Serological Evaluation of Turkey Herpesvirus Vector Vaccines Expressing the Hemagglutinin Gene of Avian Influenza Virus H5 Subtype under Three Different Promoters

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Marek’s disease virus, including turkey herpesvirus (HVT), have been utilized as vectors to express foreign antigen genes and induce immunity against the antigens in chickens. Selection of promoters in developing such vector vaccines is one of the most important factors influencing efficacy of vector vaccines. In this study, in order to find a suitable promoter for expressing the hemagglutinin gene of avian influenza virus H5 subtype in HVT vector vaccines, three HVT vector avian influenza virus H5 subtype (HVT-AI) viruses expressing the hemagglutinin gene were constructed using three promoters; the cytomegalovirus (CMV) promoter, the chicken β-actin (Bac) promoter, and CMV/Bac chimera (Pec) promoter. Of those three vector vaccines, HVT-AI with the CMV promoter induced significantly higher avian influenza virus (AIV) hemagglutinin inhibition titers than the other HVT-AI vaccines with the Bac or the Pec promoters, after inoculation into chickens at one day old. When evaluated with two of commercially available AIV enzyme-linked immunosorbent assay kits, the HVT-AI vaccines did not induce positive titers, indicating that these HVT-AI vaccines may be utilized for easy differentiation of vaccinated chickens from ones infected with field AIV.

Key words: avian influenza H5 subtype, DIVA, promoter, turkey herpesvirus, vector vaccine


Introduction

Turkey herpesvirus (HVT), or Meleagrid herpesvirus 1, belongs to the family of Herpesviridae, the subfamily of Alphaherpesvirinae, and the genus Mardivirus and is part of the Marek’s disease virus (MDV) group, designated as serotype 3 MDV. Marek’s disease virus, including HVT, have been evaluated as viral vectors expressing protective antigen gene(s) of various poultry pathogens (Morgan et al., 1993; Darteil et al., 1995; Sonoda et al., 2000; Tsukamoto et al., 2002; Ma et al., 2014). There are several elements of MDV vector vaccines that can impact efficacy of these vaccines in chickens. One of them is the choice of insertion sites. Gao et al. (2011) reported that HVT vector vaccine carrying the hemagglutinin (HA) gene of avian influenza virus (AIV) H5 subtype at US2 insertion site provided better immunity than HVT vector with the HA gene at US10 insertion site. Selection of promoters that control expression of antigen genes also appears to be an important factor. Tsukamoto et al. (2002) constructed a HVT vector infectious bursal disease vaccine expressing the infectious bursal disease virus (IBDV) VP2 gene under the cytomegalovirus (CMV) promoter or CMV/chicken β-actin chimera (Pec) promoter. The HVT vector vaccine with the Pec promoter provided better protection than the HVT vector with the CMV promoter against challenge with IBDV. Sonoda et al. (2000) reported that the MDV serotype 1 (MDV1) glycoprotein B promoter appeared to be more suitable than the simian virus 40 (SV40) late promoter for use in MDV1 vector Newcastle disease virus vaccine expressing the fusion gene, when tested in chickens with maternally derived antibodies to Newcastle disease virus. Ma et al. (2014) compared five different promoters in MDV1 vector vaccines for expression of the HA gene of AIV H9N2 subtype and found that two MDV endogenous promoters (pp38 and gB) with relatively low expression activities gave better protection than the other three promoters (CMV, SV40, and p1.8 kb).

Avian influenza (AI) is an important zoonotic disease and a major threat to the society as evidenced by the recent H5N1 outbreaks, which started in 1996 and has become endemic in several countries (FAO, 2011). Enforcement of strict bio-
security programs is the primary measure for control and eradication of AI. Vaccination has also been considered an appropriate tool to support AI eradication or as control programs for use in emergency situations as well as for routine use in endemic countries (Swayne, 2012). It has been shown that proper use of vaccination can protect chickens from clinical disease and reduce virus shedding from infected chickens (Swayne, 2003).

