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Recombinant turkey herpesvirus expressing H9 hemagglutinin providing protection against H9N2 avian influenza

Litao Liu, Tong Wang, Mingyang Wang, Qi Tong, Yipeng Sun, Juan Pu, Honglei Sun*, Jinhua Liu*

Key Laboratory of Animal Epidemiology and Zoonosis, Ministry of Agriculture, College of Veterinary Medicine, China Agricultural University, Beijing 100193, China

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ABSTRACT

H9N2 avian influenza viruses (AIVs) were prevailing in chickens, causing great economic losses and public health threats. In this study, turkey herpesviruses (HVT) was cloned as an infectious bacterial artificial chromosomes (BAC). Recombinant HVT (rHVT-H9) containing hemagglutinin (HA) gene from H9N2 virus were constructed *via* galactokinase (galK) selection and clustered regularly interspaced short palindromic repeats/associated 9 (CRISPR/Cas9) gene editing system. The recombinant rHVT-H9 showed no difference with parent HVT in plague morphology and virus replication kinetics. H9 protein expression of rHVT-H9 could be detected by western blot and indirect immunofluorescence assay (IFA) *in vitro* and *in vivo*. Immunization with rHVT-H9 could induce robust humoral and cellular immunity in chickens. In the challenge study, no chicken shed H9N2 virus from oropharynx and cloaca, and no H9N2 virus was found in viscera in vaccination groups. The result suggests that rHVT-H9 provides effective protection against H9N2 AIV in chickens.

1. Introduction

H9N2 avian influenza viruses (AIVs) were wildly spreaded and had become the most prevalent subtype of influenza virus in poultry in China. It caused reduction in egg production and increased mortality associated with co-infection with secondary pathogens (Choi et al., 2004; Dong et al., 2011; Li et al., 2005; Powder Technology Bi et al., 2011; Sun and Liu, 2015) resulting in severe economic losses. H9N2 AIVs could also transmit from avian species to mammals and humans causing public health threats (Yu et al., 2013). H9N2 AIVs could donated its gene segments to other emerging influenza avian viruses, including H5N2 (Gu et al., 2014), H6N1 (Cheung et al., 2007), H7N7 (Gu et al., 2014), H7N9 (Bi et al., 2015; Liu et al., 2013; Gao et al., 2013), and H10N8 viruses (Chen et al., 2014; Zhang et al., 2014). Among these emerging viruses, H7N9 AIVs have been circulating in domestic poultry in China and caused five waves of human infection resulted in 1625 human infections and 623 deaths until 25 August 2018 (http://www.fao.org/ag/againfo/programmes/en/empres/H7N9/Situation_update.html). Thus, control of the circulation of H9N2 viruses is essential for public health in China.

A vaccination program using inactivated vaccines has been used in China since 1998 to prevent H9N2 AIVs infection in chickens (Li et al., 2005). The inactivated vaccines provided protection from clinical signs, and minimized viral shedding. However, it could not eliminate the virus in chicken completely (Capua and Alexander, 2008). Therefore, H9N2 AIVs still circulated in farm chickens over the past two decades (Sun and Liu, 2015; Pu et al., 2015). Vaccinated chickens were investigated for H9N2 infection. The mean of H9N2 isolation rates increased from 22.08% in 2010 to 47.08% in 2013 (Pu et al., 2015), indicating that the inactivated vaccines could not provide effective protection. A new vaccine against the H9N2 is in need to protect domestic poultry in China.

Turkey herpesviruses (HVT), belonging to meleagrid herpesvirus 1, has been extensively used as a vaccine against Marek's disease (MD) for over 40 years (Okazaki et al., 1970a). Recently, HVT was considered as one of the most potent vectors for polyvalent live vaccines. It is used effectively for the induction of protective immunity against MD as well as the expressed antigen of other viral diseases (Swayne, 2009; Fuchs et al., 2009; Ge et al., 2007; Pavlova et al., 2009; Sonoda et al., 2000). The recombinant vaccine could induce lifetime protection against MD even in the presence of maternal antibodies after *in ovo* inoculation into the chicken embryo or subcutaneous administration into one-day-old chicks (Sonoda et al., 2000). HVT was used as a vector for protective antigens of other avian pathogens such as Newcastle disease virus (NDV) (Sondermeijer et al., 1993), infectious bursal disease virus (IBDV) (Darteil et al., 1995), and Eimeria acervulina (Cronenberg et al., 1999). However, these HVT recombinants were prepared using conventional genomic recombination techniques, which were extremely

* Corresponding authors.
E-mail addresses: shlei668@163.com (H. Sun), ljh@cau.edu.cn (J. Liu).

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time-consuming and labor-intensive. Recent technologies using the insertion of full-length genomes of herpesviruses into bacterial artificial chromosomes (BAC) (Shizuya et al., 1992; Messerle et al., 1997) and gene edition mediated by CRISPR/Cas9 (Cong et al., 2013) have revolutionized the field of recombinant HVT vaccine consturction.

In this study, HVT was successfully cloned as an infectious BAC (HVT-BAC). The HA gene of H9N2 virus was inserted, integrating with the human cytomegalovirus immediate-early promoter (CMV) or pec promoter into HVT-BAC. The BAC sequence was then deleted by CRISPR/Cas9 to construct the recombinant HVT (rHVT-H9). The rHVT-CMV-H9 and rHVT-pec-H9 virus were characterized *in vitro* and *in vivo*. Finally, their immune protection efficiency of rHVT-H9 in chickens were assessed. The results showed that the rHVT-pec-H9 could be a candidate vaccine for the prevention of H9N2 influenza.

2. Materials and methods

2.1. Ethics statement

All animal experiments were approved by Beijing Association for Science and Technology (approval ID SYXK [Beijing] 2007-0023) and conducted in strict accordance with Beijing Laboratory Animal Welfare and Ethics guidelines issued by Beijing Administration Committee of Laboratory Animals, as well as in accordance with China Agricultural University (CAU) Institutional Animal Care and Use Committee guidelines (ID: SKLAB-B-2010-005). Animal experimental protocol was approved by Animal Welfare Committee of CAU.

