



# Vaccine protection of chickens against antigenically diverse H5 highly pathogenic avian influenza isolates with a live HVT vector vaccine expressing the influenza hemagglutinin gene derived from a clade 2.2 avian influenza virus



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## ABSTRACT

Vaccination is an important tool in the protection of poultry against avian influenza (AI). For field use, the overwhelming majority of AI vaccines produced are inactivated whole virus formulated into an oil emulsion. However, recombinant vectored vaccines are gaining use for their ability to induce protection against heterologous isolates and ability to overcome maternal antibody interference. In these studies, we compared protection of chickens provided by a turkey herpesvirus (HVT) vector vaccine expressing the hemagglutinin (HA) gene from a clade 2.2 H5N1 strain (A/swan/Hungary/4999/2006) against homologous H5N1 as well as heterologous H5N1 and H5N2 highly pathogenic (HP) AI challenge. The results demonstrated all vaccinated birds were protected from clinical signs of disease and mortality following homologous challenge. In addition, oral and cloacal swabs taken from challenged birds demonstrated that vaccinated birds had lower incidence and titers of viral shedding compared to sham-vaccinated birds. Following heterologous H5N1 or H5N2 HPAI challenge, 80–95% of birds receiving the HVT vector AI vaccine at day of age survived challenge with fewer birds shedding virus after challenge than sham vaccinated birds. In vitro cytotoxicity analysis demonstrated that splenic T lymphocytes from HVT-vector-AI vaccinated chickens recognized MHC-matched target cells infected with H5, as well as H6, H7, or H9 AI virus. Taken together, these studies provide support for the use of HVT vector vaccines expressing HA to protect poultry against multiple lineages of HPAI, and that both humoral and cellular immunity induced by live vaccines likely contributes to protection.

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## 1. Introduction

Since 1990s, there has been a significant increase in the number of highly pathogenic (HP) avian influenza (AI) outbreaks and also in the number of birds involved in those outbreaks [1,2]. The most notable of these recent HPAI outbreaks is a series of H5N1 outbreaks started in China and Hong Kong since 1996, which then spread to

other parts of the world including South-East Asia, the Middle East, Europe and Africa. These H5N1 viruses have become endemic in several countries including China, Indonesia, Viet Nam and Egypt [3]. Since 1996, H5N1 viruses underwent significant antigenic drift and the viruses have been classified into various clades according to phylogenetic topology based on hemagglutinin (HA) gene sequences according to WHO/OIE/FAO H5N1 Evolution Working Group [4].

Vaccination has been considered a suitable and powerful tool to support AI eradication or control programs in the endemically infected countries in combination with other control measures such as good biosecurity and monitoring programs [5–7]. When used properly, vaccination has been shown to protect poultry

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against clinical signs and death and markedly reduce virus shedding in vaccinated birds, thereby reducing transmission of virus [8]. For example, the number of H5N1 outbreaks in Viet Nam dropped from 2388 in 2003 to 45 in 2011 due to increased biosecurity, depopulation and vaccine implementation [3]. It is known that antigenic differences between vaccines and field viruses affect vaccine efficacy [9–11].

Immunity against AI is largely based on the induction of neutralizing antibodies produced against the HA, although cytotoxic T lymphocytes (CTL's) have been reported as critical for clearance of virus. Antibody production against a particular virus typically will only protect against homologous or antigenically matched isolates [2,12–14]. At least one study has demonstrated cross reactive AIV CTL's in chickens against heterosubtypic AIV [15]. More recently, we have demonstrated broad cross reactive cellular immunity in birds infected with low pathogenic (LP) AI against different subtype of AIV [16].

Here, we tested a commercially available turkey herpesvirus (HVT) vector AI vaccine expressing the HA gene from a recent clade 2.2 H5N1 virus to protect chickens against lethal HPAI challenge. The efficacy of this vaccine was tested against various HPAI viruses including H5N1 viruses belonging to different clades and a Mexican-lineage H5N2 virus.

## 2. Materials and methods

### 2.1. Viral strains and culture

Three HPAI strains were used in the vaccine-challenge studies: A/Whooper Swan/Mongolia/3/2005 H5N1 (SM05), A/chicken/West Java Sbg/29/2007 H5N1 (CW07) and A/chicken/Queretaro/14588-19/95 H5N2 (CQ95) (Supplemental Table 1). Four LPAI isolates were used for determination of cellular immunity in HVT-vector-AI vaccinated birds to different subtypes of AI including, A/turkey/Wisconsin/68 H5N9, A/chicken/CA/203/03 H6N2, A/turkey/VA/4259/02 H7N2 and A/chicken/NJ/12220/97 H9N2. Viruses were propagated and titrated in 9–11 days of embryonation, specific pathogen free (SPF) chicken eggs according to standard procedure [17].

### 2.2. Vaccines

A HVT vector AI vaccine, Vectormune® AI (CEVA Animal Health, Lenexa, KS) was constructed by inserting the HA gene of the HPAI A/Swan/Hungary/4999/2006 (SH06) H5N1 clade 2.2 strain into the genome of HVT FC-126 strain, hereby referred to rHVT-Hu4999. The cleavage site of the HA gene was altered to a typical cleavage site sequence of LPAI virus strains. In experiment I, two different forms of HVT were used as the vaccine vector, a frozen, cell-associated (ca) form or a lyophilized, cell-free (cf) form. In experiment III, a HVT vector AI vaccine containing the HA gene from A/turkey/Wisconsin/68 (H5N9) strain (rHVT-Wisc68) was used for comparison. Ceva® Flu-Kem H5N2 vaccine (iH5N2) was produced by Ceva-Mexico, Cuernavaca, and is an oil emulsion inactivated vaccine containing AI virus antigen A/Chicken/Mexico/232/94 strain (H5N2) (CM94) LPAI virus. Names and gene inserts of these vaccines are summarized in Supplemental Table 2.

