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Cross-protective efficacy of baculovirus displayed hemagglutinin against highly pathogenic influenza H7 subtypes

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ABSTRACT

The outbreak of human infections with avian-origin H7N9 influenza has raised global concerns about a potential human pandemic. Therefore, the generation of simple and reliable newer vaccines is high priority for pandemic preparedness. In this study, we aimed to develop a recombinant vaccine by expressing HA of H7N9 (A/Shanghai/2/2013) on the surface of baculovirus (BacHA). Further, live or inactive form of BacHA (H7N9) vaccine was immunized twice either intranasally or subcutaneously into mice. The immunogenicity and cross-protective efficacy of the BacHA (H7N9) vaccine was assessed against H7N9 or H7N7 subtype challenge. The results showed that mice immunized subcutaneously with adjuvanted inactive BacHA (H7N9) induced robust cross-neutralizing antibody responses against H7 subtypes (H7N9, H7N7 and H7N3) compared to subcutaneous or intranasal immunization of live BacHA. In contrast, mice immunized intranasally with live BacHA stimulated higher HA-specific mucosal IgA levels in the upper airways, the port of virus entry. Also, intranasal immunization of BacHA of either H7N9 or H7N7 completely protected against 5 MLD_{50} of both H7N9 and H7N7 infections. An overall study revealed that intranasal administration of HA expressed on the baculovirus envelope is alternative way to prime the immune system against influenza infection during a pandemic situation.

1. Introduction

Over the past two decades, low and high pathogenic avian influenza viruses expressing an H7HA have been circulating in birds, causing numerous poultry outbreaks (Belser et al., 2013). However, human infections have occasionally occurred in association with poultry outbreaks of H7N2, H7N3 and H7N7 viruses (Puzelli et al., 2005; Fouchier et al., 2004; Pasick et al., 2009). Human infections with N9 subtype influenza viruses had not been reported before 2013. Avian-origin influenza A H7N9 virus was first reported in China in March 2013 has since caused more than 355 human infections with more than 112 fatalities, a mortality rate of 31% (as of February, 2014). Most patients with this infection have had severe pulmonary disease and acute respiratory distress syndrome (ARDS) (Gao et al., 2013). This is in contrast to previously reported H7 human infections, which typically show mild respiratory symptoms and conjunctivitis with one exception being a fatal case of ARDS syndrome associated with the H7N7 virus (Fouchier et al., 2004). So far, there is no evidence of sustained human to human transmission of H7N9 has been found, though possesses several molecular markers associated with human adaptation, including the presence of E627K, in the PB2 protein, Q226L at the 210 loop of HA gene (Gao et al., 2013; Shi et al., 2013). The recent mouse studies revealed that H7N9 virus is capable of using the eye as an entry for respiratory infections (Belser et al., 2013). Also, H7N9 virus is much more transmissible from poultry to humans than the H5N1 virus (To et al., 2013). The above characteristic features of the H7N9 are raising concerns for the possibility of a future influenza pandemic.

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However, most of the previous H7 vaccine studies showed less immunogenic in humans and animals (de Wit et al., 2005; Cox et al., 2009; Couch et al., 2012; Rajesh Kumar et al., 2013). Also, commercially available influenza vaccines are conventional inactivated "split" vaccines which need eggs or mammalian cell cultures to produce sufficient amount of essential vaccine antigen. The egg-based technology could be restricted by the sufficient supply of fertile chicken eggs in pandemic situation and safety issues.

As an alternative to conventional vaccine approaches, several approaches have been adopted including the use of non-human viral vectors for vaccine delivery. Among those, baculovirus Autographa californica multicapsid Nucleopolyhedrovirus (AcMNPV) has evolved as a novel tool for vaccine vector development (Madhan et al., 2010). Baculovirus expression system allows us to present large complex proteins on the baculovirus envelope in their native antigenic confirmation and functions, and also transduce efficiently foreign genes into host tissues.

Baculovirus displayed HA serves as both a subunit vaccine and a DNA vaccine, and is capable to elicit strong humoral and cellular immune responses without the need for immunological adjuvants. Baculovirus has been used as a vaccine vector for several animal and human diseases and was shown to induce humoral, mucosal and cell mediated immunity (Meng et al., 2011; Rajesh Kumar et al., 2013; Prabakaran et al., 2013; Premanand et al., 2013). Recombinant baculovirus based intranasal vaccine has opened up the way to novel approaches in vaccine development against mucosal acquired pathogens. Here, we have assessed the cross-protective efficacy of intranasal or subcutaneous vaccination of baculovirus surface displayed HA of H7N9 (BacHA) against H7N9 or H7N7 subtype in a mouse model.

2. Materials and methods

2.1. Viruses and cell lines

The reassortant influenza virus H7N3 (A/Canada/rv504/04), H7N7 (NL03: A/Netherlands/219/03), H7N7 (A/duck/Hokkaido/1/10) and H7N9 (A/Shanghai/2/2013) were generated by reverse genetics as described previously (WHO, 2005). Briefly, the hemagglutinin (HA) and neuraminidase (NA) genes of H7 subtype viruses were synthesized (GenScript, USA) based on the sequence from the NCBI influenza Database. A reassortant virus containing HA and NA from each H7 subtype virus and the internal six genes from A/Puerto Rica/8/1934 was generated (Prabakaran et al., 2009). Virus titer was determined by hemagglutination assay as described previously (Webster et al., 1991). The tissue culture infectious dose 50 ($TCID_{50}$) of reassortant virus was then calculated by the Reed and Muench method (1938). *Spodoptera frugiperda* (Sf9) cells (ATCC) were propagated at 28 °C in Sf 900 II serum free medium (Invitrogen, USA). All experiments with highly pathogenic viruses were conducted in a biosafety level 3 (BSL-3) containment facility in compliance with NIH/CDCP and WHO recommendations (NIH/CDCP, 1999; WHO, 2004).

2.2. Preparation of recombinant baculovirus subunit vaccine

The recombinant baculovirus vector was generated as described previously (Prabakaran et al., 2010). The full length HA genes from H7N9 (A/Shanghai/2/2013) and H7N7 (NL03) reassortant viruses were amplified and individually inserted into the shuttle vector pFASTBacHT A (Invitrogen, San Diego, CA, USA) for expression under the white spot syndrome virus (WSSV) immediate early (ie1) promoter (Rajesh Kumar et al., 2013). Further, the constructs were integrated into the baculovirus genome within DH10BacTM (Invitrogen, USA). The recombinant bacmids were then transfected into Sf9II cells and the supernatant containing recombinant baculovirus particles were harvested at 96 h post transfection. The supernatant was centrifuged at 500*g* for 10 min and the virus titer was determined by a standard plaque assay with Sf9 cells according to the baculovirus construction protocol (Invitrogen, No. 10359).

