Chapter 8

Development of Vaccines for Poultry Against H5 Avian Influenza Based on Turkey Herpesvirus Vector

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Additional information is available at the end of the chapter

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Abstract

Avian influenza (AI) remains a major threat to public health as well as to the poultry industry. AI vaccines are considered a suitable tool to support AI control programs in combination with other control measures such as good biosecurity and monitoring programs. We constructed recombinant turkey herpesvirus (HVT) vector vaccines expressing the hemagglutinin gene of AI virus H5 subtype (rHVT-H5) and evaluated their characteristics and efficacy against AI. We found that the cytomegalovirus (CMV) promoter is the most suitable for expression of the hemagglutinin gene among three promoters we evaluated. The rHVT-H5 vaccine did not cause any adverse reactions and did not revert to virulence after passages in chicken. Finally, efficacy of the rHVT-H5 vaccine was evaluated. We demonstrated that it provided protection against diverse AI H5 viruses belonging to different clades and reduced virus shedding from the challenged chicken. We also proved that efficacy provided by the rHVT-H5 vaccine was not significantly affected by presence of maternally derived antibodies (MDA) against AI virus. Furthermore, the rHVT-H5 vaccine could be applicable to the differentiating infected from vaccinated animals (DIVA) strategy. In summary, we successfully developed a HVT vector AI vaccine that possesses features that could be beneficial to AI control.

Keywords: avian influenza, turkey herpesvirus, vector vaccines, DIVA, hemagglutinin gene

1. Introduction

Avian influenza (AI) is an important zoonotic disease and it remains a major threat to public health and to the poultry industry. The highly pathogenic (HP) H5N1 outbreaks were first reported in China in 1996 and then spread to other parts of the world. These HP avian influenza viruses (AIV) have become endemic in several countries including China, Indonesia, Vietnam, and Egypt [1]. Between 2003 and 2015, 846 confirmed human cases of AI (H5N1) have been reported and 449 of those human patients have died (WHO, 2016). Most recently, HP H5N2 and H5N8 viruses caused outbreaks in the United States from December 2014 to June 2015, which resulted in depopulation of more than 48 million chickens and turkeys. These outbreaks cost farmers, the government, and consumers in the United States billions of dollars. France has also been hit by HP H5 viruses since November 2015 and the outbreaks have caused significant damages to its poultry industry. These recent HP H5 AIV continued to evolve into various clades as defined by the World Health Organization (WHO)/World Organisation for Animal Health (OIE)/Food and Agriculture Organization (FAO) H5N1 Evolution Working Group according to phylogenetic topology based on *hemagglutinin* (HA) gene sequences [2]. Another significant event of AI is the series of human infections in China caused by H7N9 AIV since March 2013. Although these viruses are low pathogenic (LP) for poultry, 277 human deaths have been reported out of 693 confirmed cases (WHO, 2016). These cases emphasize importance of controlling AIV in poultry for the industry as well as for public health.

Control of HP AI in poultry has been achieved traditionally through (1) education, (2) biosecurity, (3) diagnostics and surveillance, and (4) elimination of infected poultry (stamping-out) [3]. Viruses were successfully eradicated through a combination of those measures in many countries affected by HP AIV. However, in endemically infected countries, viruses had spread widely before executing effective measures, and therefore, it was impossible to identify and eliminate all of infected birds. In such endemic situations, vaccines are considered suitable and powerful tools to support AI eradication or control programs in combination with other control measures such as good biosecurity and monitoring programs [4–6]. When used properly, vaccines for AI have been demonstrated to protect poultry against clinical signs and mortality, increase resistance to infection, and reduce virus shedding markedly, thus decreasing the possibility of virus spreading among birds [7]. Most frequently used AI vaccines have been oil-adjuvanted, inactivated whole-virus vaccines and fowlpox virus or Newcastle disease virus (NDV)-vectored vaccines are also available. However, efficacy of these vaccines is limited especially against antigenically distant viruses [8–10]. Also, efficacy of these vaccines is known to be severely impaired by maternally derived antibodies (MDA) [11, 12]. Furthermore, oil-adjuvanted, inactivated whole-virus vaccines impede virus surveillance programs based on serology because serological responses elicited by the inactivated whole-virus vaccines are indistinguishable to those elicited by live field viruses. Therefore, development of novel vaccines which are efficacious in the face of MDA and are compatible with so-called differentiate infected from vaccinated animals (DIVA) strategy is necessary.

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Turkey herpesvirus (HVT), or the meleagrid herpesvirus 1, belongs to the family of *Herpesviridae*, the subfamily of *Alphaherpesvirinae*, and the genus *Mardivirus*. It is classified as part of the Marek's disease virus (MDV) group and designated as serotype 3 MDV. HVT is non-oncogenic and antigenically related to oncogenic serotype 1 MDV. The virus has been utilized extensively as a vaccine against Marek's disease for over 30 years and considered extremely safe [13, 14]. It is administered at hatcheries either to day-of-age chicks by subcutaneous (SQ) route or in ovo to chicken embryos at 18–19 days of incubation and is known to elicit strong cell-mediated immunity as well as humoral immunity [15–17]. Furthermore, since HVT becomes latent and persists in inoculated chickens [18], a longer period of protection is expected. Indeed, HVT-vectored Newcastle disease vaccine has been shown to be efficacious for 72 weeks after inoculation into day-of-age chicks [19]. For aforementioned reasons and also because of its large genome that can be potentially used for insertion of heterologous genes [20], HVT has been evaluated as vectors expressing protective antigen gene(s) of various poultry pathogens including NDV, infectious bursal disease virus (IBDV), and infectious laryngotracheitis virus (ILTV) [21–26]. These HVT vector vaccines have shown to be very safe and induce effective humoral and cellular immunity that is long lasting. Also, the efficacy of HVT vector vaccines does not appear to be excessively affected by the presence of MDA, probably because HVT replicates in a cell-associated manner [22]. Here, we intended to apply the HVT vector technology to develop an effective poultry vaccine against AI. Several HVT vector vaccines expressing *HA* gene of H5 AIV (rHVT-H5) were constructed and evaluated for their characteristics and efficacy against AI.

