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Recombinant duck enteritis virus bearing the hemagglutinin genes of H5 and H7 influenza viruses is an ideal multivalent live vaccine in ducks

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ABSTRACT

Due to the fact that many avian influenza viruses that kill chickens are not lethal to ducks, farmers are reluctant to use avian influenza inactivated vaccines on ducks. Large numbers of unvaccinated ducks play an important role in the transmission of avian influenza viruses from wild birds to domestic poultry, creating a substantial challenge to vaccination strategies for avian influenza control. To solve this problem, we constructed a recombinant duck enteritis virus (DEV), rDEV-dH5/H7, using a live attenuated DEV vaccine strain (vDEV) as a vector. rDEV-dH5/H7 carries the hemagglutinin gene of two H5 viruses [GZ/S4184/17 (H5N6) (clade 2.3.4.4 h) and LN/SD007/17 (H5N1) (clade 2.3.2.1d)] and an H7 virus [GX/SD098/17 (H7N9)]. These three hemagglutinin genes were stably inherited in rDEV-dH5/H7 and expressed in rDEV-dH5/H7-infected cells. Animal studies revealed that rDEV-dH5/H7 and vDEV induced similar neutralizing antibody responses and protection against lethal DEV challenge. Importantly, rDEV-dH5/H7 induced strong and long-lasting hemagglutinin inhibition antibodies against different H5 and H7 viruses and provided complete protection against challenges with homologous and heterologous highly pathogenic H5 and H7 influenza viruses in ducks. Our study shows that rDEV-dH5/H7 could serve as an ideal live attenuated vaccine to protect ducks against infection with lethal DEV and highly pathogenic avian influenza viruses.

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KEYWORDS Duck; H5 avian influenza virus; H7 avian influenza virus; multivalent vaccine; duck enteritis virus

Introduction

Avian influenza viruses continuously challenge the poultry industry and human health. According to the OIE World Animal Health Information System (OIE-WAHIS) and World Health Organization (WHO) websites, several subtypes of H5 and H7 viruses have caused 2634 human cases around the world, including more than 1000 deaths. These viruses have also caused multiple disease outbreaks in wild birds and poultry, killing at least 422 million poultry since 2005 [1].

Since 2003, the hemagglutinin (HA) gene of H5 viruses has evolved into 10 different phylogenetic clades, from clade 0 to clade 9, and the clade 2 viruses have further evolved into different sub-clades [2–5]. H5 viruses bearing the clade 2.3.2 or clade 2.3.4.4 HA gene have spread to multiple countries and continents [6–19]. Since 2020, the ongoing third wave of this century's H5 influenza epidemic, mainly driven by H5N8 and H5N1 viruses, has led to the death of 193.9 million poultry through infection or culling [1]. More importantly, these viruses have transmitted to humans and caused severe disease and deaths [20]. In early 2013, human infections caused by a novel H7N9 virus were reported in China [21,22]. Genetic analysis suggested an epidemiological bridge from migratory birds to farm ducks and then to market birds [21,23–25]. From 2013 to 2017, the new H7N9 virus caused five waves of human infections, resulting in 1,564 human cases and 615 deaths [26–28], which raised global concerns that the H7N9 virus could cause a new influenza pandemic[26,29,30]. Nationwide vaccination of poultry with an H5/H7 bivalent inactivated avian influenza vaccine that was initiated in September 2017 has successfully controlled H7N9 avian influenza infections in poultry and eliminated

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human infections [1,27,31–33], although the H7N9 virus has not yet been eradicated from poultry.

Wild birds, which are natural reservoirs for avian influenza viruses [6,34–38], share water sources and foods with ducks that are often farmed in open fields with no biosecurity measures [39]. Since many H5 and H7 viruses replicate asymptomatically in domestic ducks, farmers are not motivated to vaccinate their ducks with the avian influenza vaccine, which allows large numbers of unvaccinated ducks to play an important role in the transmission of avian influenza viruses from wild birds to domestic poultry [5,26,40,41]. Therefore, a vaccine strategy that protects against both a deadly pathogen in ducks and the avian influenza virus is highly desirable.