2.2. Viruses and cells

HVT Fc126 strain (NCBI accession number, AF291866.1) was kept. HVT or HVT recombinant virus was propagated in primary chicken embryonic fibroblasts (CEFs). The H9N2 virus strains A/chicken/Beijing/0701/2015 (BJ/15) were grown in 9-day-old specific pathogen free (SPF) embryonated hen eggs or in Madin-Darby canine kidney cells (MDCK). The virus titers were calculated as median egg infectious doses (EID_{50}) or median tissue culture infective dose ($TCID_{50}$) respectively. Primary CEFs and MDCK cells were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% antibiotics and 10% or 1% fetal bovine serum (FBS) respectively, at 37 °C with 5% CO_2 atmosphere.

2.3. Cloning HVT genome as an infectious BAC

PCR primers used in this study were listed in Table 1. The plasmid pBAC-US, 2.0- and 2.7-kbp fragments on either side of the HVT US gene (Fig. 1) were amplified by PCR using primers (left arm-F/R, right arm-F/R) containing appropriate restriction enzyme sites. Both fragments were cloned into pUC19 to obtain plasmid pUC-US. Green fluorescent protein (GFP) expression cassette under the control of CMV was amplified and inserted into the pBelloBAC11 (Invitrogen, USA) to construct BAC-GFP. The BAC-GFP was fragmented by BamHI and inserted into BamHI site of pUC-US to construct the transfer vector pBAC-US (Fig. 1). HVT Fc126 DNA was transfected into primary CEFs with pBAC-US to generate the recombined HVT-BAC virus. The viral DNA of HVT-BAC was prepared from infected cells and electroporated into *E. coli* DH10B to obtain HVT-BAC infectious clone (Adler et al., 2000). Electrocompetent bacteria were prepared, and electroporation was performed in 0.1-cm-wide cuvettes at 1250 V with resistance of 200 V and capacitance of 25 mF (Gene Pulser MXcell; BIO-RAD, USA). Transformed bacteria were incubated in Luria-Bertani (LB) medium and then plated on LB agar containing chloramphenicol. Single clones were picked and screened. Next-generation sequencing (NGS) combined with Sanger sequencing was used to determine the whole-genome sequences of HVT-BAC infectious clone. DNA were extracted from positive clones for NGS. The sequencing libraries with an insert size of 200 bp were prepared by end-repairing, dA-tailing, adaptor ligation, and PCR amplification according to the instructions provided by the manufacturer (Illumina, USA). The libraries were sequenced on an Illumina HiSeq. 4000 Sequencer by 101 bp paired-end sequencing. Sequencing depth was 2 G. Small-scale preparations of BAC DNA were performed by alkaline lysis of *E. coli*. Large-scale preparations of BAC DNA were achieved by silica-based affinity chromatography using commercially available kits (Macherey & Nagel, Germany).

2.4. Construction of recombinant HVT-BAC-H9 infectious clone

BJ/15 genomic RNA was extracted and purified using QIAamp Viral RNA Mini kit (QIAGEN, Germany) from the allantoic fluid generated by propagation. HA gene was amplified (H9-F/H9-R, Table 1) by RT-PCR from the RNA template. pcDNA-H9 was constructed by cloning the amplified fragment into eukaryotic expression vector pcDNA3.1 + (Invitrogen, USA) between KpnI and *Bam*HI restriction sites to form H9 expression cassette incorporated the CMV promoter at the 5′ end and bGH polyadenylation at the 3′ end. For constructing H9 expression cassette incorporated with pec promoter and bGH polyadenylation, pec promoter (Genbank: AF428265.1) was synthesized and inserted into pcDNA-H9 between *Nhe*I and *Kpn*I. H9 expression cassettes with CMV or with pec promoter were then amplified with primers (Homo-H9-F/R) containing 50 bp homology to the area flanking the insert site. A recombinant HVT-BAC containing galactokinase (galK) selection marker was developed according to recombineering protocol #3 (Warming) to construct a recombinant HVT-BAC harboring the H9 expression cassette. The galK gene cassette were amplified with primers (Homo-galK-

Table 1
List of primers used for rHVT-H9 construction.

Primer	Sequence (5′–3′)	Length
left arm-F	GCTGTCGACCACATCGGGCCACGTTCCGCC	2061 bp
left arm-R	CGCGGATCCGCTGACGTGTGGAATTATTCAATG	
right arm -F	GCAGGATCCCGGACTAATATGGGCACACCC	2696 bp
right arm -R	CTAGGTACCTGGCCCATCTAGGTGATTAT	
pec-F	CGCGCTAGCAGTTATTAATAGTAATCAAT	561 bp
pec-R	GGGGTACCCCTCTAGACGCGGTCAGTCA	
H9-F	GGGGTACCATGGAGACAGTATCACTAAT	1683 bp
H9-R	CGGGATCCTCATATACAAATGTTGCATCT	
Homo-galK-F	ATTATTATGAAGTCTACGCCTCTGTCCTCTCTAATGCGATGAGTAAATAACCTGTTGACAATTAATCATCG	1296 bp
Homo-galK-R	CTATGGTGTGATTGGATAAATAAAACACAGTAACCGTTAGAGGTGCGTTTTCAGCACTGTCCTGCTCCTT	
Homo H9-F	CTATGGTGTGATTGGATAAATAAAACACAGTAACCGTTAGAGGTGCGTTTAGTTATTAATAGTAATCAAT	2673 bp
Homo H9-R	ATTATTATGAAGTCTACGCCTCTGTCCTCTCTAATGCGATGAGTAAATAACATAGAGCCCACCGCATC	
sgRNA-F	CACCGAATGCGGATCTCTACGATAA	25 bp
sgRNA-R	AAACTTATCGTAGAGATCCGCATTC	
donor-F	GGCGGGCAAGAGTGTTCCAAACATTT	1575 bp
donor-R	GTTGGATGATATAAAATATATAG	