2. Construction of rHVT-H5 vaccines

2.1. Construction of recombinant viruses

A number of HVT vector vaccines have been constructed and evaluated for their characteristics and efficacy against avian pathogens [21–26]. There are three important elements in HVT vector vaccines that can impact efficacy of these vaccines in chicken: insertion sites, antigen genes, and promoters. Several insertion sites including US2, US10, UL39 [21], and an intergenic region between UL45 and UL46 (UL45/46) [24–26] have been evaluated. Out of these potential insertion sites, we demonstrated that insertion of extraneous genes at the UL45/46 site did not alter their capacity to replicate [26]. Furthermore, using the UL45/46 as the insertion site, we were successful in constructing HVT vector vaccines having antigen genes of NDV, IBDV, or ILTV that are highly efficacious against these pathogens [24–26]. Therefore, we decided to use the UL45/46 site for insertion of antigen genes of AIV.

Eight segments of genomic RNA of influenza A virus encode three membrane-associated proteins, HA, neuraminidase (NA), and Matrix (M) 2, five internal proteins, nucleoprotein (NP), PB1, PB2, PA, and M1, and two nonstructural (NS) proteins, NS1 and NS2. Out of these viral proteins, the HA surface glycoprotein is known to be the major antigen and elicits neutralizing antibodies that provide protection against the disease [27]. We used the *HA* gene cloned from LP A/turkey/Wisconsin/68 (H5N9) strain or HP A/Swan/Hungary/4999/2006

(H5N1) clade 2.2 strain. The cleavage site of the *HA* gene from A/Swan/Hungary/4999/2006 (H5N1) strain was altered to a typical cleavage site sequence of LP AIV strains.

Selection of promoters that control the expression of antigen genes is also an important factor. Tsukamoto et al. compared the cytomegalovirus (CMV) promoter and CMV/chicken β-actin chimera (Pec) promoter for expression of IBDV *VP2* gene in HVT vectors and found that the Pec promoter expressed more VP2 protein and provided superior protection against challenge with IBDV [24]. In MDV serotype 1 (MDV1) vectors expressing NDV *fusion* gene, less potent MDV glycoprotein B promoter gave better protection than the simian virus 40 (SV40) late promoter when tested in chickens with MDA [28]. Another group compared five different promoters for expression of the *HA* gene of AIV H9N2 subtype in MDV vectors and found that two MDV endogenous promoters (pp38 and gB) with relatively low expression activities provided better protection than the other three promoters (CMV, SV40, and p1.8 kb) [29]. In this experiment, to find the most appropriate promoter to express the *HA* gene of AIV H5 subtype in HVT vectors, we compared three different promoters: the CMV promoter, the chicken β-actin (Bac) promoter, and the Pec promoter.

To prepare for construction of recombinant HVT, HVT insertion site sequences (*UL45* and *UL46* genes) were isolated by polymerase chain reaction (PCR) and cloned into pUC18. Homology plasmids were then constructed by inserting the *HA* gene with the promoter into the insertion site (between *UL45* gene and *UL46* gene) of the HVT sequences. The homology plasmid along with HVT infectious genomic DNA was transfected into chicken embryo fibroblasts (CEF), where homologous recombination took place. Recombinant HVT with the inserted gene was identified by an in situ immunostaining assay called black plaque assay (BPA) using anti-HA (H5) antibodies. Recombinant HVT was purified from HVT parent through several rounds of screening process by the BPA.

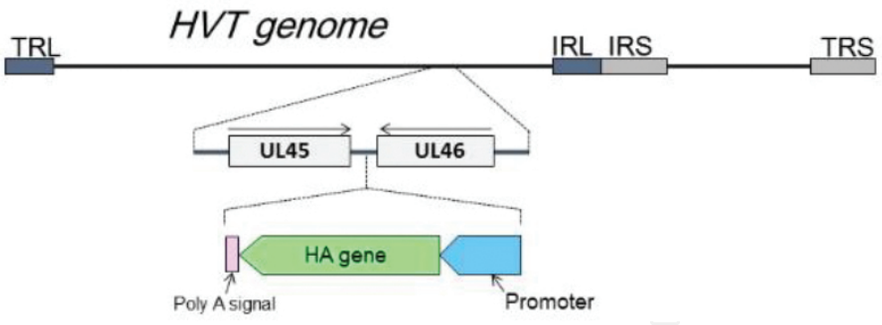

Figure 1. Genomic structure of rHVT-H5 vaccines.

