



## Vaccine

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# Squalene-adjuvanted H7N9 virus vaccine induces robust humoral immune response against H7N9 and H7N7 viruses



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## ABSTRACT

Recent cases of avian influenza H7N9 have caused great concerns that virus may become transmissible between humans. It is imperative to develop an effective vaccine to fight against the pandemic potential of this H7N9 influenza virus to protect human from the disease. This study aims to investigate an optimized formulation for the development of H7N9 vaccines. Various doses of H7N9 inactivated whole or split-virus antigens (0.5, 1.5, or 3  $\mu$ g based on hemagglutinin content) combined with squalene-based adjuvant (AddaVAX), aluminum hydroxide Al(OH)<sub>3</sub> or without adjuvant were evaluated for the efficacy of H7N9 vaccine regimens in mice. With either H7N9 whole or split-virus based vaccines, AddaVAX-adjuvanted formulations were the most immunogenic in eliciting significant humoral immune response against H7N9 virus and exhibited strong cross-reactive response in hemagglutination inhibition (HAI) and viral-neutralization assays against H7N7 virus as well. In contrast, formulations with Al(OH)<sub>3</sub> or without adjuvant were less immunogenic and elicited lower titers of HAI and microneutralization assays against both viruses. Dose-sparing experiments suggested that the formulation with as low as 0.004  $\mu$ g of split or whole virus vaccine antigens together with 50% AddaVAX provided sufficient sero-protective HAI titers and achieved essential virus-neutralizing antibody titers against H7-subtype influenza viruses in mice. Protection experiments demonstrated that the formulation of 0.004  $\mu$ g to 0.5  $\mu$ g of split-virion vaccines with AddaVAX conferred full protection against viral challenge up to 100 LD<sub>50</sub> of wild-type H7N9 virus, with 0% survival in placebo group. Taken together, our study demonstrates that squalene-based adjuvant can significantly enhance the protective efficacy of H7N9 virus vaccine and provides a useful strategy to confront the potential pandemic outbreaks of H7N9 virus.

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## 1. Introduction

Human infection with a newly emerged avian-origin influenza A/H7N9 virus was first confirmed in March 31, 2013, in China [1]. To date there have been 251 cases of human infection caused by H7N9 virus in twelve provinces and two municipalities in China, with 20–30% mortality rate among infected individuals. A case of severe illness imported from China was also confirmed in Taiwan in late summer, 2013 [2]. The clinical research reported that most of patients infected with the novel H7N9 virus exhibit severe illness,

including pneumonia and acute respiratory distress syndrome, with high rates of intensive care unit admission or death, suggesting it is highly pathogenic and high fatality rate to human [1].

Recent evidence showed that the novel H7N9 virus is originally zoonotic and may be better adapted than other avian influenza viruses such as H5N1 to infect human [1,3–6]. Although no direct evidence has indicated the human-to-human transmission of the avian-origin H7N9 virus, recent studies reported that the virus may evolve to have the human-transmissible features through mutation in receptor binding site or genetic reassortment, and possibly results in a global pandemic in the future [7–11]. Comprehensive surveillance and early diagnosis may help to reduce the number of cases and deaths in the suspected areas, but the virus spread among animal reservoirs is difficult to control since the infected poultry may display ignorable symptoms [3]. Vaccination is

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considered to be the most effective way to prevent the transmission and the subsequent huge economic loss and human sufferings caused by influenza pandemics; therefore it is urgently needed to prepare an effective H7N9 influenza vaccine for the control of potential pandemic outbreak.

Previous clinical study has shown the inactivated H7N7 subtype influenza vaccine candidate is safe but poorly immunogenic in human trial when subjects were randomized to receive two doses of 90 µg of HA of an inactivated subunit influenza A (H7N7) vaccine intramuscularly [12]. The result indicates that the making of efficacious H7N9 vaccine for human might need efforts to improve the immunogenicity of viral antigens. In this study, the H7N9 inactivated virus vaccines were prepared to investigate the optimal vaccine formulation in mice, including the different doses of antigens combined with commonly used adjuvants and dose-sparing effect of adjuvanted-H7N9 vaccines. Our results demonstrated that squalene-adjuvanted virus vaccines containing antigens from H7N7 or H7N9 are both sufficient to provide mice with high hemagglutination inhibition (HAI) titers and cross-neutralizing activity against H7 subtype viruses. Immunogenicity studies revealed that while splitted or whole H7N7 virus vaccine induced similar level of immune response, splitted H7N9 virus elicited higher immunity than whole virus against H7-subtype viruses. This study provides new insights into the cross reactivity and protective immunity conferred by squalene-adjuvanted H7 subtype virus vaccines and reveals a general strategy for H7N9 vaccine design for future clinical trials and human use.

## 2. Materials and methods

### 2.1. Cell, virus strains, and antibody

MDCK cells (CCL-34) obtained from the American Type Culture Collection were maintained in 1× DMEM supplemented with 5% fetal bovine serum (Thermo Scientific) in incubator at 37 °C with 5% CO<sub>2</sub>. The new reassortant H7 vaccine strains, containing six internal genes derived from A/PR/8/34 virus, were obtained from the Centers for Disease Control and Prevention (Atlanta, GA). The A/Shanghai/2/2013(H7N9)-IDCDC-RG32A (HA and NA were derived from A/Shanghai/2/2013(H7N9)); A/Mallard/Netherlands/12/2000(H7N7)-IBCDC-1 (HA and NA were derived from A/Mallard/Netherlands/12/2000(H7N3) and A/Mallard/Netherlands/2/2000(H10N7), respectively); the wild-type influenza virus, A/Taiwan/01/2013(H7N9) (The gene of HA and NA has been sequenced and reported to WHO), was obtained from the Centers for Disease Control, Taiwan. These viruses were propagated in chicken eggs or in MDCK cells for vaccine antigen production, challenge assay, HAI assay, and microneutralization, respectively.

### 2.2. Vaccine preparation

Virus stocks were propagated in 10-day-old specific-pathogen-free embryonated chicken eggs at 34 °C. The infected allantoic fluids were harvested at 48 h post-inoculation and concentrated for the clarification. The viruses were purified by using sucrose density centrifugation in a 20%-to-50% (W/V) sucrose gradient for 1.5 h at 25,000 rpm at 4 °C. The inactivated whole virus vaccines were prepared by treating with 0.05% β-propiolactone (BPL) at 4 °C for 48 h. The vaccines in a splitted form were prepared by ether treatment, followed by 0.01% formalin inactivation. The inactivated vaccine antigens were verified for the absence of viral infectivity by serial passages in eggs.