### 2.3. Serologic assays

Hemagglutination inhibition (HI) assays were performed according to standard protocol [18]. Homologous HPAI SM05 (H5N1) virus, heterologous HPAI CW07 (H5N1) virus, and heterologous HPAI CQ95 (H5N2) virus were used as antigen.

### 2.4. Statistical analysis

Kaplan–Meier survival curves were generated with Prism 5 (GraphPad Co., San Diego, CA). The Mantel–Cox log-rank test was used to compare survival curves between two experimental groups (Prism 5). Statistical differences in mean and standard error between HI and virus titers were analyzed using Tukey one-way ANOVA (Prism 5). The Fisher Exact test was used for pair-wise comparison on frequency of virus isolation between groups (SigmaStat 2.0.3, SPSS Inc., Chicago, IL). Lower case letters indicate statistical significance between compared groups. All statistical tests were performed using  $P < 0.05$ .

### 2.5. Animal challenge experiments

For experiments I and III, mixed-sex SPF, White Leghorn (WL) chickens were obtained from Charles River Laboratories (Wilmington, MA). For experiment II, mixed-sex SPF broiler (White Rock) chickens from Southeast Poultry Research Laboratory were used. Chickens were housed in a BSL2 facility at the University of Georgia, Poultry Diagnostic Research Center, Athens, Georgia for vaccination and grow out. Chickens were transferred to a BSL3 enhanced facility at Southeast Poultry Research Laboratory for challenge. Birds were maintained in Horsfall isolation units with feed and water ad libitum.

Serum samples were taken via wing vein prior to vaccination, prior to challenge and 14 days post-challenge (dpc), and were stored at  $-20^{\circ}\text{C}$ . Oro-pharyngeal and cloacal swab samples were collected on days 2 and 4 post-challenge (experiments I and II) or days 3 and 6 post-challenge (experiment III) for virus isolation. Swab samples were placed in 2.0 ml BHI broth as previously described [2]. Following challenge, groups were monitored twice daily for 14 days for clinical signs, and those with severe clinical signs disease were humanely euthanized by approved protocol and counted as mortalities for that day.

#### 2.5.1. Experiment I – clinical protection of vaccinated SPF chickens against homologous H5N1 challenge

Day of age SPF WL chicks were divided into three groups. The first group was vaccinated with the cell-associated form of rHVT-Hu4999 (1500 pfu) and the second group received the cell-free form of rHVT-Hu4999 (1500 pfu). The last group was inoculated with only vaccine diluent and served as a challenge control. At 6 weeks of age, chickens were challenged intranasally with HPAI SM05 H5N1 clade 2.2 virus at  $10^6$  mean embryo infectious dose ( $\text{EID}_{50}$ ) per chicken. Thirty chickens in the cell-associated rHVT-Hu4999 group, 20 chickens in the cell-free rHVT-Hu4999 group and 10 chickens in the challenge control group were challenged.

#### 2.5.2. Experiment II – clinical protection against heterologous H5N1 HPAI of Indonesian origin

Day of age SPF broiler (white rock) chicks were divided into four groups. The first group was vaccinated with rHVT-Hu4999 ca (2000 pfu) at day of age. The second group was vaccinated with rHVT-Hu4999 ca (2000 pfu) at day of age and then with iH5N2 at 10 days of age. The third group received only iH5N2 vaccine at 10 days of age. The last group received PBS and was used as a challenge control. At 4 weeks of age, chickens were challenged intranasally with HPAI CW07 H5N1 clade 2.1.3 virus at  $10^6$   $\text{EID}_{50}$  per chicken.

#### 2.5.3. Experiment III – clinical protection of vaccinated SPF chickens after heterologous H5N2 HPAI of Mexican origin

In this study, SPF WL chickens were divided into three groups. The first group received rHVT-Hu4999 ca (1500 pfu), the second group received rHVT-Wisc68 ca (1500 pfu), and the third group was

inoculated with only vaccine diluent and served as a challenge control. At 4 weeks of age, chickens were challenged intranasally with HPAI CQ95 H5N2 virus at  $10^6$  EID<sub>50</sub> per chicken. Twenty chickens in the vaccinated groups and 10 chickens in the challenge control group were challenged.

### 2.6. Cytotoxic T-lymphocyte (CTL) lysis of avian influenza infected lung-cell cultures

Major histocompatibility complex (MHC)-defined B<sub>2</sub>B<sub>2</sub> SPF chickens (gift from Avian Disease Oncology Laboratory, ARS, East Lansing, MI) received 1500 pfu rHVT-Hu4999 at 1 day of age. At 4 weeks of age, splenic lymphocytes were purified over Ficoll–Hypaque. Lung cell cultures from 2-week-old B<sub>2</sub>B<sub>2</sub> SPF birds were obtained as previously described and used as target cells for CTL assay [15]. Lung cells were infected with either H5N9, H6N2, H7N2 or H9N2 LPAI at an MOI of 2 for 16 h prior to testing. Various ratios of lymphocytes were added and centrifuged onto target cells. CTL activity was monitored using the CytoTox96 nonradioactive assay (Promega), as previously described [15].

