Vaccine 37 (2019) 484–493

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Preclinical evaluation of the efficacy of an H5N8 vaccine candidate (IDCDC-RG43A) in mouse and ferret models for pandemic preparedness

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ARTICLE INFO

Article history:
Received 13 June 2018
Received in revised form 9 November 2018
Accepted 20 November 2018
Available online 27 November 2018

Keywords:
H5N8 pre-pandemic vaccine
Preclinical evaluation
Immunogenicity
Protective efficacy

ABSTRACT

Because H5N1 influenza viruses continuously threaten the public health, the WHO has prepared various clades of H5N1 mock-up vaccines as one of the measures for pandemic preparedness. The recent worldwide outbreak of H5Nx virus which belongs to clade 2.3.4.4 and of which H5N6 subtype belongs and already caused human infection also increases the need of pandemic vaccine for such novel emerging viruses. In this study, we evaluated the protective efficacy and immunogenicity of an egg-based and inactivated whole-virus H5N8 (IDCDC-RG43A) developed by CDC containing HA and NA gene of the parent virus A/gyrfalcon/Washington/41088-6/2014. Mice vaccinated two times elicited low to moderate antibody titer in varying amount of antigen doses against the homologous H5N8 vaccine virus and heterologous intra–clade 2.3.4.4 H5N6 (A/Sichuan/26221/2014) virus. Mice immunized with at least 3.0 μg/dose of IDCDC-RG43A with aluminum hydroxide adjuvant were completely protected from lethal challenge with the mouse-adapted H5N8 (A/Environment/Korea/ma468/2015, maH5N8) as well as cleared the viral replication in tissues including lung, brain, spleen, and kidney. Vaccinated ferrets induced high antibody titers against clade 2.3.4.4 H5N8/H5N6 viruses and the antibody showed high cross-reactivity to clade 2.2 H5N1 but not to clade 1 and 2.3.4 viruses as measured by hemagglutinin inhibition and serum neutralization assays. Furthermore, administration of the vaccine in ferrets resulted in attenuation of clinical disease signs and virus spread to peripheral organs including lung, spleen, and kidney from high dose challenge with maH5N8 virus. The protective and immunogenic characteristic of the candidate vaccine are essential attributes to be considered for further clinical trials as a pre-pandemic vaccine for a potential pandemic virus.

1. Introduction

The pandemic threat posed by H5Nx virus, causing severe disease and mortality in both animals and humans, have raised the public health concern. Viruses of the H5 subtypes are continuously isolated from poultry and wild birds in Asia, the Middle East, Africa, and Europe [1,2] of which sporadic transmission in humans have also occurred [3,4]. In 2015, The WHO/OIE/FAO H5 Evolution Working Group have updated the H5 hemagglutinin (HA) clade nomenclature due to the increasing number of detection of H5Nx viruses as well as its rapid evolution that have led to the new designation clade 2.3.4.4 for H5N1, H5N2, H5N5, H5N6 and H5N8 subtype viruses [5]. This recent H5 highly pathogenic avian influenza virus (HPAIV) of clade 2.3.4.4 is found to be antigenically different from the viruses of clades 2.3.4 and 2.3.2.1, suggesting the occurrence of diverse antigenic evolution [6]. Among the H5Nx viruses, strains of HPAIV (H5N8) of clade 2.3.4.4 were first detected in 2010 in wild birds in Asia and have spread across China, Japan and South

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https://doi.org/10.1016/j.vaccine.2018.11.064
0264-410X/

Korea and recently spread to Europe and for the first time, to America, causing a severe outbreak and economic loss in poultry industry [7]. Aside from its severe effect in the poultry health, it also endangers human public health as the H5N6, its related virus also belonging to clade 2.3.4.4, has reportedly breached the interspecies barrier sporadically causing severe disease in humans [8–10].

Since these emerging H5Nx viruses present unpredictable antigenic properties and cause occasional transmission of HPAIV from infected poultry to humans, surveillance and monitoring are essential for controlling the spread of the virus and that development of HPAI vaccines is considered the primary strategy for pandemic preparedness. However, development of such vaccines presents some challenges especially in the timely development of an efficacious and safe vaccine and requires a significant early planning. Thus, some advanced studies such as evaluating "mock-up" vaccines, which differs from other candidate vaccines by containing an influenza strain that has not recently circulated in the human population [11], have greatly hastened the approval and licensing of pandemic vaccines.

In this study, we investigated the efficacy and immunogenicity of an inactivated whole-virus H5N8 containing HA and NA gene from a candidate vaccine strain A/gyrfalcon/Washington/41088-6/2014 (IDCDC-RG43A) recommended by WHO for pre-clinical trial and pandemic preparedness [2]. As a pandemic preparedness vaccine or "mock vaccine", such advance study was demonstrated to this specific H5 virus due to its most recent addition in the list of influenza candidate vaccine viruses recommended by WHO [2]. Prior to clinical trials in humans and further production, influenza vaccines are pre-clinically screened by testing on mammalian models such as mice and ferrets to evaluate vaccine efficacy and safety [12].

2. Materials and methods

2.1. Ethics statement

All animal experiments were conducted in accordance and adherence to relevant policies on animal handling as mandated in the Guidelines for Animal Use and Care of the Chungbuk National University and the experimental protocol was approved by Institutional Biosafety Committee in Chungbuk National University (CBNUA-1121-17-01 and CBNUA-1057-17-01). All experiments using HPAI viruses were performed in biosafety level-3 laboratory (BSL3) at Chungbuk National University approved by Korea Centers for Disease Control and Prevention.

2.2. Viruses

The virus strain HPAI H5N8 A/gyrfalcon/Washington/41088-6/2014 (clade 2.3.4.4, H5N8, IDCDC-RG43A, vacH5N8) used as a mock vaccine in this study was selected from the list of candidate vaccine viruses recommended by WHO [2]. The virus was isolated from the brain of an infected falcon and was characterized by CDC [13]. Master and working seeds were prepared from the parent virus stock which was kindly provided by WHO in accordance with standard procedure. Virus stocks were serially diluted 10-fold and propagated in the allantoic cavities of 10-day old embryonated chicken eggs incubated at 37 °C for 48 h and the harvested allantoic fluid after overnight chilling were stored in −80 °C until use. The viral infectivity was calculated as $\log_{10}EID_{50}$ per milliliter ($\log_{10}$ EID_{50}/ml) by the method of Reed and Muench [14]. The mouse-adapted H5N8 virus A/Environment/Korea/ma468/2015 (maH5N8) [15] was used as a challenge virus in both mice and ferret animal models. Other viruses used in serologic assays to confirm cross-reactivities were H5 vaccine strains generated using multi-cleavage peptide truncated HA and NA genes including A/Environment/Korea/W468/15 (clade 2.3.4.4, H5N8, Rgdel468/PR8), A/Sichuan/26221/2014 (clade 2.3.4.4, H5N6, IDCDC-RG42A, vacH5N6), A/Environment/Korea/W149/06 (clade 2.2, H5N1, Rgdel149/PR8), A/Viet Nam/1203/04 (clade 1, H5N1, NIBRG-14), as well as A/duck/Anhui/1/2006 (clade 2.3.4, H5N1, RgdelAN1/PR8) in PR8 background.