2.3. Analysis of antigenic conformation of HA0 expressed by the baculovirus

The HA0 expressed by the baculovirus in the insect cells was confirmed by indirect immunofluorescence assay with neutralizing monoclonal antibodies (n-mAb 62 and 98) recognizing two different conformational epitopes of HA1 of H7 subtypes (He et al., 2013). Briefly, Sf9 cells were infected with baculovirus displayed HA and incubated for 48 h at 28 °C. After fixation cells were stained with mouse anti-H7HA neutralizing monoclonal antibody, n-mAb 62 or 98 followed by FITC-conjugated rabbit anti-mouse immunoglobulin (Dako Cytomation, Denmark). Then the fluorescence signal was detected with an inverted fluorescence microscope (Olympus, UK). Further, HA0 displayed on the baculoviral particles was tested for functional hemagglutination activity with chicken red blood cells (cRBCs) (Webster et al., 1991). Further, hemagglutination inhibition (HAI) activity of the HA0 display on the baculovirus surface was tested by neutralizing monoclonal antibodies against different conformational epitopes by HAI assay as described below. The recombinant baculoviral vaccine and RG-H7N9 were inactivated with binary ethylenimine (BEI) as described previously by Rueda et al. (2001) and King (1991), respectively. Then the vaccine dose was prepared based on the hemagglutination (HA) units.

2.4. Mice immunization and challenge

Six to seven weeks old specific pathogen free female BALB/c mice were used in all experiments. Twenty mice per group were immunized subcutaneously on day 0 and 28 with 100 μl containing 256 HA units of live BacHA (H7N9) or inactive BacHA (H7N9) with or without emulsified with Montanide ISA563 adjuvant. For intranasal immunization, twenty mice per group were immunized intranasally on day 0 and 28 with 50 μl (25 μl per naris) containing 512 HA units of live or inactive BacHA (H7N9). The negative control groups were immunized PBS and wild-type baculovirus (10^7 PFU of wt-Bac) by subcutaneous and intranasal route respectively. The reference control group was vaccinated subcutaneously with 100 μl containing 256 HA units of inactive whole RG-H7N9 virus, emulsified with Montanide ISA563 adjuvant. Blood samples were collected on days 14 and 42 for serum hemagglutination inhibition (HAI) assay and virus microneutralization (VMN) assay. Four mice from each group were sacrificed on day 42 and nasal washes were collected as described previously (Wang et al., 2010a). In a separate study, the protective efficacy of baculovirus displayed HA of H7N7 (NL03) against H7N9 challenge was assessed. For this, ten mice per group were immunized intranasally with 50 μl (25 μl per naris) containing 512 HA units of live BacHA (H7N7) or subcutaneously with 100 μl containing 256 HA units of live BacHA (H7N7) or inactive BacHA (H7N7) with Montanide ISA563 adjuvant.

To assess the protective efficacy of the vaccines, eight mice from each immunized groups were intranasally challenged with 50 μl (25 μl per naris) of 5 MLD50 of RG-H7N9 or RG-H7N7 (NL03) subtype on day 49. Six mice per group were observed daily to monitor body weight and mortality. Monitoring continued until day 14 after challenge (day 63). Also, 4 days after the viral challenge, the right and left lungs from two mice were aseptically removed for virus titration and histopathology examination, respectively. Briefly, for determination of viral titers, right lungs were asepti-

cally removed. Tissues were homogenized in 1 ml Dulbecco's Minimal Essential Medium (DMEM) supplemented with antibiotic–antimycotic solution (Gibco-BRL, USA) to achieve 10-fold serially diluted suspensions of lung samples. The homogenized suspensions were titrated on monolayers of MDCK cells. The viral titers were calculated by use of the method of Reed and Muench (1938) and expressed as $\log_{10}$ $TCID_{50}$/mL of tissue ± S.D. The limit of virus detection was 1.5 $\log_{10}$ $TCID_{50}$/mL of lung tissue specimen. For histopathology, the left lungs were stored in 10% (wt/vol) neutral buffered formalin and embedded in paraffin and sectioned. Sections were stained with hematoxylin and eosin (H/E) prior to light microscopy examination and were evaluated for lung pathology (Rajesh Kumar et al., 2013).

2.5. Hemagglutination inhibition (HAI) assay

Receptor-destroying enzyme (RDE Denka Siken Co., Japan) treated sera were serially diluted two fold in V-bottom 96-well plates. Four HA units of each influenza viral antigen was added to each well of the 96-well plate and incubated with the serum for 30 min and 1% cRBCs were added, and incubated for 40 min at room temperature (Webster et al., 1991). The inhibition of hemagglutination at the highest serum dilution was considered the HAI titer of the serum.

2.6. Microneutralization assay

Neutralizing antibody titer of the sera on day 42 was determined by microneutralization assay (Rowe et al., 1999). Briefly, MDCK cells were seeded into 96-well culture plates and cultured at 37 °C to form a monolayer. Serial twofold dilutions of heat-inactivated (56 °C for 30 min) immune sera were mixed separately with 100 × 50% tissue culture infective dose ($TCID_{50}$) of H7 subtype viral strain and incubated at room temperature for 1 h. The virus antibody mixture was then added to the monolayer of MDCK cells and incubated at 37 °C for 72 h. The neutralizing antibody titer was expressed as the highest dilution of serum which showed no cytopathic effect.

2.7. Measurement of anti-H7HA specific antibodies by indirect ELISA

The HA specific mucosal IgA level was measured against inactivated H7N9 antigen by indirect ELISA described previously (Bright et al., 2008). In brief, microtiter well ELISA plates (Maxisorb, Nunc) were coated with purified inactivated RG-H7N9 virus in coating buffer (0.1 mol/L carbonate/bicarbonate, pH 9.6). Nasal wash samples were diluted 1:40 in PBS-containing 0.05% Tween 20 (PBS-T). Then, the color development was visualized by adding goat anti-mouse IgA (Bethyl Lab) conjugated with horseradish peroxidise and followed by addition of 3,3′,5,5′-tetramethyl benzidine (Sigma, USA). The optical density (OD) was measured at 450 nm using an ELISA plate reader (Sunrise, Tecan). The mean absorbance value for triplicate wells was used to express mucosal HA-specific IgA antibody levels.

2.8. Statistical analysis

The data were expressed as geometric or arithmetic mean ± standard deviation (SD) or standard Error (SE). The unpaired two tailed Student's *t*-test was performed to determine the level of significance in the difference between means of two groups. The level of significance was expressed as $P < 0.05$.

Table 1
HAI titer of neutralizing monoclonal antibodies (n-mAbs) with HA of H7N9 displayed baculovirus.

n-mAbs[b]	Antigenic site (amino acid[a])	HAI titer against BacHA	HAI titer against H7N9
62	Ser 136 & Gly 137	512	128
98	Lys 175	512	256

[a] Amino acid numbering with signal peptide.
[b] Concentration of n-mAb at 250 μg/mL.

3. Results

3.1. Antigenic conformation of HA0 displayed on the baculovirus

Hemagglutinin of H7N9 displayed on the baculovirus surface has shown functional hemagglutination activity similar to the wild type influenza virus, as evident by agglutination of erythrocytes. However, wild type baculovirus did not agglutinate with chicken erythrocytes. Further, hemagglutination activity of the HA displayed on the baculovirus was inhibited by two different neutralizing monoclonal antibodies (62 and 98) against conformational epitopes of HA1 (Table 1). The mAb 62 and 98 were previously identified as recognizing at amino acid 175 and 136 or 137 of HA1 of H7 subtype, respectively. In addition, the reactivity of BacHA infected Sf9 cells with these neutralizing antibodies against conformational epitopes of HA were also confirmed by indirect immunofluorescence assay (Fig. 1). This indicates HA displayed on the surface of baculovirus has retained its native structure and was able to translocated to the plasma membrane of infected insect cells.