Lethal duck enteritis virus (DEV), a herpesvirus, can be 100% lethal in ducks [42]. Live attenuated DEV vaccine has been widely used to control duck viral enteritis since the 1960s [43,44]. We previously established a system to generate DEV-vectored vaccine by using overlapping fosmid DNAs and developed a DEV-vectored vaccine expressing the HA gene of an early H5N1 virus. We demonstrated that the vaccine could provide solid protection in ducks against challenge with lethal DEV and different H5 viruses [45,46]. Since both H5 and H7 viruses are circulating in nature, and H5 viruses bearing different HAs have considerable antigenic difference, it would be highly desirable to construct a recombinant DEV vaccine that expresses HA genes from multiple H5 and H7 viruses to provide broader protection in ducks against different viruses. In this study, we constructed a recombinant DEV expressing three HA genes (two H5 HA and one H7 HA), and extensively evaluated the antibody responses and protective efficacy in ducks induced by the recombinant DEV against different highly pathogenic H5 and H7 influenza viruses.

Materials and methods

Viruses and cells

The lethal DEV and DEV vaccine strain (vDEV) were obtained from the China Veterinary Culture Collection and were propagated in chicken embryo fibroblasts (CEFs). H5 and H7 viruses were isolated during our influenza surveillance or diagnosis, as previously reported [26,33,47–49], and were propagated in the allantoic cavities of 10-day-old SPF embryonated chicken eggs and stored at −80°C.

Construction of the recombinant fosmid and recombinant virus rescue

The HA genes were amplified from the avian influenza inactivated vaccine seed viruses H5-Re11, H5-Re12, and H7-Re2, which have been reported previously [47]. The donor viruses of the H5 HA genes are listed in Table 1. The HA genes were introduced into the plasmid pENTRsv40 for construction of the HA gene-expressing cassettes. The three HA-expressing cassettes were then introduced into different sites of the recombinant fosmid T as previously described (Figure 1(A)) [45]. The resultant fosmid was designated T-dH5/H7 (Figure 1(B)). The five fosmid combination of D + H + J + Q + T-dH5/H7, which covered the entire DEV genome, was used for virus rescue (Figure 1(A,B)). Viral DNA released from the purified fosmids was used to co-transfect CEFs by using the calcium phosphate procedure [50]. CEFs were observed for cytopathogenic effect (CPE) for 7 days after transfection, and CPE-positive samples were harvested to identify the insertions by using PCR and sequence analysis. The recombinant virus was designated rDEV-dH5/H7.

Three recombinant DEVs, rDEV-H5re11, rDEV-H5re12 and rDEV-H7re2, each contains a single HA gene of the three influenza viruses, were constructed by using the same strategy that was described previously [45] and used as controls for immunofluorescence studies.

Expression of H5 and H7 HA genes in rDEV-dH5/H7-infected cells

HA gene expression in rDEV-dH5/H7-infected CEFs was confirmed by immunofluorescence and western blotting. The primary antibodies to HA proteins were specific chicken polyclonal serum against H5N1, H5N6, or H7N9 virus induced by a DNA vaccine, and the secondary antibodies were Alexa Fluor 488 Goat anti-Chicken IgY (H + L) (Thermo Fisher Scientific). Cells were observed with an EVOS FL microscope (AMG). Western blotting was performed as described previously [45]. The primary antibody to GAPDH was rabbit anti-GAPDH mAb (Sigma Aldrich); secondary antibodies were IRDye 800CW donkey anti-chicken IgGs (LI-COR) for HA detection, and IRDye 800CW goat anti-rabbit IgG (LI-COR) for GAPDH detection.

Stability and growth properties of rDEV-dH5/H7

To evaluate the genetic stability of the foreign genes, the recombinant virus was passaged in CEFs 15 times. The inserted genes were detected by PCR with three pairs of specific primers: Pdsorf3us2-F/R (5'ACG CAA ATT ATG TCG TTG TT and 5'TTG AGG TTC CGT AGT CTG G), Pdus78-F/R (5'AAC TGT ATA ACA ACG ATC AAT GC and 5'GAG AGT CCA ATA CAA ACA ACG C), and Pdus81-F/R (5'CGA GTT CTC CGT TCC ACC ATA and 5'AAG TTG GCA TTA ACA CAA AGC G). To investigate the growth properties of the recombinant virus, CEFs cultured in 12-well plates were inoculated with rDEV-dH5/H7 or

Table 1. Similarity of hemagglutinin gene and antigenic relationship of H5 viruses used in this study with the ones in the rDEV-dH5/H7.