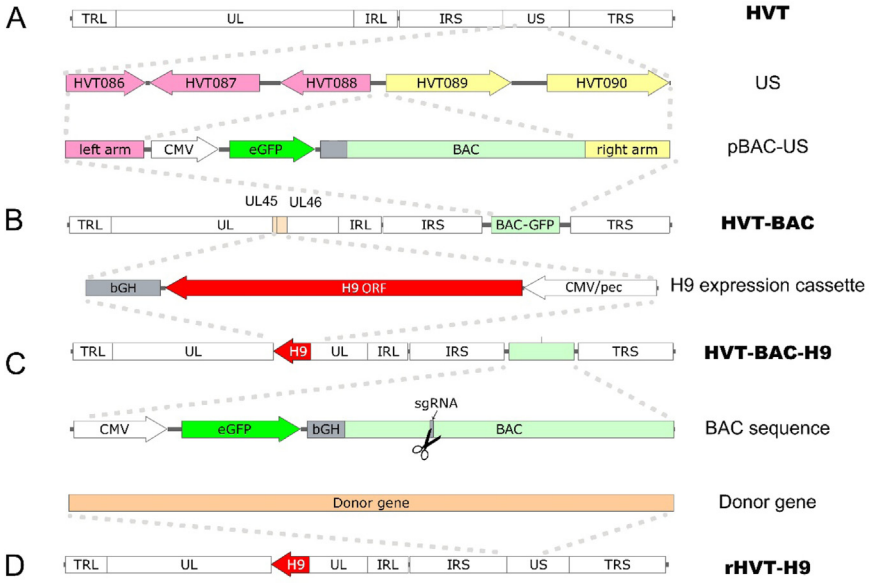

Fig. 1. Schematic presentation of the construction of rHVT-H9. (A) The organization of the approximately 159 kbp HVT genome was shown. HVT DNA was transfected into CEFs together with transfer vector pBAC-US; BAC was introduced into HVT US region and the recombinant HVT-BAC virus was constructed (B). DNA of HVT-BAC virus was extracted and electroporated into DH10B to obtain the infectious clone HVT-BAC (B) sequence which is identical to HVT-BAC virus. The expression cassette of H9 gene of BJ/15 (H9N2) virus under the control of CMV or pec promoter was inserted into HVT-BAC infectious clone between the UL45 and UL46 genes of HVT genome to construct the HVT-BAC-H9 (C) by galK selection in SW102. HVT-BAC-H9 DNA was transfected with CRISPR/Cas9 and donor gene into CEFs to reconstitute rHVT H9 (D) without BAC sequence to delete BAC sequence.

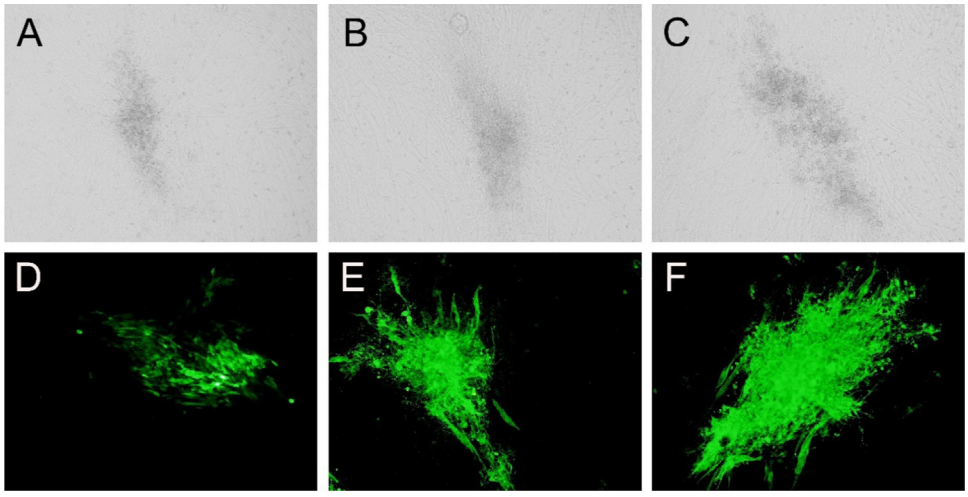

Fig. 2. Cytopathic effect and IFA detection of rHVT-H9. Morphology of infected CEFs induced by parental HVT (A), rHVT-CMV-H9 (B) and rHVT-pec-H9 (C) was observed by inverse microscopy (magnifications 100×) at 5 days p.i. (D) DNA of HVT-BAC infectious clone was extracted and transfected into primary CEFs. Plaques were checked by inverse microscopy at 4 days post transfection. CEFs infected with rHVT-CMV-H9 (E) and rHVT-pec-H9 (F) were detected by probing with H9-MS and fluorescence-conjugated anti-mouse IgG at 5 days p.i. Observation by inverse microscopy (magnifications 100×).

F/R, Table 1) containing 50 bp homology to the area flanking the insert site. Clones of HVT-BAC-UL45-galK-UL46 were screened and cultured on the M63 minimal media plates. Then, galK gene in the HVT-BAC-UL45-galK-UL46 clones were substituted by H9 gene expression cassettes with CMV or with pec promoter (UL45-bGH polyadenylation-H9HA-CMV-UL46) after transforming PCR applications flanking with homology into electrocompetent SW102 bacteria (Warming et al., 2005). Positive clones HVT-BAC-H9 were grown on 2-deoxy-galactose (DOG) and chloramphenicol-containing minimal media plates with glycerol as the carbon source. They were then screened and confirmed by sequence analysis.

2.5. Generation of recombinant rHVT-H9 virus

The CRISPR/Cas9 mediated gene editing system was used to completely remove the BAC insertion. The sequences containing the targeting regions were submitted to CRISPR Design Tool (http://crispr.mit.edu/, Zhang Feng's Lab). sgRNA with highest scores was chosen. The oligos of the sgRNA (sgRNA-F/R, Table 1) were synthesized and cloned into sgRNA/Cas9 cloning vector pX458 (catalog no. 48138; Addgene, USA) to construct pX458-sgRNA in accordance with the manufacturer's instructions. To delete the inserted BAC sequence, PCR applications (donor-F/R, Table 1) identical to HVT sequences cover the target site were transfected together as a donor gene (Fig. 1) for homologous recombination. pX458-sgRNA, donor gene and HVT-BAC-H9 DNA were cotransfected into CEFs using lipofectamine®2000 transfection reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The cells were kept at 37 °C in 5% CO_2 incubator for 6 days. The plaques without expressing GFP were screened using inverted fluorescence microscope and purified.

