly pathogenic avian influenza viruses (HPAIVs) with different neuraminidase (NA) subtypes have emerged in China and spread globally through multiple genetic reassortments of clade 2·3·4·4 H5N1 and other subtypes [1–4]. Clade 2·3·4·4 H5N8 HPAIV was first reported in Korea in early 2014 [4] and since then, several subclades (a–d) have been reported as of 2017 [5]. H5N8 HPAI clades 2·3·4·4a and b were detected in 2014. Clade 2·3·4·4a viruses are predominant and have caused substantial economic loss in the Korean poultry industry [6]. Five different reassorted H5N6 HPAI clade 2·3·4·4c viruses were isolated from poultry and wild birds in Korea in 2016. The clade 2·3·4·4b H5Nx viruses spread worldwide from 2014 to

Abbreviations: HPAI, highly pathogenic avian influenza; ECEs, embryonated chicken eggs; HA, hemagglutinin; NA, neuraminidase; HI, hemagglutination inhibition; FBS, fetal bovine serum; EID, embryo infectious disease; $TCID_{50}$, tissue culture Infective Dose; BEI, binary ethylenimine; HPAI, highly pathogenic avian influenza; NA, neuraminidase; ECEs, embryonated chicken eggs; HA, hemagglutinin; HI, hemagglutination inhibition; FBS, fetal bovine serum; d-o, day-old; RBCs, red blood cells; WHO, World Health Organization; EID_{50}, embryo infectious disease; hpi, hours post-inoculation; $TCID_{50}$, Tissue culture Infective Dose; w-o, week-old; dpi, days-post-inoculation; RDE II, Receptor Destroying Enzyme II; BEI, binary ethylenimine; wpi, weeks post inoculation; wpv, week-post-vaccination; wpc, week-post-challenge; dpc, days-post-challenge; pdmH1N1, pandemic H1N1.

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2018 [5,7,8]. Clade 2·3·4·4 HPAI viruses cause asymptomatic infection in Muscovy ducks and fatal infections in chickens and humans [9–11].

A/Puerto Rico/8/1934(H1N1) (PR8)-derived clade 2·3·4·4 (6:2 reassortant) and H5N8 vaccines have been developed for poultry and humans. They have been evaluated concerning their efficacy in protecting from death caused by homologous and heterologous viruses [12–15]. PR8-derived vaccine strains contain six internal protein coding genes of PR8 that confer high replication efficiency to embryonated chicken eggs (ECEs). The PB2 gene is one of the most important mammalian pathogenicity-determinants in AIVs and various mutations have been identified in this gene [16,17]. The PB2 gene in PR8 has even more mammalian pathogenicity-related mutations (E627K, A199S, A674T, T271A, and A588T) than those of the 1918 pandemic H1N1 virus (E627K, A199S, and K702R). Thus, the potential mammalian pathogenicity of conventional PR8-derived vaccine strains against HPAI need to be improved [17,18]. In addition, the E627K mutation negatively affects efficient replication in ECEs [18]. The PB2 gene of the A/chicken/Korea/01310/2001(H9N2) (01310) was successfully used for the generation of PR8-derived vaccine strains with high replication efficiency in ECEs and mammalian non-pathogenicity [19–22].

In order to increase virus titers of HPAIVs, successive virus passage through ECEs has been used and various mutations in hemagglutinin (HA) and neuraminidase (NA) have been identified [22–24]. HPAIVs replicate with high efficiency in ECEs due to polybasic amino acid residues at the cleavage site of HA. Few studies have sought to improve HA and NA genes in HPAIVs for better replication efficiency in ECEs [25]. The H103Y mutation increases the acid stability of HA by decreasing the pH at which HA undergoes irreversible conformational changes. This mutation is regarded as a mammalian pathogenicity-related mutation, which facilitates airborne transmission of H5N1 AIVs between mammals [26–29]. However, H103Y was also postulated to increase replication efficiency in ECEs, and a restricted role of H103Y in mammalian hosts was suspected [24,30].

In this study, we aimed to engineer an optimized PR8-derived clade 2·3·4·4 H5N8 HPAI vaccine strain to be highly replicative in ECEs and non-pathogenic to mammalian hosts. We synthesized the consensus HA and NA genes from clade 2·3·4·4 H5Nx HPAIV genes, and inserted single point mutations into genes related to high replication efficiency (H103Y, K161E, and L317P in HA; S369N in NA) [24]. We generated PR8-derived parent and mutant H5N8 recombinant viruses with PB2 genes of PR8 or 01310, and compared the replication efficiency in ECEs and mammalian pathogenicity in mouse and mammalian cells. We also evaluated the immunogenicity of the selected vaccine strain in specific pathogen-free (SPF) chickens and commercial ducks, and the protective efficacy in SPF chickens by infecting them with a heterologous clade 2·3·4·4c H5N6 HPAIV.