2.3. Production of H5N8 mock-up vaccine for preclinical use

The H5N8 vaccine virus (IDCDC-RG43A) was propagated in 10- to 11-days old embryonated eggs at 37 °C for 72 h. The harvested allantoic fluid was clarified by the centrifugation and concentrated by ultracentrifugation at 100,000*g* for 2 h and then finally purified by sucrose density gradient centrifugation. The purified vaccine viruses were inactivated by the addition of formalin (formaldehyde, 36.5 to 38%; Sigma) with a final concentration of 0.025% and stored at 4 °C for 3 days. To confirm the inactivation of the vaccine virus, loss of infectivity was observed after 2 passages in eggs. To determine the HA protein content in the purified vaccine bulk, the standard single radial immunodiffusion (SRID) technique was performed using the H5N8 standard antibody. The standard antibody against H5N8 virus was produced by multiple immunizations to ferret with 300 μg of HA plasmid originated from homologous A/gyrfalcon/Washington/41088-6/2014 (H5N8) virus via intramuscular electroporation method followed by intramuscular injection with 15 μg of HA protein of A/CK/Netherlands/14015526/14 (H5N8) purchased from Sino Biological Inc. (China).

2.4. Protective efficacy study in the mouse model

The protective efficacy of the mock-up vaccine was evaluated by vaccinating intramuscularly (IM) groups of six-week-old BALB/c mice (Samtako, Korea) (22 per group) with 2 doses each of the inactivated (IDCDC-RG43A) vaccine containing 1.5 and 3.0 μg/dose of HA without AlOH adjuvant as well as 3 μg/dose of HA with 250 μg AlOH adjuvant administered in 3 weeks interval. The control group received 250 μg alum only in 0.20 ml of sterile PBS. All groups were challenged after 2 weeks from last vaccination by inoculating intranasally (i.n.) with 10 MLD_{50} of the maH5N8 and survival and signs of disease were monitored for 14 days. Lung, brain, spleen, and kidney tissues were harvested from 3 mice per group per time point at 2, 5, 7 and 9 dpi for virus titration. For antibody titration, sera from the blood of vaccinated mice were collected via the retro-orbital plexus 20 days after first vaccination and 14 days after the second vaccination and stored at −80 °C until use.

2.5. Protective efficacy study in the ferret model

Twenty to twenty-four-week-old ferrets were purchased from IDbio Co. Ltd. (Korea) and tested seronegative against seasonal H1N1, H3N2, and H5-like viruses by hemagglutination inhibition (HI) assays prior to this study. Groups of 6 ferrets were vaccinated intramuscularly with the inactivated vaccine containing 3.0, 7.5 and 15 μg HA/dose with 500 μg of AlOH adjuvant while the control group received AlOH only. Two doses of the H5N8 mock vaccines were administered 3 weeks apart and sera were collected 3 weeks post-vaccination and 2 weeks after the second vaccination. After 2 weeks from last vaccination, the vaccinated ferrets were lightly anesthetized with Zoletil and Xylazine and were challenged with 10^7 $TCID_{50}$ of maH5N8 virus and signs of disease were monitored for 14 days. Lung, spleen, and kidney tissues were harvested from 2 ferrets per group at 2 and 5 dpi for viral titration while nasal wash collection was done at 1, 2, 3, 5, 7, 9, 11 and 13 dpi. Harvested lung samples at 5 dpi were fixed in formalin and further processed

to histopathologically examine the hematoxylin-and-eosin (H&E)-stained sections.

2.6. Serology and antibody assays

Antibody titers of post-vaccinated mouse and ferret sera were determined by hemagglutination inhibition (HI) assay as described elsewhere [16]. Briefly, the sera were treated with receptor-destroying enzyme (RDE, Denka Seiken, Japan) for 18 h to inactivate non-specific inhibitors of HA and further diluted at a final serum dilution of 1:10 with phosphate-buffered saline. In addition, potential non-specific HA activity present in sera were removed by hemadsorption against 4.5% turkey RBC for 30 min at room temperature as previously described [17] but with slight modifications. 25 μl of the RDE-treated sera were 2-fold serially diluted in PBS in V-bottom 96 well microtiter plates and incubated with equal volumes of 4–8 HA units/50 μl of the virus. To determine the homologous antibody titer of the serum collected from the vaccinated mice and ferrets, the intra-clade (2.3.4.4) H5Nx viruses including vacH5N8, vacH5N6 and, Rgdel468/PR8 were used. In addition, the different clades (cross-clade) of H5N1 viruses including NIBRG-14 (clade 1), Rgdel149/PR8 (clade 2.2), and RgdelAN1/PR8 (clade 2.3.4) were also used to determine the cross-reactivity of the serum elicited from animals vaccinated with the H5N8 vaccine strain. The plate was incubated at room temperature to give time for the reaction of the serum and virus for 30 min. The HI titers were then observed after 30 min of incubation with 0.5% turkey erythrocytes and recorded as the reciprocal of the highest dilution of the serum that did not exhibit agglutination of the erythrocytes.

Neutralizing antibody titers of post-vaccinated ferret were determined by serum neutralizing (SN) assay as described previously [18] with intra-clade 2.3.4.4 H5N8 and H5N6 vaccine viruses and to determine its cross-reactivity to cross-clades of H5N1 viruses. Initial serum dilutions of 1:10 were 2-fold serially diluted and incubated with 100 $TCID_{50}$ of the homologous intra-clade and heterologous viruses and incubated for 1 h at 37 °C in 5% CO_2. A monolayer of confluent MDCK (1.5×10^4 cells/well) in 96-well plates were infected with the virus and serum mixtures for an hour for virus absorption and after replacement of supernatant with infection media, the cells were incubated for 72 h at 37 °C in 5% CO_2. Neutralizing antibody titers were defined as the reciprocal of the highest dilution of serum that completely neutralized the infectivity of 100 $TCID_{50}$ of the virus determined by the absence of CPE and confirmation of viral replication in the supernatant by hemagglutination test.

2.7. Statistical analysis

The Kaplan-Meier survival curves calculated by Prism 5 (GraphPad) between the experimental groups were compared using the Mantel-Cox log-rank test (Prism 5). ANOVA was used for comparison of virus titers from lung, brain, spleen, kidney and nasal washes between the control and experimental mice and ferrets.