3.2. Systemic antibody responses

Intranasal or subcutaneous immunization of BacHA (H7N9) vaccine on the systemic immunity was evaluated by measuring the activity of serum hemagglutination inhibition (HAI) and virus microneutralization (VMN) assays. The results showed that mice immunized intranasally with live BacHA (H7N9) vaccine significantly ($P < 0.001$) enhanced serum HAI titer compared to mice immunized with inactivated BacHA (H7N9) vaccine (Fig. 2A). Moreover, mice immunized subcutaneously with adjuvanted inactive BacHA (H7N9) or live BacHA (H7N9) significantly ($P < 0.01$) elicited HAI titer compared to mice immunized with adjuvanted inactive RG-H7N9 vaccine (Fig. 2B). Mice immunized intranasally or subcutaneously with BacHA sera also cross-reacted with H7N7 (NL03) while the titers were one fold lower. However, mice vaccinated subcutaneously with RG-H7N9 showed very low HAI titer against H7N7 (NL03).

Further, virus microneutralization assay was performed to determined functional antibodies responsible for the protective immunity to influenza. The cross-neutralizing antibody titer of the vaccinated sera was determined against 100 $TCID_{50}$ of RG-H7N9, RG-H7N7 (NL03), RG-H7N3 and RG-H7N7 (07) on day 42 (Fig. 2C). The results showed that mice immunized intranasally or subcutaneously with live BacHA (H7N9) vaccine induced neutralizing antibody titer against H7N9 (1:360) and other H7 subtypes (>1:200 against H7N7 and 1:140 against H7N3). Moreover, mice immunized subcutaneously with adjuvanted inactive BacHA (H7N9) significantly ($P < 0.001$) induced cross-neutralizing antibody titer against H7 subtypes (1:720 against H7N9, 1:320 against H7N7/03, 1:400 against H7N7/07 and 1:360 against H7N3) (Fig. 2C) compared to mice immunized with adjuvanted inactive H7N9 whole viral vaccine, showed neutralizing antibody

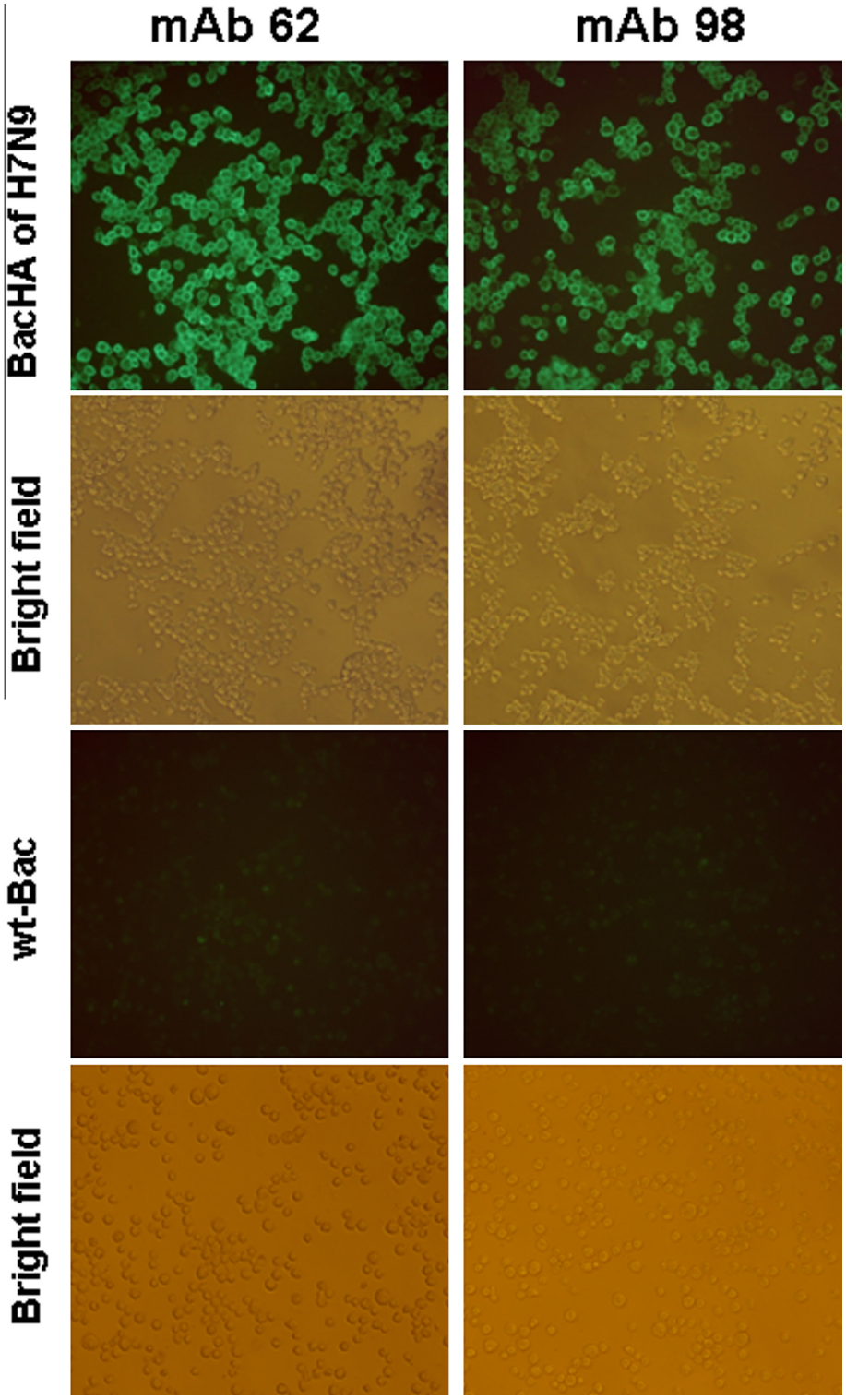

Fig. 1. Indirect immunofluorescence assay of insect cells infected with BacHA of H7N9. The Sf9 cells were infected with baculovirus expressed HA0 from A/Shanghai/2/2013 (BacHA) or wild type baculovirus (wt-Bac). Infected cells were fixed and stained with anti-HA neutralizing monoclonal antibody (mAb 62 or 98) against conformational neutralizing epitopes followed by FITC-conjugated secondary antibody.

titer of 1: 280 against H7N9 and around 1: 200 against H7 subtypes (Fig. 2C). Similarly, neutralizing antibody titer of HA of H7N7 (NL03) displayed on the baculovirus vaccines were also tested against H7 subtypes. The results showed that mice immunized subcutaneously with live BacHA (H7N7) or adjuvanted inactive BacHA (H7N7) induced very low neutralizing antibody titer ($\leqslant$1:45 and $\leqslant$1:71) against H7 subtypes compared to intranasally immunized with live BacHA (H7N7) vaccine, which showed 1:142 against homologous H7N7, 1: 101 against H7N9 and 1:63 against H7N3 subtypes (Table 2). The overall results revealed that mice vaccinated either intranasally or subcutaneously with HA of H7N9 expressed on the recombinant baculovirus induced robust systemic immune responses against H7 subtypes.