2. Materials and methods

2.1. Plasmids, cells, and eggs

Recombinant H5N8 viruses were generated using the Hoffmann reverse genetics vector system and the bidirectional pHW2000 vector [31]. The 293 T, MDCK, and A549 cells were purchased from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea). HEK293T and MDCK cells were maintained in Dulbecco's modified Eagles' medium (DMEM) containing 10% fetal bovine serum (FBS, Life Technologies Co., Carlsbad, CA, USA) and were used for plasmid transfection and recombinant virus generation. A549 cells were maintained in DMEM/F12 supplemented with 10% FBS (Life Technologies). The recombinant viruses generated were used in experiments after three passages in 10 day-old SPF ECEs (Charles River Laboratories, North Franklin, CT. USA). All recombinant viruses were confirmed by RT-PCR and genome sequencing.

2.2. Synthesis and cloning of consensus HA and NA genomes and site-directed mutagenesis

The Global Initiative on Sharing All Influenza Data (GISAID) and Influenza Research Database (IRD) databases were used to obtain the HA and NA genome sequences of subtype H5N8 clade 2.3.4.4 HPAIVs isolated in Asia from 2014 to 2016 (n = 80). They are listed in Supplementary Table 1. The BioEdit program (v7.2.5) was used for nucleotide sequence translation, amino acid comparison, and calculation of amino acid identity. The HA and NA sequences were compared and the consensus amino acid sequences were synthesized on the basis of the HA and NA genomes of A/broiler duck/Korea/Buan2/2014(H5N8) (Buan2) (Cosmo Genetech, Seoul, Korea). The multi-basic cleavage site of the HPAI clade 2.3.4.4 HA gene was substituted by a nucleotide sequence coding for ASGR in the synthetic HA genome for virulence attenuation, as previously reported [19]. Amino acid at position 499 (H5, Buan2, numbering) was mutated from asparagine (N) to threonine (T) to remove putative N-glycosylation. This likely decreases immunogenicity of the universal epitope in the stalk region of HA2 without affecting replication efficiency [32,33]. Synthetic HA and NA genomes were cloned into the pHW2000 vector using universal primer sets. Site-directed mutagenesis was performed to introduce mutations related to replication efficiency of HA (H103Y, K161E and L317P) and NA (S369N) with a commercial kit (iNtRON Biotechnology, Gyeonggi-do, Korea) as previously reported [24,34]. The mutagenesis primers used in this study are listed in Table 1.

2.3. Recombinant virus generation

PR8-derived H5N8 recombinant viruses were generated by combining eight genome segments and cloning them into a bidirectional reverse genetics vector (pHW2000) as previously described [20]. The PB2 genome of PR8 was replaced using the pHW2000 plasmid containing the 01310 PB2 gene, as previously described [35]. Briefly, 300 ng each of the eight plasmids was mixed with Lipofectamine 2000 and Plus reagents (Life Technologies) and transfected into 293 T cells (1×10^6 cells/well in a 6-well plate). Following overnight incubation, 1 ml of Opti-MEM (Life Technologies) and 4 μg/well of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich, St. Louis, MO, USA) were added and incubated for another 24 h. 200 μl of the harvested culture media were injected into 10-day-old SPF ECEs via the allantoic cavity. Inoculated ECEs were incubated for 3 days with candling twice a day, after which the allantoic fluid was harvested. The presence of recombinant virus was confirmed by the HA assay using 1% (v/v) chicken red blood cells (RBCs), according to the World Health Organization (WHO) Manual

Table 1
Primer sets used for the single point mutagenesis of the consensus H5N8 genome.

Primer[a]	Sequence (5′–3′)
H5N8–hmH103Y–F	TGAAGA ACTGAAATACCTATTGAGCAGAATA
H5N8–hmH103Y–R	TATTCTGCTCAATAGGTATTTCAGTTCTTCA
H5N8–hmK161E–F	CATACCCAACAATAGAAATAAGCTACAATAA
H5N8–hmK161E–R	TTATTGTAGCTTATTTCTATTGTTGGGTATG
H5N8–hmL317P–F	CTTGCGACTGGGCCCAGAAATAGTCCTC
H5N8–hmL317P–R	GAGGACTATTTCTGGGCCCAGTCGCAAG
H5N8–nmS369N–F	GTCGAACCTCCAGAAATGGATTTGAAATAATAAG
H5N8–nmS369N–R	CTTATTATTTCAAATCCATTTCTGGAGGTTCGAC

[a] hm primer sets were used for HA mutation, and nm primer sets were used for NA mutation.

on Animal Influenza Diagnosis and Surveillance, and specific nucleotide sequences of recombinant viruses were confirmed by RT-PCR and sequencing [24,35].