In this study, in order to find an appropriate promoter for expression of the HA gene of AIV H5 subtype in HVT vector vaccines, we compared three different promoters; the CMV promoter, the chicken β-actin (Bac) promoter and the Pec promoter. Three HVT vector avian influenza virus H5 subtype (HVT-AI) vaccines expressing the HA gene under one of these promoters were constructed and evaluated for the ability to induce humoral immunity after inoculation into chickens. To our knowledge, this is the first report investigating a suitable promoter for expression of the HA gene of AIV H5 subtype in HVT vectors.

**Materials and Methods**

**Viruses**

Turkey herpesvirus FC-126 strain (Witter et al., 1970) was propagated in chicken embryo fibroblasts (CEF). A/turkey/Wisconsin/68 (H5N9) strain of AIV was propagated in the allantoic fluid of 9 to 11-day old embryonated specific pathogen free (SPF) chicken eggs. The AIV A/turkey/Wisconsin/ 68 (H5N9) strain was inactivated with binary ethyleneimine and used as an antigen in the AIV hemagglutination inhibition (HI) test. For chicken inoculation, the inactivated antigen was mixed with an oil adjuvant.

**Preparation of Antiserum Against AIV HA Protein**

Antiserum against the HA protein of AIV H5 subtype was produced in SPF chickens by immunization with eukaryotic expression plasmid DNA expressing the HA protein as described by Lee et al. (2006). Briefly, 3-week-old SPF chickens were inoculated intramuscularly with one hundred micrograms of the plasmid DNA mixed with Lipofectin. Chickens were held for 5 to 7 days, plaques expressing the HA protein were visualized by a black plaque assay. Briefly, cells were fixed with a mixture of methanol and acetone (volume ratio=1:2) and incubated with chicken anti-AIV HA serum. Next, after incubated with biotinylated anti-chicken IgG antibody (Vector Laboratories, USA) and then with streptavidin-alkaline phosphatase conjugates (Vector Laboratories, USA), plaques expressing HA protein were stained by addition of 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) solution (Bio-Rad Laboratories, USA). The purification procedure was repeated until all plaques were stained positively in the black plaque assay. Purified recombinant virus with the HA gene under the CMV promoter was designated as HVT-CMV-H5. Recombinant viruses with the Bac promoter or the Pec promoter were designated as HVT-Bac-H5 and HVT-Pec-H5, respectively.

**Serological Evaluation of HVT-AI**

One-day-old SPF White Leghorn chicks (Charles River Laboratories, USA) were vaccinated subcutaneously with one of the HVT-AI 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 HI test and AIV enzyme-linked immunosorbent assay (ELISA). The AIV HI tests were conducted using four hemagglutination units (HAU) of an inactivated AIV homologous antigen of the A/turkey/Wisconsin/68 (H5N9) strain according to the standard procedure (OIE, 2014). Briefly, before the HI assay, the number of the hemagglutination units in the inactivated A/turkey/Wisconsin/68 (H5N9) antigen was determined as the highest dilution of the antigen giving complete agglutination, and the antigen was diluted to contain four HAU in 25 μl. In U-bottom 96 well plates, the sera were initially diluted 1:5 and then serially diluted by two-fold across the plates with phosphate buffered saline (PBS) to contain 25 μl per well. Four HAU of the antigen in 25 μl was
added to each well and incubated for 30 minutes at room temperature. Finally, 50 μl of 0.5% chicken erythrocytes in PBS was added to each well and incubated for approximately 40 minutes at room temperature. HI titers are the highest dilution of the sera exhibiting inhibition of hemagglutination. HI titers of equal to or more than 10 were considered positive. The ELISA assays were conducted using two of commercial AIV ELISA kits, FlockChek™ AIV Ab kit (Idexx Laboratories, USA) and ProFLOK® AIV Ab test kit (Zoetis, USA) according to the manufacturers’ recommendations. For FlockChek™ AIV Ab kit, S/P ratios of equal to or greater than 0.5 are considered positive. For ProFLOK® AIV Ab test kit, ELISA titers of equal to or greater than 570 are considered positive.

