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# Progress toward the development of polyvalent vaccination strategies against multiple viral infections in chickens using herpesvirus of turkeys as vector

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**Vaccination is the most cost effective strategy for the control and prevention of the plethora of viral diseases affecting poultry production. The major challenge for poultry vaccination is the design of vaccines that will protect against multiple pathogens via a single protective dose, delivered by mass vaccination. The Marek disease virus and the highly pathogenic avian influenza virus cause severe disease outbreaks in chickens. Vaccination with live herpesvirus of turkeys protects chickens from Marek disease and inactivated influenza viruses are used as antigens to protect chickens against influenza virus infections. We developed herpesvirus of turkeys (HVT) as a vaccine vector that can act as a dual vaccine against avian influenza and Marek disease. The HVT vector was developed using reverse genetics based on an infectious bacterial artificial chromosome (BAC) clone of HVT. The BAC carrying the HVT genome was genetically modified to express the haemagglutinin (HA) gene of a highly pathogenic H7N1 virus. The resultant recombinant BAC construct containing the modified HVT sequence was transfected into chicken embryo fibroblast (CEF) cells and HVT recombinants (rHVT-H7HA) harbouring the H7N1 HA were recovered. Analysis of cultured CEF cells infected with the rHVT-H7HA showed that HA was expressed and that the rescued rHVT-H7HA stocks were stable during several in vitro passages with no difference in growth kinetics compared with the parent HVT. Immunization of one-day-old chicks with rHVT-H7HA induced H7-specific antibodies and protected chickens challenged with homologous H7N1 virus against virus shedding, clinical disease and death. The rHVT-H7HA vaccine also induced strong and long-lasting antibody titers against H7HA in chickens that were vaccinated in ovo 3 d before hatching. This vaccine supports differentiation between infected and vaccinated animals (DIVA), because no influenza virus nucleoprotein-specific antibodies were detected in the rHVT-H7HA vaccinated birds. The rHVT-H7HA not only provided protection against a lethal challenge with highly pathogenic H7N1 virus but also against highly virulent Marek disease virus and can be used as a DIVA vaccine.**

**Keywords:** multivalent vaccine, in ovo vaccination, poultry, vector-based vaccine, avian influenza, recombinant herpesvirus of turkeys, highly pathogenic H7N1 avian influenza virus, Marek disease, HVT BAC, avian

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## Avian Influenza and Marek Disease Infections in Poultry

An armoury of vaccines is employed during the short life span of a chicken to reduce production losses and mortality.[1] Among the many diseases, avian influenza (AI) virus and Marek disease (MD) virus cause severe losses.[2-4] Control of avian influenza virus (AIV) infections in poultry remains a major challenge to animal and public health and the world economy.[5,6] Avian influenza outbreaks in poultry are caused primarily by H5, H7 and H9 subtype viruses that naturally exist as low pathogenicity (LP) phenotypes in wild aquatic birds.[7] The transfer of LPAI H5 and H7 viruses into poultry often results in the spontaneous emergence of viruses with high pathogenicity

(HP) phenotypes causing very high morbidity and mortality in infected birds.[8] In recent years, the widespread outbreaks of HPAI H5N1 virus have had an incalculable social and economic impact on millions of people globally. Similarly, epizootic outbreaks in poultry due to H7N1, H7N2, H7N7 and H7N3 viruses in Italy, the Netherlands, Spain, Canada and the USA in recent years have caused severe economic losses with nearly 100 human infections, of which one was fatal.[7,9,10] Although the mass culling of infected and susceptible birds usually reduces the spread of infection, these sanitary measures alone may be impractical due to the enormous economic costs, particularly when the viruses have spread over wide areas, infecting multiple avian species. In these circumstances, vaccination against AIV provides invaluable support to increase host resistance and reduce environmental contamination.[11] Currently, conventional inactivated whole virus AI vaccines are used for routine preventative vaccination as well as in target vaccination programmes. Such vaccines provide protection from clinical disease and also minimise viral shedding, but in most circumstances the virus remains prevalent in affected areas where vaccination is practised.[12] These vaccines also present inherent problems such as incompatibility with diagnostic tests that are unable to differentiate infected from vaccinated animals and do not confer lifelong immunity necessitating a booster vaccination. In addition, biohazards associated with manufacturing the vaccines and low vaccine yield from embryonated fowl eggs, when high pathogenicity strains are used as the vaccine seed virus, has hindered their usefulness.[13] Inactivated vaccines using heterologous neuraminidase (NA) subtypes and the development of viruses through reassortment and reverse genetic techniques have overcome some of these challenges.[12,14] Unlike with other live attenuated virus vaccines, the potential use of live LPAI viruses as vaccines is severely hampered, as these viruses can emerge as HP strains through mutation in vaccinated animals, or reassortant viruses could arise due to co-infection of vaccinated birds with other prevalent AIV strains, with unpredictable consequences.[15]