2.4. Virus titration

The rescued viruses were inoculated into 10-day-old SPF ECEs and harvested (embryo passage 2, E2). The E2 viruses were serially diluted from 10^{-1} to 10^{-9} and injected into five 10-day-old SPF ECEs to determine viral titer. The 50% chicken embryo infectious dose (EID_{50}) was calculated using the Spearman-Karber method [36].

2.5. Comparative replication efficiency in ECEs and mammalian cells

Replication efficiency in ECEs was compared by inoculating 100 EID_{50}/0.1 ml of each recombinant virus (E2) into 10-day-old ECEs via the allantoic cavity route. Following incubation for 3 days, the inoculated virus was harvested and EID_{50} of each virus was measured as described above. The growth kinetics in MDCK and A549 cells were estimated by harvesting supernatants of cells inoculated with 5×10^5 EID_{50}/0.5 ml of virus in a 12-well plate, at 0, 24, 48, and 72 h post inoculation (hpi) in MDCK cells, and 51 and 71 hpi in A549 cells. The 10-fold diluent of each supernatant was inoculated into confluent MDCK cells in 96-well plates and the 50% tissue culture infective dose ($TCID_{50}$) was calculated by the Spearman-Karber method.

2.6. Mouse pathogenicity test

The mouse pathogenicity test was approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (IACUC-SNU-171214–1-1) and conducted in a biosafety level 2 facility at the Animal Center for Pharmaceutical Research of Seoul National University (Seoul, Korea) according to the national guidelines for the care and use of laboratory animals. Eight 6-week-old female BALB/c mice (KOATEC, Pyeongtaek, Korea) of each group were sedated by an intraperitoneal injection of Zoletil 50 (15 mg/kg; Virbac, Carros, France), and 10^6 EID_{50}/0.1 ml of each recombinant virus was inoculated via the intranasal route. The negative control group (mock) was inoculated with the same volume of sterile phosphate buffered saline (PBS). Three of eight mice in each group were euthanized through CO_2 asphyxiation to test virus replication and virus titers in the lungs at 3 days-post-inoculation (dpi). The remaining mice were observed for weight loss and mortality over 2 weeks. The mice were euthanized by CO_2 asphyxiation when the body weight of each mouse was reduced by more than 20%. The collected lungs were homogenized using a TissueLyzer 2 (Qiagen, Valencia, CA, USA) equipped with 5 mm diameter stainless steel beads and mixed with PBS (10 volumes of lung weight). Following centrifugation at 13,000 rpm for 10 min, 0.1 ml of each supernatant and 10-fold diluted pooled supernatants were inoculated into ECEs as described above. The presence of virus in harvested allantoic fluid was tested by the plate HA test with 1% chicken RBCs. The virus titer of pooled lung specimens was calculated as described above.

2.7. Immunogenicity of inactivated recombinant vaccine in chickens and ducks

Ten milliliters of the candidate vaccine strain in undilute allantoic fluid was inactivated with 1 ml of 1 M binary ethylenimine (BEI; final concentration 0.1 M) and incubated at 37 °C for 24 h. The virus inactivation reaction was completed by adding 0.1 M sodium thiosulfate and was confirmed by inoculation into 10-day-old ECEs. The inactivated virus was mixed with ISA70 at an oil-to-virus ratio of 7:3 to make an inactivated oil emulsion vaccine. Allantoic fluid of uninfected 10-day-old ECEs was mixed with ISA70 and used as the negative control group.

The duck vaccination experiment was approved by the Institutional Animal Care and Use Committee of Seoul National University (IACUC-SNU-181205–6). Seven 1-day-old ducks and 2-week-old ducks were vaccinated with 0.5 ml of the oil-adjuvant inactivated vaccine, and serum was collected at 0, 3, and 4 weeks post vaccination (wpv).

2.8. Protection efficacy test of inactivated oil emulsion vaccine in chickens

Protection efficacy test in chickens was approved by the IACUC of Konkuk University (IACUC-KU18179) and was conducted in a biosafety level 3 facility at Konkuk University. Nine 3-week-old SPF chickens (Namduck Sanitek, Korea) in each group were vaccinated with the inactivated oil emulsion vaccine (0.5 ml/chicken) and challenged intra-nasally with the 10^6 EID of A/Mandarin duck/Korea/K16-187-3/2016 (H5N6) (K16-187-3) at 3 wpv. The serum of vaccinated chickens was collected at 0 and 3 wpv and 1 week-post-challenge (wpc) for serum antibody analysis. Oropharyngeal and cloacal swab samples were collected at 1, 3, 5, and 7 days-post-challenge (dpc) and virus shedding through oropharyngeal and cloacal routes was evaluated by RT-PCR using an Applied Biosystems 7500 Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) as previously described [37].