2.2. In vitro characterization of rHVT-H5

Figure 1 shows genomic structures of the constructed rHVT-H5 vaccines. The genomic structures of the rHVT-H5 vaccines were confirmed by PCR assays with one primer binding to the insert sequence and the other primer binding to the HVT insertion site sequences (data not shown). The genomic structures were further confirmed by Southern blot analysis using

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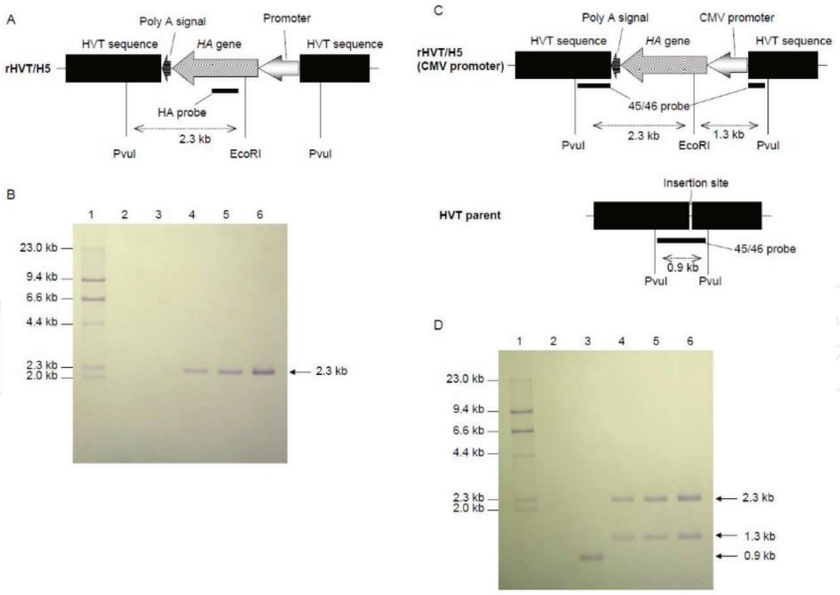

Figure 2. (A) Gene structure of rHVT/H5 and expected annealing site of the HA probe. (B) Result of Southern blot using the HA probe. Lane 1 = DNA molecular weight marker II, DIG-labeled (Roche); Lane 2 = uninoculated CEF control; Lane 3 = HVT parent; Lane 4 = rHVT/H5; Lane 5 = rHVT/H5 passage 5; Lane 6 = homology plasmid. (C) Gene structure of rHVT/H5 with CMV promoter and HVT parent and expected annealing site of the 45/46 probe. (D) Result of Southern blot using the 45/46 probe. Lane 1 = DNA molecular weight marker II, DIG-labeled (Roche); Lane 2 = uninoculated CEF control; Lane 3 = HVT parent; Lane 4 = rHVT/H5 with CMV promoter; Lane 5 = rHVT/H5 with CMV promoter passage 5; Lane 6 = homology plasmid.

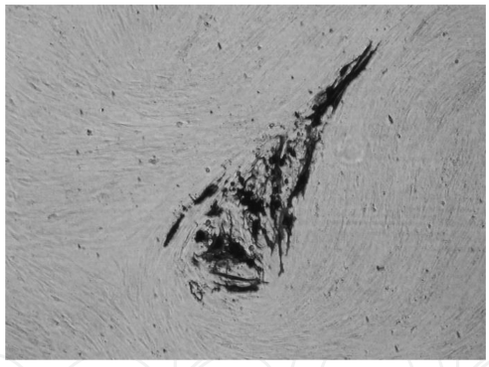

Figure 3. Black plaque assay on a rHVT/H5 plaque detecting expression of the HA protein of AIV H5 subtype. CEF monolayer infected with rHVT/H5 was incubated for 5 days and fixed with methanol:acetone. The monolayer with rHVT/H5 plaques was reacted with chicken anti-AIV HA serum, then with biotinylated anti-chicken IgG antibody and finally with streptavidin-alkaline phosphatase conjugates. Plaques expressing HA protein were stained by addition of BCIP/NBT solution.

digoxigenin-labeled probes specific to the *HA* gene or HVT insertion site sequences (**Figure 2**). Expression of the HA protein by the rHVT-H5 vaccines was confirmed by BPA using the chicken anti-HA serum (**Figure 3**). Western blot analysis using the chicken anti-HA serum detected a 75-kDa band with CEF infected with the rHVT-H5 (**Figure 4**). This band corresponds to non-cleaved HA protein that is produced by the rHVT-H5. Since the cleavage sites of the expressed HA proteins are those of LP AIV strains, the HA protein was not cleaved to HA1 and HA2 subunits in CEF.

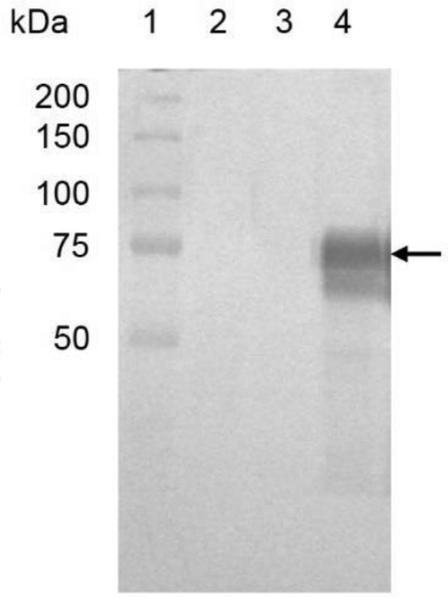

Figure 4. Western blot assay detecting expression of the HA protein by rHVT/H5. Lane 1 = Precision Plus Protein All Blue Standards (Bio-Rad Laboratories); Lane 2 = CEF control; Lane 3 = HVT FC126 parent strain; Lane 4 = rHVT/H5. An arrow indicates the HA protein with a molecular weight of 75 kDa.

In order to assess genetic and phenotypic stability of the rHVT-H5 vaccines, the viruses were passed in CEF 20 times. Viruses after the passages were characterized by PCR, Southern blot, BPA, and western blot. Results obtained with the passed viruses were identical to those obtained with the viruses before the passage (data not shown), and therefore, it was concluded that these viruses were genetically and phenotypically stable.