3. Results

3.1. Immunogenicity and protective efficacy of IDCDC-RG43A pre-pandemic vaccine in mice

The immunogenicity of the IDCDC-RG43A pre-pandemic H5N8 vaccine was evaluated in each mice immunized with varying doses of the vaccine antigen including 1.5 and 3.0 μg HA with or without 250 μg AlOH through i.m. No HI antibody against homologous H5N8 (A/gyrfalcon/Washington/41088-6/2014, clade 2.3.4.4.) and heterologous intra-clade H5N6 (A/Sichuan/26221/2014, clade 2.3.4.4.) vaccine strains were detected in the sera of mock-vaccinated mice and mice that received AlOH only (Fig. 1). No detectable HI titers against both clade 2.3.4.4 H5N8 and H5N6 vaccine strains was observed in mice after the primary vaccination. However, after the second dose of vaccine, the HI titers against homologous H5N8 substantially elevated in all vaccinated mice dose-dependently and the average antibody titers elicited by mice immunized with 3.0 μg HA/dose with AlOH exhibited significantly higher antibody titer than that elicited by 3.0 μg HA/dose without AlOH group ($p < 0.05$) indicating that the AlOH enhanced the antibody production (Fig. 1A). Comparable or higher cross-reactivity than homologous H5N8 vaccine virus was observed against intra-clade 2.3.4.4. H5N6 in sera of mice immunized with at least 1.5 μg HA/dose.

Two weeks following the second immunization, all groups of mice were challenged with 10 MLD_{50} of mouse-adapted A/Environment/Korea/ma468/2015 (maH5N8) to evaluate the protective efficacy of the IDCDC-RG43A pre-pandemic vaccine in mice and were monitored for survival for 14 days. All mock-vaccinated mice and mice vaccinated with only AlOH died within 8 dpi of maH5N8 virus challenge (Fig. 2) while a slight decrease in weight (up to 12% from their initial weight) was observed in group 3 mice vaccinated with 1.5 μg HA. Mice immunized with at least 3 μg/dose with or without AlOH (Groups 4 and 5) obtained 100% protection with no significant weight loss against homologous maH5N8 virus challenge. On the other hand, the mice immunized with a lower concentration of 1.5 μg HA/dose had 88% survival rate against the same virus. Most of the infected mice in mock and AlOH only groups exhibited ruffled fur after infection and they exhibited labored breathing and convulsion particularly 2 days prior to death, indicative of a severe influenza-like illness (Table 1). Although the mice vaccinated with 1.5 μg HA/dose demonstrated weight loss and death rate, they showed no apparent clinical signs. In contrast, mice vaccinated with at least 3 μg HA/dose with or without AlOH did not show any clinical signs of illness which correlated with its survival rate.

3.2. Viral replication in mice after challenge

The ability of the IDCDC-RG43A pre-pandemic vaccine to suppress viral systemic infection in the peripheral organs including lung, brain, spleen, and kidney of mice, which is a typical characteristic of HPAI H5 infection was also evaluated. The maH5N8 virus persistently replicated in the lungs of mock- and AlOH-vaccinated mice at high titer (higher than $10^{6.0}$ $TCID_{50}$/g) until 7 dpi prior to death. Meanwhile, the inhibitory effect of the vaccine against the virus replication in the lungs of all vaccinated mice was significantly seen at 5 dpi despite of high replication observed in all vaccine groups earlier at 2 dpi (Fig. 3). Although the inoculated virus was isolated from brain, spleen, and kidney of mock-vaccinated mice at more than 2-time points, no viral titers in brain and kidney were detected from all the vaccinated mice (Fig. 3). Modest viral titers were detected in the spleen of 1.5 μg HA/dose vaccine group at 2 dpi while no viral titers had reached the detection limit in the spleen of all the vaccinated groups at 5, 7, and 9 dpi. The result demonstrated that 3 μg HA of vaccine dose formulated with AlOH efficiently suppressed viral replication in pulmonary organ and completely inhibited viral systemic infection in mice.

3.3. Immunogenicity of IDCDC-RG43A in ferrets challenged with homologous virus

To determine the immunogenicity of IDCDC-RG43A vaccine in the ferret model which has been widely used for influenza virus animal study [12], groups of 6 ferrets were i.m. immunized twice with various antigen doses including 3.0, 7.5, and 15 μg of HA pro-

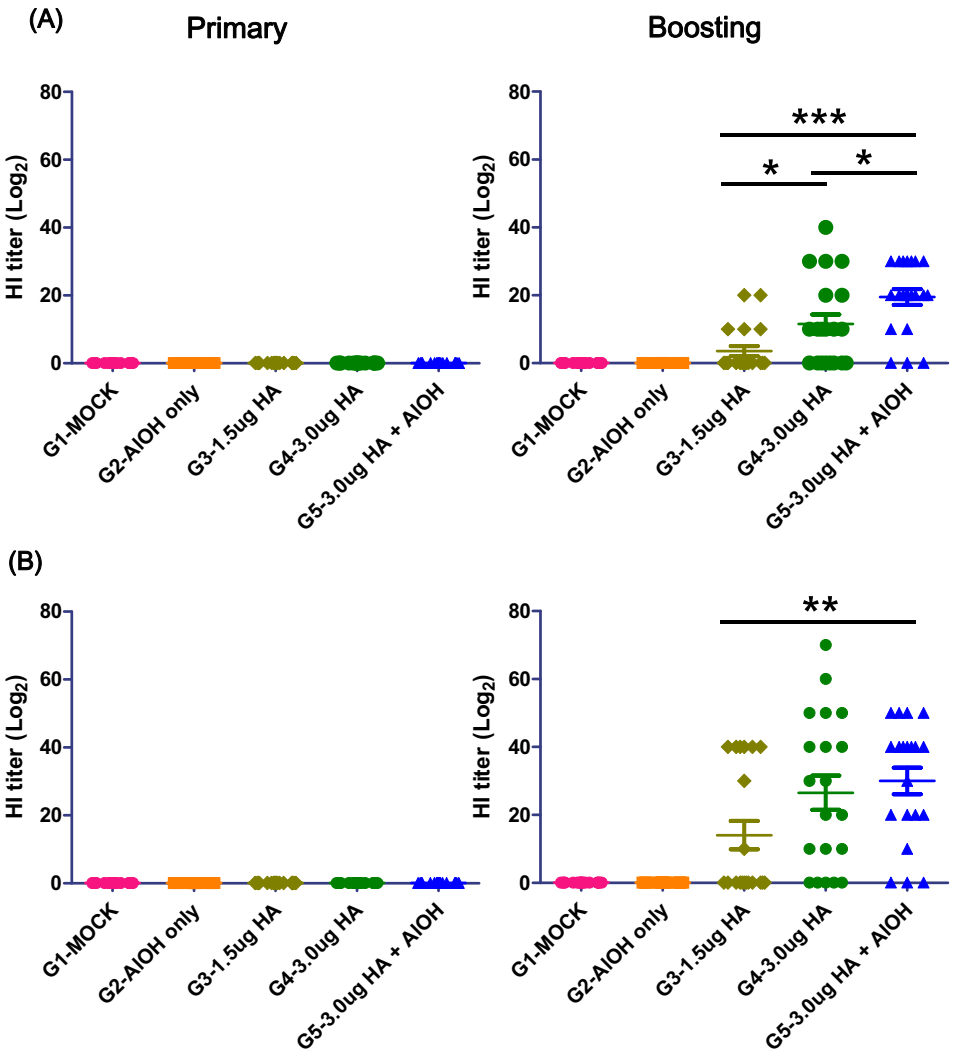

Fig. 1. Antibody response in mice serum administered with different doses of the H5N8 vaccine. Differences of mean hemagglutinin inhibition (HI) assay titer induced in mice immunized twice with different doses of 1.5 μg and 3.0 μg HA antigen/dose with or without 250 μg aluminum hydroxide (AlOH). Mice serum were collected at three weeks after primary vaccination and two weeks after second vaccination. The collected sera from the vaccinated mice were used to perform HI assays against (A) vacH5N8, (B) vacH5N6. *p-values are indicated by stars **$p < 0.05$, ***$p < 0.001$.