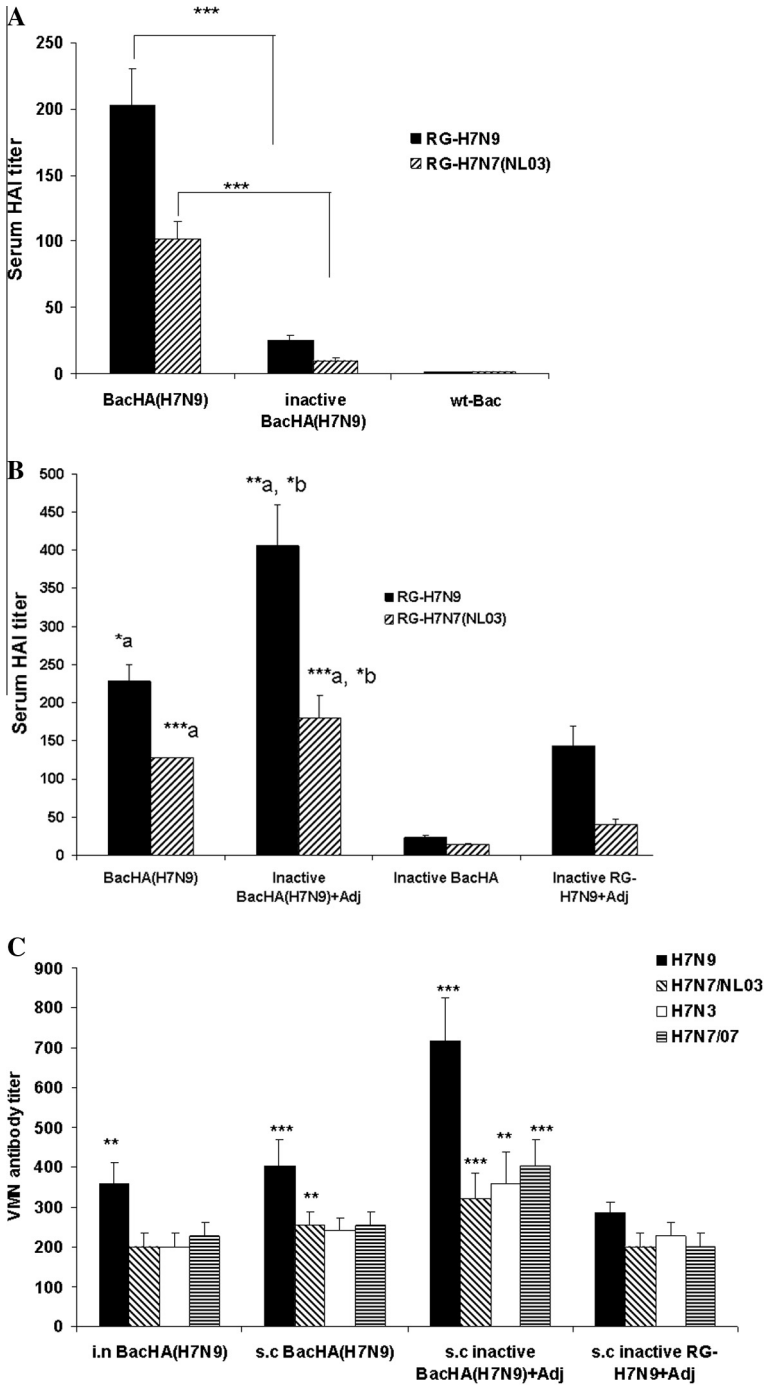

Fig. 2. Serum hemagglutination inhibition (HAI) assay. (A) Groups of mice were intranasally immunized on days 0 and 28 with 100 μl of 128 HA units of live or inactive BacHA (H7N9). The negative control group was intranasally immunized with 100 μl containing 10^7 PFU of wild type baculovirus (wt-Bac). (B) Groups of mice were subcutaneously (s.c) immunized on days 0 and 28 with 50 μl of 256 HA units of live or inactivate BacHA (H7N9) with or without adjuvant. The reference control group was subcutaneously immunized with 100 μl containing 256 HA units of inactivate RG-H7N9 with adjuvant (inactive RG-H7N9 + Adj). The serum HAI titer against RG-H7N9 was measured on day 42. Each point represents the geometric mean value ($n = 6$) ± SE, (a-when compared with adjuvanted inactive RG-H7N9 vaccine group, b-when compared with live BacHA vaccine, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). (C). Serum cross-neutralizing antibody titers against H7 subtype in mice. Microneutralization titers of vaccinated mouse sera on day 42 against influenza H7 subtypes from RG-H7N9 (A/Shanghai/2/2013), RG-H7N3 (A/Canada/rv504/04) and RG-H7N7 (A/Netherlands/219/03 and A/duck/Hokkaido/1/10) were used for this assay. Each point represents the geometric mean value ($n = 8$) ± SD ($^{**}P < 0.01$, $^{***}P < 0.001$, compared with mice subcutaneously vaccinated with adjuvanted inactive RG-H7N9 vaccine).

3.3. HA specific mucosal antibody responses

The HA specific mucosal IgA antibody response in the nasal washes of vaccinated mice was measured by indirect ELISA on day 42. As shown in Fig. 3, mice immunized nasally with live BacHA (H7N9) significantly ($P < 0.001$) enhanced mucosal IgA levels compared to inactive BacHA (H7N9). Moreover, subcutaneous immunization with either live BacHA (H7N9) or adjuvanted inactive bivalent BacHA (H7N9) vaccine failed to induce mucosal antibody responses (Fig. 3). The results revealed that mice immunized intranasally with live BacHA induced robust HA specific mucosal IgA levels.

3.4. Mice challenge study

Four weeks after the final immunization, mice were challenged intranasally with 5MLD$_{50}$ of RG-H7N7 (NL03) or RG-H7N9. Mice immunized intranasally with live BacHA (H7N9) showed complete protection from weight loss against RG-H7N9 challenge (Fig. 4A) and showed only 8% body weight loss against RG-H7N7 (NL03) challenge (Fig. 4C) while as mice immunized intranasally with inactive BacHA (H7N9) showed incomplete protection against H7N9 (Fig. 4B) and H7N7 (Fig. 4D) infections. Also, mice immunized subcutaneously with live BacHA (H7N9) lost around 9% and 13% of their bodyweight on day 5 post challenges against RG-H7N9 and RG-H7N7 respectively (Fig. 5A and C), and showed 100% survival after the infections (Fig. 5B and D). Mice immunized subcutaneously with adjuvanted whole RG-H7N9 showed complete protection against RG-H7N9 challenge (Fig. 5B), but showed insufficient protection against RG-H7N7 (NL03) challenge (Fig. 5D). However, mice vaccinated with adjuvanted inactive BacHA (H7N9) obtained complete protection from weight loss and death against 5MLD$_{50}$ of RG-H7N9 (Fig. 5A and B) and lost around 10% of body weight against RG-H7N7 (NL03) (Fig. 5C). All other groups of mice lost more than 25% of their original body weight or died from complications associated with influenza infection, showing incomplete protection against both RG-H7N9 and RG-H7N7.