2.3. Evaluation of promoters for expression of HA gene

In order to identify the most suitable promoter among the CMV promoter, the Bac promoter, and the Pec promoter for expression of the *HA* gene, we compared three rHVT-H5 vaccines harboring the *HA* gene from A/turkey/Wisconsin/68 (H5N9) strain with one of these promoters. The rHVT-H5 vaccines with the CMV promoter, the Bac promoter, and the Pec promoter were designated as HVT-CMV-H5Wis68, HVT-Bac-H5Wis68, and HVT-Pec-H5Wis68, respectively.

One-day-old specific pathogen free (SPF) White Leghorn chicks were vaccinated subcutaneously with one of the rHVT-H5 vaccines. A group of chickens was held as a non-inoculated negative control group and another group of chickens was vaccinated subcutaneously with inactivated A/turkey/Wisconsin/68 (H5N9) vaccine at 3 weeks of age as an inactivated vaccine control group. Chickens in each group were bled each week between 3 and 7 weeks of age (6 and 7 weeks of age for the inactivated vaccine control group) and obtained sera were evaluated by the AIV hemagglutination inhibition (HI) test and AIV enzyme-linked immunosorbent assay (ELISA). The AIV HI tests were conducted using four hemagglutination units of an inactivated AIV homologous antigen of the A/turkey/Wisconsin/68 (H5N9) strain according to the standard procedure [30].

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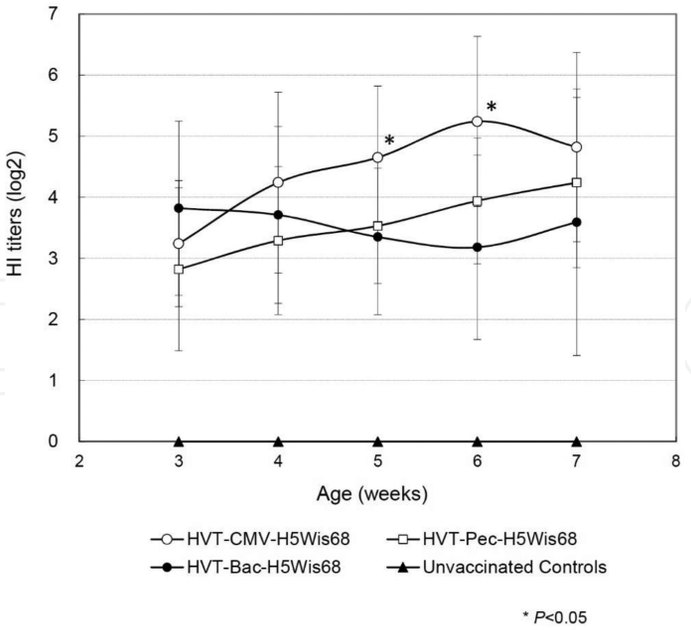

Figure 5. HI titers in chickens vaccinated with rHVT-H5 vaccines with different promoters.

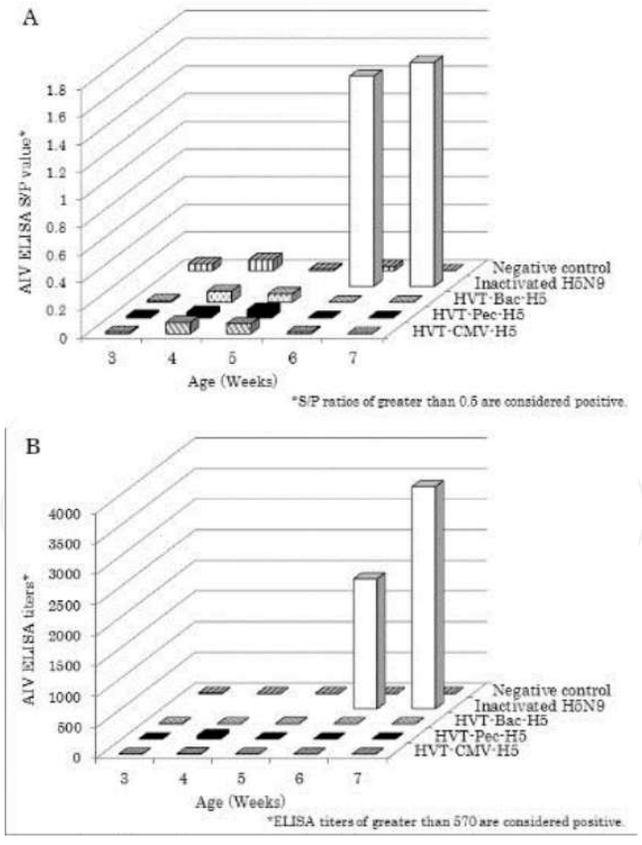

Figure 6. ELISA titers in chickens vaccinated with three rHVT-H5 vaccines using (A) FlockChek™ AIV Ab kit (Idexx Laboratories) and (B) ProFLOK® AIV Ab test kit (Zoetis).