### 2.3. Hemagglutination inhibition assay (HAI)

To determine HAI titers, mice sera were treated with a receptor-destroying enzyme (RDE) overnight and heat-inactivated for 1 h. The sera were tested in 2-fold dilutions starting with an initial dilution of 1:10, and then admixed with 4 HA units of H7N9 or H7N7 viruses individually. After incubation at room temperature for 1 h, the fresh prepared 0.5% suspension of Turkey red blood cells was added and hemagglutination was assessed by observation after 1 h. HAI titer is defined as the reciprocal of the highest dilution that showed ≥50% inhibition of hemagglutination. A titer of 5 was recorded if no inhibition at a serum dilution of 1:10.

### 2.4. Microneutralization assay

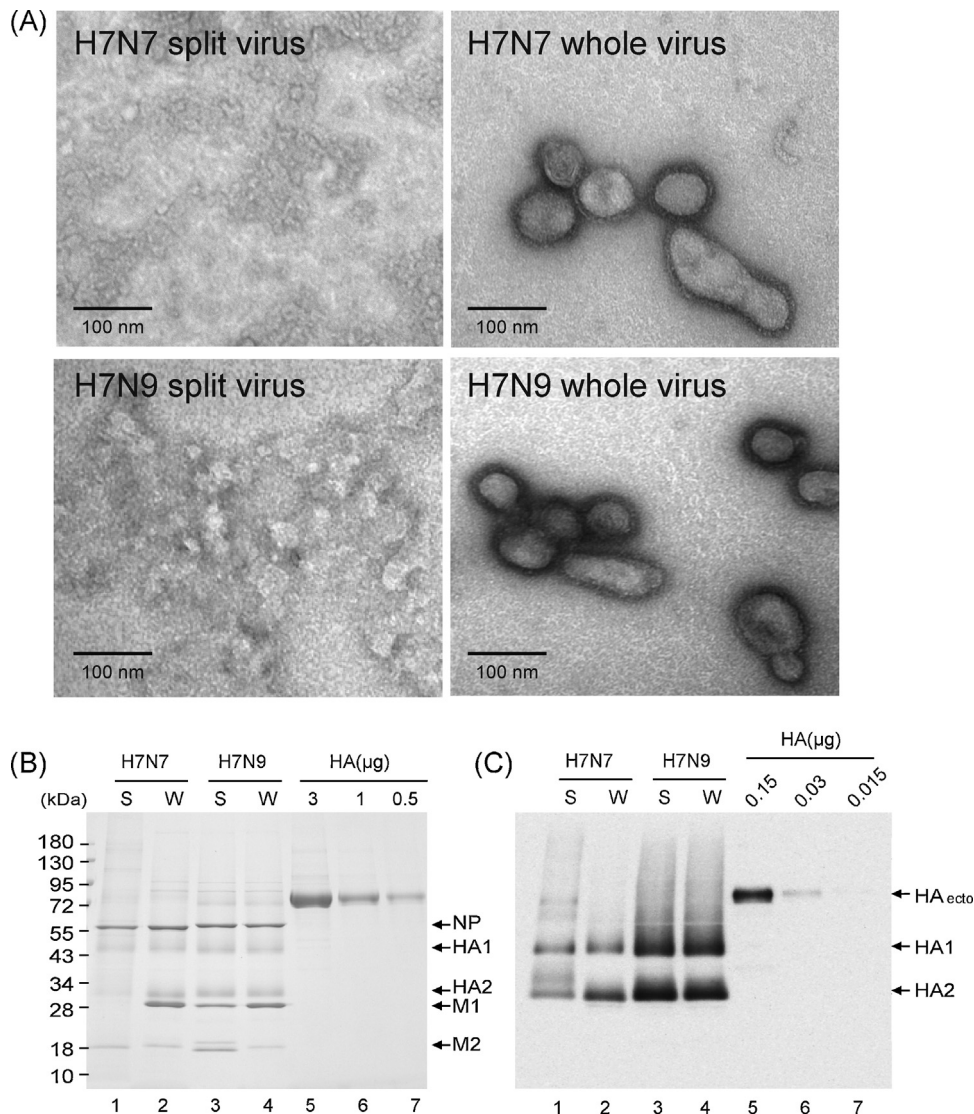
The detection of vaccine-induced neutralizing antibody titers against influenza viruses were performed with a World Health Organization recommended protocol. Each RDE-treated serum performed two-fold serial dilutions in a 96-well microtiter plate was co-incubated with equal volume of virus diluents (100 TCID<sub>50</sub>/well) at 37 °C for 1 h and then added 1.5 × 10<sup>4</sup> MDCK cell into each well to allow virus replication overnight at 37 °C in a 5% CO<sub>2</sub> incubator. After fixation of the cells, the presence of virus was detected by enzyme-linked immunosorbent assay (ELISA) with specific antibody against NP protein. After tracing with HRP-conjugated secondary antibody and developed with TMB substrate, the absorbance was measured at 450 nm with a Multi-Detection Microplate Reader (Synergy HT, Bio-Tek). Untreated virus control (VC), uninfected cell control (CC), and back titration of virus infectivity are included on each plate. Half cell infection was calculated by the following equation:  $X = (\text{average OD of VC wells} - \text{average OD of CC wells}) / 2 + (\text{average OD of CC wells})$ . Microneutralization titer is expressed as the reciprocal of the highest serum dilution that showed ≤50% of the cells are infected.

### 2.5. Mice immunization and virus challenge

Six-weeks-old female BALB/c mice were immunized intramuscularly with inactivated virus vaccines (based on HA content of 0.004 µg, 0.02 µg, 0.1 µg, 0.5 µg, 1.5 µg, or 3 µg) containing adjuvants or without adjuvants at weeks 0 and 2. AddaVAX is an oil-in-water emulsion, consisting of the 5% oil squalene, 0.5% Tween 80, and 0.5% Span-85 in a sodium citrate buffer, with a formulation similar to MF59 adjuvant (Novartis). To prepare Al(OH)<sub>3</sub>-formulated vaccine, each dose of vaccine consisted of indicated amount of HA was mixed with 15 µg of Al(OH)<sub>3</sub> in sterile phosphate-buffered saline (PBS; pH 7.1), in a final volume of 50 µL. Mice antisera were collected at week 2 or 4 and virus challenge was performed at week 5. The immunized mice were challenged intranasally with a lethal dose (100 LD<sub>50</sub>) of wild-type A/Taiwan/01/2013(H7N9) influenza virus and monitored daily for 14 days for survival and weight loss. All animal experiments were evaluated and approved by the Institutional Animal Care and Use Committee of Adimmune Corporation. Mice were euthanized if they exceeded 30% loss of body weight.