We also tested HA of H7N7 (NL03) expressed on the baculovirus vaccine against 5MLD$_{50}$ of RG-H7N9 viral challenge. The results showed that mice immunized subcutaneously with live or inactive BacHA (H7N7) with adjuvant showed 20% loss of body weight (Fig. 6A) and provided only 33% and 50% protection against H7N9 infection (Fig. 6B). Importantly, mice immunized intranasally with BacHA (H7N7) showed 100% survival after infection with RG-H7N9 virus, and showed only 9% decrease in body weight on day 5 and regained their body weight rapidly (Fig. 6A and B).

Viral titers in lungs were analyzed to determine the replication of challenge virus on day 4 post challenge. As shown in Fig. 7, mice immunized intranasally with live BacHA (H7N9) or subcutaneously immunized with live BacHA (H7N9) vaccine had significantly lower lung viral titers upon H7N9 ($P < 0.001$) and H7N7 ($P < 0.001$) challenge when compared to mice vaccinated with wt-Bac or inactive BacHA. However, mice vaccinated subcutaneously with adjuvanted inactive whole RG-H7N9 viral vaccine did not have any significant difference in the lung viral titer against H7N7 challenge when compared with wt-Bac group (Fig. 7). Additionally, the histopathology studies were performed for the mice vaccinated and challenged with H7N9 and H7N7 subtype viruses. On day 4 post infection, lungs of negative control (wt-Bac) had pulmonary lesions consisting of severe necrotizing bronchitis and histiocytic alveolitis with associated pulmonary edema (Fig. 8G and H). Mice i.n immunized with live BacHA (H7N9) had only minimal bronchitis and an absence of lesions against H7N9 and H7N7 subtypes (Fig. 8A and B) compared to mice immunized subcutaneously with adjuvanted BacHA, which showed minimal lesions (Fig. 8C and D). Mice immunized subcutaneously with adjuvanted inactive RG-H7N9 vaccine had minimal bronchitis against H7N9 viral challenge (Fig. 8E) and showed moderate to severe bronchitis and alveolitis against H7N7 subtype (Fig. 8F). Overall challenge studies revealed that mice intranasally immunized with live BacHA of either H7N7 or H7N9 showed complete protection against both H7N9 and H7N7 subtypes.

Table 2
Virus microneutralizing antibody titers (VMN) of BacHA of H7N9 and BacHA of H7N7 vaccines.

Route of immunization	Vaccine	VMN GMT titer against 100 TCID$_{50}$ of H7N9, H7N7 and H7N3		
		RG-H7N9	RG-H7N7 (NL03)	RG-H7N3
Subcutaneous	Live BacHA of H7N9	403 ± 67.4	254 ± 33.7	241 ± 32.1
	Live BacHA of H7N7	40 ± 0	45 ± 20.7	22 ± 10.3
	Inactive BacHA of H7N9 + Adj	718 ± 107	320 ± 64.2	359 ± 80
	Inactive BacHA of H7N7 + Adj	63 ± 20.6	71 ± 32.7	45 ± 16.3
Intranasal	Live BacHA of H7N9	360 ± 53.3	202 ± 33.7	202 ± 33.7
	Live BacHA of H7N7	101 ± 41.3	142 ± 32.7	63 ± 20.7

The results of neutralizing antibody titers were expressed as geometric mean titers (GMT) ± SD.

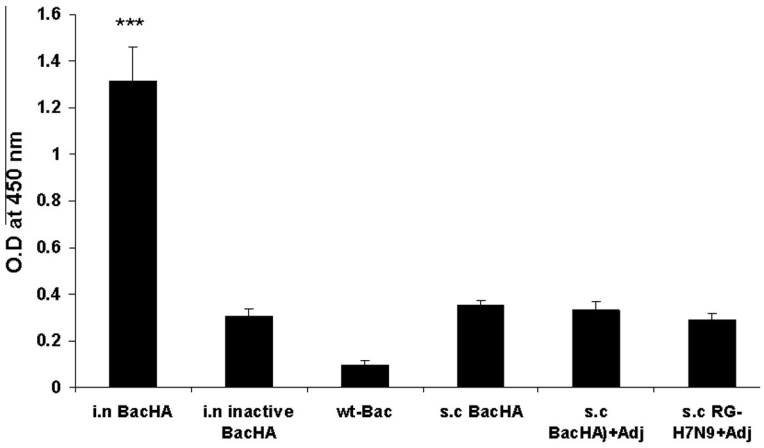

Fig. 3. Measurement of mucosal anti-HA specific IgA antibody levels by indirect ELISA on day 42. Groups of mice ($n = 4$) were intranasally (i.n) or subcutaneously (s.c) vaccinated with live or inactive BacHA (H7N9) or subcutaneously immunized with adjuvanted inactive BacHA (H7N9) or inactive RG-H7N9 whole viral vaccine on day 0 and 28. Each point represents the arithmetic mean value ($n = 4$) ± SD (***$P < 0.001$).