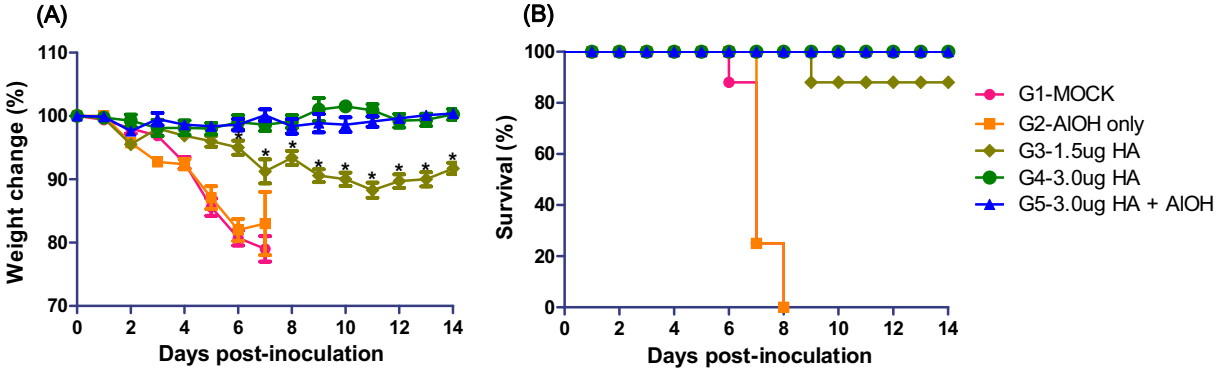

Fig. 2. The evaluation of the protective efficacy of the H5N8 vaccine in mice model. Efficacy of the vaccination was verified by weight change (A) and survival rate (B). 5 Groups with ten 6-week-old Balb/c mice were vaccinated intramuscularly (IM) and challenged with maH5N8 (10 MLD_{50}). *$p < 0.05$ for comparison between weight changes of 1.5 μg HA group and other dose groups.

tein formulated with 500 μg of AlOH with 3 weeks interval. Due to the significant suppression of viral replication previously observed in mice vaccinated with the AlOH-formulated vaccine, all groups of ferrets were also immunized with the antigen formulated with AlOH. The homologous H5N8 vaccine strain demonstrated the highest geometric mean antibody titers (GMT) both in first and

Table 1
Clinical signs of maH5N8 infection observed in vacH5N8-vaccinated mice.[a]

Group	Antigen	Days post-inoculation	Clinical sign[b]	Mobility[c]
G1	Mock	3–5	1	1
		6–7	1, 2, 3	2
		8	–[†]	–[†]
G2	AlOH only	3–6	1	1
		7	1, 2, 3	2
		8	†	–[†]
G3	H5N8 1.5 μg[*]	0–14	0	0
G4	H5N8 3.0 μg			
G5	H5N8 3.0 μg + AlOH			

[a] Groups of mice were intranasally challenged with 10 MLD_{50} of the mouse-adapted H5N8 virus two weeks after the last immunization. Clinical signs of mice were monitored daily for 14 days after challenge.
[b] Clinical signs: 0, Normal; 1, Ruffled fur; 2, Labored breathing; 3, Convulsion.
[c] Mobility: 0, Normal; 1, Low; 2, Very row.
[†] All mice were dead.
[*] One of the infected mice was dead at 9 dpi without detectable clinical signs.