### 2.6. Statistic analysis

The significance in differences between vaccine groups was statistically computed applying t-test using GraphPad Prism software, Version 6.0.



**Fig. 1.** Characterization and identification of inactivated split and whole virus vaccine. (A) The electron micrograph of negatively stained A/Mallard/Netherlands/12/2000(H7N7)-IBDC-1 and A/Shanghai/2/2013(H7N9)-IDDC-RG32A inactivated split or whole viruses. (B) SDS-PAGE analysis of 5 μg total proteins in H7N7 and H7N9 virus vaccines. Purified HA protein of indicated amount was loaded to calibrate the HA content. S, split virus vaccine; W, whole virus vaccine. (C) Total viral proteins (1 μg) and indicated amount of HA was analyzed by western blotting using anti-HA antibody to identify the H7 proteins as shown in panel B. HA<sub>ecto</sub>, 293-F cell-expressed HA of H7N9 virus. Polyclonal antibody used in the western blotting was prepared by immunizing rabbit with human cell-expressed H7N9 HA protein. Images of SDS-PAGE or western blot were acquired by FluorChem<sup>®</sup> HD2 (Alpha Innotech) and Alpha VIEW AS software.

### 3. Results

#### 3.1. Characterization of H7-type vaccine candidates

In this study, the H7-subtype vaccine candidates were produced by egg-based process, which has been used as standard method since the 1950s to manufacture current licensed influenza vaccines. The morphologies of inactivated H7-subtype whole and split virus vaccines were negatively stained with 2% uranyl acetate and observed using TEM (Fig. 1A). To evaluate the abundance of HA in vaccine antigen, the amounts of proteins of each vaccine candidate and purified HA<sub>ecto</sub> protein as determined by BCA protein assay were resolved by SDS-PAGE in a 7.5–17.5% gradient gel and then subjected to either Coomassie blue staining (Fig. 1B) or western blot analyses by specific antibodies against H7 protein (Fig. 1C). By using the scanning densitometry, the HA standard curve constructed by HA<sub>ecto</sub> protein ranging from 3 μg to 0.5 μg was used to calibrate the HA content in vaccines. Further, the amounts of HA protein as located by western blotting in vaccine antigens were estimated by

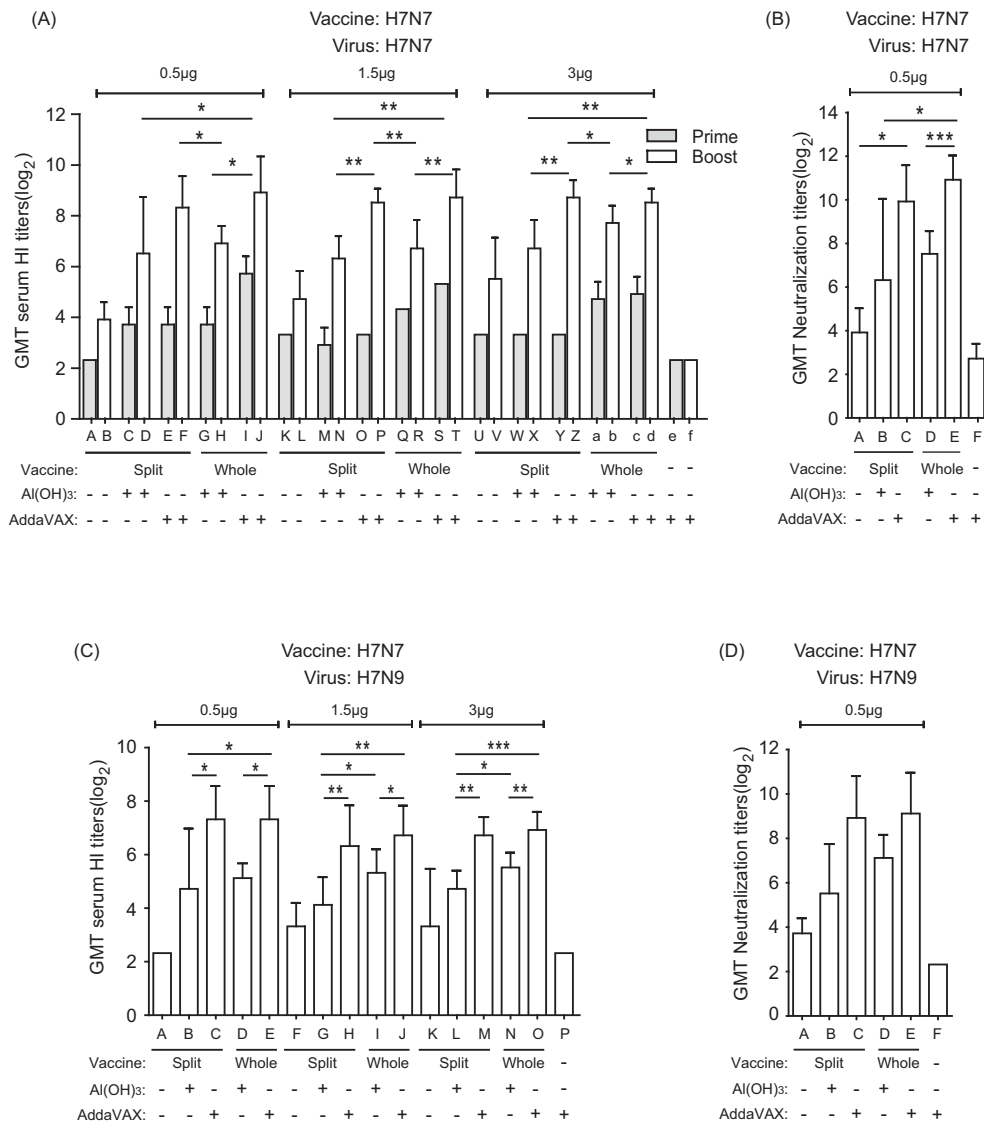
**Table 1**

The relative abundances of HA protein to total protein of vaccine bulks.