2.6. Virus growth and plaque assays

Virus infectivity and growth characteristics of rHVT-CMV-H9 or rHVT-pec-H9 *in vitro* were examined by infecting CEFs and checked by IFA. For virus growth detection, CEFs were seeded in 6-well plates. HVT, rHVT-CMV-H9 and rHVT-pec-H9 virus was inoculated into CEFs with 100 plaques forming unit (PFU) per well respectively. Cells were harvested and titrated at 24, 48, 72, 96 and 120 h post inoculation (p.i.). To titrate HVT viruses, CEFs seeded in 96-well plates were inoculated with 2-fold dilutions of HVT, rHVT-CMV-H9 or rHVT-pec-H9 virus-infected CEFs. On day 4 p.i., the cells were checked by IFA. CEFs were fixed with ice-cold ethanol-acetone 3:2 (v/v) for 20 min at room temperature, then washed twice with phosphate buffered saline (PBS). Plaques were identified by incubating the cells with monoclonal antibody (BD8) against HVT and mouse antiserum against BJ/15 (H9-MS)

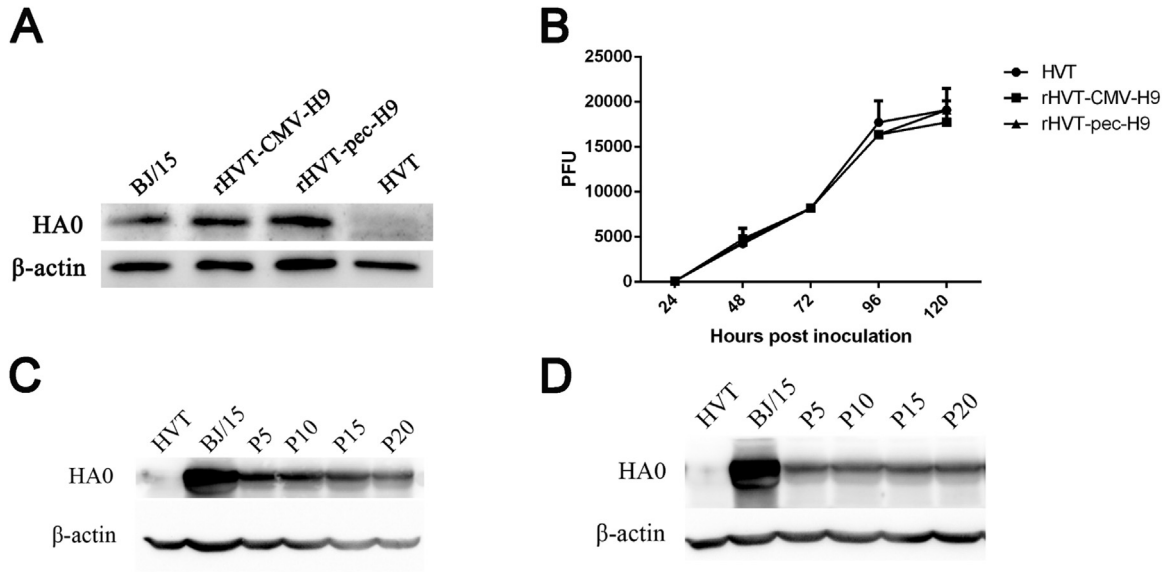

Fig. 3. Western blot analysis and growth curve of rHVT-H9. (A) Western blot analysis of rHVT-H9. CEFs were inoculated with BJ/15, rHVT-CMV-H9, rHVT-pec-H9 and HVT virus. Cells were harvested to examine HA0 expression by western blot at 4 days p.i. (B) Growth curve of rHVT-H9. CEFs were seeded in 6-well plates. HVT, rHVT-CMV-H9 or rHVT-pec-H9 virus was inoculated into CEFs with 100 PFU per well. Cells were harvested and titrated at 24, 48, 72, 96 and 120 h p.i. The error bars indicate standard deviations. rHVT-CMV-H9 (C) or rHVT-pec-H9 (D) was grown sequentially in CEF cells for 20 passages. Expression of HA glycoprotein was examined every five passages by western blot analysis.

for 1 h, respectively. Then, washed three times with PBS, and incubating for 1 h with fluorescence conjugated goat anti-mouse immunoglobulin G in 37 °C incubator. The cells were washed twice with PBS containing 0.1% Tween-20 (PBS-T). Finally, plaques were analyzed and counted using inverted fluorescence microscope at the dilution giving the easiest distinction between plaques.

2.7. Western blot analysis

Expression of HA in rHVT-H9 infected CEFs was determined by western blot analysis using H9-MS. CEFs grown in 6-well plates were inoculated at a multiplicity of infection (MOI) of 0.1 with BJ/15, rHVT-CMV-H9, rHVT-pec-H9 and HVT virus. After 4 days p.i., the cells were harvested and treated with SDS-PAGE loading buffer. 10 μl sample was subjected to SDS-PAGE, and the separated proteins were electroblotted on polyvinylidene fluoride (PVDF) membranes. After blocking with 5% (w/v) skimmed milk dissolved in PBS containing 0.1% Tween-20 (PBS-T), the membrane was probed with H9-MS and horseradish peroxidase-conjugated anti-mouse IgG. The HA glycoprotein bands were visualized using ECL detection reagents.

2.8. Stability analysis of rHVT-H9

To test genetic stability, rHVT-CMV-H9 or rHVT-pec-H9 was grown sequentially in CEF cells for 20 passages. Expression of HA glycoprotein was examined every five passages by western blot analysis and the integrity of HA gene insert was examined by PCR with Homo H9-F/R primers using DNA extracted from every five passages. The PCR product was analyzed on 1% w/v agarose gel and stained with ethidium bromide.

2.9. Protective efficacy of the rHVT-H9 virus in chickens

To examine protective efficacy of the rHVT-H9, one-day-old white Leghorn SPF chicks (sex sale linked) provided by Beijing Boehringer Ingelheim Vital Biotechnology Co., Ltd., were randomly divided into 3 groups (n = 9) and vaccinated subcutaneously with 3000 plaque forming units (PFU) rHVT-CMV-H9, rHVT-pec-H9 and parental HVT, respectively. Randomly, blood samples were collected weekly from five birds in each group until 5 weeks post vaccination (WPV). Serum antibody titers specific for H9 were measured using haemagglutinin inhibition (HI) and virus neutralization (VN) (Cox et al., 2005; Cottey et al., 2001). The re-isolation of HVT, rHVT-CMV-H9 or rHVT-pec-H9 in vaccinated birds was conducted by inoculating peripheral blood lymphocytes (PBL), isolated weekly from whole blood by centrifugation over 70% percoll (Solarbio, China), into CEFs. Then cells inoculated with PBL from chickens vaccinated with HVT or rHVT-H9 were harvested, and checked by IFA using BD8 or H9-MS, respectively, at 6 days p.i. In 35 days (0 day post challenge, DPC), chickens were challenged intranasally with BJ/15 virus at 0.1 mL of 10^4 EID_{50}. Oropharyngeal and cloacal swabs, and viscera including trachea, lung, kidney and spleen were collected on 3 and 5 DPC for viral detection and titrated by EID_{50}. A portion of the trachea and lung tissue challenged with BJ/15 at 3 DPC was preserved in 10% phosphate-buffered formalin. Samples were processed for paraffin embedding and were cut into 5 μm sections and stained with hematoxylin and eosin (H&E).