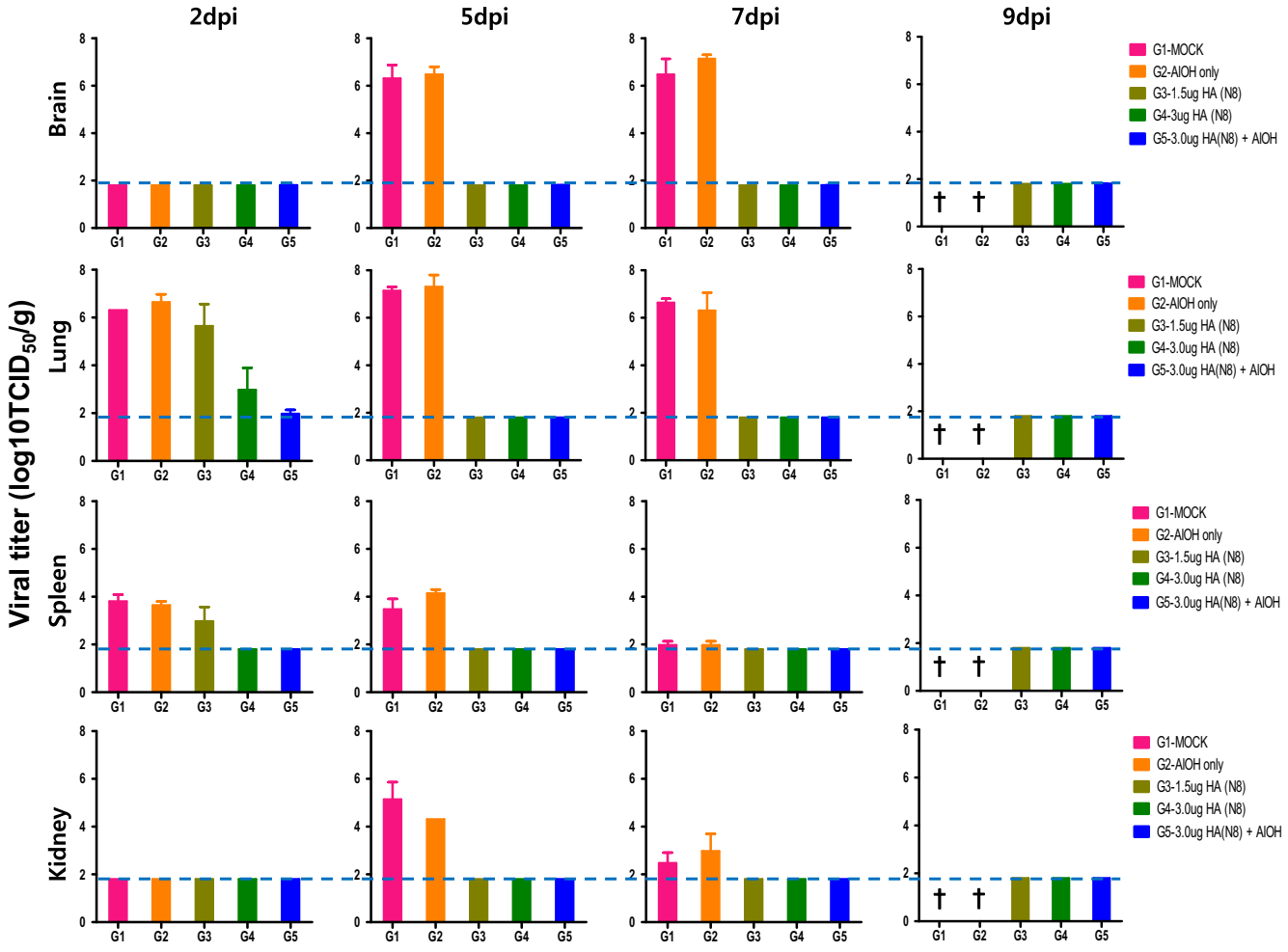

Fig. 3. Replication of maH5N8 challenge virus in the peripheral organs of vacH5N8-vaccinated mice. After infection (intra-nasally) with a lethal dose (10MLD_{50}) of maH5N8, various organs such as brain, lung, spleen, and kidney were harvested at 2, 5, 7 and 9 dpi for virus titration. Each bar represents the viral titer measured in the specified tissue organ of the different dose groups on the indicated dpi. Viral titrations are expressed as $log_{10}TCID_{50}$ per gram of tissue collected according to the method of Reed and Muench [14]. The dashed horizontal line indicates the lower limit of detection. Data are mean titers ± standard deviation. † indicates that all mice were dead.

second collected sera with 320 HI GMT at 15 μg/dose but was only about 120 HI GMT at the lowest concentration of 3.0 μg/dose (Fig. 4) after the second vaccination. Although the higher GMT against homologous vaccine strain than heterologous intra-clade 2.3.4.4 viruses after first vaccination was observed, in general, the comparable GMT at the equal antigen doses was shown between the homologous vaccine and heterologous intra-clade 2.3.4.4 viruses at two weeks after second vaccination. Additionally, the sera obtained from ferrets after the second vaccination were also tested by serum neutralizing (SN) assays to test whether the sera can neutralize *in vitro* the homologous vaccine and heterologous intra-clade 2.3.4.4 viruses used for testing serological cross-

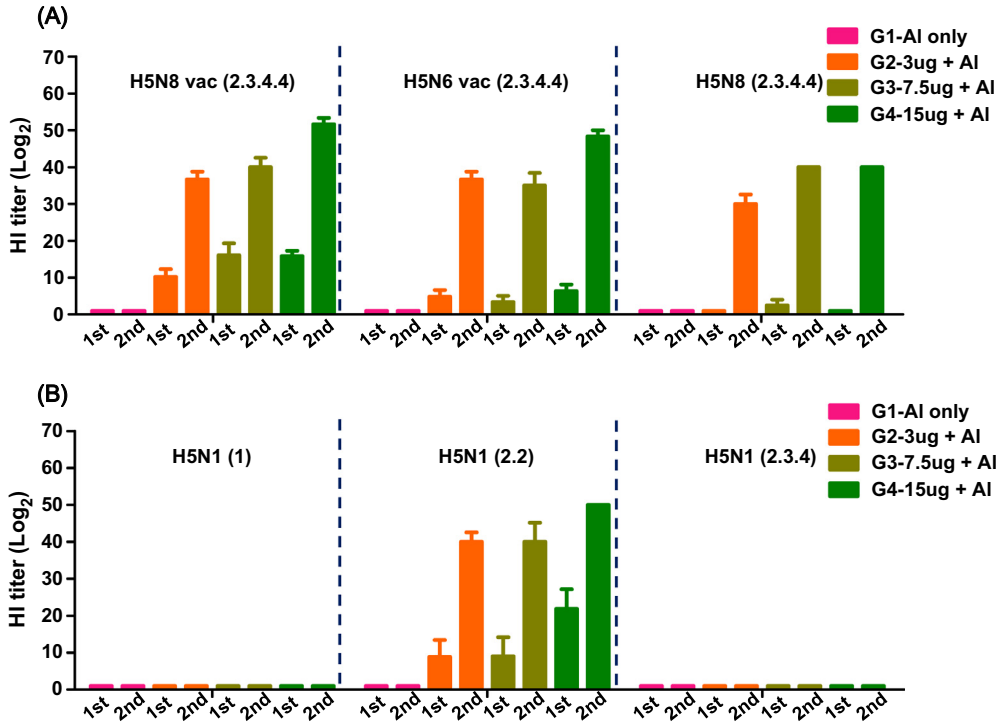

Fig. 4. Antibody titers in ferrets vaccinated with different doses of the vacH5N8. Differences of mean hemagglutinin inhibition (HI) assay titer induced in mice immunized twice with different doses (3.0 μg, 7.5 μg, and 15 μg) of H5N8 vaccine with 500 μg aluminum hydroxide (AlOH). Ferret serum was collected at three weeks after first vaccination (1st) and two weeks after second vaccination (2nd). The collected serum from the vaccinated ferret was used to perform HI assays against (A) the vaccine strain and intra-clade 2.3.4.4 viruses and (B) heterologous cross-clade viruses. Data are mean titers ± standard deviation.

reactivities. High virus-neutralizing antibody titers (up to 320 neutralizing units) were detected in sera from ferrets that received at least 15 μg/dose HA, particularly against the H5N8 vaccine strain and intra-clade 2.3.4.4 H5N8/H5N6 viruses (Fig. 5).

Similar sera collected after the first and second vaccination from ferrets were tested for cross-reactivity against heterologous cross-clades of H5N1, particularly clade 1, 2.2 and 2.3.4. Of note, moderate to high HI titers ranged from 80 to 320 HI units were observed against Rgdel149/PR8 (H5N1, clade 2.2) in sera collected from ferrets vaccinated with two doses of H5N8 vaccine in a dose-dependent manner, which was comparable to the HI titers against the homologous vaccine strain. In contrast, none of the vaccine doses could induce HI titers beyond the positive limit of detection (≤20 HI titers) against NIBRG-14(H5N1, clade 1) and RgdelAN1/PR8 (H5N1, clade 2.3.4) after the first and second vaccination. Additionally, comparable cross-neutralization *in vitro* of sera obtained from ferrets that received 3.0, 7.5 and 15 μg HA was observed against Rgdel149/PR8 (H5N1, clade 2.2) with marked increases in virus-neutralizing antibody titers (≥160 neutralizing units). However, none of the collected sera could strongly neutralize the H5N1 viruses of clade 1 and 2.3.4. Thus, the cross-neutralization of the tested viruses *in vitro* appeared to correlate with the cross-reactivity observed in the HI assays (Fig. 5).