Vaccine type	H7N7		H7N9	
	Split (S)	Whole (W)	Split (S)	Whole (W)
HA(%) <sup>a</sup>	37.0 ± 4.5	35.2 ± 2.3	32.0 ± 1.5	35.5 ± 1.8

<sup>a</sup> Data are presented as means ± standard deviations from triplicate samples.

interpolation from the calibration curve. After three independent quantifying experiments, we estimated that the HA protein contributes approximately 32–35.5% and 37–35.2% of total protein of split/whole H7N9 and H7N7 vaccine, respectively (Table 1). At the time of this experimentation, the qualified standard reagents for single radial immunodiffusion conventionally used to evaluate the H7N9 vaccine potency were not available. We employed quantitative sandwich ELISA to further quantify the amount of HA antigen in purified H7N9 vaccine (Fig. 1, Supplemental). HA protein was estimated to constitute 33.6% of the total protein in H7N9 split virus



**Fig. 2.** Humoral immune response elicited by H7N7 vaccine and cross-reaction of antisera with H7N9 virus. BALB/c mice ( $n = 5$ ) were immunized with two injections of various doses of inactivated virus vaccines as labeled or adjuvant alone as a control. All mice antisera collected at week 2 and week 4 post-priming were tested for seroconversion by HAI and microneutralization assays. (A) Antisera were analyzed for HAI titers against H7N7 virus. Comparing the vaccination effect among various formulations, asterisk (\*\*), (\*\*), and (\*) indicates a significant difference ( $P < 0.001$ ), ( $P < 0.01$ ), and ( $P < 0.05$ ), by unpaired *t*-test. The unlabeled significant differences between two groups are presented as follows, BF: \*\*, LN: \*, LP: \*\*\*, VZ: \*\*\*, Hb: \*, Xb: \*. (B) Antisera from the mice immunized with 0.5 µg vaccines were analyzed for neutralizing antibody titers against H7N7 virus. (C) Antisera were analyzed for cross-reactivity of HAI titers against H7N9 virus. The unlabeled significant differences between two groups are presented as follows, AC: \*\*, FH: \*\*, KM: \*\*. (D) Antisera from the mice immunized with 0.5 µg vaccines were analyzed for cross-neutralizing antibody titers against H7N9 virus.

vaccine from representative results, consistent with that shown in Table 1.

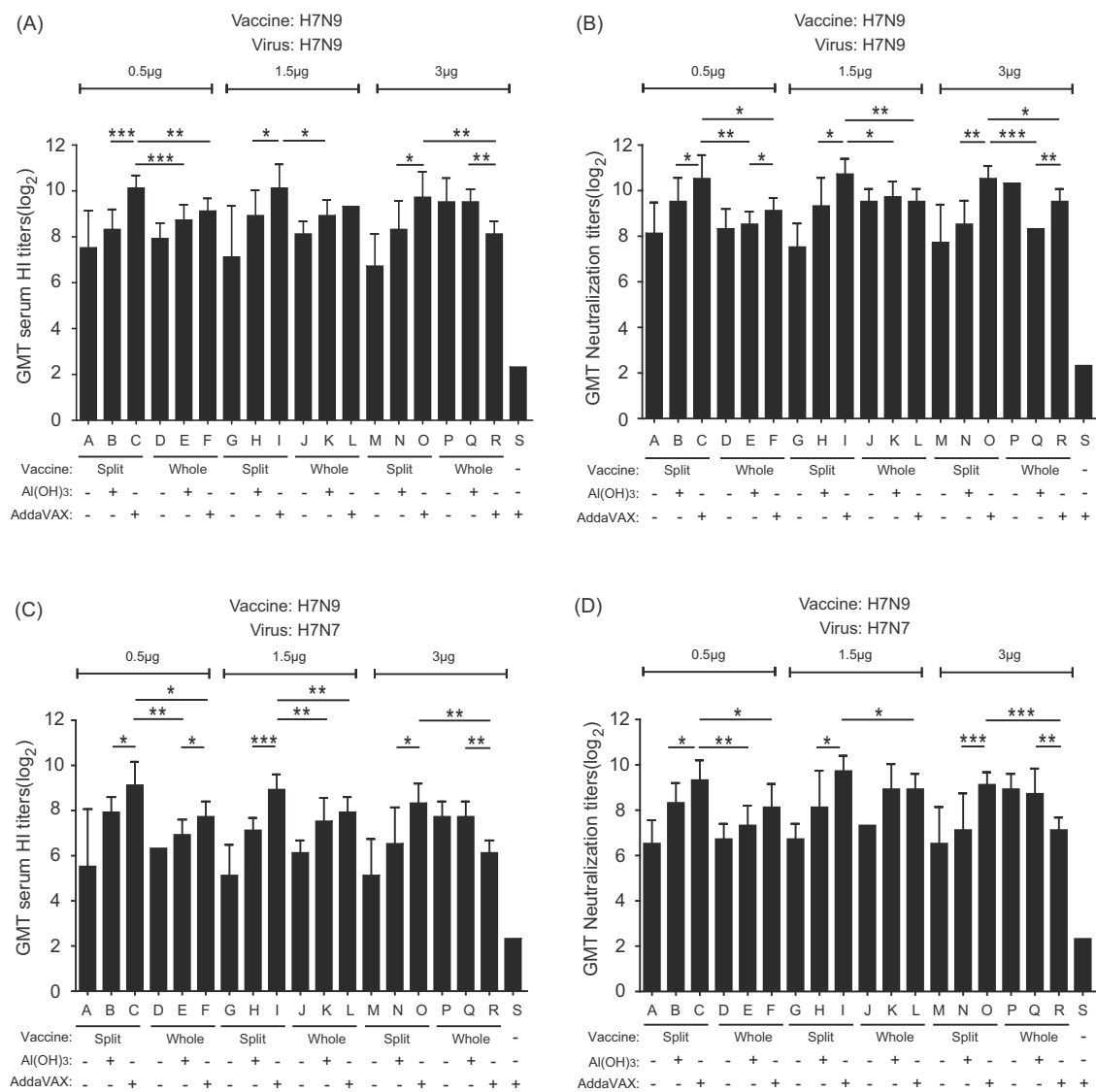
### 3.2. The immunogenicity of H7N7 antigen combined with various adjuvants