2.10. Examination of T lymphocytes expressing IFN-γ in lungs

T lymphocytes expressing IFN-γ were isolated and screened as previously reported (Wei et al., 2016). Lung tissues were digested with Dnase I and collagenase A for homogenization. Red blood cells were lysed. Cell suspensions from the lung tissues were incubated for 6 h at 37 °C under 5% CO_2 in the presence of BJ/15 at MOI of 1, compared with rhIL-2, and Brefeldin A at 0 h for CD8 + responses, or 2 h for CD4 + responses. At 6 h, cells were washed and stained with anti-CD8-SPRD and anti-CD4-PE (Southern Biotech, USA) for 30 min on ice. For intracellular IFN-γ staining, cells were permeabilized using Cytofix/Cyoperm (BD Biosciences, USA), first stained with rabbit anti-chicken IFN-γ antibody (AbD Serotec, USA) for 30 min on ice, then stained with FITC-labeled anti-rabbit antibody (Abcam, UK) for 30 min on ice. The stained cells were evaluated by flow cytometry (BD Biosciences, USA) for expression of CD4, CD8, and IFN-γ. Data were analyzed using the FlowJo Software (Tree Star, USA).

2.11. Statistical analyses

Statistically significant differences between experimental groups

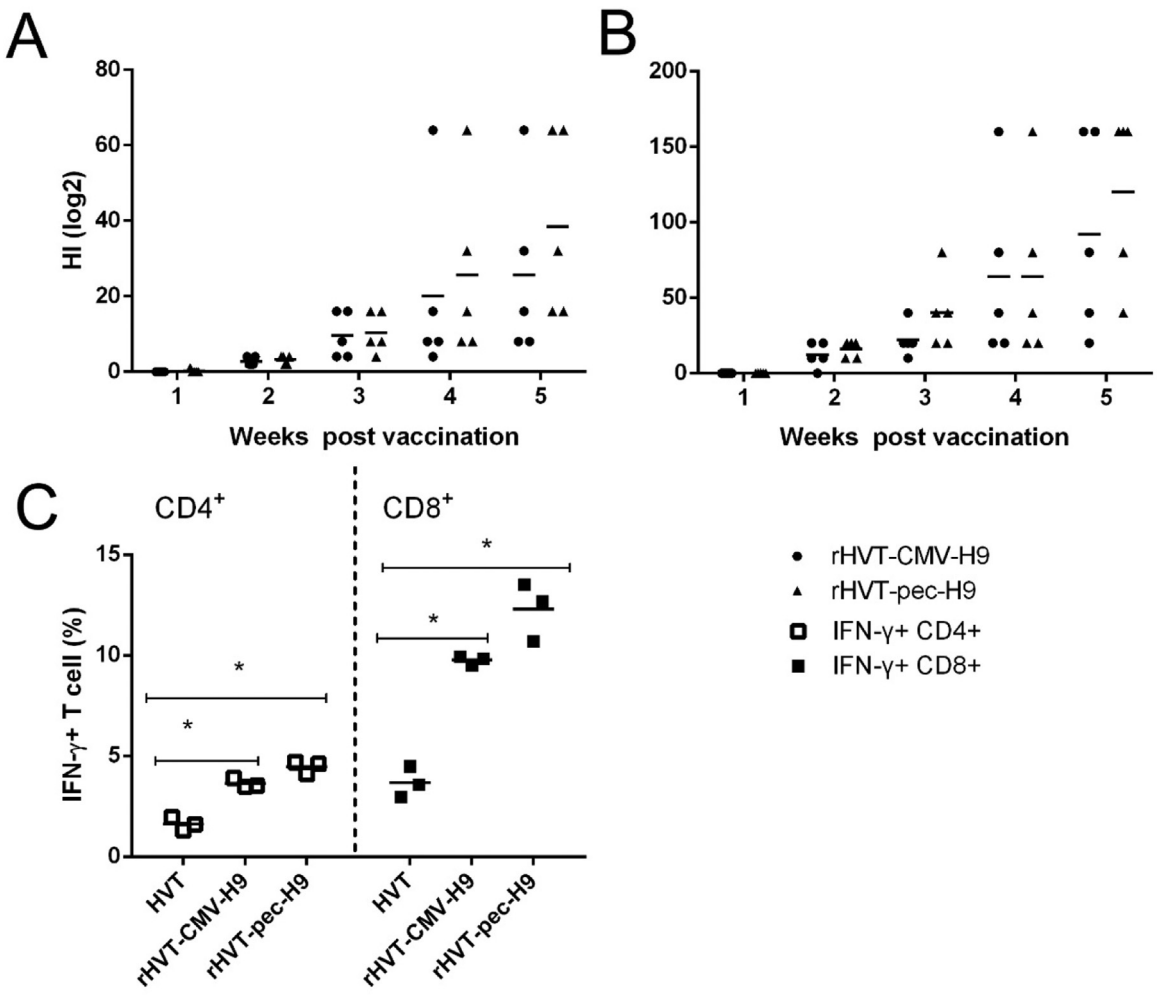

Fig. 4. Immune responses induced by rHVT-H9. Sera were collected from the rHVT-CMV-H9 and rHVT-pec-H9 immunized chickens (n = 5) weekly after vaccination. (A) HI antibody analysis. Sera collected weekly were detected by HI assays response to BJ/15 H9N2 IAV. (B) VN antibody analysis. Sera collected weekly were analyzed by VN assays response to BJ/15 virus in MDCK cells. (C) Cellular immune response analysis. Chicks were vaccinated with rHVT-CMV-H9, rHVT-pec-H9 or HVT at One-day-old. Lungs (n = 3) were harvested at 35 DPV. IFN-γ + CD4 + and IFN-γ + CD8 + T cells in lungs stimulated with BJ/15 were identified by intracellular cytokine staining assay. Percentages of IFN-γ + CD4 + or IFN-γ + CD8 + T cells within CD4 + or CD8 + T cells were analyzed, $*P < 0.05$, by one-way analysis of variance of the difference between rHVT-CMV-H9, rHVT-pec-H9 and HVT vaccine group at 35 DPV.

were determined using one-way analysis of variance with the GraphPad Prism software (Version 6.01). Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Generation of rHVT-H9 recombinant virus

The recombinant HVT-BAC virus was made by transferring plasmid pBAC-US into primary CEFs together with DNA of HVT Fc126. DNA extracted from recombinant HVT-BAC virus was transformed to *E. coli* DH10B (Fig. 1). The transformed bacteria were plated on agar containing chloramphenicol. Single clones were picked and screened. Clones containing high molecular mass extrachromosomal DNA were selected for further analysis. DNA of positive clones was extracted and transfected into primary CEFs. Plaques expressing GFP (Fig. 2 D) were visible about 4 days post transfection demonstrating their infectivity. To detect whether sequences were mutated during the construction of HVT-BAC, deep sequence analysis was used to scan the genome of HVT-BAC. No mutation was detected. The sequences were fully consistent with the expectation.