Virus (abbreviation)	Subtype (HA clade)	Gene identity (%) of hemagglutinin				Cross-reactive HI antibody titer of antiserum[b]	
		GZ/S4184/17		LN/SD007 /17			
		Nucleotide level	Amino acid level	Nucleotide level	Amino acid level	GZ/S4184/17	LN/SD007/17
A/duck/Guizhou/S4184/2017 (GZ/S4184/17)[a]	H5N6 (2.3.4.4 h)	**100**	**100**	88.5	90.7	**512**	8
A/chicken/Liaoning/SD007/2017 (LN/SD007/17)[a]	H5N1 (2.3.2.1d)	88.5	90.7	**100**	**100**	8	**512**
A/duck/Hunan/S11553/2020 (HuN/S11553/20)	H5N6 (2.3.4.4 h)	98.2	98.6	88.2	90.3	128	8
A/broiler/Hunan/2/2020 (HuN/2/20)	H5N1 (2.3.2.1d)	88.1	90.5	93.6	95.8	8	64
A/duck/Guangdong/S4525/2021 (GD/S4525/21)	H5N1 (2.3.4.4b)	91.6	94.0	87.8	91.5	16	16

[a]Donor of H5 HA gene of rDEV-dH5/H7.
[b]Antisera were generated by vaccinating SPF chickens with the oil-emulsified inactivated viruses as indicated in the table. The homologous titers are shown in bold.

vDEV at a multiplicity of infection (MOI) of 0.01, and the cells were harvested at different timepoints for virus titration as described previously [45].

Animal challenge studies

Total 263 two-week-old specific pathogen free (SPF) ducks (Shaoxing shelduck, a local breed) were used in this study. Ducks were intramuscularly inoculated with 0.1 mL of phosphate-buffered saline (PBS) diluted rDEV-dH5/H7 or vDEV, and were intramuscularly challenged with 0.1 mL of lethal DEV (100-fold 50% duck lethal dose (DLD_{50})) or intranasally challenged with 0.1 mL of influenza virus (10^6 50% embryo infective dose (EID_{50})). The vaccine dosage used in different experiments are indicated in the Results section. Oropharyngeal and cloacal swabs were collected from the challenged ducks on days 3, 5, and 7 post-challenge (p.c.) for virus titration in eggs. Animals were observed for signs of disease and death for two weeks after challenge.

Serological tests

Antibodies against influenza virus were evaluated by using the hemagglutination inhibition (HI) assay with 1.0% chicken erythrocytes. Antibodies against DEV were evaluated by using the neutralization (NT) assay in CEFs.

Results

Generation of recombinant DEV co-expressing two H5 and one H7 HA genes

We previously identified an area between the us8 and us7 of the DEV genome as a nonessential region for DEV replication [45]. Using a similar strategy, we identified two more new areas as nonessential regions in the genome of DEV: one between the us8 and us1, and the other between the sorf3 and us2 (Figure 1(A)). The Dsred2 gene encoding the red fluorescent protein was stably maintained at these two sites without affecting DEV replication or immunogenicity (date no shown). We therefore constructed a recombinant fosmid T-dH5/H7 by inserting the HA genes of A/chicken/Guangxi/SD098/2017 (H7N9) (GX/SD098/17), GZ/S4184/17 (H5N6), and LN/SD007/17 (H5N1) between the sorf3 and us2, the us7 and us8, and the us8 and us1, respectively (Figure 1(B)). Recombinant DEV was rescued by co-transfection of CEFs with overlapping fosmid DNA fragments as described previously [45]; the resultant virus was designated rDEV-dH5/H7. Insertion of the HA genes into the virus was confirmed by sequencing and expression of the HA genes the rDEV-dH5/H7 in infected CEFs was confirmed by immunofluorescence as described previously (Figure 1(C)) [45].

Stability of the HA genes in rDEV-dH5/H7

To investigate the stability of the three HA genes in the genome of rDEV-dH5/H7, we passaged the recombinant virus 15 times in CEFs. The three HA genes were amplified from different generations of rDEV-dH5/H7 by PCR (Figure 2(A)), and the expression of the three HA proteins in rDEV-dH5/H7-infected CEFs was detected by western blotting (Figure 2(B)). Moreover, rDEV-dH5/H7 had similar replication properties to those of vDEV in CEFs (Figure 2(C)). These results indicate that the three HA genes can be stably inherited in rDEV-dH5/H7 and properly expressed in rDEV-dH5/H7-infected CEFs, and that the insertion of the three HA genes did not affect the replication of recombinant DEV.