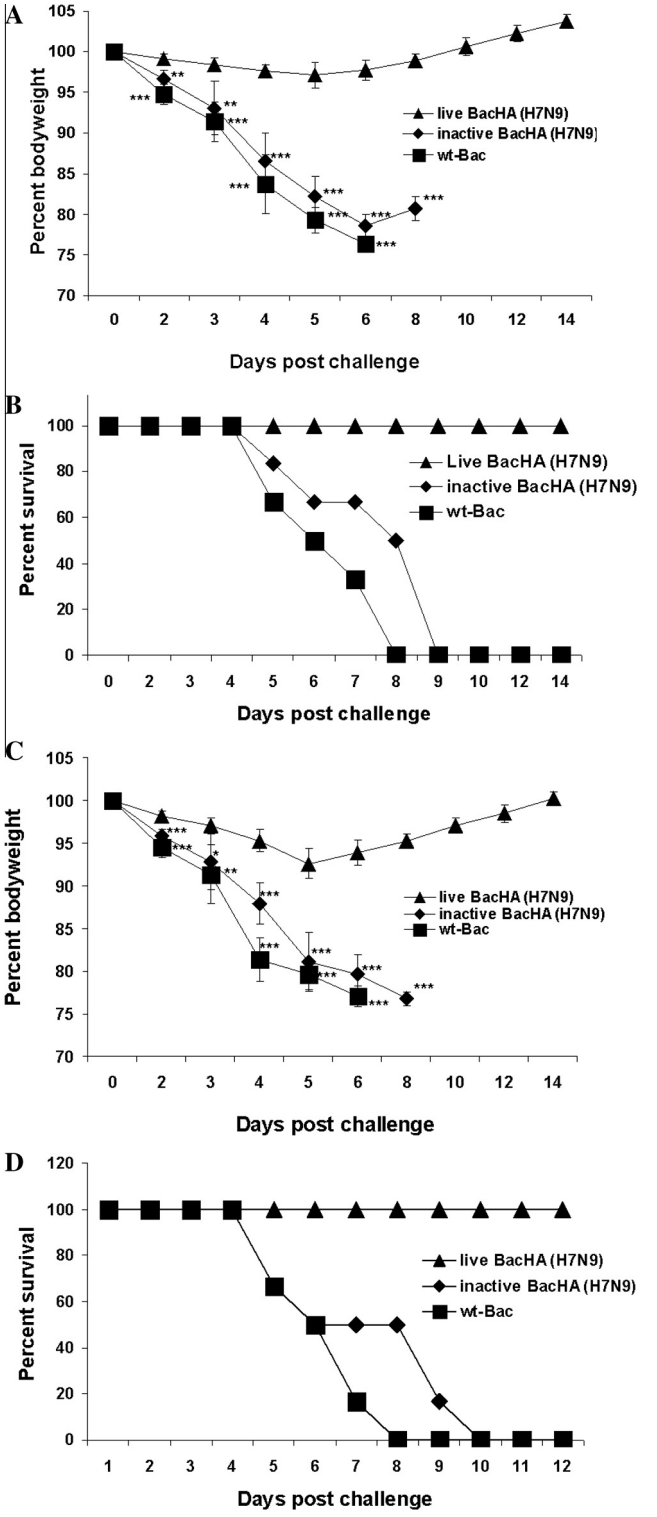

Fig. 4. Protection of i.n immunized mice against lethal H7N9 or H7N7 viral challenge. Each group of mice ($n = 8$) was intranasally (i.n) vaccinated with live or inactive BacHA (H7N9) on day 0 and 28. The negative control group was intranasally immunized with wild type baculovirus (wt-Bac). Three weeks after the second vaccination, mice were intranasally infected with 5 MLD_{50} of RG-H7N9 (A/Shanghai/2/2013) or RG-H7N7 (NL03: A/Netherlands/219/03) viral strain. Mice ($n = 6$) were monitored for weight loss throughout a 14-day observation period after H7N9 (A) or H7N7 (C) challenge. The results are expressed in terms of percent body weight compared to the beginning of the viral challenge. Each point represents the arithmetic mean value ± SD ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Mice were monitored for survival after H7N9 (B) or H7N7 (D) challenge. Kaplan–Meier curves represent percent survival after infection.

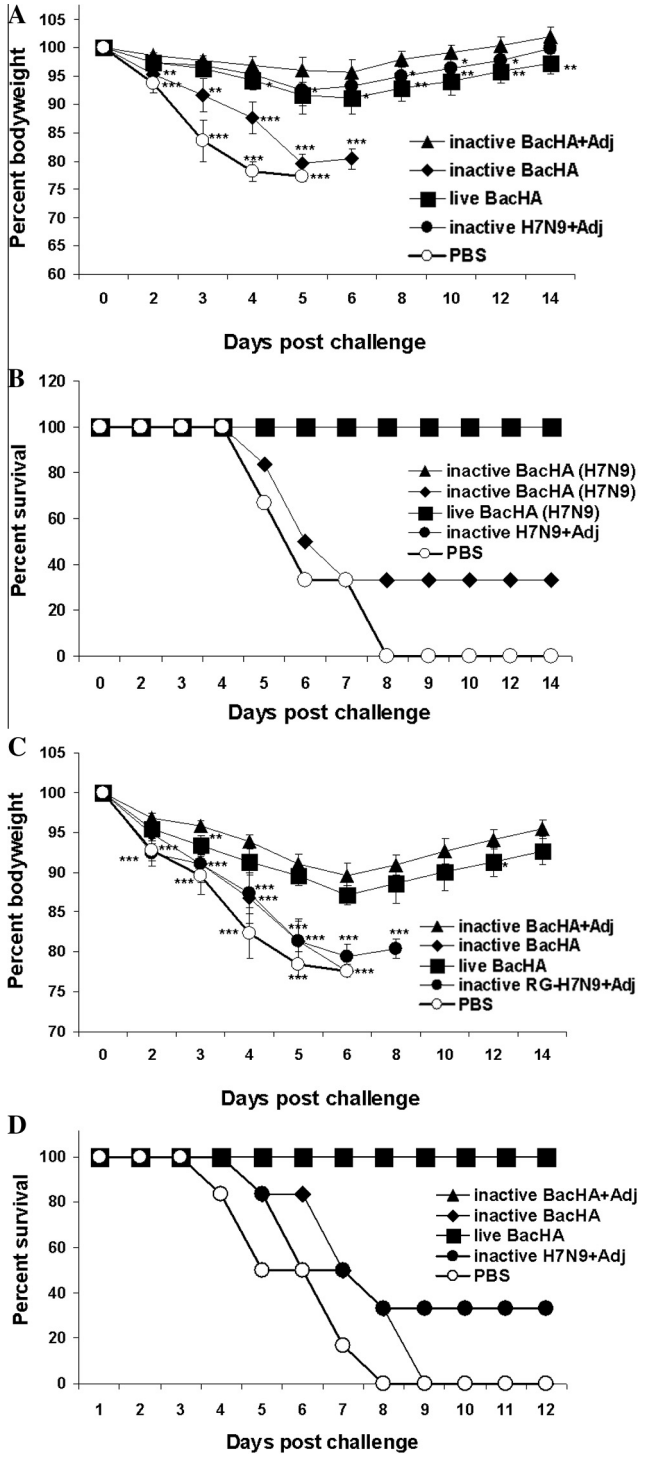

Fig. 5. Protection of s.c immunized mice against lethal H7N9 or H7N7 viral challenge. Each group of mice ($n = 8$) was subcutaneously (s.c) vaccinated with live or inactive BacHA (H7N9) with or without adjuvant. The reference control group was subcutaneously immunized with adjuvanted inactive RG-H7N9 whole viral vaccine on day 0 and 28. Three weeks after the second vaccination, mice were intranasally infected with 5 MLD_{50} of RG-H7N9 (A/Shanghai/2/2013) or RG-H7N7 (NL03: A/Netherlands/219/03) viral strain. Mice ($n = 6$) were monitored for weight loss throughout a 14-day observation period after H7N9 (A) or H7N7 (C) challenge. The results are expressed in terms of percent body weight compared to the beginning of the viral challenge. Each point represents the arithmetic mean value ± SD ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Mice were monitored for survival after H7N9 (B) or H7N7 (D) challenge. Kaplan–Meier curves represent percent survival after infection.