The development of new AI poultry vaccines produced by manipulating attenuated avian viruses for use as vectors to deliver protective HA and NA antigens of AIV is gaining support. This is because single vaccination regimes could confer solid immunity against multiple diseases, be administered through mass application and support the DIVA strategies. Production of these vaccines is also cost effective compared with conventional vaccines. Among candidates, fowl pox virus (FPV), Newcastle disease virus (NDV), infectious laryngotracheitis virus (ILTV), Marek disease virus (MDV-1) and herpesvirus of turkeys (HVT) are receiving the most attention as potential vectors, since these viruses have restricted host ranges among avian species.[14,16-19] The recombinant FP vector-based vaccine "Trovac AI H5" was licensed in the USA in 1998 for the immunization of poultry and an NDV-based live attenuated vaccine "NDV-H5HA" is commercially available in China.[20] However, there are some inherent shortcomings, such as lower susceptibility in very young birds due to maternally derived antibodies, that limit their effectiveness and commercial use.

The MDV vaccine viruses are considered some of the most potent vectors for polyvalent live vaccines in expressing recombinant foreign antigens and the induction of protective immunity against both the vector virus and the expressed antigen of other viral diseases. This is because this vaccine can induce lifetime protection against MDV even in the presence of maternal antibodies after in ovo inoculation of the embryo or subcutaneous administration into one-day-old chicks.[19] Herpesvirus of turkeys is a naturally occurring apathogenic MDV strain (MDV-3) originally isolated from domestic turkeys and has been widely used as a vaccine against MD for several decades. Herpesvirus of turkeys vaccine is still widely used for the control of MD in countries where MDV infection is endemic.

## Engineering HVT as a Bivalent Vaccine for Avian Influenza and Marek Disease

Herpesvirus of turkeys causes persistent infection even in the presence of maternal HVT/MDV antibodies and has a host-range restricted to avian species. Therefore, HVT has been among the most widely used as a safe and potent vaccine against MD by the poultry industry worldwide. Herpesvirus of turkeys is also widely recognized as a vector for expressing protective antigens of other avian pathogens such as NDV, infectious bursal disease virus (IBDV) and *Eimeria acervulina*. Such recombinant HVT-based vaccines confer excellent and long-lasting simultaneous protective immunity in chickens against more than one pathogen, following the "one stone, two birds" principle.[21-24] However, these HVT recombinants have been prepared using conventional genomic recombination techniques, which are extremely time-consuming and labor-intensive. Additionally, it has never been possible to recover purified recombinant viruses through plaque selection methods, leading to decreased immunogenicity after continuous passage in cultured cells. Recent technologies involving the insertion of full-length genomes of herpes viruses into BAC have revolutionized the field for the development of more efficient and versatile recombinant MDV vaccine vectors, including HVT[25] and the virulent strains of MDV; MDV-1 [26-28] and MDV-2.[29] We have successfully constructed a BAC vector for HVT strain FC126.[25] In our recent paper in Vaccine,[30] we generated a recombinant HVT (rHVT) as a multivalent vaccine vector by manipulating the HVT genome within a BAC following insertion of the HA gene of a HPAI H7N1 virus (**Fig. 1**). The rHVT-H7HA expressed the heterologous HA glycoprotein in infected avian cells. We demonstrated that the rHVT-H7HA bivalent vaccine administrated to one-day-old chicks induced HA-specific antibodies and conferred protection and a reduction in viral shedding in vaccinated chicks subsequently challenged with HPAI H7N1 virus with concomitant protection against MDV. In this study, the efficacy of the

