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# A highly immunogenic vaccine against A/H7N9 influenza virus

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ABSTRACT

Since the first case of human infection in March 2013, continued reports of H7N9 cases highlight a potential pandemic threat. Highly immunogenic vaccines to this virus are urgently needed to protect vulnerable populations who lack protective immunity. In this study, an egg- and adjuvant-independent adenoviral vector-based, hemagglutinin H7 subtype influenza vaccine (HAd-H7HA) demonstrated enhanced cell-mediated immunity as well as serum antibody responses in a mouse model. Most importantly, this vaccine provided complete protection against homologous A/H7N9 viral challenge suggesting its potential utility as a pandemic vaccine.

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## 1. Introduction

Until the first case reported in China on March 2013, H7N9 influenza infections were confined primarily to avian species [1]. As of March 2015, however, there have been a total of 631 human infections. Many patients have had severe respiratory illness with a 35% case fatality rate [2]. Most of these infections are believed to result from the exposure to infected poultry or contaminated environment. Although direct human-to-human transmission is very limited, the continued circulation of the H7N9 virus poses a potential pandemic threat to the human population. Although prior infection or vaccination with A/H3N2 influenza A strains induce H7 cross-reactive antibodies [3], they are less frequent as only 6 monoclonal antibodies (mAb) of a total of 83 mAb isolated from 28 individuals showed reactivity against H7N9 virus. Furthermore, only 3 of these 6 mAb demonstrated virus neutralizing activity against A/H7N9 virus both *in vitro* and *in vivo* in mice. Hence, the majority of the human population still lacks protective immunity against H7N9 virus. Although vaccination is the most cost-effective intervention strategy, several independent studies have shown that H7N9 vaccines are poorly immunogenic [4,5]. Furthermore, the circulation of H5N2 and H5N8 viruses in the United States is threatening the supply of embryonated chicken eggs, the substrate for current egg-derived inactivated and live attenuated influenza vaccines production [6]. Hence, an egg-independent vaccine production technology is beneficial for pandemic preparedness. In our earlier studies, we developed a replication-incompetent human adenoviral (HAd) vector-based, adjuvant-, and egg-independent pandemic influenza vaccine strategy and demonstrated that an HAd vaccine expressing the gene encoding hemagglutinin (HA) from A/Hong Kong/156/97 H5N1 viruses conferred long-lasting immunity and cross-protection in mice against challenge with more-recent strains of highly pathogenic H5N1 viruses [7,8]. Therefore, in this study, we explored the potential utility of an

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Adenoviral vector-based delivery system expressing H7HA from A/Anhui/1/2013 influenza virus and assessed its immunogenicity and efficacy to confer protection in BALB/c mice against a homologous challenge compared to a recombinant H7HA vaccine.

## 2. Materials and methods

### 2.1. Cell culture and vector purification

293, 293Cre and bovine-human hybrid (BHH2C) cell lines were grown in minimum essential medium (MEM) containing 10% FetalClone III (Thermo Fisher Scientific Inc., Waltham, MA) and gentamicin (50 μg/ml). HAd vector purification was done by cesium chloride density gradient centrifugation and virus titration was done in BHH2C by plaque assay.

### 2.2. Generation and characterization of replication deficient HAd-H7HA vector

A Cre-recombinase-mediated site-specific recombination technique [9] was used to insert the full-length coding region of the HA gene of the A/Anhui/1/2013 (A/H7N9) influenza virus under the control of the cytomegalovirus (CMV) promoter and bovine growth hormone (BGH) polyadenylation signal (polyA). An HAd gene with deletions of the early regions E1 and E3 (HAd-ΔE1E3) served as a negative control [10]. The recombinant virus was plaque purified, and its genome was analyzed to confirm the presence of the HA gene cassette without any other major deletion or insertion. 293 cells were mock-infected or infected with an empty vector (HAd-ΔE1E3) or HAd-H7HA at a multiplicity of infection (MOI) of 10 plaque-forming units (PFU) per cell. Thirty-six hours (h) post-infection, cells were harvested, and cell lysates were examined for the expression of H7HA protein using the ferret anti-A/Netherland/219/03 (H7N7)-specific antibody by Western blot as described [11].