Protective efficacy of rDEV-dH5/H7 against lethal DEV challenge

To investigate whether the insertion of the HA genes affects the protective efficacy of the recombinant

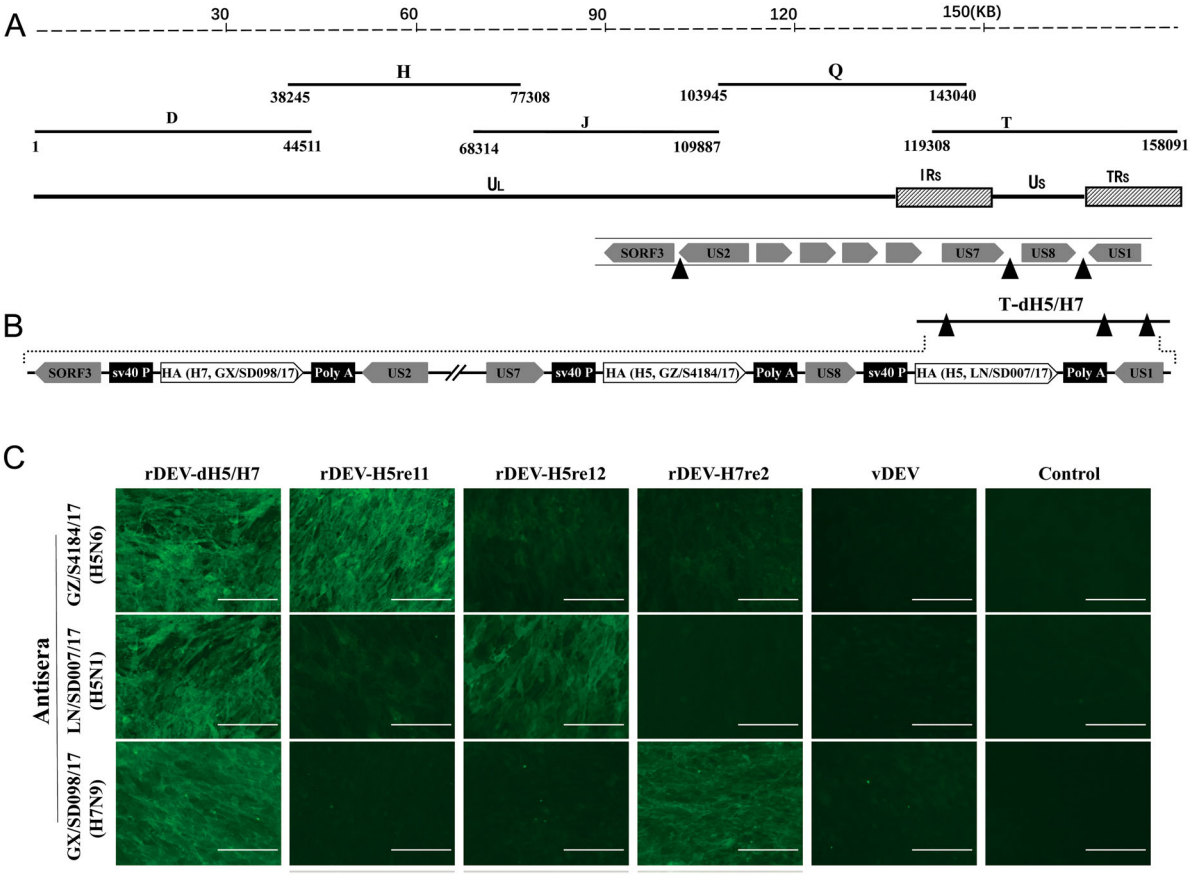

Figure 1. Construction of the recombinant DEV virus rDEV-dH5/H7. (A) Genomic structure of the DEV vaccine strain and the five fosmid DNAs used for DEV regeneration. Numbers show the location of each fosmid fragment in the DEV genome. Triangles indicate the insertion sites of the foreign genes. (B) Construction of fosmid T-dH5/H7. The three HA genes were respectively inserted into the three replication nonessential areas as indicated. (C) Expression of the HA proteins in the fifth generation recombinant virus-infected CEFs. The recombinant viruses of rDEV-H5re11, rDEV-H5re12, and rDEV-H7re2 were constructed by respectively inserting the HA gene of GZ/S4184/17(H5N6), LN/SD007/17(H5N1), and GX/SD098/17(H7N9) between the US7 and US8 genes of DEV and were used as controls. The expression of the HA proteins was detected by immunofluorescence using the indicated antiserum. Scale bar = 75 μm.