2.9. Serological test

Serum antibodies of vaccinated chickens and ducks were estimated by the hemagglutination inhibition (HI) assay following the WHO Manual on Animal Influenza Diagnosis and Surveillance. Chicken and duck serum samples were treated at 56 °C for 30 min, and duck serum samples were mixed with three volumes of Receptor Destroying Enzyme II (RDE II; Denka Seiken Co., Ltd., Tokyo, Japan) and incubated for 24 h before heat treatment. The treated serum was diluted 2-fold with PBS, and 25 μl of the diluted serum was mixed with the same volume of rH5N8-H103Y-310 PB2 antigen with a hemagglutination titer (HAT) of 4. After incubation for 30 min at 4 °C, 25 μl of 1% (v/v) chicken RBCs was added and the serum antibody titer was recorded after 40 min of incubation at 4 °C.

2.10. Heat stability test

The HAT of each virus was measured before heat treatment as described above. Each virus was diluted to an HAT of 64 with PBS. Aliquots of each dilution were dispensed into three tubes and incubated for 30 min at 50, 55, and 60 °C. The HAT of each virus was determined after the heat treatment.

2.11. Statistical analyses

The significance of replication efficiency and body weight changes were evaluated by one-way analysis-of-variance, and the difference of average body weight was assessed by the Student's *t*-test (log-rank test, 95% confidence intervals) using SPSS statistical software (IBM, Armonk, NY, USA). A p-value <0.05 indicated statistical significance.

3. Results

3.1. Genetic characteristics of synthesized consensus HA and NA genes of clade 2.3.4.4 H5N8 recombinant viruses

The variable amino acid residues in the synthesized consensus HA and NA sequences from other clade 2·3·4·4 viruses are summarized in Tables 2 and 3. The amino acid sequences of representative H5N8 Korean isolates Buan2 (clade 2·3·4·4a) and Gochang1 (clade 2·3·4·4b) from 2014, the more recent H5N8 strain from 2017, and the challenge strain (H5N6, clade 2·3·4·4c) were compared with the synthetic consensus HA and NA sequences. The sequence identity with the HA1 amino acid sequence of Buan2, Gochang1, the recent H5N8, and the challenge strains bearing the cleavage site sequences was 99.1%, 97.6%, 97.3%, and 95.9%, respectively (Table 2). Among the variable amino acid residues, 145 and 156 residues were the components of known epitopes and were variable in the challenge virus (145L deletion), Gochang1, and the recent H5N8 strain (A156T) [38,39].

The sequence identity of Buan2, Gochang1, and the recent H5N8 strain with the NA amino acid sequence was 99.4%, 98.5%, and 98.9%, respectively (Table 3). The NA stalks of the viruses were compared, including the synthetic consensus NA sequence. The stalks were intact with no amino acid deletion when compared with the earliest H5N8 isolate, A/turkey/Ireland/1378/1983 (H5N8) (accession no. EPI129564).

3.2. Comparison of replication efficiency of H5N8 recombinant viruses in ECEs

PR8-derived parent (rH5N8) and mutant H5N8 recombinant viruses containing a single mutation in HA (rH5N8-hmH103Y, rH5N8-hmK161E, and rH5N8-hmL317P) and NA (rH5N8-nmS369N), combined mutations in HA and NA (rH5N8-wm), and single point HA mutation and 01310 PB2 (rH5N8-hmH103Y-310 PB2) were successfully generated (Table 4). The virus titer of rH5N8 of $10^{8.3}$ EID_{50}/ml was slightly higher than that of rH5N8-hmL317P ($10^{8.0}$ EID_{50}/ml), rH5N8-nmS369N ($10^{8.1}$ EID_{50}/ml), and rH5N8-wm ($10^{7.8}$ EID_{50}/ml). However, the difference was not significant ($p > 0.05$). Similarly, rH5N8-hmK161E showed a slightly higher virus titer than that of rH5N8, which was also not significant. However, rH5N8-hmH103Y and rH5N8-hmH103Y-310 PB2 showed significantly higher virus titers than that of rH5N8 ($p < 0.05$) (Table 4).

3.3. Comparison of replication efficiency of the H5N8 recombinant viruses in mammalian cells

The replication efficiency of recombinant viruses, rH5N8, rH5N8-hmH103Y, rH5N8- rH5N8-hmH103Y-310 PB2, and rPR8 in mammalian cells was compared on the basis of the growth kinetics in MDCK and A549 cells. In MDCK cells, only rH5N8-hmH103Y-310 PB2 did not replicate, and rH5N8-hmH103Y replicated better than rH5N8 did, with a significantly higher virus titer. Moreover, rPR8 virus replicated efficiently in A549 cells, while rH5N8 and rH5N8-hmH103Y-310 PB2 did not. Interestingly, rH5N8-hmH103Y replicated in A549 cells and showed significantly higher virus titer at 24 hpi ($10^{2.50}$ $TCID_{50}$) and 51 hpi ($10^{2.33}$ $TCID_{50}$) compared to rH5N8 ($10^{0.67}$ $TCID_{50}$) and rH5N8-hmH103Y-310 PB2 (undetectable level of $TCID_{50}$) ($p < 0.05$, Fig. 1).