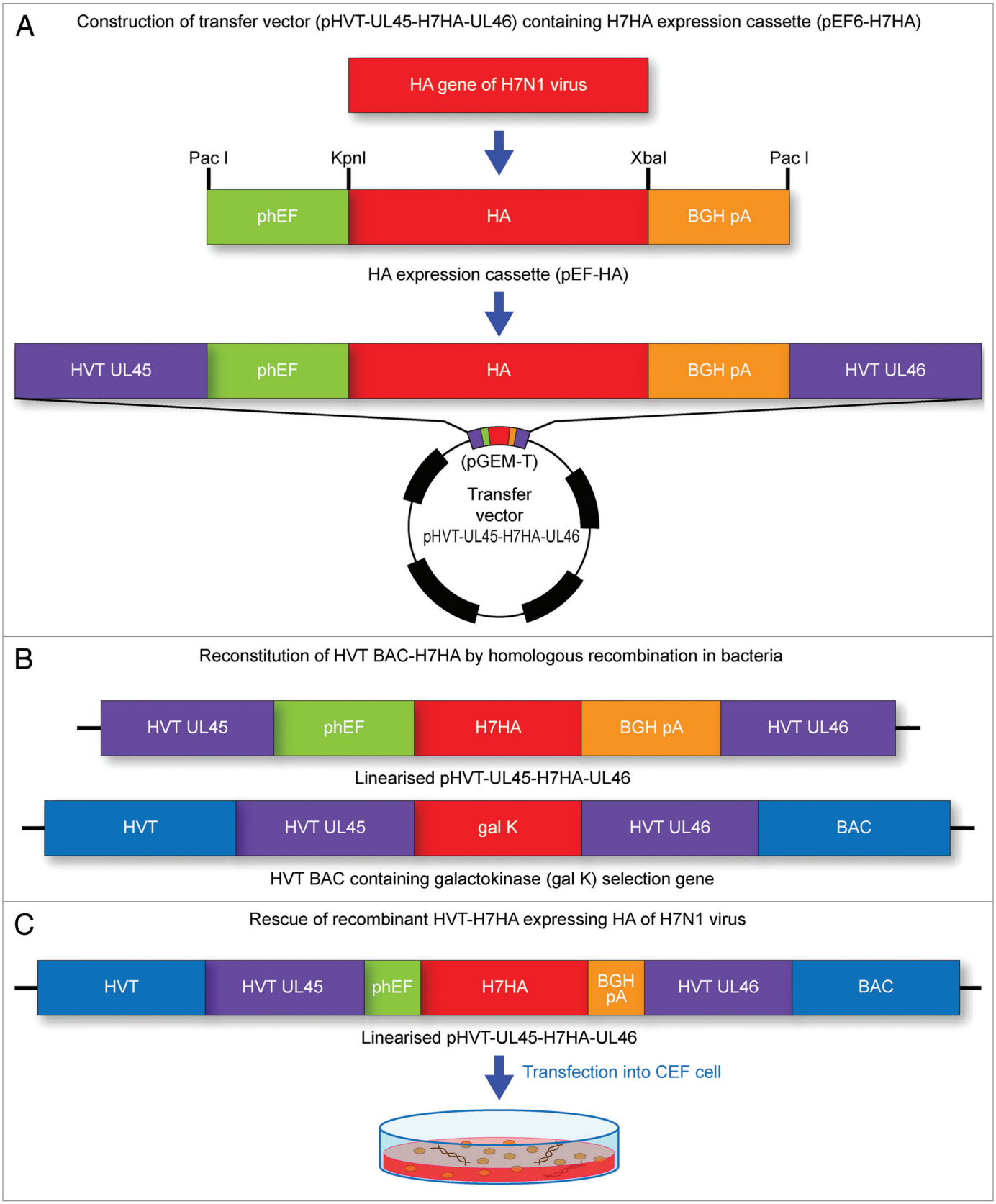


**Figure 1.** For figure legend, see page 225.

**Figure 1 (See opposite page).** Development of recombinant HVT-based Influenza virus vaccine. (A) The construction of transfer vector (pHVT-UL45-H7HA-UL46) containing H7HA expression cassette (pEF6-H7HA) was achieved by incorporating human elongation factor 1α-subunit promoter (hEF1α), H7HA and the polyadenylation signal of bovine growth hormone gene (BGH pA) between the UL45 and UL46 genes of HVT genome. (B) The HVT BAC-H7HA was reconstituted by replacing *gal K* gene in HVT BAC with H7HA expression cassette. The linearized transfer vector (pHVT-UL45-H7HA-UL46) was electroporated into SW105 competent bacteria containing the HVT-*gal K* BAC DNA. (C) The recombinant HVT-H7HA vaccine stocks were rescued by transfecting newly reconstituted HVT BAC-H7HA DNA into chick embryo fibroblast cells.