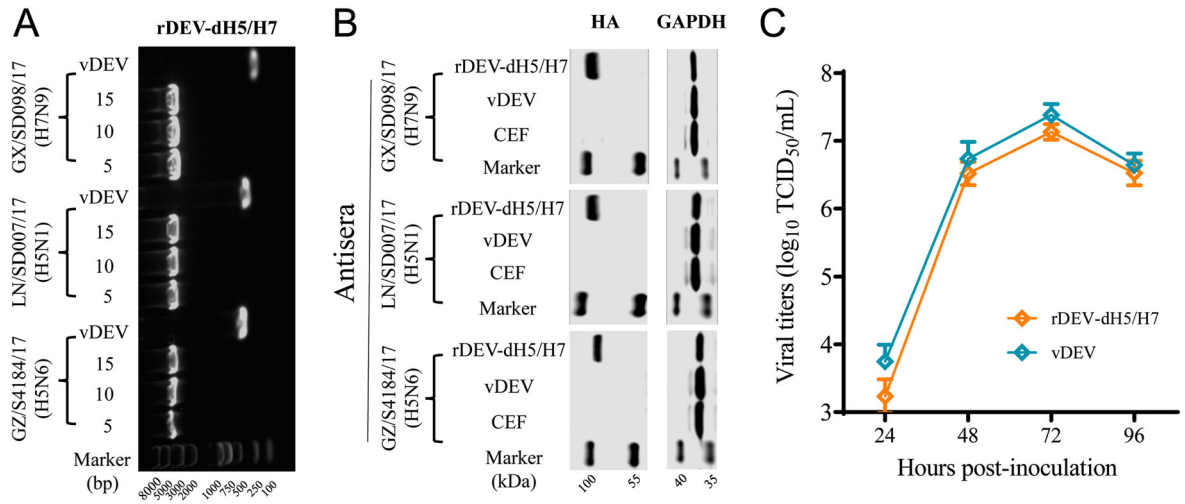

Figure 2. Genetic stability and growth property of rDEV-dH5/H7 *in vitro*. (A) Detection of the three inserted HA gene cassettes in the recombinant virus. The numbers show the passages of the recombinant virus. (B) Detection of the expression of the three HA proteins in the fifteenth generation rDEV-dH5/H7-infected CEFs by western blotting. (C) One-step growth curves of rDEV-dH5/H7 and its parental virus in CEFs. Infected cells and supernatants were collected, and viral titers were determined at the indicated timepoints post-infection.

virus against lethal DEV, we evaluated the protective efficacy of different dosages of rDEV-dH5/H7 and vDEV against lethal DEV challenge. Groups of eight two-week-old ducks were inoculated with 10^3TCID$_{50}$, 10^4TCID$_{50}$, or 10^5TCID$_{50}$ of rDEV-dH5/H7 or vDEV, one group of ducks were inoculated with PBS as control. The ducks were challenged with 100DLD$_{50}$ of lethal DEV at 2 weeks post-vaccination. All the ducks inoculated with 10^4TCID$_{50}$ and 10^5TCID$_{50}$ of rDEV-dH5/H7 or vDEV remained healthy during the 2-week observation period (Figure 3(A)). However, two ducks in the 10^3TCID$_{50}$ of rDEV-dH5/H7-inoculated group, four ducks in the 10^3TCID$_{50}$ of vDEV-inoculated group, and all eight PBS-inoculated control ducks showed signs of disease and died during the observation period (Figure 3(A)). These results indicate that rDEV-dH5/H7 and vDEV have similar protective efficacy against lethal DEV challenge.