### 2.7. HA sequence analysis

Avian influenza HA amino acid sequences were obtained from GenBank and Influenza Research Database (<http://www.fludb.org/brc/home.do?decorator=influenza>). Comparison of HA sequence from isolates used in these studies to putative H5<sub>(246–260)</sub> MHC class I and II T cell epitope identified in A/turkey/Ireland/1378/83 (H5N8) were performed using Jotun Hein method with MegAlign (DNA STAR Lasergene 8, Madison, WI) [19].

## 3. Results

### 3.1. Experiment I – comparison of cell-associated or cell-free rHVT-Hu4999 vaccine-induced protection of chickens against matched homologous H5N1 HPAI challenge

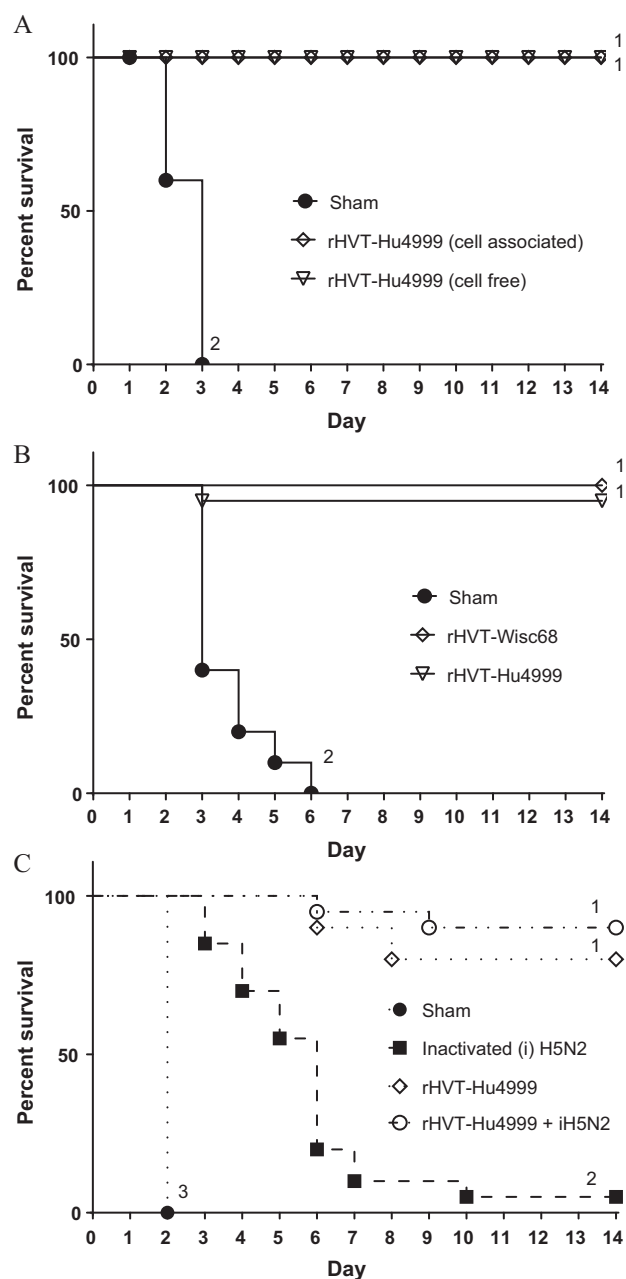
Efficacy of rHVT-Hu4999 vaccine against challenge with homologous H5N1 HPAI virus was evaluated. The HPAI SM05 H5N1 clade 2.2 virus used for challenge has 100% HA gene sequence homology with the vaccine. All chickens vaccinated subcutaneously at day of age with rHVT-Hu4999, survived the homologous challenge at 6 weeks of age, while all of the challenge control chickens died within 3 days (Fig. 1A). No clinical signs were observed in the vaccinated groups.

Elevated HI titers were detected in both of the rHVT-Hu4999 vaccinated groups before challenge with mean titers of  $2^{5.1}$  for the cf group and  $2^{6.3}$  for the ca group (Fig. 2A). After challenge, mean HI titer of the cf group increased to  $2^{6.4}$ , while mean HI titer of the ca group decreased to  $2^{5.6}$  (Fig. 2B).

The rHVT-Hu4999 vaccinated chickens shed little challenge virus at 2 and 4 dpc. From oro-pharyngeal swabs at 2 dpc, virus was isolated from 4/30 (13%) chickens in the ca rHVT-Hu4999 group and 3/20 (15%) chickens in the cf rHVT-Hu4999 group, with minimal virus titers ( $10^1$ – $10^3$  EID<sub>50</sub>/ml), while all the challenge controls shed significant amounts ( $10^5$ – $10^7$  EID<sub>50</sub>/ml) of virus (Fig. 3 and Table 1). No virus was isolated from cloacal swabs of the rHVT-Hu4999 vaccinated chickens at 2 dpc, while all the challenge controls shed virus.