3.4. Comparison of pathogenicity of H5N8 recombinant viruses in mice

Pathogenicity of rH5N8, rH5N8-hmH103Y, rH5N8-hmH103Y-310 PB2, and rPR8 was compared in 6-week-old female BALB/c mice on the basis of virus titer in the lungs and changes in body weight (Table 5 and Fig. 2). All recombinant viruses, except rPR8, showed no loss in body weight, but rH5N8 and rH5N8-hmH103Y were detected in all inoculated mice at 3 dpi. The virus titer of rH5N8-hmH103Y ($10^{4.5}$ EID_{50}/0.1 ml) was significantly higher than that of rH5N8 ($10^{3.7}$ EID_{50}/0.1 ml), but rH5N8-hmH103Y-310 PB2 was not detected in any of the inoculated mice.

Table 2
Comparison of variable amino acid sequences between clade 2·3·4·4 H5 proteins and the synthetic consensus H5 used in this study.

Variable residue[a]	Buan2	Gochang1	Recent H5N8[b]	Challenge strain[c]	Consensus H5[d]
Subgroup	a	b	b	c	a
10	V	I	I	V	I
16	S	S	G	S	S
30	K	E	E	E	K
110	T	S	S	N	T
130	T	I	I	T	I
140	N	D	N	N	N
145[e]	L	L	L	–[f]	L
149	A	A	A	S	A
156[e]	A	T	T	V	A
157	S	P	P	P	S
167	I	I	I	T	I
178	I	I	I	M	I
185	R	R	R	G	R
201	A	A	E	A	A
211	D	T	T	T	N
214	V	V	I	V	V
239	R	R	R	Q	R
285	V	V	V	M	V
298	I	V	V	I	I
336	S	N	S	S	S
% identity with the consensus HA1[g]	99.1	97.6	97.3	95.9	100.0

[a] H5 numbering including signal peptide.
[b] Recent H5N8: recently isolated H5N8 strains in Korea since 2017.
[c] Challenge strain: A/Mandarin duck/Korea/K16-187-3/2016 (H5N6).
[d] Consensus H5: synthetic consensus H5 sequence used in this study.
[e] Amino acid residue in reported epitope.
[f] –: Deletion.
[g] Homology with the consensus H5 sequence barring the cleavage site sequences.

Table 3
Comparison of the variable amino acid sequences between natural N8 and the synthetic consensus N8 sequence used in this study.

Variable residue	Strain			
	Buan2	Gochang1	Recent H5N8	Consensus N8
8	V	M	V	V
18	V	A	V	V
30	I	T	I	I
46	N	K	K	N
136	S	A	A	S
190	A	T	T	T
264	R	Q	T	G
303	I	V	V	I
329	T	A	A	T
397	S	L	L	L
% identity with the consensus N8 sequence	99.4	98.5	98.9	100.0

Table 4
Genome segments used for recombinant virus generation and the viral titer.

Recombinant virus	Mutation		PB2	EID_{50}/ml[b]
	HA[a]	NA		
rH5N8	–[a]	–	PR8	8.3 ± 0.3
rH5N8-hmH103Y	H103Y[b]	–	PR8	9.3 ± 0.3*
rH5N8-hmK161E	K161E	–	PR8	8.8 ± 0.3
rH5N8-hmL317P	L317P	–	PR8	8.0 ± 0.4
rH5N8-nmS369N	–	S369N	PR8	8.1 ± 0.5
rH5N8-wm	H103Y, K161E, L317P	S369N	PR8	7.8 ± 0.4
rH5N8-hmH103Y-310 PB2	H103Y	–	01,310	9.3 ± 0.1*

[a] H3 numbering.
[b] Viral titer measurement after inoculation of each virus with 100 EID_{50} into 10 do SPF ECEs.
* Significant difference compared to rH5N8 ($p < 0.05$).