3.4. Protective and virus inhibitory efficacy of IDCDC-RG43A in ferrets

The protective efficacy of the vaccine in ferrets was also evaluated by monitoring the body temperature and weight changes following viral challenge with $10^{7.0}$ $TCID_{50}$ of maH5N8 via the intranasal route. The clear weight loss and increased body temperature were observed in the mock-vaccinated group compared to the vaccinated groups. A decreased in weight until 9 dpi was observed in ferrets that received AlOH only, while fast recovery was observed in all vaccinated groups as early as 2 dpi (Fig. 6). In addition, decreased food intake and mobility, unlike the vaccinated groups which only exhibited a slight increase of body temperature for 2–3 days after virus infection, was observed in mock-vaccinated ferrets (Table 2).

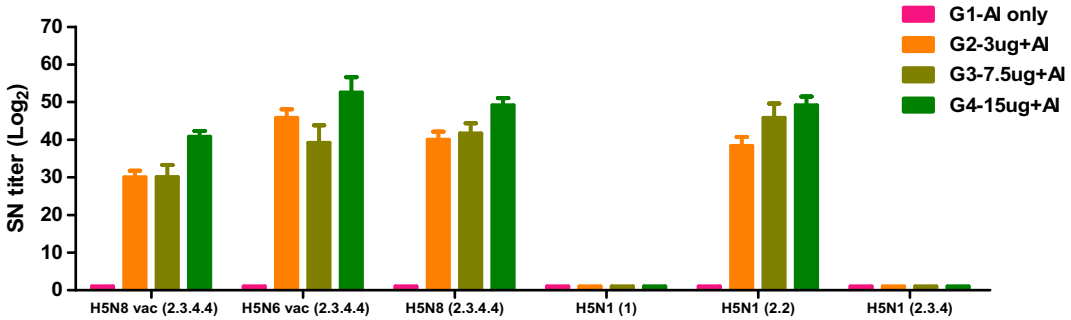

Fig. 5. In vitro neutralization of the homologous and heterologous H5NX strain by ferret serum vaccinated with vacH5N8. Differences in the serum neutralization titer of serum collected from vacH5N8 vaccinated ferrets with different doses of vacH5N8 containing 500 μg aluminum hydroxide adjuvant. SN titer was determined by the *in vitro* microneutralization of homologous (2.3.4.4) and heterologous (1, 2.2, 2.3.4) H5NX virus in MDCK cells. Data are mean titers ± standard deviation.

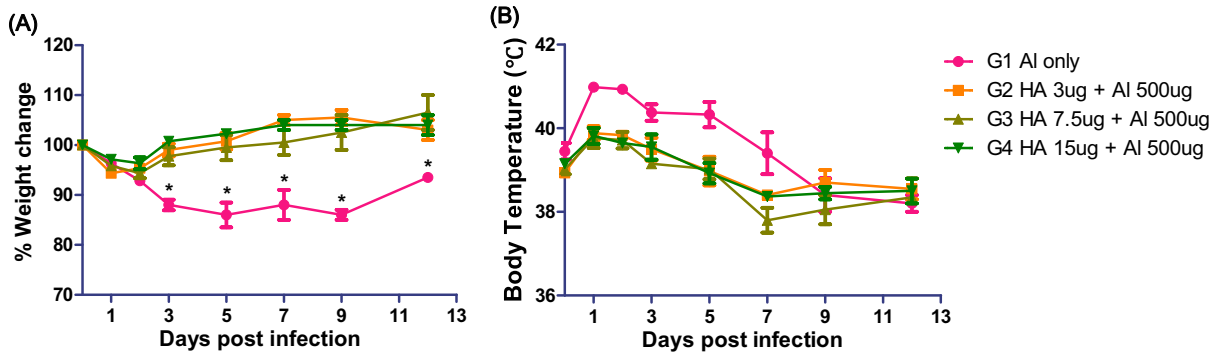

Fig. 6. Percentage weight change and body temperature of vacH5N8-vaccinated ferrets. Efficacy of the vaccination was verified by weight change (A) and body temperature (B). 4 Groups with six 20 to 24-week-old ferret each group were vaccinated intramuscularly (IM) and challenged with maH5N8 (10^7 $TCID_{50}$). $^*p < 0.05$ for comparison between weight changes of alum only group and other dose groups.

Table 2
Clinical signs of maH5N8 infection observed in vacH5N8-vaccinated ferrets.[a]

Group	Antigen	Days post-inoculation	Clinical sign[b]	Mobility[c]
G1	Al only	0	0	0
		1	1, 2	0
		2	1, 2	1
		3	1, 2	1
		4	1, 2	1
		5	1, 2	1
G2	H5N8 3.0 μg + Al 500 μg	0	0	0
		1	1	0
		2	1	0
		3	1	0
		4	0	0
		5	0	0
G3	H5N8 7.5 μg + Al 500 μg	0	0	0
		1	1	0
		2	1	0
		3	0	0
		4	0	0
		5	0	0
G4	H5N8 15 μg + Al 500 μg	0	0	0
		1	1	0
		2	1	0
		3	1	0
		4	0	0
		5	0	0

[a] Groups of ferrets were intra-nasally challenged with 10^7 $TCID_{50}$ of the mouse-adapted H5N8 virus two weeks after the last immunization. Clinical signs of mice were monitored daily for 14 days after challenge.
[b] Clinical signs: 0, Normal; 1, Increased body temperature; 2, Decreased food intake; 3, Frequent coughing; 4, Nasal congestion.
[c] Mobility: 0, Normal; 1, Decreased mobility.

Nasal washes were obtained from vaccinated ferrets at 1, 2, 3, 5, 7 and 9 dpi to determine the inhibitory effect of viral replication in the upper respiratory tract by vaccination. In addition, ferrets were sacrificed at 2 and 5 dpi (2 animals/group/day) after nasal wash collection and tissue samples (lung, kidney, and spleen) were harvested for viral titration to determine the inhibitory effect of viral replication in lower respiratory tract as well as viral systemic infection. Except for the control group (Group 1), there were no detectable viral titers from lungs, kidney and spleen of all vaccinated ferrets regardless of dose (Fig. 7). All vaccinated animals cleared viral replication in nasal washes within 5 dpi, while the highest viral titer was observed in the control group at 5 dpi (Fig. 7D).

Histopathological examination of lung samples from the vaccinated ferrets was also performed on day 5 post-infection to examine changes and differences among groups (Fig. 8). Severe signs of pneumonia, degenerating epithelia, and presence of inflammatory cells were observed in the lungs of the mock-vaccinated ferrets (Fig. 8A). In contrast, moderate pathological lesion was observed in lung tissue samples from the vaccinated ferrets with at least 7.5 μg/dose with AlOH (Group 3 and 4). Although ferrets vaccinated with 3.0 μg with AlOH showed rapid recovery and complete inhibition of viral replication in tissues, histopathological lesions were still often observed in the H&E stained lung sample (Fig. 8B). Even though the viral replication of the challenge virus in various organs of ferret were observed to be attenuated when immunized with at least 3 μgHA/dose with AlOH, less pathological changes were observed in the lung tissue section of ferret vaccinated with the highest dose (15 μg/dose with AlOH) as presented by marginal pulmonary virus replication after virus challenge, stipulating viral spread suppression in ferrets (Fig. 8D). Thus, collectively, the H5N8 vaccine sufficiently suppressed the clinical disease outcome and viral replication in the ferret model in a dose-dependent manner.