HA gene of BJ/15 was inserted into HVT-BAC under the control of CMV or pec promoter through galK selection to construct HVT-BAC-H9 in a modified bacterial strain, SW102 (Fig. 1). To delete the BAC sequences, CRISPR/Cas9 mediated gene editing system was used to recovery the gene scar. DNA of HVT-BAC-H9 was transfected into primary CEFs together with pX458-sgRNA expressing Cas9 and sgRNA, and a donor gene containing the homologous sequences identical to the US of HVT (Fig. 1). Positive plaques of recombinant rHVT-H9 virus without expressing GFP were screened by inverted fluorescence microscope and purified by picking and propagating them into fresh CEFs.

3.2. Characterization of rHVT-H9 in vitro and in vivo

To characterize the rHVT-H9 viruses, rHVT-CMV-H9 or rHVT-pec-H9 was inoculated into CEFs. H9-primed PCR analysis of the rHVT genomic DNA extracted from infected CEFs was conducted. The sequencing analysis showed that the H9 gene was integrated into the HVT. The plaques of rHVT-CMV-H9 and rHVT-pec-H9 had similar size and morphology with parental HVT (Fig. 2 A, B & C). CEFs infected with rHVT-CMV-H9 or rHVT-pec-H9 were harvested. IFA and western blot confirmed the H9 gene expression. The results showed plaques could be detected by probing with H9-MS (Fig. 2 E & F). Western blot analysis demonstrated distinct bands of HA0 approximately 78 kDa in the positive control sample infected with BJ/15 virus. The cell lysates of both rHVT-CMV-H9 and rHVT-pec-H9 showed band of HA0

Table 2
Vaccine protection against H9N2 influenza.

Vaccination	Virus shedding of challenged chickens				Virus replication in viscera of challenged chickens								
	3 DPC		5 DPC		3 DPC				5 DPC				
	Oropharynx	Cloaca	Oropharynx	Cloaca	Trachea	Lung	Kidney	Spleen	Trachea	Lung	Kidney	Spleen
rHVT-CMV-H9	0/6	0/6	0/6	0/6	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
rHVT-pec-H9	0/6	0/6	0/6	0/6	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
HVT	6/6[a] (5.17 ± 0.56)	3/6 (1.08 ± 0.58)	3/3 (5.91 ± 0.29)	2/3 (2.0)	3/3 (4.6 ± 0.57)	3/3 (5.8 ± 0.57)	0/3	0/3	3/3 (4.5 ± 0.35)	3/3 (3.9 ± 0.52)	1/3 (1.75)	2/3 (2.0)

One-day-old SPF chicks were immunized intranasally with 3000 PFU rHVT-CMV-H9, rHVT-pec-H9 or HVT viruses. Chickens were challenged intranasally with 10^4 EID_{50} of BJ/15 virus at 35 DPV. Titers of H9N2 virus shedding in oropharyngeal and cloacal swabs, and H9N2 virus replication in organs including trachea, lung, kidney and spleen on 3 and 5 DPC were determined.

[a] Number of chickens positive for virus/total number of chickens tested (mean titers [log_{10} EID_{50}/mL] ± standard errors).

glycoprotein. No detectable band was observed in CEFs lysates infected with parental HVT (Fig. 3 A).

Growth kinetics of rHVT-CMV-H9, rHVT-pec-H9 and parent HVT were analyzed in CEFs. Viruses were inoculated into primary CEFs with 100 PFU per well in 6 wells-plate. Cells were harvested at different time points and titrated. The results showed that rHVT-H9 virus grew well in CEFs and had no difference in replication ability compared with parent HVT virus *in vitro* (Fig. 3 B). rHVT-H9, rHVT-CMV-H9 or rHVT-pec-H9 was grown sequentially in CEF cells for 20 passages for genetic stability analysis. No variability in size and amount of HA gene product following repeated passages of the virus in cultured CEF were found indicating the H9 gene inserted HVT genome was stable. Similarly, no difference in the expression of the recombinant HA glycoprotein was observed when rHVT-H9-infected cell lysates were analyzed after every five passages by western blot analysis (Fig. 3 C & D). Peripheral blood lymphocytes were isolated from whole chicken blood by centrifugation over 70% percoll, and inoculated into CEFs. Cells were harvested and checked by IFA at 6 days p.i. Weekly detection of parent HVT virus, rHVT-CMV-H9 and rHVT-pec-H9 virus demonstrated its well replication *in vivo*.

3.3. Immune responses induced by rHVT-H9 in chicken

To assess the immunogenicity of rHVT-H9 in chickens, sera were collected weekly and checked by HI and VN after vaccination at one day old. The earliest immune responses to the HA glycoprotein was at 2 WPV. The geometric mean titers (GMT) of antibody induced by rHVT-CMV-H9 reached 1:2.8 (range 1:2–4) for HI and 1:12 (range 1:0–20) for VN. GMT of antibody induced by rHVT-pec-H9 were slightly higher, reaching 1:3.2 (range 1:2–4) for HI and 1:16 (range 1:10–20) for VN. At 5 WPV, the GMT of antibody induced by rHVT-CMV-H9 reached 1:25.6 (range 1:8–64) for HI and 1:92 (range 1:20–160) for VN. GTM of antibody induced by rHVT-pec-H9 reached 1:38.4 (range 1:16–64) for HI and 1:120 (range 1:40–160) for VN (Fig. 4 A & B). The results showed that rHVT-H9 was highly immunogenic to induce humoral immunity, and pec promoter could induce higher antibody titers than CMV.