As a preparatory research before acquiring the H7N9 vaccine strain for manufacturing production, we first studied its closely related virus, H7N7, in terms of immunogenicity and optimization of vaccine formulation. A serial of vaccinations in mice were performed to address the dose response and adjuvant effects on H7N7 vaccine efficacy which may serve as references to calibrate better vaccine formulation for the pandemic H7N9 strain. Briefly, groups of mice were immunized intramuscularly twice in two-week interval with inactivated split or whole virus H7N7 vaccine containing Al(OH)<sub>3</sub>, AddaVAX, or without adjuvant. The sera from the mice received 0.5 µg (low-dose), 1.5 µg and 3 µg (high-dose) vaccines based on HA content were collected at week 2 and

week 4 post-priming for further serological assays. As shown in Fig. 2, only vaccine formulations with the 0.5 µg and 1.5 µg antigens in AddaVAX-adjuvanted H7N7 whole-virus (lane I and lane S) can elicit the HAI titers over 40 after first vaccination (Fig. 2A, prime). After the second immunization, the resulting HAI titers against H7N7 virus illustrated that adjuvants indeed enhanced the immunogenicity of H7N7 vaccine either with a low-dose or high-dose vaccination (Fig. 2A). In addition, the squalene-adjuvanted H7N7 antigens elicited the highest geometric mean with HAI titers ranging from 320 to 640 among the three experimental groups, suggesting the squalene emulsion is the most efficacious in stimulating specific HA antibodies (Fig. 2A). The determination of neutralizing antibody titers elicited by vaccination may be more relevant to the assessment of vaccine efficacy because it is not clear that all HAI antibodies can accomplish viral-neutralization activity. To this end, microneutralization assay, as a measurement of antisera ability to neutralize viral infections to MDCK cells, were performed. The results showed that the mice immunized with vaccines







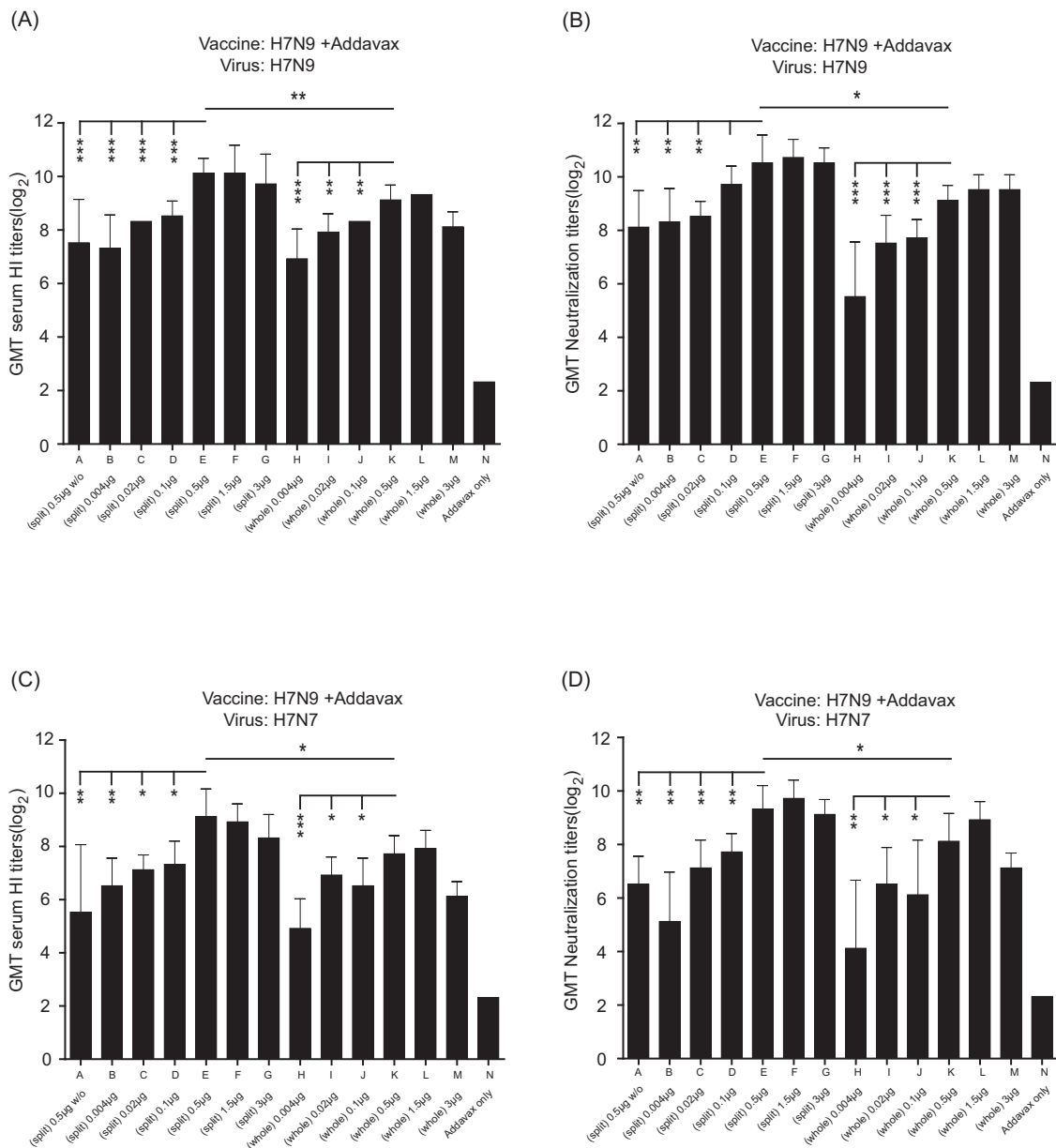
**Fig. 4.** Humoral immune response elicited by H7N9 vaccine and cross-reaction of antisera with H7N7 virus. BALB/c mice ( $n = 5$ ) were immunized with different doses of H7N9 split or whole virus vaccines combined with or without adjuvants as indicated. (A) Vaccine-induced HAI titers against H7N9 virus were detected in antisera. The unlabeled significant differences between two groups are presented as follows, DE: \*; AC: \*\*\*; GH: \*; GI: \*; MN: \*; DF: \*\*; MO: \*\*; JK: \*; JL: \*\*\*; PR: \*; MP: \*\*; EQ: \*; BF: \*; MQ: \*\*\*; MR: \*; NP: \*; NQ: \*. (B) Antisera from the mice immunized with vaccines were analyzed for microneutralization titers against H7N9 virus. The unlabeled significant differences between two groups are presented as follows, AB: \*; BE: \*; GJ: \*\*; AC: \*\*; GH: \*; GI: \*\*\*; DJ: \*; DF: \*; MO: \*\*\*; JP: \*\*; BN: \*; EK: \*\*; NR: \*; MP: \*\*\*; MR: \*\*; NP: \*\*\*; PR: \*\*; GK: \*\*; GL: \*\*; IJ: \*\*. (C) Vaccine-induced cross-reactive HAI titers against H7N7 virus were detected in antisera. The unlabeled significant differences between two groups are presented as follows, AB: \*\*; BE: \*\*; GF: \*\*; EQ: \*; GH: \*\*\*; AC: \*\*; GI: \*\*\*; IR: \*\*\*; DF: \*\*; JK: \*; MO: \*\*; CR: \*\*; JL: \*\*\*; BH: \*; HL: \*; PR: \*\*; BN: \*; KG: \*\*; GL: \*\*\*; HJ: \*\*; IJ: \*\*\*; MP: \*\*; MQ: \*\*. (D) Antisera were analyzed for cross-neutralizing antibody titers against H7N7 virus. The unlabeled significant differences between two groups are presented as follows, AB: \*\*; BE: \*; NQ: \*; GJ: \*; EQ: \*; GH: \*; AC: \*\*; GI: \*\*\*; IR: \*\*\*; DJ: \*; DF: \*; MO: \*\*\*; JL: \*\*; PR: \*\*\*; EK: \*; IO: \*; KG: \*; GL: \*\*\*; IJ: \*\*\*; KJ: \*; MP: \*\*; MQ: \*\*; NP: \*\*; PR: \*\*\*.