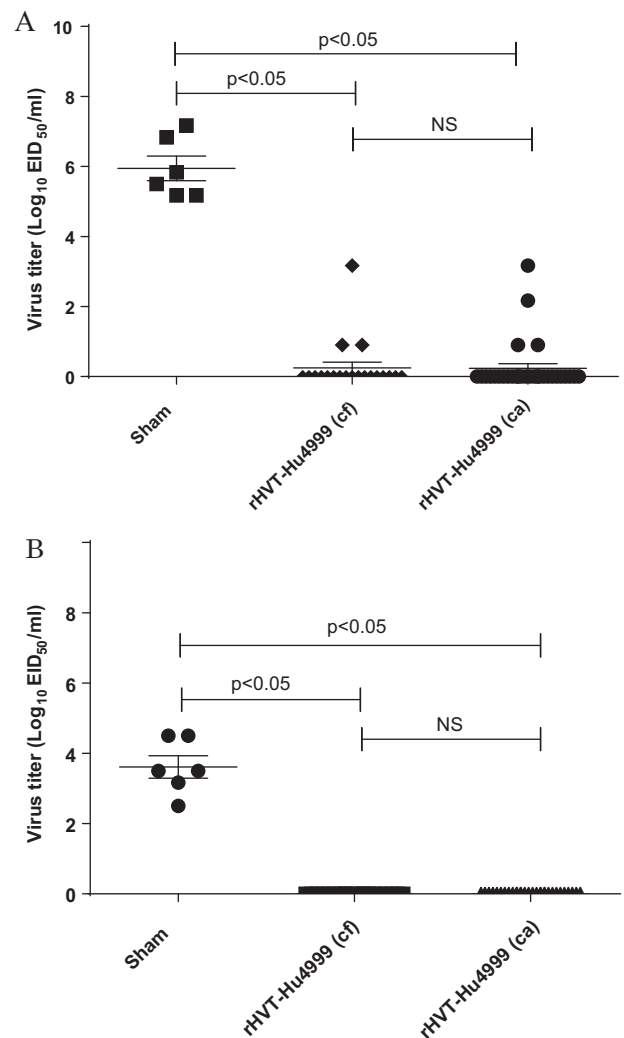
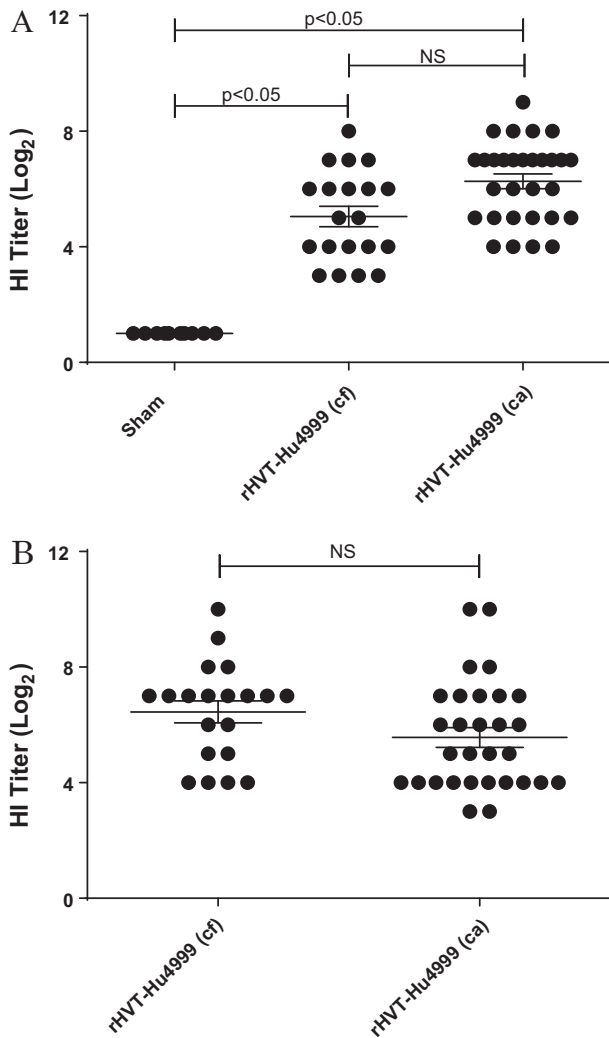
### 3.2. Experiment II – protection following challenge with Indonesian origin H5N1 HPAI

Efficacy of rHVT-Hu4999 vaccine against heterologous CW07 H5N1 clade 2.1.3 virus of Indonesian origin was evaluated in SPF broilers. Sequence similarity of the HA genes between the vaccine



**Fig. 1.** Kaplan–Meier survival plots for protection of vaccinated chickens against homologous or heterologous HPAI H5 subtype challenge. Survival of chickens from unvaccinated (Sham) or vaccinated with rHVT-Hu4999 in cell-associated (ca) or cell-free (cf) form at 1 day-of-age and challenge at 6 weeks with homologous HPAI H5N1 in experiment I (A). Survival of chickens vaccinated with either rHVT-Hu4999 at one day-of-age, inactivated (i) H5N2 (CM94) at 10 days-of-age, or a combination of both. Birds were challenged with heterologous HPAI of Indonesian H5N1 (CW07) (clade 2.1.3) at 4 weeks-of-age in experiment II (B). Survival of chickens following vaccination with rHVT-Hu4999 or rHVT-Wisc68 at day-of-age with heterologous challenge from Mexican lineage H5N2 (CQ94) at 4 weeks-of-age in experiment III (C). Different numbers next to survival lines within an individual graph represent statistical difference in survival curves between the vaccine groups ( $p < 0.05$ ).

insert SH06 and the CW07 virus was 93%, but only 87% and 84%, respectively, to the CM94 isolate in the iH5N2 vaccine. We used broiler chickens in this trial because broilers have been affected greatly by H5N1 HPAI as well as layer chickens. Also, we conducted challenge at 4 weeks of age rather than 6 weeks of age in experiment I, in order to see if the HVT vector AI vaccine provides protective immunity at an earlier time than 6 weeks of age. After the challenge,



**Fig. 2.** Individual HI titers ( $\log_2$ ) and standard error for bird groups in experiment I, (A) 5 weeks post-vaccination (prechallenge) and (B) 2 weeks post-challenge. Statistical significance between mean titers was determined with ANOVA using the Tukey's multiple comparison test ( $p < 0.05$ ). Sham: unvaccinated/HPAI challenged; rHVT-Hu4999 cell associated (ca) vaccinated/HPAI challenged; rHVT-Hu4999 cell free (cf) vaccinated/HPAI challenged. NS = no significant difference.