The DEV vaccine induces rapid protection [45,46,51]. To investigate whether rDEV-dH5/H7 also provides rapid protection against lethal DEV challenge, groups of two-week-old ducks were inoculated with 10^5TCID$_{50}$ rDEV-dH5/H7, vDEV, or PBS and challenged with 100 DLD$_{50}$ of lethal DEV at one week post-vaccination. All of the PBS-inoculated control ducks died within five days of challenge, whereas all rDEV-dH5/H7- and vDEV-inoculated ducks were healthy and survived during the 2-week observation period (Figure 3(B)). These results further indicate that insertion of the three HA genes did not affect the protective efficacy of recombinant DEV against lethal DEV challenge.

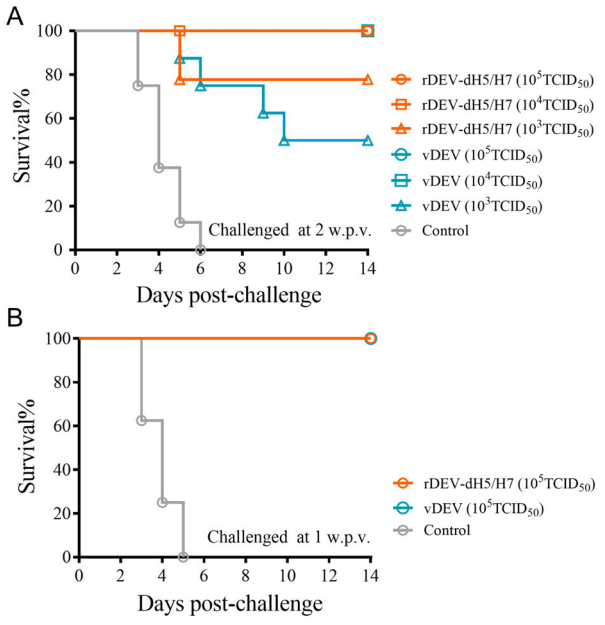

Figure 3. Protective efficacy of rDEV-dH5/H7 and rDEV against lethal DEV challenge. (A) Groups of eight ducks were inoculated intramuscularly with the indicated dose of rDEV-dH5/H7 or vDEV and challenged with lethal DEV at 2 weeks post-vaccination (w.p.v.). The ducks were monitored daily for 2 weeks after challenge. (B) Groups of eight ducks were inoculated intramuscularly with 10^5TCID$_{50}$ of rDEV-dH5/H7 or vDEV and challenged with lethal DEV at one w.p.v.. The ducks were monitored daily for 2 weeks after challenge.

Antibody responses induced by rDEV-dH5/H7

Ducks need three doses of the DEV vaccine to be protected against deadly DEV infections in the field, and they are vaccinated at 2, 5, and 15 or 16 weeks of age (just before they lay eggs) [52]. To evaluate the antibody response induced by rDEV-dH5/H7 against H5 virus, H7 virus, and vDEV, we inoculated groups of eight two-week-old ducks with three doses of 10^5TCID$_{50}$ rDEV-dH5/H7, vDEV, or PBS at the ages of 2-, 5-, and 16-weeks, and collected sera from all ducks at the indicated timepoints to test for HI antibodies against influenza viruses and NT antibodies against DEV.

After the first dose of rDEV-dH5/H7, only one duck had detectable HI antibodies against GX/SD098/18 (H7N9) at one week post-vaccination, and low HI titers to all three viruses were detected in a limited number of ducks at 2 and 3 weeks after the first dose (Figure 4(A–C)). At one week after the second dose, the HI antibody titers had increased sharply and all animals had detectable HI antibodies with mean titers >6.2log$_2$ against the three viruses; these titers gradually declined to 3.1log$_2$ at 11 weeks after the second dose (Figure 4(A–C)). The HI antibodies against all three tested viruses sharply increased after inoculation with the third dose, with mean peak titers ranging from 7.6log$_2$ to 8.0log$_2$ at one week after the third dose, and the titers were still about 4.6log$_2$ when the ducks reached 40 weeks old (Figure 4(A–C)). The vDEV- and PBS-inoculated ducks did not raise any HI antibodies against these influenza viruses (data not shown). The pattern of NT antibodies against DEV in the rDEV-dH5/H7-inoculated ducks was similar to that in the vDEV-inoculated ducks (Figure 4(D)). These results indicate that rDEV-dH5/H7 induces a solid antibody response against H5 and H7 viruses in ducks, with comparable HI antibody levels to all three HA gene donor viruses. In addition, rDEV-dH5/H7 and vDEV induced similar levels of anti-DEV NT antibodies.