As shown in **Figure 5**, the rHVT-H5 vaccines induced increased HI titers as early as 3 weeks of age and the increased titers were maintained through 7 weeks of age. Chickens vaccinated with HVT-CMV-H5Wis68 had higher mean HI titers than HVT-Pec-H5Wis68 and HVT-Bac-H5Wis68 vaccinated groups had between 4 and 7 weeks of age, with the differences statistically significant at 5 and 6 weeks of age. As expected, the inactivated A/turkey/Wisconsin/68 (H5N9) vaccine induced high HI titers at 6 and 7 weeks of age (3 and 4 weeks post-vaccination), confirming validity of the assay. When tested with the commercial AIV ELISA kits, Flock-Chek™ AIV Ab kit (Idexx Laboratories) and ProFLOK® AIV Ab test kit (Zoetis), sera collected from the rHVT-H5 vaccinated chickens were negative between 3 and 7 weeks of age, whereas sera collected from the inactivated vaccine control chickens showed highly positive ELISA titers with both kits (**Figure 6**).

This result demonstrated that the CMV promoter was the most suitable for expression of the *HA* gene in HVT vector vaccines. Furthermore, sera from the rHVT-H5 vaccinated chickens were found to be negative by the commercially available ELISA kits, although they were highly positive by the AIV HI test. This is most likely because these ELISA kits are designed to detect antibodies to more conserved internal protein, NP, of AIV, in order to detect antibodies to many different subtypes of AIV. This feature of the rHVT-H5 vaccines will be useful in differentiating chicken infected with field AIV viruses from vaccinated chicken (DIVA).

3. Safety of rHVT-H5 vaccine

Based on the results described above, the CMV promoter was selected for the expression of the *HA* gene. The rHVT-H5 vaccine with the CMV promoter attached to the *HA* gene from A/Swan/Hungary/4999/2006 (H5N1) strain (rHVT-H5h) was evaluated further for its safety and efficacy.

Safety of the rHVT-H5h vaccine was evaluated by an overdose study and a backpassage study. For the overdose study, SPF embryos at 18 days of incubation or day-of-age SPF chicks received rHVT-H5h at 10 times the typical field dose. In ovo application of rHVT-H5h at overdose did not affect hatchability and the vaccinated chickens remained free from any clinical signs or adverse reactions until 18 weeks of age. Chickens were necropsied at 18 weeks of age and no gross lesions were observed in any of the vaccinated chickens. Similarly, rHVT-H5h did not cause any clinical signs, adverse reactions, or gross lesions in other avian species such as turkeys, quail, pheasants, and pigeons.

For the backpassage study, to confirm that the rHVT-H5h vaccine will not revert to virulence, rHVT-H5h was passed five times in SPF chickens by using heparinized blood from chickens in the previous passage to inoculate a new set of SPF chicks. Chickens inoculated with the virus at the fifth passage, that is, inoculated with the heparinized blood from the fourth passage, were observed closely for any clinical signs for 45 days. Chickens were then necropsied and observed for grossly observable lesions. No chickens had any clinical signs, adverse reactions, or gross lesions. Therefore, it was concluded that rHVT-H5h did not revert to virulence after backpassages in chickens.

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We conducted another study to evaluate the ability of the rHVT-H5h vaccine to transmit from vaccinated chickens to non-vaccinated chickens in contact. Day-of-age SPF chickens vaccinated in ovo with rHVT-H5h or parental HVT were commingled in isolators with non-vaccinated contact chickens. Virus isolation was attempted from the peripheral mononuclear blood lymphocytes collected from chickens at 10, 14, and 21 days of age. No virus was isolated from any of the contact chickens at any time points while the viruses were isolated from the vaccinated chickens, indicating that neither rHVT-H5h nor parental HVT spread from vaccinated chickens to non-vaccinated contact chickens. It appears that transmissibility of HVT parent and the rHVT-H5h vaccine between chickens is negligible.

In summary, similar to parent HVT vaccines and other HVT vector vaccines, the rHVT-H5h vaccine is extremely safe causing no adverse effects and does not revert to virulence after passages in chickens. The lack of transmission of the rHVT-H5h vaccine further strengthens the safety profile of this vaccine in terms of containment of the genetically modified organism.

4. Efficacy of rHVT-H5 vaccine

4.1. Efficacy against homologous challenge

Finally, efficacy of the rHVT-H5h vaccine was evaluated. After vaccination, humoral immune response was evaluated by the AIV HI tests. After challenge with HP AIV, vaccine efficacy was evaluated for (1) protection against mortality and clinical signs and (2) reduction of challenge virus shedding from challenged birds. Both the criteria are important for AI vaccines because these features will ensure that the vaccine will be beneficial as a measure to contain spread of field AI viruses as well as to reduce economical burdens in both endemic and emergent situations [4, 31].

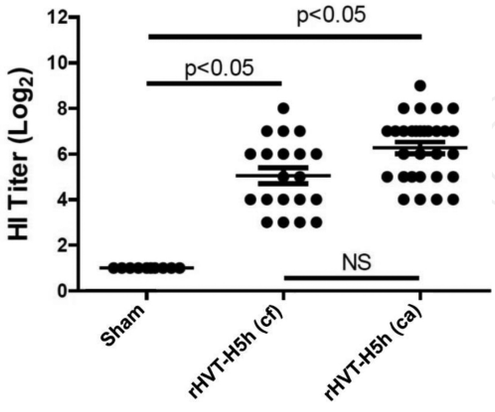

Figure 7. Individual HI titers (log_2) and standard error for bird groups at 5 weeks post-vaccination (prechallenge). Statistical significance between mean titers was determined with ANOVA using the Tukey's multiple comparison test ($p < 0.05$). NS = no significant difference. Modified from "Vaccine 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" by D.R. Kapczynski et al., 2015, Vaccine, 33, p1200.