**Fig. 3.** Viral titers from oral (A) and cloacal (B) on day 2 post-challenge in experiment I. Birds were vaccinated with a single dose rHVT-Hu4999 in cell-associated (ca) or cell-free (cf) form at one day-of-age and challenge at 6 weeks with homologous HPAI H5N1. Viral titers are expressed as  $\log_{10}$  EID<sub>50</sub> per milliliter. The lower limit of detection is 0.9  $\log_{10}$  EID<sub>50</sub> per milliliter. Statistical significance between mean titers was determined with ANOVA using the Tukey's multiple comparison test ( $p < 0.05$ ). NS = no significant difference.

all the challenge controls died within 2 days (Fig. 1B). In the group vaccinated with rHVT-Hu4999 only, 80% (16/20) of the chickens survived. When rHVT-Hu4999 vaccinated chickens received boost with iH5N2 vaccine at 10 days of age, protection increased to 90% (18/20). Only one bird in a group that received only iH5N2 vaccination at 10 days of age survived. Survived chickens did not show any clinical signs of AI.

HI titers were evaluated using homologous SM05 antigen and heterologous CW07 antigen (Fig. 4). When the homologous antigen was used, rHVT-Hu4999, either with or without boost with iH5N2

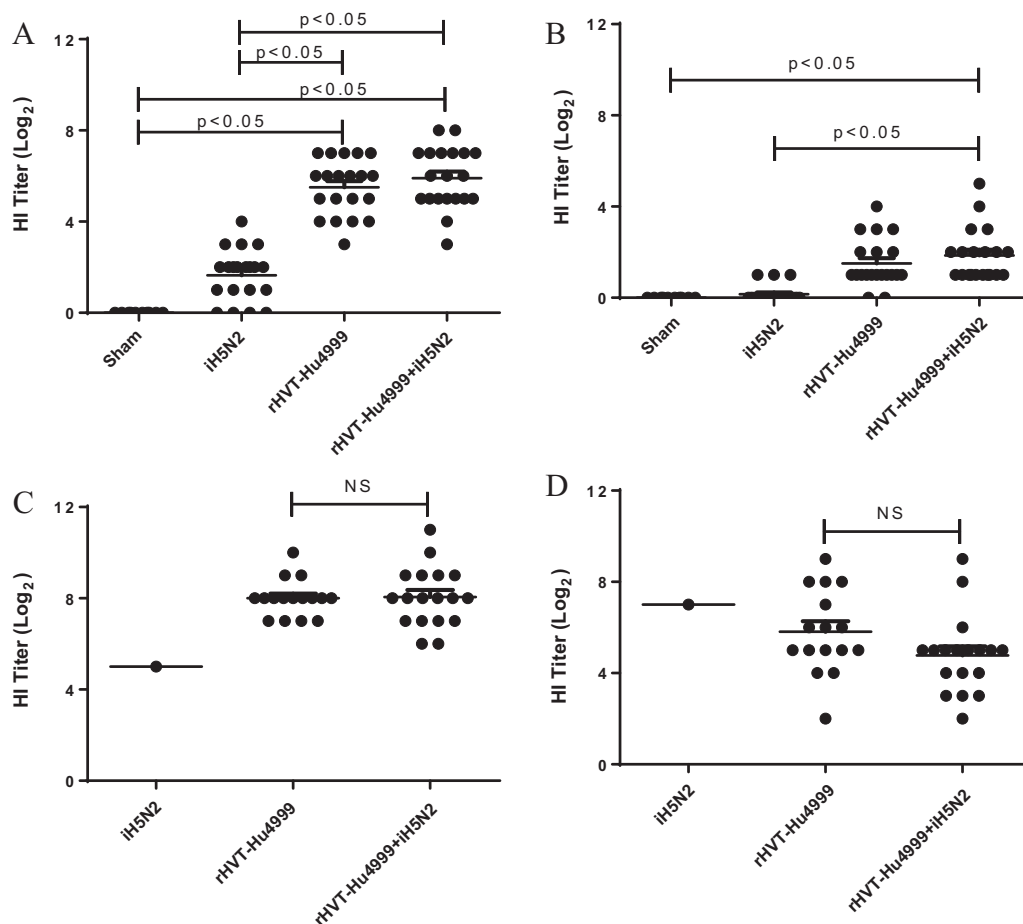
vaccine, induced average HI titers of  $2^5$ – $2^6$  prior to challenge. HI titers were much lower with the heterologous antigen with average between  $2^1$  and  $2^2$ . iH5N2 alone induced lower HI titers than rHVT-Hu4999, with average titers of  $2^{1.7}$  against the homologous antigen and  $2^{0.2}$  against the heterologous antigen. After challenge, significant HI increase was observed with all the groups.

Virus shedding from rHVT-Hu4999 vaccinated chickens, with or without boost with iH5N2 vaccine was significantly decreased compared to the challenge control and iH5N2 alone (Fig. 5

**Table 1**  
Frequency of virus isolation from groups of chickens in experiment I after homologous H5N1 challenge with A/whooper swan/Mongolia/3/2005. Oral and cloacal swab samples were collected on days 2 and 4 post-challenge from birds vaccinated with cell-associated (ca) or cell-free (cf) rHVT-Hu4999.