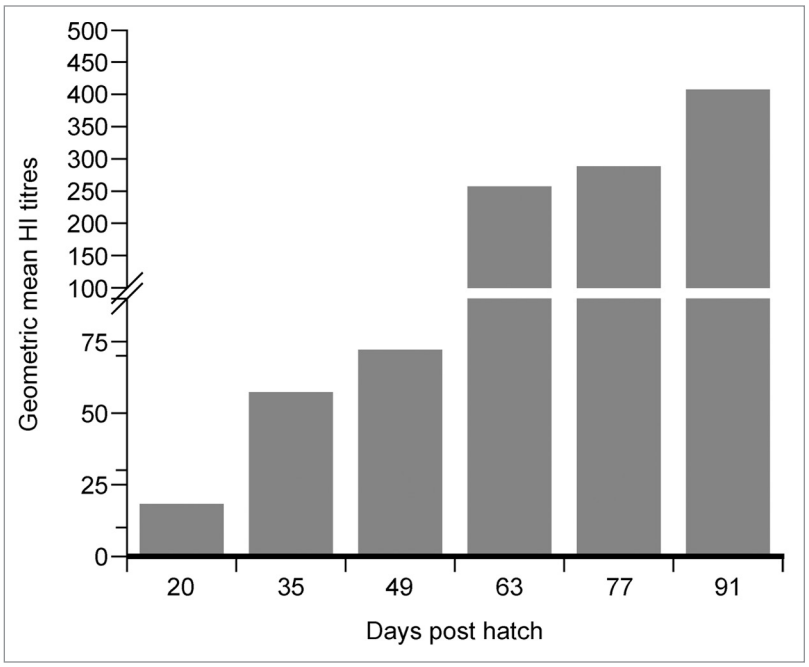


**Figure 2.** Induction of HA-specific immune responses in chickens vaccinated in ovo 3 d before hatching with rHVT-H7HA. Sera collected from vaccinated chickens (n = 8) at various time points until 91 d post-hatching and each sample was analyzed by HI assays and presented as geometric mean titers.

rHVT-H7HA vaccine was further examined for the induction of H7HA specific immune responses in chickens vaccinated in ovo 3 d before hatch.

## Immunity to H7HA Following In Ovo Vaccination with rHVT-H7HA

HVT vaccine has been widely employed in ovo.[4,31] To test the immunogenic efficacy of rHVT-H7HA in ovo, groups of ten 18 d embryonated eggs were injected with rHVT-H7HA or parental HVT as a control at 4,000 pfu/0.1 ml per egg. All vaccinated embryos were hatched and serum samples were collected at designated time points from 20 d post vaccination (dpv) until the termination of the experiment at 91 dpv. Seroconversion against H7HA was observed in 8 of 10 birds. The levels of HA-specific antibodies were measured with haemagglutination inhibition (HI) assays using homologous H7N1 virus. The HI titers at 20 dpv were 1:18 geometric mean titer (GMT) (range 1:8:1:32) and continuously and steadily increased to 1:406 GMT (range 1:128–2,048) until the termination of the experiment (**Fig. 2**). These results indicated that in ovo vaccination with rHVT-H7HA induced protective immune responses similar to that observed in post-hatch vaccination. Although we only monitored the duration of immune responses to 13 weeks, the results convincingly dictate that the higher levels of immunity produced by rHVT-H7HA should have persisted during the lifetime of the chicken, as was seen in HVT-based vaccines for MDV and IBDV.[24]

## Potential for Development of Multivalent HVT-Based Vaccines

The HVT genome contains many regions that are not critical for virus replication and could be available to be modified for expression of additional antigens of other subtypes (H5 and H9) of AI viruses or protective antigens of other viral pathogens affecting poultry such as NDV, IBDV and infectious bronchitis virus. These next generation HVT vector-based vaccines hold great promise to afford lifelong immunity in chickens by delivering a single dose of vaccine in ovo and providing simultaneous protection against multiple viral diseases affecting poultry worldwide.


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