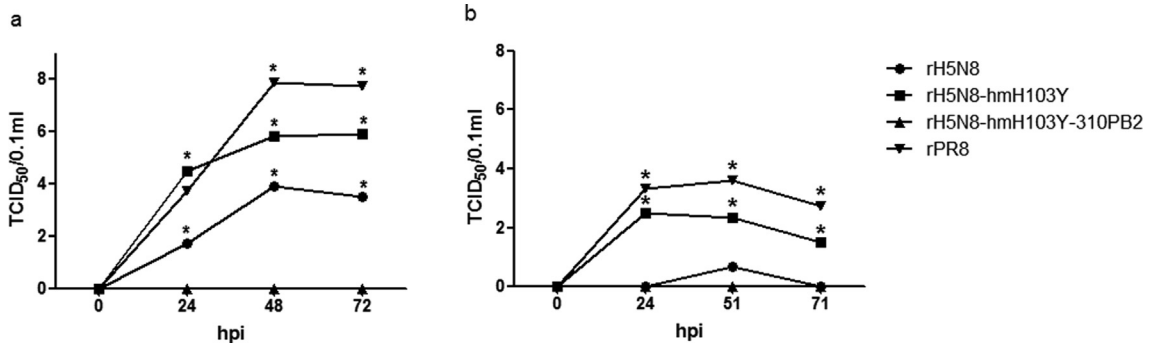

Fig. 1. Growth kinetics of recombinant viruses in mammalian cells. Each recombinant virus (5×10^5 EID_{50}/0.5 ml) was inoculated into MDCK and A549 cells. Supernatants were harvested at 0, 24, 48, and 72 hpi in MDCK cells and at 51 and 71 hpi in A549 cells following 72 h incubation at 37 °C in a CO_2 chamber. The viral titer at each time point was titrated as $TCID_{50}$ in MDCK cells. Significant difference is denoted by an asterisk ($p < 0.05$). The data was the average of three independent experiments.

Table 5
Virus isolation rate and viral titer isolated from mouse lung at 3 dpi.

Recombinant virus	Virus isolation rate	Log_{10} EID_{50}/0.1 ml[a]
rH5N8	3/3	3.70
rH5N8-hmH103Y	3/3	4.50
rH5N8-hmH103Y-310 PB2	0/3	0.00
rPR8	3/3	7.25
mock	0/3	0.00

[a] EID_{50}/0.1 ml was measured with pooled lung samples.

3.5. Effect of H103Y mutation on heat stability of HA protein

HAT of 64 of rH5N8-hmH103Y did not decrease, but the HAT of 2 of rH5N8 decreased after heat treatment at 55 °C for 30 min. Moreover, the HAT of rH5N8 decreased to an undetectable level, but rH5N8-hmH103Y showed residual hemagglutination activity (HAT of 4) after incubation at 60 °C for 30 min (Fig. 3).

3.6. Heterologous protection efficacy of inactivated oil emulsion vaccine in chickens

Chickens inoculated with the inactivated oil emulsion vaccine of rH5N8-hmH103Y-310 PB2 showed geometric mean HI titers with 95% confidence interval (CI) at 3 wpv (before challenge) of 174.2 (95% CI, 85.60–354.4) and at 1 wpc (after 1 week of challenge) of 94.06 (95% CI, 46.23–191.4). Surprisingly, the HI titer decreased after challenge, instead of increasing. The vaccinated chickens were challenged with heterologous clade 2·3·4·4c H5N6 HPAI virus, K16-187-3, at 3 wpv. Eight chickens in the mock group died at 2 dpc and one at 3 dpc. All chickens in the vaccine group were protected from mortality. However, virus shedding through the oro-pharynx and cloaca was not protected and virus was detected in the oro-pharynx (5/9) and cloaca (1/9) until the end of the experiment at 7 dpc (Table 6).

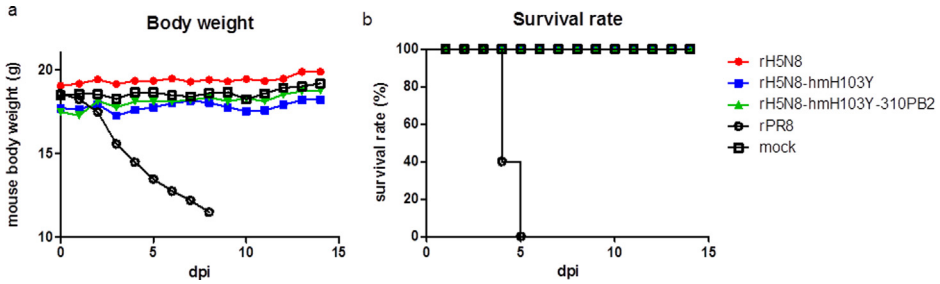

Fig. 2. Pathogenicity of recombinant viruses in mice. Five 6-week-old BALB/c mice were challenged with 10^6 EID_{50} of each virus or PBS (mock) via the intranasal route and body weight was measured for 2 weeks. (a) Change in the body weight of mice and (b) survival rate of virus infected mice groups. Statistical significance was assessed by Student's *t*-test ($p < 0.05$).