**Statistical Analysis**

Statistical differences in mean HI titers between HVT-AI vaccine groups were analyzed using one-way analysis of variance (ANOVA) test followed by Tukey’s HSD test (SigmaStat; SPSS Inc., USA). Different lowercase letters (a, b) indicate statistical significance between compared groups in Table 1. All statistical tests were performed using \( P < 0.05 \).

**Results**

**In vitro Evaluation of HVT-AI**

Genomic structures of three HVT-AI vaccines were confirmed by Southern blot analysis using Digoxigenin-labeled probes specific to the HA gene or HVT insertion site sequences (data not shown). Expression of the HA protein by the HVT-AI vaccines was confirmed by the black plaque assay using the chicken anti-HA serum (Fig. 1).

**Serological Evaluation of HVT-AI**

To investigate the ability of the three HVT-AI vaccines with different promoters to elicit humoral immune responses in vaccinated chickens, SPF chickens vaccinated with one of the HVT-AI vaccines at one day old were bled each week between 3 and 7 weeks of age and evaluated by the AIV HI tests and the AIV ELISA. SPF chickens used in this study were negative for AIV antibodies by the HI test and the ELISA at one day old. As shown in Table 1, the HVT-AI vaccines induced increased HI titers as early as 3 weeks of age, which were significant between the HVT CMV H5 and the other two vaccines. The HI GMTs at 7 weeks were higher than those at 3 weeks for all three vaccines. The HI GMTs of the HVT Bac H5 and HVT Pec H5 vaccines were significantly higher than those of the HVT CMV H5 vaccine at 7 weeks.

![Fig. 1. Black plaque assay detecting expression of the HA protein of AIV H5 subtype by HVT-CMV-H5. CEF monolayer infected with HVT-CMV-H5 was incubated for five days and fixed with methanol:acetone. The monolayer with HVT-CMV-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. Similar staining was observed with HVT-Pec-H5 and HVT-Bac-H5 plaques and no staining was observed with HVT FC-126 plaques (not shown).](image)

![Table 1. HI titers in chickens vaccinated with three HVT-AI vaccines](table)

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>3 weeks</th>
<th></th>
<th>4 weeks</th>
<th></th>
<th>5 weeks</th>
<th></th>
<th>6 weeks</th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PositiveA</td>
<td>HI GMTB</td>
<td>PositiveA</td>
<td>HI GMT</td>
<td>PositiveA</td>
<td>HI GMT</td>
<td>PositiveA</td>
<td>HI GMT</td>
<td>PositiveA</td>
<td>HI GMT</td>
</tr>
<tr>
<td>HVT-CMV-H5</td>
<td>16/17 (94%)</td>
<td>23.5</td>
<td>15/17 (88%)</td>
<td>47.1</td>
<td>17/17 (100%)</td>
<td>62.6a</td>
<td>17/17 (100%)</td>
<td>94.2a</td>
<td>17/17 (100%)</td>
<td>70.8</td>
</tr>
<tr>
<td>HVT-Pec-H5</td>
<td>15/17 (88%)</td>
<td>17.7</td>
<td>16/17 (94%)</td>
<td>24.5</td>
<td>16/17 (94%)</td>
<td>28.9b</td>
<td>17/17 (100%)</td>
<td>38.4b</td>
<td>16/17 (94%)</td>
<td>47.1</td>
</tr>
<tr>
<td>HVT-Bac-H5</td>
<td>16/17 (94%)</td>
<td>35.4</td>
<td>16/17 (94%)</td>
<td>32.6</td>
<td>15/17 (88%)</td>
<td>25.5b</td>
<td>14/17 (82%)</td>
<td>21.4b</td>
<td>13/17 (76%)</td>
<td>28.5</td>
</tr>
<tr>
<td>H5N9 killed</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>17/17 (100%)</td>
<td>294.9</td>
</tr>
<tr>
<td>Negative control</td>
<td>0/10 (0%)</td>
<td>N/A</td>
<td>0/10 (0%)</td>
<td>N/A</td>
<td>0/10 (0%)</td>
<td>N/A</td>
<td>0/10 (0%)</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

A Positive=HI titers of equal to or more than 10 were considered positive.