Protective efficacy of rDEV-dH5/H7 against homologous H5 and H7 viruses

To evaluate the protection of rDEV-dH5/H7 against lethal avian influenza virus challenge, groups of eight 2-week-old ducks were inoculated with two 10^5TCID$_{50}$ doses of rDEV-dH5/H7 or with PBS, at a three-week interval, and were then challenged with 10^6EID$_{50}$ of GZ/S4184/17(H5N6), LN/SD007/17 (H5N1), or A/duck/Fujian/SE0195/2018 (H7N2) (FJ/

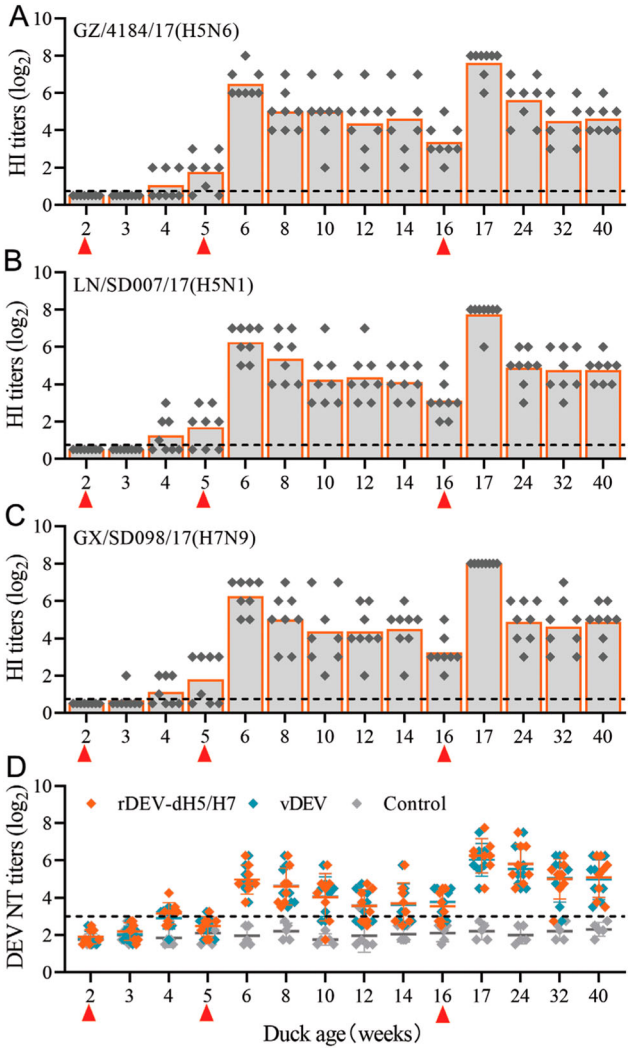

Figure 4. Antibody responses induced by rDEV-dH5/H7 or vDEV in ducks. Groups of eight ducks were inoculated intramuscularly with three 10^5TCID$_{50}$ doses of rDEV-dH5/H7 or rDEV at the indicated timepoints. HI antibodies against antigens of GZ/S4184/17 (H5N6) (A), LN/SD007/17(H5N1) (B), and GX/SD098/17(H7N9) (C), and neutralizing antibodies against DEV (D) were evaluated at the indicated timepoints. The dashed lines in A, B, and C show the limits of detection, whereas the dashed lines in D show the detection limits for a positive response. The red triangles indicate the vaccine inoculation time.

SE0195/18) at three different timepoints: one week after dose one, two weeks after dose one, and 10 weeks after the second dose (Table 2).

All the vaccinated ducks were completely protected against the challenge from any of the three lethal viruses: there was no detectable virus shedding and all ducks survived during the two-week observation period (Table 2). All the control ducks shed virus through the oropharynx and/or cloaca (Table 2). When the challenge was performed at one week after inoculation dose one, only one of the control ducks survived (a duck in the GZ/S4184/17(H5N6)-challenged group) (Table 2). When the challenge was performed at two weeks after inoculation dose one, one, zero, and one of the eight control ducks survived in the GZ/S4184/17(H5N6)-challenged group, LN/SD007/17(H5N1)-challenged group, and FJ/SE0195/18 (H7N2)-challenged group, respectively (Table 2). When the challenge was performed at 10 weeks after inoculation dose two, four, eight, and six of the eight control ducks survived in the GZ/S4184/17(H5N6)-challenged group, LN/SD007/17(H5N1)-challenged group, and FJ/SE0195/18(H7N2)-challenged group, respectively (Table 2). These results suggests that the older ducks were more tolerant to infection with the deadly avian influenza viruses than younger ducks.