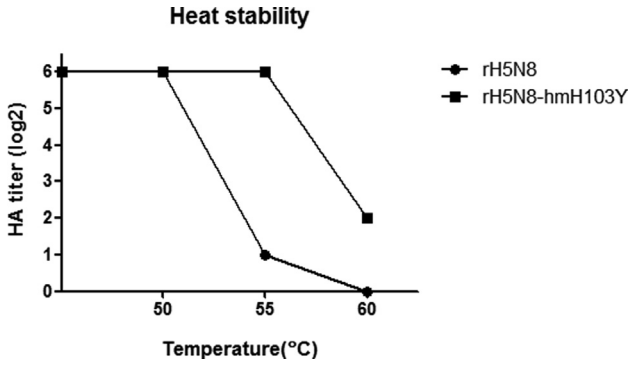

Fig. 3. Heat stability of rH5N8 and rH5N8-hmH103Y. Each virus was diluted with PBA to an HAT of 64, and three aliquots of each virus were incubated at 50, 55, and 60 °C for 30 min. After heat treatment, HAT was determined by the HA assay using 1% (v/v) chicken RBCs.

3.7. Immunogenicity of inactivated oil emulsion vaccine of rH5N8-hmH103Y-310 PB2 in ducks

Ducks that were vaccinated at 1-day-of-age showed undetectable HI titers at 3 and 4 wpv. However, ducks vaccinated at 2-weeks-of-age showed significantly higher HI titers of 21.5 (95% CI, 15.3–30.3) at 3 wpv and 23.8 (95% CI, 9.7–58.2) at 4 wpv. HI antibody was not detected in any chicken and duck before vaccination in the vaccine groups and before and after vaccination in the mock groups (Tables 6 and 7).

4. Discussion

The HA and NA surface glycoproteins are the main targets of host humoral immunity [40]. Clade 2·3·4·4b is currently prevalent in wild birds and poultry, and clade 2·3·4·4c has evolved to become more pathogenic in mammals [11,41]. The synthesized consensus HA gene showed higher amino acid identity of HA1 to clade 2·3·4·4a than clades 2·3·4·4b and c (Supplementary Table 2). In addition, clades 2·3·4·4b and c include NA subtypes other than N8. Therefore, updating HA and NA to match the outbreak virus may be better than using only consensus HA [42,43].

A/wild duck/Korea/SNU50-5/2011 (H5N1) (50-5) showed increased viral titer in ECEs after 20 passages and acquired multiple mutations in PA, HA, NA, M1, and M2 genes. The mutations in HA and NA genes were sufficient to increase the titer of PR8-derived recombinant viruses [24]. In this study, the single H103Y and K161E mutations increased virus titers, but the single L317P and S369N mutations and all combined mutations decreased virus titer only slightly in ECEs (Table 3). The H103Y mutation has been reported to enhance the air-borne transmission of H5N1 viruses in mammalian hosts by enhancing thermo- and pH stability [26–29], but it was also related to high virus titers in ECEs [24]. AIVs and recombinant viruses were typically passaged and incubated at 37.3–37.8 °C, and the pH of the allantoic fluid may be decreased at higher incubation temperature [44]. The H103Y mutation is related to thermo- and pH stability, which may have helped in maintaining active HA and virus infectivity during temperature and pH variations in the incubation environment of the ECEs. Lower incubation temperatures of 33–37 °C achieve higher virus titers of PR8 than those achieved at 37–39 °C [45]. However, the optimal temperature for chicken embryo development is 37.8 °C [46], and the H103Y mutation may support virus replication at the optimal embryonic cell condition by buffering the negative effect of acidic condition at higher incubation temperatures. Although we did not test pH stability, the correlation of H103Y with thermo-stability was also verified in this study.

In the Y161F mutation in pandemic H1N1 (pdmH1N1) in 2009, H3N2 and H3N8 viruses increased virus titer in MDCK and ECEs and also increased thermo-stability of HA [47]. The K161E mutation in this study [24] is located three amino acids from Y161F [47], but their proximal locations may be noteworthy. However, our 161 residue is close to the HA1 epitope site group 2, and there-

Table 6
Serum antibody titer, survival rate and viral shedding rate after wild-type H5N6 HPAI virus challenge in chickens.

Inactivated vaccine stain	GMT HI titer [a]			Survival rate	Viral shedding rate							
					Oro-pharynx[b]				Cloaca			
	0	3 wpv	1 pc		1[c]	3	5	7	1	3	5	7
rH5N8-hmH103Y-310 PB2	<2[d]	174.2 (85.60–354.4)	94.06 (46.23–191.4)	9/9 (100%)	0/9	3/9	6/9	5/9	1/9	4/9	4/9	1/9
Mock	<2	<2	<2	0/9 (0%)	9/9	1/1[e]	nt[f]	nt	7/9	1/1[e]	nt	nt

[a] Geometric mean HI titer with 95% confident interval
[b] Viral shedding was confirmed by real-time RT-PCR with oro-pharyngeal and cloacal swab samples.
[c] dpc: day post challenge.
[d] <2: undectectable titer
[e] Eight and one chickens were dead at 2 and 3 dpc, respectively.
[f] nt: not tested.