Group	2 dpc <sup>a</sup>		4 dpc <sup>b</sup>	
	Oropharyngeal swab	Cloacal swab	Oropharyngeal swab	Cloacal swab
1: rHVT-Hu4999 ca	4/30 <sup>a</sup>	0/30 <sup>a</sup>	0/30	0/30
2: rHVT-Hu4999 cf	3/20 <sup>a</sup>	0/20 <sup>a</sup>	1/20	0/20
3: Sham	6/6 <sup>b</sup>	6/6 <sup>b</sup>	Not done	Not done

<sup>a</sup> # Shedding virus/total at 2 days post-challenge. Lower case letters indicate a significant difference among groups by Fisher's exact test ( $p < 0.05$ ).  
<sup>b</sup> # Shedding virus/total at 4 days post-challenge.



**Fig. 4.** Individual HI titers ( $\log_2$ ) and standard error for bird groups in experiment II. HI titers were evaluated using homologous SM05 antigen (A), (C) and heterologous CW07 antigen (B), (D). Titters obtained prechallenge (A), (B) and 2 weeks post-challenge (C), (D) are shown. Statistical significance between mean titers was determined with ANOVA using the Tukey's multiple comparison test ( $p < 0.05$ ). NS = no significant difference.

and Supplemental Table 3). At 2 dpc from oro-pharyngeal swabs, there was a 3  $\log_{10}$  reduction in the rHVT-Hu4999 groups compared to the challenge control and 1  $\log_{10}$  reduction compared to iH5N2 alone. Both differences were statistically significant. There was no difference between rHVT-Hu4999 alone and rHVT-Hu4999 + iH5N2.

### 3.3. Experiment III – clinical protection of vaccinated chickens against heterologous H5N2 HPAI challenge

Finally, efficacy of rHVT-Hu4999 vaccine against heterologous H5N2 HPAI challenge was evaluated. Homology of the HA genes between the insert SH06 strain and HPAI CQ95 H5N2 virus was 82%. In this trial, rHVT-Wisc68 expressing HA gene of A/turkey/Wisconsin/68 H5N9 (TW68) strain was also used for comparison. The HA gene of the TW68 strain was 91% similar to that of HPAI CQ95 H5N2 virus. After challenge at 4 weeks of age, all of the challenge control chickens died by 6 dpc. In the rHVT-Hu4999 vaccinated group, 95% (19/20) of the chickens survived the challenge (Fig. 1C). All chickens vaccinated with rHVT-Wisc68 survived. Survived chickens did not show any clinical signs of AI.

Because previous experiments demonstrated HI titers after challenge to the homologous challenge virus (Fig. 2B), HI titers were evaluated using heterologous CQ95 H5N2 virus as an antigen. Both rHVT-Hu4999 and rHVT-Wisc68 induced somewhat increased HI titers before challenge (Fig. 6A), although HI titers were not as high as what were observed when using the homologous antigen. Titters in rHVT-Wisc group were higher at mean titer of  $2^{4.5}$  than those in

rHVT-Hu4999 group at  $2^{2.8}$  probably because TW68 strain is more similar to the CQ95 H5N2 strain than the SH06. HI titers increased to  $2^8$  after challenge (Fig. 6B).

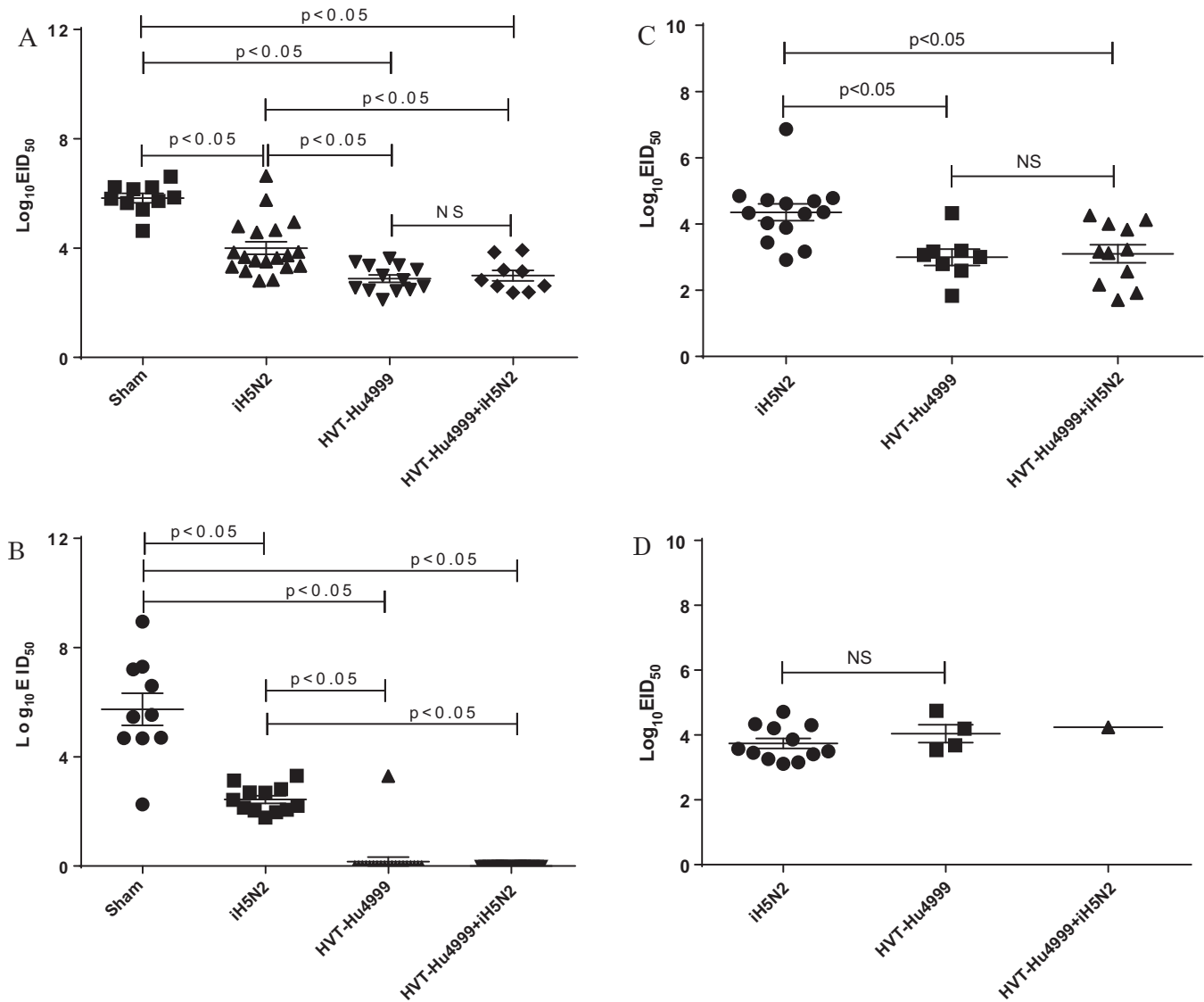
Both rHVT-Hu4999 and rHVT-Wisc68 reduced challenge virus shedding significantly (Supplemental Table 4). At 3 dpc, only 2 out of 19 chickens (11%) in the rHVT-Hu4999 group shed virus from oro-pharyngeal swabs and none in the rHVT-Wisc68, while 6 out of 7 (86%) of the challenge controls shed virus.