with this purpose (Fig. 4A). In contrast, the HAI titers moderately decreased in mice when the receiving dosage reduced from 3 to 0.5 µg whole-virus antigen in the presence of Al(OH)<sub>3</sub> adjuvant (lane E vs. lane Q,  $p < 0.05$ ), indicating a better immune response elicited by Al(OH)<sub>3</sub>-adjuvanted H7N9 whole-virus vaccine may need a higher-dose administration (Fig. 4A).

In parallel, the ability of H7N9 virus vaccine to induce the neutralizing antibodies against H7N9 and H7N7 virus were evaluated by microneutralization assay. AddaVAX-adjuvanted split vaccine (lane C) elicited significantly higher neutralizing antibody titers than Al(OH)<sub>3</sub>-adjuvanted split vaccine (lane B,  $p < 0.05$ ) and adjuvanted whole-virus vaccine (lane E,  $p < 0.01$  and lane F,  $p < 0.05$ ) (Fig. 4B). Similar results were found in the H7N9 vaccines to induce the cross-reactive HAI and neutralizing antibodies against H7N7 virus (Fig. 4C and D). The strong correlation between neutralization

and HAI titers for respective H7N9 and H7N7 viruses was significant at 0.5 µg H7N9 vaccine groups, suggesting the HA antibody is predominantly responsible for impeding the infectivity of H7N9 and H7N7 viruses (Fig. 4).

To examine the dose-sparing effect of H7N9 vaccine combined with AddaVAX formulation, additional mice were immunized with lower-dose of antigen ranging from 0.004 µg to 0.1 µg to observe the minimal dose requirement for eliciting significant immune response. The presence of AddaVAX adjuvant in low-dose antigens from 0.004 µg to 0.1 µg substantially enhanced the H7N9 vaccine efficacy and elicited an adequate immune response against both H7-subtype viruses similar to the group of 0.5 µg antigen without adjuvant (Fig. 5A–D). Nevertheless, induction of HAI titers ( $\geq 1:40$ ) in immune sera are widely accepted as indicators for protection of 50% subjects was achieved by vaccination as little



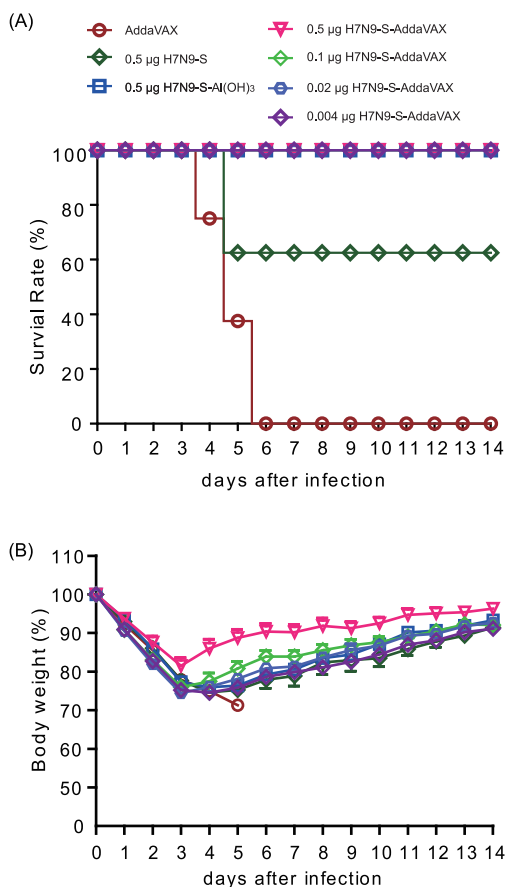
**Fig. 5.** The dose-sparing effect of AddaVAX on antibody responses to H7N9 inactivated whole or split virus vaccines. Mice were immunized with different doses of H7N9 inactivated split or whole virus vaccines combined with AddaVAX as labeled. (A) and (C) Antisera were collected to analyze the HAI titers against H7N9 and H7N7 viruses. (B) and (D) The neutralizing antibodies against H7N9 and H7N7 viruses were measured by microneutralization assay. The unlabeled significant differences between two groups are presented as follows, (A) BC: \*, HM: \*, BD: \*, CF: \*, BF: \*\*, CG: \*\*, IL: \*\*\*, AF: \*, BG: \*\*, HJ: \*\*, AG: \*\*, DF: \*, DG: \*, HL: \*\*\*, MK: \*\*, ML: \*\*\*, JL: \*\*, BD: \*, JM: \*\*, AD: \*\*, CF: \*\*, BF: \*\*, CG: \*\*, IL: \*\*, AF: \*\*, BG: \*\*, HJ: \*\*, IM: \*\*, AG: \*\*, DF: \*, DG: \*, HL: \*\*\*, FL: \*\*, GM: \*\*, BH: \*, CJ: \*, DJ: \*\*. In panel (C) HM: \*, JL: \*\*, CF: \*\*, BF: \*\*, CG: \*, HI: \*\*, IL: \*\*, KM: \*\*, AF: \*\*, BG: \*\*, HJ: \*, IM: \*\*, LM: \*\*, AG: \*\*, DF: \*\*, DG: \*, HL: \*\*, FL: \*\*, GM: \*\*, BH: \*. In panel (D) BC: \*, HM: \*\*, JL: \*\*\*, BD: \*\*, AD: \*, CF: \*\*, FG: \*, BF: \*\*, CG: \*\*, IL: \*\*, KM: \*, AF: \*\*, BG: \*\*, LM: \*\*, AG: \*\*, DF: \*\*, DG: \*\*, HL: \*\*, FL: \*, GM: \*\*, DJ: \*.