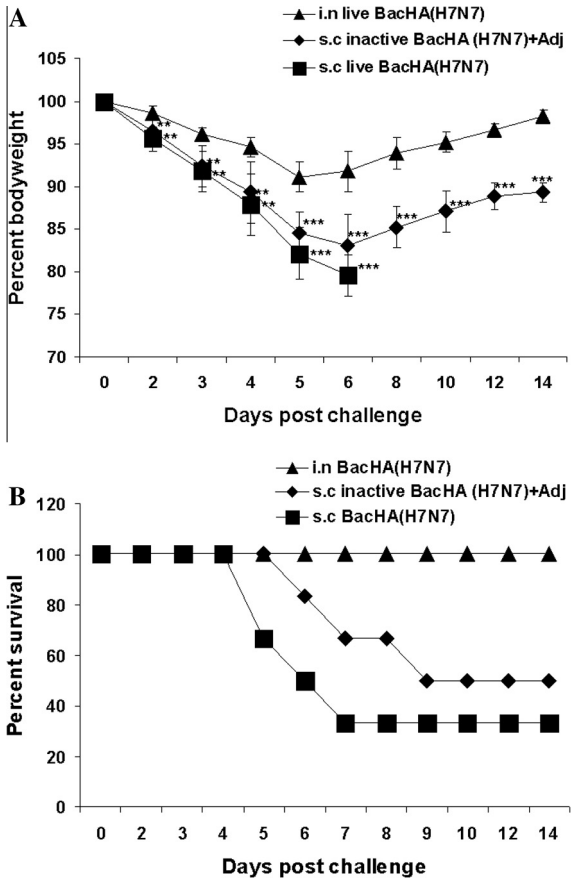

Fig. 6. Protective efficacy of BacHA of H7N7 against H7N9 challenge. Each group of mice ($n = 8$) was intranasally (i.n) or subcutaneously (s.c) vaccinated with live or inactive BacHA (H7N9) or subcutaneously immunized with adjuvanted inactive BacHA (H7N7) on day 0 and 28. Three weeks after the second vaccination, mice were intranasally infected with 5 MLD_{50} of RG-H7N9 (A/Shanghai/2/2013) viral strain. Mice ($n = 6$) were monitored for weight loss throughout a 14-day observation period (A). The results are expressed in terms of percent body weight compared to the beginning of the viral challenge. Each point represents the arithmetic mean value ± SD ($^{**}P < 0.01$, $^{***}P < 0.001$). Mice were monitored for survival after H7N9 challenge (B). Kaplan–Meier curve represents percent survival after infection.

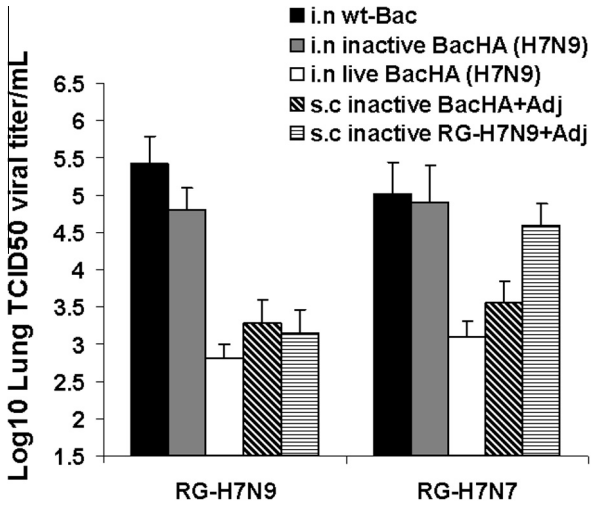

Fig. 7. Measurement of viral infectivity titers in the lungs of vaccinated mice experimentally infected with RG-H7N9 (A/Shanghai/2/2013) or RG-H7N7 (NL03: A/Netherlands/219/03) subtype. The viral loads were measured in the lungs of the infected animals on day 4 post challenge. The results are expressed in terms of mean value of log $TCID_{50}$/mL ± standard deviation (S.D). The lower limit of detection was 1.5 $\log_{10}$ $TCID_{50}$/mL.

4. Discussion

The emerging epidemic of H5N1 in Asia and the recent emergence of avian-origin H7N9 influenza in humans in Eastern China highlight the need for safe and effective vaccines. However, most of the H7 vaccine studies reported that vaccine induces inadequate immunogenicity in humans (Cox et al., 2009; Couch et al., 2012) and also in animal models (de Wit et al., 2005; Rajesh Kumar et al., 2013). Furthermore, most of the currently licensed inactivated influenza vaccine requires high-biocontainment facility, expensive downstream processes and stable supply of specific-pathogen-free fertile eggs. Hence the generation of effective cross-protective vaccines against H7 subtypes is highly desirable. In this present study, we have expressed HA of H7N9 on the baculovirus under the control of an immediate early promoter 1 (ie1) of white spot syndrome virus (WSSV) by using baculovirus expression system.

The baculovirus system has already been shown to display several immunogenic proteins in their native functional forms and have been used as vaccine against several diseases including influenza subtypes (Madhan et al., 2010; Prabakaran et al., 2011; Rajesh Kumar et al., 2013). Also, the HA expressed on the baculovirus envelope showed hemagglutination activity and its hemagglutination activity was inhibited by neutralizing antibodies against conformational epitopes of HA1. This confirmed that HA0 displayed on the baculovirus envelope retained its native antigenic structure.

Further, the immunogenicity and cross-protective efficacy of baculovirus displayed HA of H7N9 was evaluated in a mouse model. The results showed that intranasal immunization of live BacHA (H7N9) elicits comparable levels of serum humoral immune responses with subcutaneous immunization of live BacHA (H7N9) in mice. Furthermore, mice immunized subcutaneously with adjuvanted inactive BacHA (H7N9) stimulated robust serum humoral immune responses and enhanced higher serum neutralizing antibody titer against 100 $TCID_{50}$ of H7N9 and other H7 subtypes (H7N7 and H7N3) compared to adjuvanted with inactive whole RG-H7N9 or intranasal immunization of live BacHA. In contrast, intranasal immunization of live BacHA (H7N9) induced high level of HA-specific mucosal immune responses which provide an important first line of defense against influenza infection. Also, mice immunized intranasally with inactivated BacHA were able to induce only low-level of specific humoral and mucosal immune responses compared to live BacHA. This might be due to inability to transduce the HA gene into the host tissue by inactive baculovirus due to the degradation of its genome caused by inactivation with BEI (Prabakaran et al., 2010; Bahnemann, 1990). Also, inactivation of baculovirus strongly abolished their adjuvant properties resulted in a poor immune responses (Hervas-Stubbs et al., 2007).

Previously, Cox et al. (2009) reported that parenteral immunization of inactivated H7N3 and H7N1 subunit vaccines induced only low HAI and microneutralization titers in humans even at high doses with adjuvant. Similarly, our previous study also showed that intranasal or subcutaneous immunization of same amount of H7N7 HA (NL03) displayed on the baculovirus induced only very low level of HAI titer and serum neutralizing antibody responses in mice (Rajesh Kumar et al., 2013). The differences between the immunogenicity of HA of H7N9 and HA of H7N7 (NL03) might be due to amino acid difference at A143T that introduced a potential glycosylation site at position 141N of HA which masking the epitopes of H7HA (NL03) (Munster et al., 2007; Wang et al., 2010b). However, further studies are warranted to understand why H7N7 (NL03) or some of the H7 subtype vaccines at high dosage was so poorly immunogenic in humans or animals.