Initial evaluation of the rHVT-H5h vaccine efficacy was conducted using homologous AIV as a challenge virus [32]. Day-of-age SPF chicks were vaccinated subcutaneously with either a frozen, cell-associated (ca) form or a lyophilized, cell-free (cf) form of the rHVT-H5h vaccine. At 5 weeks of age before challenge, increased HI titers were observed in all the rHVT-H5h vaccinated chicken with mean titers of $2^{6.3}$ for the ca group and $2^{5.1}$ for the cf group (**Figure 7**) when using homologous antigen.

Chickens were challenged with 10^6 mean embryo infectious dose (EID_{50}) HP H5N1 AIV A/Whooper Swan/Mongolia/3/2005 clade 2.2 strain at 6 weeks of age. The *HA* gene from the A/Whooper Swan/Mongolia/3/2005 strain has 100% gene homology with the A/Swan/Hungary/4999/2006 (H5N1) *HA* gene sequence. All chickens in the diluent-vaccinated, challenge control group died within 3 days. However, none of the rHVT-H5h vaccinated chickens succumbed to challenge and all the chickens were free from clinical signs of AI.

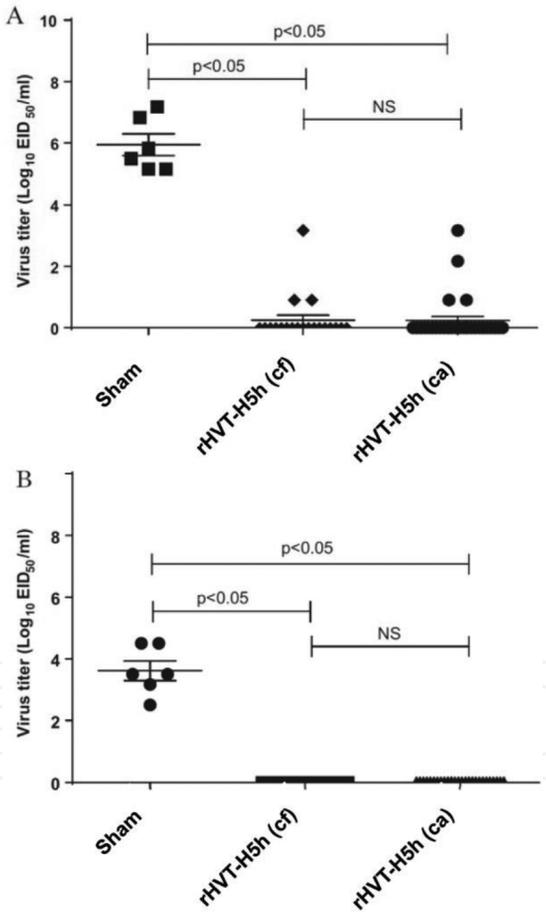

Figure 8. Viral titers from oral (A) and cloacal swabs (B) on day 2 post-challenge. Birds were vaccinated with a single dose rHVT-H5h in cell-associated (ca) or cell-free (cf) form at 1 day-of-age and challenge at 6 weeks with homologous HPAI H5N1. Viral titers are expressed as $log_{10}EID_{50}$ per milliliter. The lower limit of detection is 0.9 $log_{10}EID_{50}$ per milliliter. Statistical significance between mean titers was determined with ANOVA using Tukey's multiple comparison test ($p < 0.05$). NS = no significant difference. Modified from "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" by D.R. Kapczynski et al., 2015, Vaccine, 33, p1200.

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We observed 3–6 $\log_{10}$ reduction of challenge virus shedding in the rHVT-H5h vaccinated chickens. From oropharyngeal swabs at 2 days post-challenge (dpc), only 4/30 (13%) chickens in the ca rHVT-H5h group and 3/20 (15%) chickens in the cf rHVT-H5h group shed virus with minimal virus titers (10^1–10^3 EID_{50}/ml), while all the challenge controls shed significant amounts (10^5–$10^7 EID_{50}$/ml) of virus (**Figure 8A**). From cloacal swabs, no virus was isolated from any of the rHVT-H5h vaccinated chickens at 2 dpc, while all the challenge controls shed virus at 10^2–$10^5 EID_{50}$/ml (**Figure 8B**).

These results demonstrated high potential of the rHVT-H5h vaccine as an excellent tool to support control of AI. Therefore, we proceeded to further evaluation of the rHVT-H5h, especially for protection against heterologous virus challenge and effects of MDA. Since the ca form appeared to provide slightly better immunogenicity than the cf form, the following evaluation was conducted using the ca form of the vaccine.

4.2. Efficacy against heterologous challenge

Since HP H5 viruses have become diverse and have evolved into various different clades, it is highly important that AI vaccines exert broad "cross-clade" efficacy against diverse AIV strains. Indeed, one of the limitations of conventional oil-adjuvanted, inactivated vaccines is that they are not as effective against heterologous viruses as homologous viruses [8–10]. Therefore, we tested efficacy of the rHVT-H5h vaccine against various AIV HP H5 strains.

Day-of-age SPF broiler chicks were vaccinated with the rHVT-H5h vaccine. A commercially available inactivated vaccine based on H5N2 Mexican strain (iH5N2) was used as a control and injected into birds at 10 days of age. Challenge was conducted at 4 weeks of age using the Indonesian A/chicken/WestJava Sbg/29/2007 H5N1 strain (CW07). The CW07 virus belongs to clade 2.1.3 and sequence similarity of the HA gene between the rHVT-H5h insert and the CW07 virus was 93%. After challenge, all chickens in the sham-vaccinated, challenge control group died within 2 days. In the group vaccinated with rHVT-H5h, 80% (16/20) of the chickens survived the challenge, while only one bird (5%) vaccinated with iH5N2 survived the challenge. When rHVT-H5h vaccinated chickens received boost with iH5N2 vaccine at 10 days of age, protection increased to 90% (18/20). Reduction in virus shedding up to 3 $\log_{10}$ was observed in rHVT-H5h vaccinated chickens compared to the challenge control. When the homologous antigen was used, rHVT-H5h, either with or without boost with iH5N2 vaccine, it induced average HI titers of 2^5–2^6 prior to challenge. HI titers were much lower with the heterologous CW07 antigen with average between 2^1 and 2^2. These results indicate that cell-mediated immunity as well as mucosal immunity provided by HVT vector vaccines might be involved in protective efficacy against heterologous CW07 strain.