as 0.004 μg in AddaVAX-adjuvanted split vaccine against both H7-subtype influenza viruses (Fig. 5A and C). To test whether the vaccines offered protective efficacy, the immunized mice were challenged with lethal dose (100 LD<sub>50</sub>) of wild-type H7N9 virus and the efficacy of vaccine protection was evaluated over 14 d based on survival rate and the body weight change. The result showed mice immunized with all dosages of split vaccine with adjuvants provided fully protection against a lethal H7N9 challenge, in contrast to immunization with split antigen only provided mice with 60% protection (Fig. 6A). The mice immunized with 0.5 μg of AddaVAX split vaccine provided a better protection with a less loss of mice body weight than other groups and recovered quickly after virus challenge (Fig. 6B). On the other hand, lower dose (0.004 μg to 0.1 μg)

of split vaccine with AddaVAX and 0.5 μg split vaccine with Al(OH)<sub>3</sub> compromised the body weight of mice more than 20% loss at Day 3 post-infection and most survivors recovered slower than those receiving 0.5 μg of AddaVAX-split vaccine (Fig. 6B). In summary, these results indicates the adjuvation of squalene emulsion in H7N9 split virus vaccine is the most promising way to optimize the formulation, achieves better antigen-sparing effect, and provides a potent protection against H7N9 virus.

#### 4. Discussion

In this study, we systematically investigated the H7N9 vaccine efficacy and its improvement by combining various doses of



**Fig. 6.** At week 5 post-priming, vaccinated mice were challenged intranasally with a lethal dose (100 LD<sub>50</sub>) of wild-type H7N9 virus and monitored daily for survival rate (A) and weight loss (B). The percentages of survival rate and changes of body weight were recorded. The used vaccine formulations for challenge studies are as indicated.

antigen with Al(OH)<sub>3</sub> or squalene-based adjuvants in mice vaccination. To our knowledge, there are no published data on improvement of H7-subtype vaccines with squalene adjuvants, as yet. In addition to Al(OH)<sub>3</sub> adjuvant, the safety and potency of squalene-based immunogenic adjuvants such as MF59 has been discussed in many human clinical trials [14,15]. To date, it has been extensively used as formulation for preparedness of current licensed influenza virus vaccines [16,17]. Current study not only proposed a practicable approach but also an alternative formulation to develop effective H7N9 vaccine.

The highly pathogenic avian influenza A viruses have caused global outbreaks and raised a great concern that further changes in the viruses may occur to bring about a deadly pandemic [6]. In March 2013, H7N9 avian influenza virus, like all newly emerged strains that people have not been exposed to and acquired preexisting immunity, has caused the outbreak of human infections with sickness and mortality in China. Until now, it's not fully understood what risk factors are involved in the bird-to-human cross-species transmission, as well as what might cause pandemics through viral adaptation to human population. The most cost-effective way to prevent the spread of highly pathogenic avian influenza diseases is to induce human immunity by extensive vaccination. Most of the clinical studies indicated avian influenza vaccines are less immunogenic than seasonal flu vaccine or induce less immunological memory in human, thus requiring adjuvantation or two-dose administration to improve the vaccine efficacy [18–21]. Although previous study showed that Al(OH)<sub>3</sub>-adjuvanted H7N7 whole virus vaccine was highly immunogenic, elicited substantial HAI titers,

and protected the immunized mice from H7N7 viral challenge [22]. However, the clinical study showed the unadjuvanted split H7N7 vaccine induced fairly low antibody response with a 36% seroconversion rate even at high dosage, arguing that H7N7 virus vaccine antigen is poorly immunogenic in human [12]. Moreover, the unique low immunogenicity of H7N9 HA has been predicted by immunoinformatics tool owing to less T-cell epitopes in protein sequence than circulating influenza A strains [23]. These reports highlight the need for more immunogenic vaccine formulations in H7-subtype vaccine preparations.

For the initial development of H7N9 vaccine, we first determined the kinetics of the humoral immune response to different doses of H7-subtype influenza vaccine formulations, including whole and split virus vaccines combined with or without adjuvants (Figs. 2–4). Based on previous studies, it is well known that HA is the major immunogen of vaccines to elicit HAI and viral-neutralization titers against influenza viruses. Although the HA sequence of H7N9 is similar to H7N7 with a high homology of 97%, split HA antigens from these two viruses presented a very different ability to elicit effective humoral immune response. In this study, H7N7 and H7N9 inactivated whole virus vaccines induced very similar level of antibody responses against the same or different type of H7 viruses (Fig. 2A, lane J vs. Fig. 4A, lane F; Fig. 2C, lane E vs. Fig. 4C, lane F). In contrast, while H7N7 split vaccine is a very poor immunogen, which induced negligible HAI titers, and neutralizing antibodies against both H7-subtype viruses except combined with adjuvants (Fig. 2), H7N9 split vaccine induced much stronger immune response either in the presence of or without adjuvants (Fig. 4). The low immune response to H7N7 split vaccine was also observed in previous studies in humans and further clarified by conducting the comparison of HA antigen uptake, processing, presentation, and trimer conformation as well as the EM morphology among influenza vaccines [24]. Interestingly, our TEM observations showed the H7N7 split vaccine primary exhibited the small round (5–20 nm) structures and consistent with the recent report (Fig. 1A vs. Fig. 5, H7N7 [24]). In contrast, the H7N9 split vaccine showed the predominant pieces of viral particles of varying sizes, most of that with external projections of HA and NA (Fig. 1A). This morphology observed in our H7N9 split vaccine is similar to that of H9N2 split vaccine described in previous findings, which also indicated that H9N2 split antigen is the most immunogenic to induce immune response among the avian vaccines [24]. All of above observations support the suggestion that the morphology of vaccine may influence immunogenicity of split-virion vaccine in human.