### 3.4. Cross reactive CTL response from rHVT-Hu4999 vaccinated birds

To examine cellular immunity induced by the rHVT-Hu4999 vaccine, we evaluated the *in vitro* CTL activity of splenic T-cells recovered from vaccinated birds. Splenic T cells demonstrated the highest level of lysis against H5 AIV infected target cells (Fig. 7). At an effector:target (E:T) ratio of 40:1, 67% of target cells infected with H5N9 AIV were lysed. Lysis of target cells was also observed heterologous subtype AIVs. Decreased lysis was observed in H6, H7 or H9 infected target cells, demonstrating a decrease in specificity to the heterologous viruses. Splenic T cells did not lyse uninfected target cells, and the percent of spontaneous lysis was  $<5\%$  (data not shown).

### 3.5. Sequence comparison of HA H5<sub>(246–260)</sub> peptide

Since vaccination with live virus stimulates cellular immunity, we sought to compare previously published MHC I and II amino



**Fig. 5.** Viral titers at day 2 (A), (B) and day 4 (C), (D) post-challenge in experiment II. Birds were vaccinated with a single dose rHVT-Hu4999 alone, rHVT-Hu4999 at day of age and then with iH5N2 at 10 days of age, inactivated H5N2 alone at 10 days of age, or with PBS (sham). At 4 weeks of age, chickens were challenged intranasally with HPAI Indonesian CW07 H5N1 virus at  $10^6$  EID<sub>50</sub>. Viral titers from oral (A), (C) and cloacal (B), (D) swabs are expressed as log<sub>10</sub> EID<sub>50</sub> per milliliter. The lower limit of detection is 0.9 log<sub>10</sub> EID<sub>50</sub> per milliliter. Statistical significance between mean titers was determined with ANOVA using the Tukey's Multiple Comparison Test ( $p < 0.05$ ). NS = no significant difference.

acid residues found in the HA of A/turkey/Ireland/1378/83 H5N8 to the avian influenza viruses used in these studies. The MHC I/II peptide, H5<sub>(246–260)</sub>, shared similarity between 50% and 88% with the HA from the various H5, H6, H7, H9 isolates tested here. Not surprisingly, the highest similarity was observed with the H5

viruses at 88% for the H5N1 and H5N2 subtypes which correlated with increased CTL activity (Table 2). Interestingly, three residues, tryptophan (W) at position 246, leucine (L) at position 249, and proline (P) at position 251 were conserved in the HA of all viruses examined.

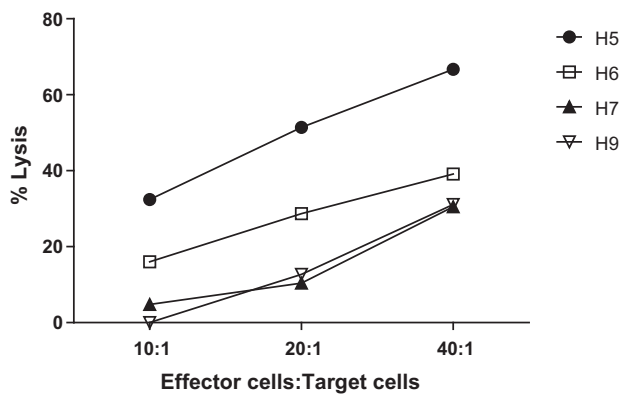
**Table 2**

Sequence alignment of known MHC I and II peptide from HA of A/turkey/Ireland/1378/83 (H5N8) with H5 HA epitopes from isolates used in these studies.