In the next trial, another AIV HP H5 strain, A/Viet Nam/1203/04 (H5N1) (VN04), was used for challenge. The VN04 virus belongs to clade 1 and shares 96.5% HA gene similarity with the rHVT-H5h insert. Vaccination was conducted at the day of age to SPF layer chicks and challenge was conducted at 4 weeks of age. Protection was 85% (17/20) while all challenge control chickens died and reduction of virus shedding of 3 $\log_{10}$ was observed in the rHVT-H5h group.

Since these two trials using heterologous HP H5 strains demonstrated that the rHVT-H5h provided "cross-clade" efficacy, we went further and conducted another efficacy trial using Mexican H5N2 HP strain which shares only 82% HA gene similarity with the rHVT-H5h insert. Day-of-age chicks vaccinated with rHVT-H5h were challenged with A/chicken/Queretaro/14588-19/95 (H5N2) strain at 4 weeks of age. Nineteen out of 20 (95%) chickens survived the challenge, while all the challenge control died. This result demonstrated very broad cross-protective efficacy provided by rHVT-H5h.

We further evaluated efficacy of the rHVT-H5h vaccine against various HP AIV H5 isolates including several Egyptian isolates and a recent 2014 H5N8 isolate from Germany, as summarized in **Figure 9**. Protection in rHVT-H5h vaccinated chickens ranged between 60 and 100% with significant reduction in virus shedding, further strengthening evidence of very broad "cross-clade" efficacy provided by the rHVT-H5h vaccine. In one study where layer chickens were vaccinated and raised under field conditions in Egypt, a single rHVT-H5h vaccination at day of age conferred a high level of protection (60–73%) for a relatively extended period (up to 19 weeks of age) against an Egyptian isolate [33].

Type of bird	with MDA against:		HPAIV used for challenge			Challenge	% Protection		Reference
	HVT	AIV	Strain	S.type	Clade	at:	Vaccinated	Not vaccinated	
SPF CK	No	No	A/CK/Viet Nam/1203/2004	H5N1	1	4 woa	85%	0%	Perez D. 2012
COM BR	Yes	No	A/Duck/Hungary/11804/2006	H5N1	2.2	2 woa	90%	0%	De Vriese J. et al. 2009
COM BR	Yes	Yes (H5N2)	A/Duck/Hungary/11804/2006	H5N1	2.2	2 woa	100%	20%	De Vriese J. et al. 2009
COM BR	Yes	Yes (H5N2)	A/Duck/Hungary/11804/2006	H5N1	2.2	3 woa	90%	0%	De Vriese J. et al. 2009
SPF CK	No	No	A/CK/Egypt/1709-1 VIR08/2007	H5N1	2.2.1	3 woa	100%	0%	Rauw F. et al. 2011
SPF CK	No	No	A/CK/Egypt/1709-6/2008	H5N1	2.2.1.1	3 woa	100%	0%	Rauw F. et al. 2011
COM BR	Yes	No	A/CK/Egypt/1709-6/2008	H5N1	2.2.1.1	4 woa	90%	0%	Rauw F. et al. 2012
COM BR	Yes	Yes (H5N1)	A/CK/Egypt/1709-6/2008	H5N1	2.2.1.1	4 woa	100%	0%	Rauw F. et al. 2012
COM BR	Yes	Yes (H5N1)	A/CK/Egypt/1709-1 VIR08/2007	H5N1	2.2.1	4 woa	90%	0%	Rauw F. et al. 2012
COM BR	Yes	Yes (H5N1)	A/CK/Egypt/1709-6/2008	H5N1	2.2.1.1	4 woa	70%	0%	Rauw F. et al. 2012
COM BR	Yes	Yes (H5N1)	A/CK/West Java Subang/29/2007	H5N1	2.1.3	4 woa	80%	0%	Soejoedono R. D. et al. 2012
COM BR	Yes	Yes (H5N1)	A/CK/Puwakarta-Cilingga/142/2010	H5N1	2.1.3	4 woa	95%	0%	Soejoedono R. D. et al. 2012
SPF CK	No	No	A/Whooper Swan/Mongolia/3/2005	H5N1	2.2	6 woa	100%	0%	Kapczynski D.R. et al. 2015
SPF CK	No	No	A/CK/West Java Subang/29/2007	H5N1	2.1.3	4 woa	80%	0%	Kapczynski D.R. et al. 2015
SPF CK	No	No	A/CK/Queretaro/14588/1995	**H5N2**	-	4 woa	95%	0%	Kapczynski D.R. et al. 2015
SPF CK	No	No	A/CK/Egypt/1709-6/2008	H5N1	2.2.1.1	4 woa	100%	0%	Rauw F. et al. 2012
SPF CK	No	No	A/CK/Egypt/1709-6/2008	H5N1	2.2.1.1	8 woa	100%	0%	Rauw F. et al. 2012
COM BR	Yes	Yes (H5N1)	A/CK/Egypt/1709-6/2008	H5N1	2.2.1.1	4 woa	93%	0%	Kilany W. H. et al. 2012
COM BR	Yes	Yes (H5N1)	A/CK/Egypt-63/2010 "variant"	H5N1	2.2.1.1	5 woa	80%	0%	Kilany W. H. et al. 2012
SPF CK	No	No	A/CK/Germany/2014	**H5N8**	2.3.4.4	4 woa	100%	0%	Steensels M. et al. 2015
SPF CK	No	No	A/CK/Bangladesh/11RS1 984-33/2011	H5N1	2.3.2.1	4 woa	100%	0%	Bonfante F. et al. 2013
COM LY	Yes	Yes (H5N1)	A/CK/Egypt/128s/2012	H5N1	2.2.1	19 woa	73%	0%	Kilany W. H. et al. 2014
COM LY	Yes	Yes (H5N1)	A/CK/Egypt/128s/2012	H5N1	2.2.1	19 woa	60%	0%	Kilany W. H. et al. 2014
SPF TK	No	No	A/WS/Mongolia/2005	H5N1	2.2	4 woa	96%	0%	Kapczynski et al. 2012