Lung cells from chickens were harvested after rHVT-H9 vaccination and stimulated with BJ/15 AIV *in vitro* for measurement of their cellular immune responses. IFN-γ-secreting T cells were identified by intracellular cytokine staining assay. The results showed that the influenza virus-specific IFN-γ + CD4 + in lungs vaccinated with rHVT-CMV-H9 shared 3.64% of CD4 + , and IFN-γ + CD8 + T cells shared 9.77% of CD8 + T cells at 35 days post vaccination (DPV). The IFN-γ + CD4 + or IFN-γ + CD8 + T cells in lungs vaccinated with rHVT-pec-H9 were 4.47% and 12.30% respectively, which is higher than rHVT-CMV-H9. IFN-γ + CD4 + and IFN-γ + CD8 + levels of rHVT-CMV-H9 and rHVT-pec-H9 vaccinated lung cells were significantly ($P < 0.05$) higher than HVT vaccinated samples (IFN-γ + CD4 + = 1.63% and IFN-γ + CD8 + = 3.68%), indicating that rHVT-CMV-H9 and rHVT-pec-H9 vaccination could induce efficacious cellular immune responses. The results showed that rHVT-H9 was highly immunogenic that it could induced humoral and cellular immunity, and pec promoter could induce higher level humoral and cellular immune response than CMV.

3.4. Protective efficacy of rHVT-H9 in chickens

One-day-old chicks were immunized subcutaneously with HVT, rHVT-CMV-H9 and rHVT-pec-H9, respectively. At 35 DPV, chickens were challenged with BJ/15 H9N2 influenza viruses intranasally. Chickens vaccinated with HVT showed obvious symptoms with depression and drowsiness after challenge. H9N2 viruses were not detected in oropharynx or cloaca in chickens vaccinated with rHVT-CMV-H9 or rHVT-pec-H9 virus at 3 or 5 DPC. No H9N2 virus was detected in viscera (trachea, lung, spleen and kidney). In contrast, chickens vaccinated with HVT shed H9N2 virus with mean titer of 5.17 log_{10} EID_{50}/

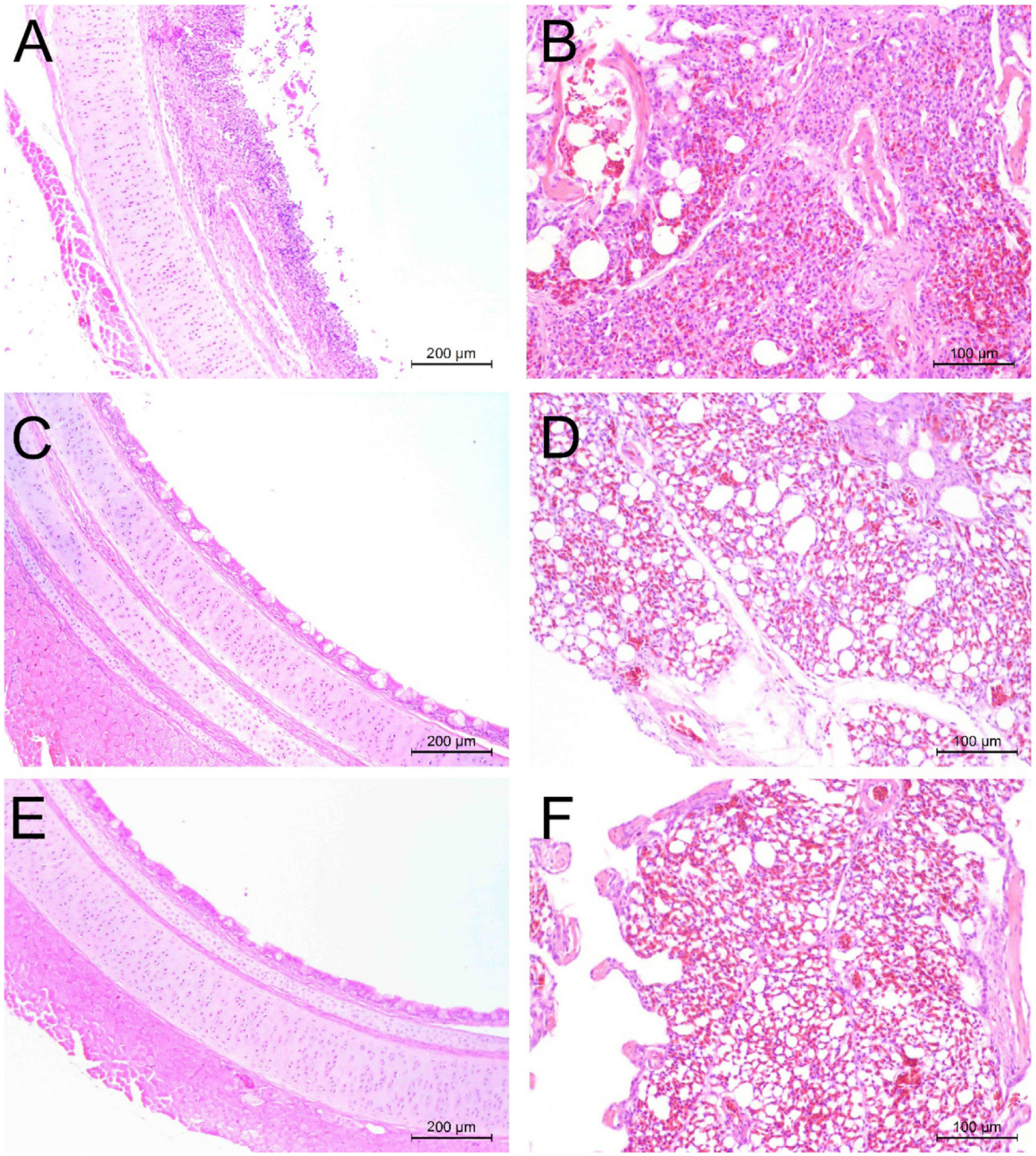

Fig. 5. Histopathological analysis of trachea and lungs vaccinated rHVT-H9. (A) Trachea and lungs (B) vaccinated with HVT showed serious damage at 3 DPC. (C) Trachea and lungs (D) vaccinated with rHVT-CMV-H9 showed no histopathological changes at 3 DPC. (E) Trachea and lungs (F) vaccinated with rHVT-pec-H9 showed no histopathological changes at 3 DPC. Sections were stained with hematoxylin and eosin.

0.1 mL in oropharynx, 1.08 $\log_{10}$ EID_{50}/0.1 mL in cloaca at 3 DPC, and 5.91 $\log_{10}$ EID_{50}/0.1 mL in oropharynx and 2.00 $\log_{10}$ EID_{50}/0.1 mL in cloaca at 5 DPC. H9N2 virus was also detected in viscera of chickens vaccinated with HVT (Table 2). The results showed that rHVT-H9 provided efficient protection against H9N2 influenza.