B HI GMT=geometric mean hemagglutinin inhibition titer. Different lowercase letters (a, b) following mean values in each column indicate statistically significant differences between groups by one-way ANOVA test followed by Tukey’s HSD test (\( P < 0.05 \)).

C N/A=not applicable.
age and the increased titers were maintained through 7 weeks of age. In chickens vaccinated with HVT-CMV-H5, 16 out of 17 chickens (94%) had positive HI titers (equal to or more than 10) with geometric mean titer (GMT) of 23.5 at 3 weeks of age and the HI titers continued to increase up to GMT of 94.2 with 100% of chickens positive at 6 weeks of age, while mean HI titers of HVT-Pec-H5 and HVT-Bac-H5 stayed between 17.7 and 47.1. Mean HI titers of the HVT-CMV-H5 vaccinated group were statistically higher than mean HI titers of HVT-Pec-H5 and HVT-Bac-H5 vaccinated groups 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. Sera from the non-inoculated negative control group were negative throughout the observation period.

When tested with the commercial AIV ELISA kits, FlockChek™ AIV Ab kit (Idexx Laboratories) and ProFLOK® AIV Ab test kit (Zoetis), sera collected from the HVT-AI-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 (Fig. 2).
Discussion

Many viral vectors have been evaluated for use as veterinary vaccines in recent years. For poultry, MDV, including HVT, have both been studied extensively as vectors because of their many advantages. They have a large DNA genome of 140–180 kb and therefore contain many potential insertion sites that may not affect viral replication. MDV vaccine strains, including HVT, are very safe and can be applied either in ovo or to one-day-old chicks at the hatchery. It is also known to elicit strong cell-mediated immunity along with humoral immunity (Parvizi et al., 2010; Rauw et al., 2010). Furthermore, a longer period of immunity can be expected, since MDV persists in inoculated chickens (Witter et al., 1978; Palya et al., 2014). Also, efficacy of MDV vector vaccines does not appear to be excessively affected by the presence of maternally derived antibodies, probably because MDV replicates in a cell-associated manner (Morgan et al., 1993; Sonoda et al., 2000). Along with other factors, selection of an appropriate promoter for expression of antigen genes in MDV vectors appear to be very important for providing optimum immunity.

In this study, we compared three different promoters, the CMV promoter, the Bac promoter and the Pec promoter for expression of the HA gene of AIV H5 subtype in HVT vectors. Of HVT-AI vaccines using one of those promoters, the HVT-AI vaccine with the CMV promoter provided significantly higher HI titers in vaccinated chickens than the HVT-AI vaccines with other promoters. Tsukamoto et al. (2002) compared the CMV promoter and the Pec promoter for expression of IBDV VP2 gene in HVT vectors and found that the HVT vector VP2 with the Pec promoter provided better humoral immunity as well as better protection against IBDV challenge than the HVT vector VP2 with the CMV promoter. It appears that one promoter that provides good immunity in combination with a particular antigen gene does not necessarily work well with another antigen gene combination. Therefore, promoters in HVT vector vaccines should be evaluated carefully for each antigen gene.

Sera from HVT-AI vaccinated chickens in this study were found to be negative by two of 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 proteins of AIV, such as nucleocapsid and matrix antigens, in order to detect antibodies to many different subtypes of AIV. This feature of the HVT-AI vaccines will be useful in differentiating chickens infected with field AIV viruses from vaccinated chickens.

In summary, we demonstrated that the HVT vector vaccine expressing the HA gene of AIV H5 subtype under the CMV promoter provided vaccinated chickens with better humoral immunity by AIV HI titers than the HVT vectors with the Bac promoter or the Pec promoter. The sera collected from chickens vaccinated from the HVT-AI vaccines were negative by commercially available AIV ELISA kits although the sera were highly positive by the AIV HI test, thus allowing easy differentiation of infected from vaccinated animals (“DIVA”).

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References


Sonoda K, Sakaguchi M, Okamura H, Yokogawa K, Tokunaga E, Tokiyoshi S, Kawaguchi Y and Hirai K. Development of an effective polyvalent vaccine against both Marek’s and Newcastle diseases based on recombinant Marek’s disease virus type 1 in commercial chickens with maternal antibodies. Jour-