### 2.3. Animal immunization, immunogenicity and viral challenges

Six to eight week old BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were anesthetized with Avertin (2,2,2-tribromoethano; Sigma) by intraperitoneal (i.p.) injection and immunized (5 animals/group) with HAd-H7HA or HAd-ΔE1E3 intranasally (i.n.). As controls, mice were immunized by the intramuscular (i.m.) route with 3 μg of the recombinant H7HA (rH7HA) from A/Shanghai/2/2013 (SH2) which has an identical HA amino acid sequence to AH1 or PBS using 50 μl in each thigh. Four weeks later, mice were boosted with the same immunization regimen. Sera were obtained three weeks post-primary and again three weeks post-boost to determine antibody responses. Mice were challenged with 50× lethal dose 50% ($LD_{50}$) of wild type H7N9 virus (AH1) and monitored for weight loss and mortality. Animal research was conducted under the guidance of the CDC's Institutional Animal Care and Use Committee in an Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) International-accredited animal facility. Mice that lost >20% of their pre-infection body weight were euthanatized.

### 2.4. Cell-mediated immune responses

Single cell suspensions were prepared from the lung, spleen, lymph node and bone marrow tissues one week post-booster immunization. To detect intracellular cytokine production by cells from the lung, spleen and lymph node, $1 \times 10^6$ cells were stimulated *in vitro* with HA peptide (5 μg/ml) or A/Shanghai/2/2013(H7N9)-PR8 reverse genetic virus (SH2/PR8) virus (MOI = 1) overnight with GolgiPlug™ (BD Bioscience, San Jose, CA) added during the last 6 h of incubation. Cells were surface stained with anti-CD44 antibody and with either anti-CD4 or anti-CD8 antibody (BD Bioscience), followed by intracellular staining with anti-IFN-γ, anti-IL-2 or anti-TNF-α antibodies (BD Bioscience). Samples were analyzed using LSRII Flow cytometer (BD Biosciences), and the cytometric data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

The percentage of H7N9 virus or HA-specific Antibody-Producing Cells (ASCs) in the spleen or bone marrow was detected by ELISPOT assay. Briefly, $1 \times 10^6$ cells were added onto antigen-coated plates and incubated overnight at 37 °C in a humidified atmosphere with 5% $CO_2$. The plates were incubated with biotinylated anti-mouse IgG (Southern Biotech, Birmingham, AL) followed by alkaline phosphatase-conjugated streptavidin and developed with Vector Blue alkaline phosphatase substrate kit III (Vector Laboratories, Burlingame, CA). Spot forming units were counted using ImmunoSpot® (Cellular Technology Ltd., Shaker Heights, OH) and expressed as the number of antigen-specific IgG or IgA secreting B cells/$10^6$ cells.

### 2.5. Serum HAI assay

Sera from all mice were subjected to overnight treatment with receptor-destroying enzyme from *Vibrio Cholerae* (Denka Seiken, Tokyo, Japan) at 37 °C to destroy non-specific serum inhibitor activity. Serial dilutions of RDE-treated sera were mixed with 4 hemagglutination units of SH2/PR8 virus for 60 min, followed by addition of 50 μl 1% horse RBC. The highest serum dilution inhibiting hemagglutination was taken as the HAI titer [12].

### 2.6. Serum microneutralization assay

RDE-treated sera were serially diluted in 96-well plates and incubated with SH2/PR8 viruses at a dose of $2 \times 10^3$ $TCID_{50}$/ml for 2 h at 37 °C. MDCK cells were added and incubated overnight. Cells were then fixed with 80% acetone and incubated with biotinylated anti-nucleoprotein Ab (EMD Millipore, Billerica, MA), followed by streptavidin-HRP (Southern Biotech, Birmingham, AL). Bound HRP was visualized using 1× TMB substrate solution (eBioscience, San Diego, CA) and the developed color was assessed using a microplate reader. The highest serum dilution that generated >50% specific signal was considered to be the neutralization titer; 50% specific signal = ($OD_{450}$ virus control − $OD_{450}$ cell control)/2 + $OD_{450}$ cell control.

### 2.7. ELISA

ELISA was performed to detect H7HA- or A/H7N9 virus-specific IgG antibody levels in the sera. Briefly, Immunol plates (Thermo Fisher Scientific, Waltham, MA) were coated overnight with 1 μg/ml H7HA or 50 HAU SH2/PR8 virus at 4 °C and then blocked for 1 h with PBS/0.05%Tween-20 (PBST) containing 4% BSA at room temperature. Sera were serially titrated 4 fold in PBST and incubated with antigen-coated plates for 2 h at room temperature. After washing with PBST, wells were probed with HRP-anti-mouse IgG for 1 h at room temperature. Signal was developed using 1× TMB substrate solution (eBioscience) and the developed color was assessed using the microplate reader.

### 2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA). Groups were compared by one-way ANOVA followed by Tukey's multiple comparison test.

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