4. Discussion

As influenza viruses have the capability to rapidly evolve into new sublineages, the probability of the occurrence of pandemic by a novel strain is increased. The amino acid variations within the antigenic sites of HA in influenza viruses lead to the emergence of antigenically distinct highly pathogenic influenza H5N1 virus, infecting domestic and wild birds in many countries, which further evolved and generated multiple clades (clades 0–9) [19,20]. Out of these different clades, the majority of the viruses circulating worldwide and causing human infections were identified in 2 clades (1 and 2) such as subclades 1.1, 2.1.3.2, 2.2.1, 2.3.2.1, 2.3.4 and 2.3.4.4 [20–22]. Thus, the H5Nx viruses, causing sporadic infections in humans, were considered to pose the greatest pandemic threat to humans when adaptive mutations and reassortments were acquired [23], leading to the need of generating influenza candidate vaccine viruses which can elicit a cross-clade immunogenicity, a critical point for pandemic preparedness.

In this study, we particularly evaluated the protective efficacy of one of the CVVs recommended by WHO containing HA and NA genes from H5N8 A/gyrfalcon/Washington/41088-6/2014 (IDCDC-RG43A), using mice and ferrets as animal models for the pre-clinical assessment. Mice vaccinated with at least 1.5 μg HA/dose of IDCDC-RG43A showed HI titers below the detectable titer after the primary dose. However, a drastic elevation of mean HI titers at 14 days was observed after vaccination with the 2nd dose, suggesting that second dose of vaccination is needed to elicit higher HI titers compared to a single dose. The higher dose of 3.0 μg HA with or without AlOH elicited higher mean HI titers against the homologous H5N8 and the heterologous intra-clade

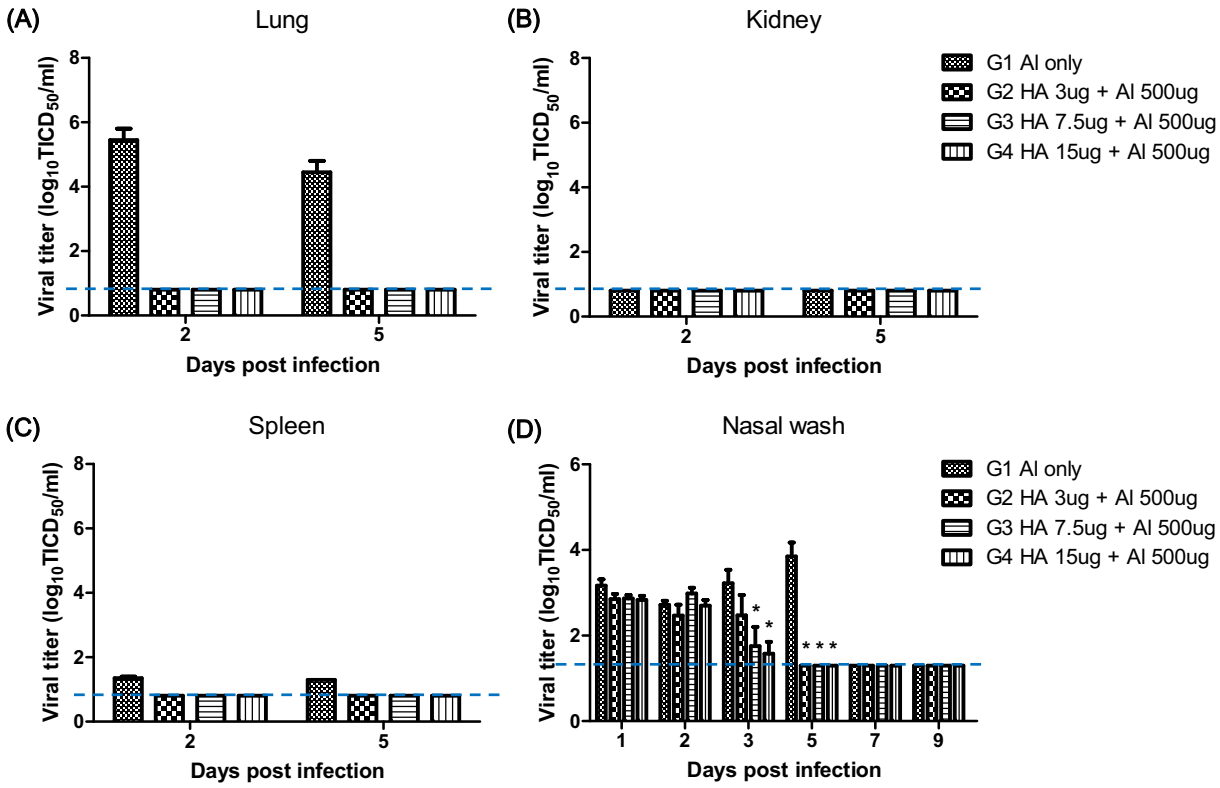

Fig. 7. Replication of maH5N8 challenge virus in the vacH5N8-vaccinated ferret. Various tissue organs (A) Lung, (B) kidney, (C) spleen were collected at 2 dpi and 5 dpi from infected ferrets with maH5N8 virus and (D) Nasal wash samples were collected at 1, 2, 3, 5, 7, 9 days after infection for virus titration determined by the $TCID_{50}$ in MDCK cells. Viral titrations are expressed as $log_{10}TCID_{50}$ per milliliter of tissue collected according to the method of Reed and Muench [14]. The dashed horizontal line indicates the lower limit of detection. Data are mean titers ± standard deviation. $^{*}p < 0.05$ for comparison between AI only group and other doses groups at 3 and 5 dpi.