Pathological studies were performed on trachea and lung samples from chickens challenged with BJ/15 on 3 DPC. Pathological changes of chickens vaccinated with HVT showed severe lesions. Extensive consolidation in lungs were found with hyperemia, edema and extensive infiltration of mesenchyme and inflammatory cells (Fig. 4 B). Tracheitis were observed characterized by infiltration of inflammatory cells in the tracheal mucosa accompanied with necrosis and exfoliation of the epithelial cells (Fig. 4 A). No obvious damage was found in lungs and trachea in the chickens vaccinated with rHVT-CMV-H9 (Fig. 4 C & D) or rHVT-pec-H9 (Fig. 4 E & F). Overall, the results showed that rHVT-H9 provided efficient protection against H9N2 influenza. (Fig. 5).

4. Discussion

H9N2 avian influenza viruses were wildly spread and had become the most prevalent subtype of influenza virus in poultry in China suggesting that the H9N2 inactivated vaccine was not effective enough (Sun et al., 2012). New vaccines to control H9N2 AIVs circulation is urgently needed. In this study, HVT-BAC infectious clones were recombined with HA of H9N2 influenza virus using galK selection. rHVT-H9 recombinant virus was generated by deleting BAC sequences *via* CRISPR/Cas9 mediated gene-editing system. After vaccination, rHVT-CMV-H9 and rHVT-pec-H9 recombinant virus could induce robust humoral and cellular immunity, which provided efficient protection against BJ/15 after challenging at 10^4 EID_{50}. The results showed that

rHVT-H9 protected chickens from H9N2 influenza virus infection effectively, and rHVT-pec-H9 could be a live vaccine candidate against H9N2 AIVs.

H9N2 inactivated vaccines provided protection from clinical disease and minimized viral shedding. However, there were some inherent shortcomings of the inactivated vaccine limited its protective efficacy. Inactivated vaccines could induce humoral immunity, but lack cellular immunity. The level of systemic and local T-cell responses was inversely correlated with the level of heterologous virus replication in the upper respiratory tract (Cheng et al., 2013). The recombinant HVT virus could induce humoral as well as cellular immunity, which provided efficient protection. Rauw et al. (2010) analyzed the immune responses induced by the vaccination prime with rHVT-ND and boosted with live ND-chitosan. They found an increasing in cell-mediated immune responses in spleen and blood, indicating that rHVT-ND could stimulate cellular immunity. Darrell R. Kapczynskia et al. Kapczynski et al. (2015) demonstrated cross reactive CTL activity against the HA induced by the HVT-H5 vector vaccine that recognized different subtypes of AIVs. In their study, T lymphocytes in lungs were isolated and stimulated with BJ/15 AIV following by staining with specific antibody. IFN-γ + CD4 + and IFN-γ + CD8 + T cells were determined to assess the level of cellular immune response induced by rHVT-H9. The results showed that rHVT-H9 could induce robust cell immune responses at 35 DPV, which was significantly higher than HVT. In the challenging study, vaccination with rHVT-H9 provided nice protection against H9N2, in which the strong cellular response played an important role. Antibodies induced by inactivated vaccines are maintained only for a short period of time, which require repeated immunization to maintain high antibody levels. Repeated vaccination process brings massive stress to animals and is a huge workload to farms workers, therefore, reduces productivity and increases management costs. HVT as an ideal avian vaccine vector candidate can induce lifetime protection against MD as well as the diseases which the antigens were recombined into the HVT (Palya et al., 2014). Moreover, inactivated vaccines induced slower antibody production. The delayed antibody production could miss the best protection time against H9N2. HVT vector vaccine could also provide protection against infectious bursal disease of chickens in the face of high-titred maternally derived antibody (Bublot et al., 2007). In short, rHVT-H9 may be an ideal live vaccine candidate to control the H9N2 AIV circulation.

The activity of promoters has been one of the major factors affecting the antigen expression and protective efficacy of recombinant vaccines. Sonoda et al. (2000) reported that recombinant Marek's disease virus (rMDV) containing the simian virus 40 promoter expressed more NDV-F proteins *in vitro*. rMDV containing MDV glycoprotein B promoter induced much stronger and faster antibody response and provided complete protection against NDV challenge ignore maternal antibodies. Tsukamoto et al. (2002) constructed recombinant HVT virus expressing a viral protein (VP2) of IBDV under controls of different promoters (rHVT-pecVP2 with pec promoter and rHVT-cmvVP2 with CMV promoter). They found that rHVT-pecVP2 expressed four times more of the VP2 antigen than rHVT-cmvVP2 *in vitro*. rHVT-pecVP2 also induced complete protection against a lethal IBDV challenge in chickens, whereas rHVT-cmvVP2 induced 58% protection. Consistently, Li et al. (2016) showed that recombinant MDV with VP2 insertion under the control of pec promoter harbored higher VP2 expression and had stronger antibody responses against IBDV in chickens with CMV promoter. In our study, rHVT-H9 was constructed under the control of CMV and pec promoter. rHVT-pec-H9 induced higher level of HI and VN antibody titers than rHVT-CMV-H9. IFN-γ + CD4 + and IFN-γ + CD8 + T cell levels from lung tissues immunized with rHVT-pec-H9 were also higher than rHVT-CMV-H9, indicating a stronger cellular immune response. The results suggested that rHVT-pec-H9 was a better vector vaccine candidate for protection against H9N2 influenza.

HVT as a vaccine against MDV have been used since 1970s (Okazaki et al., 1970b). No human health threat reported yet implying the safety of the HVT virus. Now, some HVT recombinant vaccines, such as VAXXITEK® HVT + IBD (Merial, USA) were widely used in poultry industry. These recombinant vaccines were safe to poultry and human. rHVT-H9 shared similar biological characteristics with HVT, suggesting that rHVT-H9 might also pose no potential threat to human health. In summary, in this study, HVT vector vaccine expressing HA of H9N2 AIV, rHVT-CMV-H9 and rHVT-pec-H9 virus were created. The recombinant viruses could induce humoral as well as cellular immunity, which provided efficient protection against H9N2 influenza. The recombinant rHVT-H9 may be a candidate for prevention and control of H9N2 influenza in the future.

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Conflict of interest

The authors declare they have no conflicts of interest.

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