SPF CK = SPF Chickens
SPF TK = SPF Turkeys
COM BR = Commercial broilers
COM LY = Commercial Layers
S.type = serotype
woa = weeks of age

Figure 9. Summary of challenge experiments conducted with the rHVT-H5 vaccine. Modified from "Experimental and field results regarding immunity induced by a recombinant HVT-H5 vector vaccine against H5N1 and other H5 Highly Pathogenic Avian Influenza Virus challenge." by Y. Gardin et al., 2016, Avian Dis, in press.

To see if cellular immunity indeed is involved in this broad protective efficacy, we conducted in vitro cytotoxicity assay. Splenic lymphocytes collected from chickens vaccinated with the rHVT-H5h vaccine were incubated with chicken lung cells infected with either H5N9, H6N2, H7N2, or H9N2 low pathogenic AIV. The highest level of lysis by splenic cytotoxic T cells was observed with H5-infected target cells. Lysis was also observed with other heterologous AIV (H6, H7, or H9), although to a lower degree. Negligible lysis was observed with naïve uninfected lung cells. These results indicated that the rHVT-H5h vaccine induced AIV-specific cytotoxic T-cell activity and it may contribute in part to broad protective immunity induced by the rHVT-H5h.

4.3. Efficacy of rHVT-H5 in chicken with maternally derived antibodies

In endemic countries, most breeders are vaccinated with AI vaccines and/or exposed to field AIV challenge and therefore, their progeny possess MDA against AIV. Efficacy of oil-adjuvanted, inactivated AIV vaccines and fowlpox-vectored AI vaccines has been shown to be significantly impaired in the presence of MDA. On the other hands, HVT vector vaccines have been shown not to be excessively affected by the presence of MDA against inserted antigens. Indeed, with the rHVT-H5h vaccine, several studies demonstrated lack of significant interference on its protective efficacy by the presence of MDA when administered to day-of-age chicks [10, 34].

4.4. DIVA

AI surveillance is conducted through serological assays including ELISA and HI. Except in endemic countries, positive serological response in those assays will lead to immediate and extreme actions including "stamping-out." Also, there are trade implications because many countries ban importation of poultry from AI-positive countries. Therefore, when AI vaccination is introduced, it is critical that AI vaccines do not interfere with the AI surveillance. It is highly favorable that serological responses elicited by AI vaccines may be distinguished from those elicited by infection of field AIV. Since conventional inactivated vaccines elicit humoral immune responses that lead to positive titers in both ELISA and HI tests, these vaccines do interfere with the surveillance [7].

The rHVT-H5h vaccine elicits antibodies against the HA protein and the antibody responses can be detected by the HI tests. However, those sera from vaccinated chickens were negative in commercial ELISA kits because the ELISA kits are designed to detect antibodies against more conserved internal protein (NP) in order to offer coverage over different subtypes of AIV. When we examined serological responses in chickens that were vaccinated with the rHVT-H5h and then challenged with HP AIV, we found positive ELISA titers in chickens that excreted challenge viruses. These results demonstrated that the rHVT-H5h vaccines may be applied to the DIVA strategy and do not interfere with AI surveillance.

5. Conclusions

Our studies demonstrated that the rHVT-H5h vaccine possesses characteristics that could be beneficial to control of AI in endemic countries and in emergency situations. Those characteristics are (1) broad "cross-clade" protective efficacy against diverse AIV H5 isolates, (2) lack of interference by MDA, (3) applicability to hatchery vaccination, and (4) applicability to DIVA. The rHVT-H5h vaccine has been approved by authorities in Egypt, Mexico, and Bangladesh and is in use in the field. An independent survey conducted by FAO, General Organization for Veterinary Services in Egypt, and Centre International de Recherche en Agriculture pour le Développement in France concluded that day-of-age vaccination utilizing the rHVT-H5h vaccine at hatcheries is more efficient than the program using the inactivated vaccines at farms and it would have a positive impact for disease control in Egypt [35].

It is clear that vaccines alone cannot solve all the problems associated with AI. However, we believe that in conjunction with active and efficient surveillance and strict biosecurity measures, the rHVT-H5h vaccine can contribute to disease control by increasing resistance against infection and decreasing the amount of virus shed to the environment. It would also remove economical burdens from farmers and consumers and improve animal welfare by protecting chickens from mortality and clinical signs. In conclusion, we successfully developed a HVT vector AI vaccine that possesses many features that could be beneficial to AI control. It remains to be seen whether this vaccine is truly useful in the field.

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