Table 7
Serum antibody titer of duck vaccination groups.

Host	Vaccinated age	Vaccine	GMT HI titer[a]		
			0 wpv[b]	3 wpv	4 wpv
Duck	1-Day-old	rH5N8-hmH103Y-310 PB2	<2[c]	<2	<2
		Mock	<2	<2	<2
	2-Week-old	rH5N8-hmH103Y-310 PB2	<2	21.5 (15.3–30.3)	23.8 (9.7–58.2)
		Mock	<2	<2	<2

[a] Serum antibody titer was measured against 4 HAU of rH5N8–hmH103Y–310 PB2 antigen.
[b] wpv: week-post-vaccination.
[c] <2: undectectable titer.

fore, we excluded the K161E mutation for the generation of the vaccine strain [39]. T318I mutation was reported to stabilize fusion peptide and helix A resulting in thermo- and pH stability, and it is located in close proximity to the 52 residue of HA2 [26,48,49]. The L317P mutation occurs immediately before the 318 residue and was first observed together with the R51K mutation in HA2 of 50-5 [24]. As the R51K mutation was not included, a single L317P mutation and the combined mutations may be not enough to increase the virus titer without the epistatic R51K or in the context of different amino acids of the clade 2·3·4·4 HA [50].

Although PR8 PB2 gene already possesses multiple mammalian pathogenicity-related mutations, including the fatal E627K mutation, it has been used for the generation of vaccine strains against human fatal AIVs [16,51]. Biosecurity and biosafety have been well-controlled during vaccine strain development and vaccine production, but efforts to reduce potential risks should be continued. The PB2 gene of 01310 is a prototypic PB2 gene without mammalian pathogenicity-related mutations, and also increases the viral titer of recombinant viruses in ECEs [18,19,21,35]. As expected, replacement of PR8 PB2 gene with the 01310 PB2 gene (rH5N8-hmH103Y-310 PB2) removed the replication capacity of rH5N8-hmH103Y in mammalian cells and the lungs of BALB/c mice, while maintaining high viral titer in ECEs. Therefore, the broad applicability of the 01310 PB2 gene to generate more productive and safer vaccines was demonstrated again [19,21].

Vaccination with rH5N8-hmH103Y-310 PB2 completely protected against the lethal challenge of heterologous clade 2·3·4·4c viruses, but did not prevent virus shedding. Several clade 2·3·4·4 vaccine strains have been evaluated. None has shown any defense from virus shedding or high mortality after challenge (mostly heterologous) [12,15,42]. The virus shedding after vaccination observed in this study may be related to an antigenic mismatch between the vaccine and challenge strains, relatively low amino acid identity (95.9%), and heterologous NA (N8 vs. N6) [42,51]. All the reported clade 2·3·4·4 vaccine strains possess an HA that is identical to the corresponding parent field strains, except at the cleavage site. However, information on vaccine virus titers was not available. As different experimental conditions may affect vaccine efficacy including inactivation methods, vaccine strain titer and inoculation volume per dose, we could not directly compare the relative efficacy of our vaccine to others. However, our vaccine formulation showed higher mean HI titer at 3 wpv than previous reports [12,42]. In addition, the mean HI titer did not change after challenge at 1 wpc. Therefore, the humoral immunity induced by single inoculation of rH5N8-hmH103Y-310 PB2 may be sufficient to be no longer activated by the challenge virus [42].

Ducks are more resistant than chickens to HPAI viruses and excrete virus asymptomatically [52,53]. Since asymptomatic ducks can transmit HPAI viruses to chickens, vaccination of ducks can be a strategy to control HPAI infection in domestic poultry. However, ducks show poor antibody responses to influenza viruses and the immune systems of ducks and chickens react differently to AIV infection [54–58]. Earlier induction of acquired humoral immunity in ducks is desirable, but different and age-dependent humoral immunity of ducks was apparent in this study. Although our vaccine formulation induced HI antibody in 2 w-o ducks, protective behavior of the HI antibody titers could not be predicted [59,60]. Since efficacious live vector vaccines are available, prime-boost strategy by using rH5N8-hmH103Y-310 PB2 needs to be verified in the future protection efficacy study [42,59].

In this study, we successfully bioengineered a PR8-derived highly productive in ECEs and mammalian-nonpathogenic clade 2.3.4.4 H5N8 recombinant vaccine strain by introducing H103Y mutation and replacing PR8 and 01310 PB2 genes. The established molecular formulation to bioengineer better vaccine strains may be useful for vaccine development.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.08.074.

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