Peptide sequence <sup>a</sup>	Virus strain	Subtype	Similarity
<b>WTILKPSDTINFESN</b>	A/turkey/Ireland/1378/83	H5N8	100
<b>WTILKPND<sup>u</sup>AINFESN</b>	A/Swan/Hungary/4999/06	H5N1	88
<b>WTILKPND<sup>u</sup>AINFESN</b>	A/Whooper Swan/Mongolia/3/05	H5N1	88
<b>WTILKPND<sup>u</sup>AINFESN</b>	A/chicken/West Java Sbg/29/07	H5N1	88
<b>WTILRPND<sup>u</sup>SINFEST</b>	A/chicken/Queretaro/14588-19/95	H5N2	75
<b>WTILRPND<sup>u</sup>AINFESN</b>	A/turkey/Wisconsin/68	H5N9	75
<b>WSVLKPG<sup>u</sup>ETLN<sup>u</sup>VSEN</b>	A/chicken/California/203/03	H6N2	50
<b>WLLD<sup>u</sup>PN<sup>u</sup>DTV<sup>u</sup>TFIFN</b>	A/turkey/Virginia/4259/02	H7N2	50
<b>WSVLKPG<sup>u</sup>QL<sup>u</sup>LR<sup>u</sup>RSN</b>	A/chicken/New Jersey/12220/97	H9N2	50

<sup>a</sup> Bold-faced residues represent sequences found at same position in all isolates examined. Underlined residues represent differences in peptide sequence to A/turkey/Ireland/1378/83 sequence.





**Fig. 7.** Cytotoxicity of splenic lymphocytes recovered from B<sup>2</sup>B<sup>2</sup> chickens vaccinated with rHVT-Hu4999. Effector cells were taken from spleens of chickens at 4-weeks post-vaccination. Target B<sup>2</sup>B<sup>2</sup> lung cells (target cells) were infected with either H5N9, H6N2, H7N2 or H9N2 LPAI for 16 h prior to addition of effector cells. CTL activity was measured by release of lactic dehydrogenase in lysed cells.

controls. Because virus shedding from infected birds is a primary cause of transmission to susceptible cohorts, the decreased shedding would also reduce transmission potential. Previous research has demonstrated the efficacy of this vector vaccine against homologous challenge from clade 2.2.1 Egyptian HPAI H5N1 viruses [10].

The cell-associated form of HVT vaccine is considered more immunologically efficacious than the cell-free form especially in the presence of maternally derived antibodies [25]. Therefore, we tested both forms of rHVT-Hu4999 in this experiment because the cell-free form has an advantage in transportation and storage over the cell associated form, which requires liquid nitrogen. In this experiment, both forms protected completely and there was no difference in protection between the two forms of the vaccine.

Our results also demonstrate that rHVT-Hu4999 is capable of providing heterologous protective immunity against different clades of H5N1 and an unrelated H5N2 isolate from Mexico. The vaccine induced immunity that included significant reduction in shedding in those groups compared to the challenge control. However, the significant increase in HI titers following heterologous challenge is indicative of increased virus replication compared to homologous challenge. In addition, by changing the HA component of the vaccine to the Wisc68 HA, we demonstrated complete protection with minimal virus shedding against Mexican H5N2 isolate.

rHVT-Hu4999 elicited positive HI antibodies with average titers between 2<sup>5</sup> and 2<sup>6</sup> prior to challenge, when using the homologous HA antigen. However, when using heterologous HA antigens such as CQ95 H5N2 virus and CW07 H5N1 virus, much lower HI titers with average between 2<sup>1</sup> and 2<sup>3</sup> were observed with rHVT-Hu4999. Nonetheless, rHVT-Hu4999 provided good protection against challenge with those heterologous viruses, which may indicate contribution from other immune factors in protection against AIV than antibodies.

Little is known about the induction of cellular mediated immunity (CMI) against AIV in chickens. Seo and Webster [15] demonstrated adaptive transfer of the AIV-primed T-cells from H9N2-infected birds protected naïve-birds against lethal H5N1 challenge (A/Chicken/Hong Kong/97). HVT is a known inducer of cell-mediated immunity and has been shown to induce specific cell-mediated immunity [26,27]. Our studies agree with those, in that specific cellular immunity against the HA was observed.

Our studies also demonstrate cross reactive CTL activity induced against the HA by the HVT vector vaccine that recognized different subtypes of AIV. Recently Haghghi et al. [19] identified a T cell

epitope from an H5 AIV recognized by chicken T cells. Interestingly, this epitope was determined to be on the HA protein and was recognized by both CD4<sup>+</sup> and CD8<sup>+</sup> chicken T cells. Amino acid alignment of the HA from isolates used in our studies clearly demonstrated the presence of this peptide motif. However, further research is necessary to confirm that these epitopes are responsible for the observed CMI induced by the rHVT-Hu4999 vaccine.

In conclusion, this study demonstrated that HVT vector AI vaccine expressing HA gene of A/Swan/Hungary/4999/2006 (H5N1) provided protection against a wide range of HP AIV including H5N1 clade 2.2 and clade 2.1.3 viruses and a H5N2 virus. Although antibody titers were not predictive for protection against heterologous challenge, we observed significant protection from challenge that appears to be aided in some part to broadly cross reactive cellular immunity. This vaccine appears to have the potential to be a useful tool to control multiple lineages of HPAI along with biosecurity measures and monitoring.

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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.2014.12.028>.

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