Further, the protective efficacy of the vaccine was evaluated by challenging the vaccinated mice with H7N9 or H7N7 subtype. The

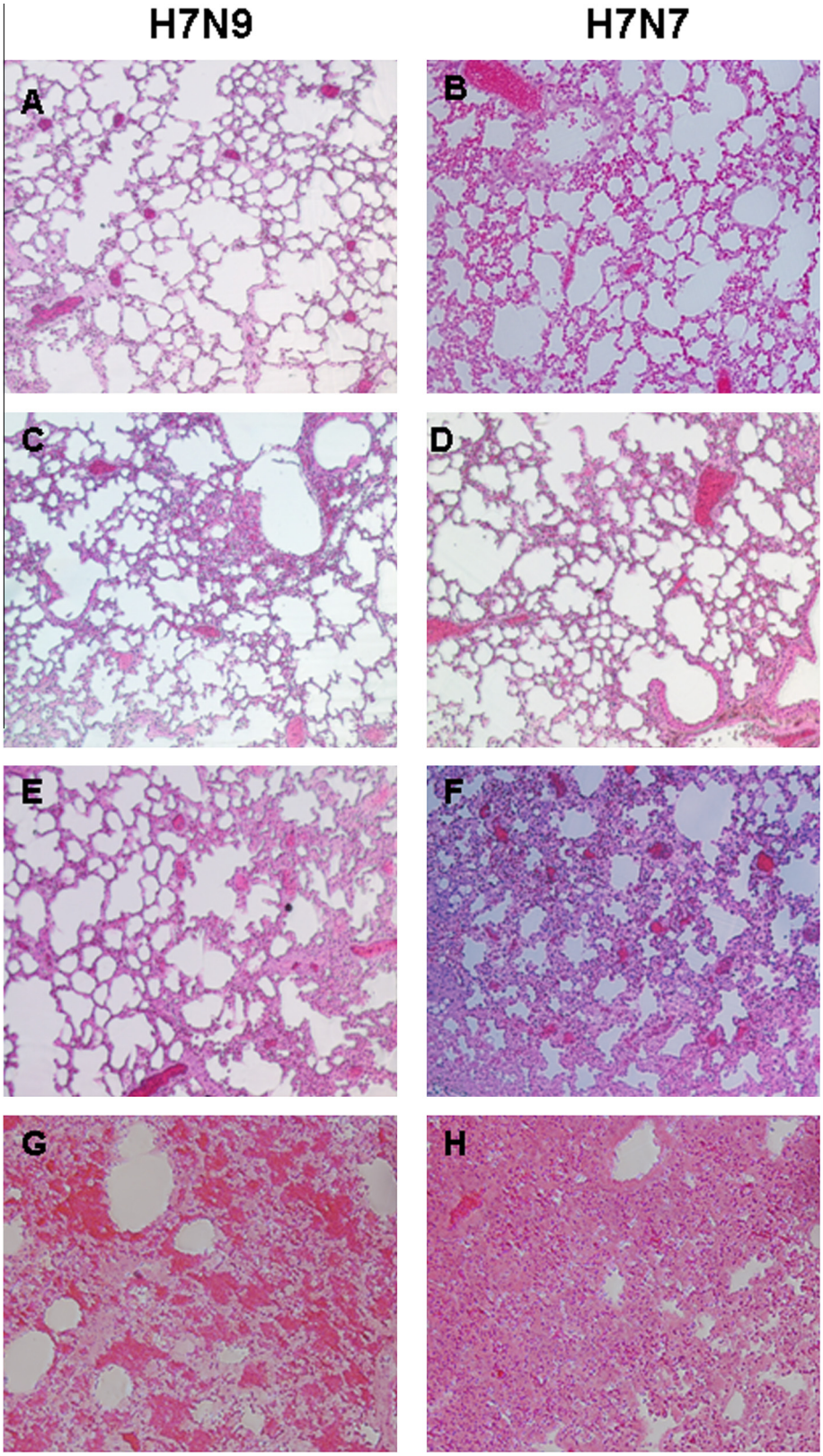

Fig. 8. Histopathology of lung tissue in vaccinated mice: Photomicrographs of hematoxylin and eosin-stained lung sections of mice 4 days after challenge with H7N9 and H7N7 subtypes. (A and B) Mice vaccinated intranasally with BacHA (H7N9) and challenged with H7N9 (A) and H7N7 (B) subtypes. (C and D) Mice vaccinated with adjuvanted inactive BacHA (H7N9) and challenged with H7N9 (C) and H7N7 (D) subtypes. (E and F) Mice vaccinated with adjuvanted inactive RG-H7N9 vaccine and challenged with H7N9 (E) and H7N7 (F) subtypes. (G and H) Mice vaccinated intranasally with wt-Bac and challenged with H7N9 (G) and H7N7 (H) subtypes.

results demonstrated that mice immunized intranasally with live BacHA (H7N9) or subcutaneously with either live BacHA (H7N9) or inactive BacHA (H7N9) with adjuvant obtained complete protection against 5 MLD_{50} of RG-H7N9 and RG-H7N7 influenza subtypes. However, subcutaneous immunization with inactive whole H7N9 vaccine provided complete protection against only

RG-H7N9 challenge and showed insufficient protection against RG-H7N7 (NL03) subtype. Mice subcutaneously immunized with live and inactive baculovirus displayed HA of H7N7 (NL03) showed only 33% and 50% protection against H7N9 challenge, respectively. Interestingly, mice immunized intranasally with live BacHA (H7N7) were protected against H7N9 infection. Previously, we tested the protective efficacy of BacHA (H7N7) vaccine against a lethal H7N7 virus in mice (Rajesh Kumar et al., 2013). The results showed that mice vaccinated intranasally with BacHA (H7N7) provided complete protection against H7N7 influenza challenge whereas incomplete protection was reported in mice vaccinated by the subcutaneous route (Rajesh Kumar et al., 2013). Hence, the induction of mucosal immunity plays an important role in recovery and protection during H7 subtype infection, especially when protective antibody titers are low.

Overall study revealed that intranasal immunization with live baculovirus displayed HA of either H7N9 or H7N7 vaccine induces antigen specific humoral and mucosal immunity, and provides protection against antigenically related H7N9 and H7N7 subtypes. However, further investigations are needed to evaluate the broadly protective efficacy of BacHA vaccine against antigenically distinct H7 subtypes. The level of immune responses and protective immunity obtained with the subcutaneous immunization of adjuvanted inactive baculovirus was comparable with live baculovirus, suggesting the potential of baculovirus as live and inactivated vaccine in humans. However, the limitation of inactive baculovirus vaccination requires adjuvant to increase their efficacy. Live baculovirus-based delivery system is advantageous over other human viral vectors due to its low cytotoxicity, inability to replicate in mammalian cells and absence of preexisting immunity in humans (Strauss et al., 2007; Madhan et al., 2010). However, a concern is that repeated annual vaccinations of baculovirus displayed or vectored vaccine in humans may induce vector-specific immune responses that interfere with the elicitation of vigorous immune responses to the desired antigen. Interference of anti-vector immunity during booster or annual immunization in humans needs further investigation.

Recently, a recombinant hemagglutinin (rHA) seasonal influenza vaccine Flublok® produced in insect cells using baculovirus expression system has been approved by the US FDA for use in adults aged 18–49 years. This approval represents a technical advance in the manufacturing of an influenza vaccine. However, recombinant baculovirus displayed vaccine provides a number of advantages over insect cells expression system based subunit vaccine such as intrinsic adjuvant properties and efficient vaccine delivery (Abe et al., 2009; Suzuki et al., 2010). Also, several previous reports revealed that baculoviruses are safe to animals and humans (Burges et al., 1980; Jin et al., 2008), however environmental and ecological impacts of the baculovirus in invertebrates will be minimized by modification of baculovirus genes required for replication and budding.

In conclusion, baculovirus displayed HA of H7N9 exhibited robust humoral and mucosal immune responses, which provide protection against influenza H7N9 and H7N7 subtypes. Also, intranasal vaccination is simple, needle-free and affordable with improved logistics, thereby enabling mass vaccination coverage during pandemic or pre-pandemic situation.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgements

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