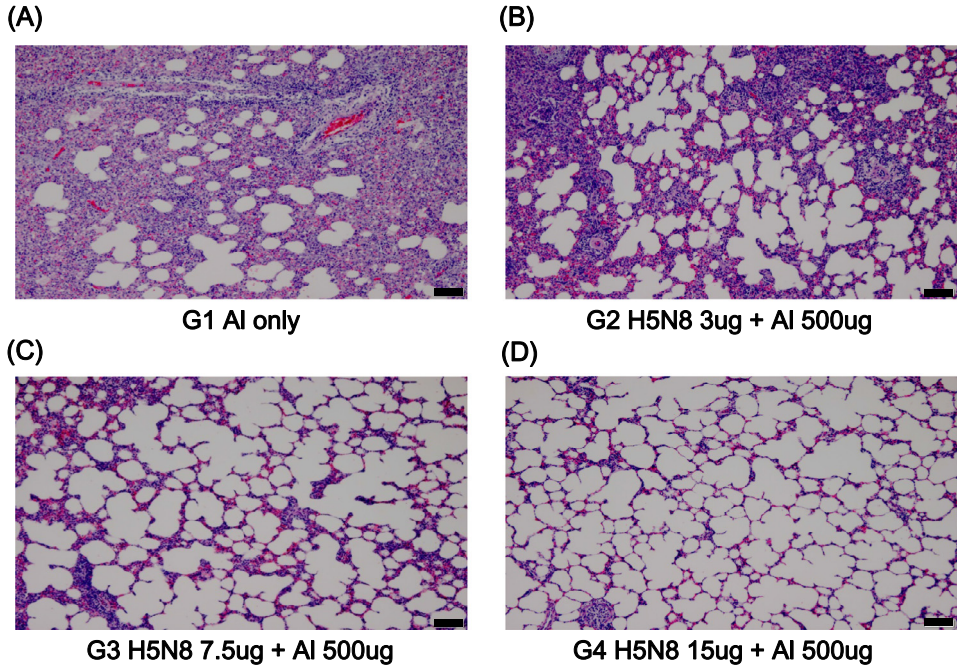

Fig. 8. Histopathology of lung tissue in vacH5N8-vaccinated ferrets. 5 days after challenge of maH5N8, collected lung tissue samples were processed for H&E (hematoxylin and eosin) staining. Shown are photomicrographs at magnification 40× of H&E-stained ferret lung sections: (A) aluminum hydroxide only group; (B) 3.0 μg HA antigen with AI group; (C) 7.5 μg HA antigen with AI group; (D) 15 μg HA antigen with AI group. *ranged in size 20 μm.

H5N6 2.3.4.4 vaccine strains, showing that the elevated levels of mean HI titers is dose-dependent. Sera obtained from the immunized mice also reacted with a distantly related strain of the same clade (H5N6) as shown by the elevated HI titers against this virus. The HI test result from mice experiments was similarly observed in another study using ferrets, an animal model widely used and suit-

able for influenza virus studies [12], with which immunization with varying dose of HA antigen also raised HI titers against both homologous and heterologous viruses. Notedly, higher GMT HI titers were elicited against the H5N8 vaccine strain virus as well as the heterologous H5N1 clade 2.2 at 21 days after the primary vaccination, while low HI titers were elicited against other viruses. However, after the second vaccination, antibody titers drastically increased with almost equal titers against the vaccine strain virus, homologous intra-clade viruses and heterologous cross-clade 2.2, suggesting that 2 doses of the vaccine are needed to elicit a higher antibody as well as cross-reactivity with H5N1 clade 2.2.

The IDCDC-RG43A vaccine virus also induced neutralizing antibodies in immune sera from ferrets with comparable cross-reactivity to other clades as shown in serum neutralization assay. Specifically, we noted a closely related antigenicity between the clade 2.3.4.4 and clade 2.2 viruses but not with other clades such as clade 1 and 2.3.4. The cross-reactivity with the heterologous intra-clade (H5N6 2.3.4.4) and cross-clade reactivity with clade 2.2 as observed in both HI and SN assays could be due to the presence of common antigenic epitopes between these viruses. Contrarily, the failure to cross-react with clade 1 and 2.3.4 could be due to the existence of distinguishable antigenic differences in hemagglutinin (HA) sequences among the different lineages of H5N1 which apparently restricts the development of universal vaccines against different H5N1 lineages [24], although further study will be needed to verify the high cross-reactivity between clade 2.2 and 2.3.4.4 viruses. Unexpectedly, a low virus replication of the H5N8 vaccine strain (IDCDC-RG43A) in MDCK cells was observed (6.0×10^3 $TCID_{50}$/ml) but high viral titer in 10 day-old embryonated chicken egg (6.0×10^9 EID_{50}/ml), which might be due to presence of relative low fraction of infective viral particles in MDCK cells in comparison to other viruses used. This could mean that differing from other prepared 100 $TCID_{50}$ viruses used in the SN assay, the vaccine strain virus might have a prevailing higher amount of viral particles to achieve a 100 $TCID_{50}$ as required in the methods. Thus, this could be a factor for the relatively result of lowered neutralizing antibody titer against the vaccine strain compared to the other viruses tested, in which similar amount of serum was also added (Fig. 5). The result seemed to show that the elicited antibody against the vaccine strain has a lower neutralizing activity compared to the other clades, but this could be due to the lower proportion of the antibody to neutralize the higher amount of infective viral particles present. Although the H5N8 vaccine strain has high viral growth property in eggs and thus, has a high potential to be used as an egg-based vaccine strain, the low infectivity of the vaccine strain to MDCK cell can limit its use as a cell culture-based vaccine strain for pandemic.

Moreover, the efficacy of the IDCDC-RG43A vaccine was evaluated in mice and ferrets. In order to fully evaluate the viral attenuation in these animal models immunized with the pre-pandemic candidate vaccine, we used a highly virulent mouse-adapted H5N8 virus strain acquired from our previous study [15] for the virus challenge to both animal models. The groups of mice immunized twice with 3.0 μg HA antigen with or without AlOH were completely protected from the challenge virus in comparison with the mock-vaccinated mice group which all died within 8 days. However, immunization with lower antigen concentration of 1.5 μg did not efficiently protect the mice as shown by presence of mortality, clearly showing that the protective efficacy of IDCDC-RG43A vaccine is dose-dependent. These results could be further substantiated by the almost similar observation in ferrets immunized with varying doses of antigen wherein at least 3.0 μg with AlOH also protected the ferrets from challenge with maH5N8, as well as, minimized morbidity, decreased virus shedding and attenuated viral replication in the lungs, while a higher dose of antigen of 15 μg caused less pathological changes in the lungs of ferrets.

Lastly, we used aluminum hydroxide as the vaccine adjuvant which has been widely used for human vaccines and were found in some studies to be effective in enhancing the protective effect of vaccine, reducing the immunizing dose of the vaccine and provided a long-term protective effect in mice [25]. Similarly, in this study, we were able to determine that alum-adjuvanted antigen can efficiently induce a higher HI GMT than the same vaccine without any adjuvant which correlated with the lowered virus replication in the lungs of mice (Figs. 1 and 3). Thus, this result could be explained by the effect of an adjuvant such as aluminum hydroxide in increasing the humoral immune response as also observed in other related studies [26,27].

In summary, the inactivated whole-virus H5N8 (IDCDC-RG43A) vaccine when given in 2 doses was immunogenic in mice and ferrets. It also effectively protected the mice and ferrets from challenge with the heterologous intra-clade virus (maH5N8), elicited cross-reactivity and cross-neutralization against a heterologous cross-clade virus (H5N1, clade 2.2), and limited the viral replication in the target organ, which are valuable features of a pre-pandemic vaccine. Although the candidate vaccine viruses need further studies and tests prior to clinical trials, this pre-clinical study using animal models may contribute in the evaluation of these candidate vaccine viruses, which could be potentially used against the growing threat of these emerging H5Nx viruses to cause the next pandemic.

Acknowledgments

This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea [grant number: HI16C1032] and by the Ministry of Science, ICT & Future Planning [NRF-2016M3A9B6918676].

Conflict of interest

The authors declare no conflict of interest.

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