The whole virus vaccines were usually used and shown to be more immunogenic than split virus vaccines [25]. In this study, we found that without adjuvants, both H7N9 split and whole virus antigens have compatible immunogenicity (Fig. 4A, lane A vs. lane D). However, with AddaVAX, the H7N9 split virus vaccine exhibited higher HAI titers and neutralizing capacity to both H7-subtype viruses than whole virus vaccine (Fig. 4, lane C vs. lane F). No obvious difference of vaccine potency was observed among split and whole virus H7N7 vaccines when combined with individual adjuvants (Fig. 2A, lane D vs. lane H and lane F vs. lane J). Overall, the AddaVAX-adjuvanted H7N9 or H7N7 vaccines elicited the highest HAI and neutralizing antibodies titers when compared to Al(OH)<sub>3</sub> or without adjuvant (Figs. 2 and 4). Our results illustrated that squalene-based adjuvant may confer the superior formulation to enhance the H7 subtype vaccine efficacy.

To address the cross-reactivity of H7 subtype vaccines, we demonstrated that 0.5 µg of both AddaVAX-H7N7 vaccines strongly confer potent cross-reactive HAI and viral neutralizing titers against H7N9 virus, suggesting the AddaVAX-adjuvantation strategy can enhance the cross-reactivity of H7N7 vaccine (Fig. 2C and D). On the other hand, the antisera from 0.5 µg split- or whole-virion H7N9 antigen exhibited compatible HAI titer (≥1:40)



and neutralization titers ( $\geq 1:100$ – $300$ ) against both H7-subtype viruses (Fig. 4). It illustrated that even no adjuvantation, the both H7N9 vaccines also provided adequate HAI titer against H7N7 virus in mice might due to their highly structure similarity [26] and more immunogenic characteristic of HA antigen. Taken together, this study provided the detailed preliminary assessment of conventional H7-subtype virus vaccines efficacy in terms of finding optimal formulation and evaluating the cross-reactivity that may help to alleviate the situation of urgent need of effective H7N9 vaccine during high wave of H7N9 outbreak.

During pandemic situations, the adjuvants may play a critical role in reducing the dose requirement to induce protective immunity in subjects, thereby allowing more people to be vaccinated with limited supply. In this study, a dose-sparing effect afford by squalene-based adjuvant was evaluated by reducing the vaccine dose ranging from  $3 \mu\text{g}$  to  $0.004 \mu\text{g}$ . All of the formulations attained an adequate immune response, achieved theoretically protective HAI titers against H7N9 in mice, and afford substantial cross-reactive HAI titers against H7N7 viral strain (Fig. 5A–D). To further address the vaccine potency, we also evaluate the protection efficacy in animals. As the humoral immune response induced by AddaVAX-adjuvanted H7N9 vaccines have reached plateau level at the doses of  $1.5 \mu\text{g}$  and above (Fig. 5, lanes F, G, L, and M), the protection of mice against virus challenge were only investigated at the doses of  $0.5 \mu\text{g}$  or less. Virus challenge result showed that  $0.5 \mu\text{g}$  or lower dose ( $0.004$ – $0.1 \mu\text{g}$ ) of AddaVAX-adjuvanted H7N9 split vaccine were sufficient to provide 100% protection from death in mice (Fig. 6A). However, the group of mice vaccinated with lower dose of H7N9-AddaVAX split vaccines exhibited a dramatically body weight loss (more than 20% of body weight change) in contrast to the mice group receiving  $0.5 \mu\text{g}$  AddaVAX-H7N9 split vaccine (Fig. 6B). This result is consistent with that the  $0.5 \mu\text{g}$  AddaVAX-H7N9 split vaccine exhibited significantly predominant immune response against H7N9 virus compared with lower-dose groups (Fig. 5A and B, lane E vs. lanes A–D). All above evidences indicate the squalene-based adjuvantation is a promising way to prepare for effective H7N9 vaccine for surged demand. Accordingly, we highlight that  $0.5 \mu\text{g}$  AddaVAX-H7N9 split virus vaccine is the optimal formulation relevant to providing potent immune response to cross-reaction with H7N7 virus and better protection of mice against H7N9 challenge.

Our results also showed that  $\text{Al}(\text{OH})_3$  can modestly enhance the H7-subtype antigens immunogenicity to move the dose-response curve to lower antigen concentration and works slightly better with high-dose of whole virus (Fig. 2A, lane H vs. b ( $p < 0.05$ ) and Fig. 4A, lane E vs. Q ( $p < 0.05$ )) while the squalene-based adjuvant shifts the optimum immunogenic dose of H7N9 split vaccine at least 10-fold lower (Fig. 5) and could be proven experimentally in a mouse model. This phenomenon of squalene-based adjuvant enhancing the immune response of poorly immunogenic split antigen is in line with the observation of previous pre-clinical and clinical studies. Based on these positive results, we suggested that H7N9 inactivated virus vaccines combined with squalene-based adjuvant is effective and may increase production capacity to support the demand of pandemic H7N9 influenza preparedness for human use and further studies in humans were warranted to elucidate the vaccine potency and optimal formulation.

#### Author contributions

C.-Y.W., C.-C.C., C.-H.C., C.-C.L., and J.-R.C. designed research; C.-Y.W., C.-Y.C., H.-H.M., C.-W.W., Y.-T.C., and J.-R.C. performed research; C.-Y.W., C.-Y.C., H.-H.M., C.-W.W., Y.-T.C., and J.-R.C. analyzed data; and C.-Y.W., P.-W.H., C.-H.C., C.-C.L., and J.-R.C. wrote the paper.

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#### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2014.06.043>.

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