Our data indicate that rDEV-dH5/H7 bearing two H5 HA genes and one H7 HA gene can induce complete protection against a challenge with homologous or antigenically similar lethal influenza viruses in ducks, as early as one week after a single vaccine dose.

Protective efficacy of rDEV-dH5/H7 against heterologous H5 virus challenge

The two H5 HA genes in rDEV-dH5/H7 were from viruses that were isolated in 2017 and belong to clade 2.3.4.4 h and clade 2.3.2.1d, respectively

Table 2. Protective efficacy of rDEV-dH5/H7 against homologous lethal H5 and H7 viruses challenge in ducks.

Challenge time	Challenge virus	Group	Virus isolation from swabs: shedding/total (titer, $log_{10}EID_{50}$/ml)[a]						
			Day 3 *p*.c.		Day 5 *p*.c.		Day 7 *p*.c.		
			Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	Survival/Total
One week post dose one	GZ/S4184/17 (H5N6)	Vaccinated	0/8	0/8	0/8	0/8	0/8	0/8	8/8
		Control	8/8 (2.1 ± 0.8)	8/8 (3.2 ± 0.6)	3/4 (2.3 ± 0.9)	3/4 (2.7 ± 1.0)	1/1 (0.8)	1/1 (2.5)	1/8
	LN/SD007/17 (H5N1)	Vaccinated	0/8	0/8	0/8	0/8	0/8	0/8	8/8
		Control	8/8 (2.1 ± 0.7)	8/8 (2.1 ± 0.9)	5/6 (1.9 ± 0.8)	5/6 (1.4 ± 0.4)	/	/	0/8
	FJ/SE0195/18 (H7N2)	Vaccinated	0/8	0/8	0/8	0/8	0/8	0/8	8/8
		Control	4/7 (1.9 ± 0.9)	7/7 (1.3 ± 0.3)	/	/	/	/	0/8
Two weeks post dose one	GZ/S4184/17 (H5N6)	Vaccinated	0/8	0/8	0/8	0/8	0/8	0/8	8/8
		Control	8/8 (3.5 ± 0.0)	8/8 (3.4 ± 0.3)	3/3 (2.7 ± 1.0)	3/3 (2.6 ± 0.7)	1/1 (1.3)	1/1 (0.8)	1/8
	LN/SD007/17 (H5N1)	Vaccinated	0/8	0/8	0/8	0/8	0/8	0/8	8/8
		Control	8/8 (3.5 ± 0.0)	8/8 (3.3 ± 0.3)	/	/	/	/	0/8
	FJ/SE0195/18 (H7N2)	Vaccinated	0/8	0/8	0/8	0/8	0/8	0/8	8/8
		Control	8/8 (2.9 ± 0.6)	8/8 (2.5 ± 0.9)	1/1 (2.5)	1/1 (1.8)	1/1 (0.8)	1/1 (1.3)	1/8
Ten weeks post the second dose[b]	GZ/S4184/17 (H5N6)	Vaccinated	0/8	0/8	0/8	0/8	0/8	0/8	8/8
		Control	8/8 (3.4 ± 0.3)	7/8 (2.6 ± 1.0)	8/8 (3.3 ± 0.3)	8/8 (2.3 ± 0.9)	3/4 (0.8 ± 0.0)	2/4 (1.6 ± 0.9)	4/8
	LN/SD007/17 (H5N1)	Vaccinated	0/8	0/8	0/8	0/8	0/8	0/8	8/8
		Control	8/8 (3.5 ± 0.0)	8/8 (3.1 ± 0.4)	8/8 (2.8 ± 0.6)	8/8 (2.4 ± 0.8)	1/8 (2.5)	0/8	8/8
	FJ/SE0195/18 (H7N2)	Vaccinated	0/8	0/8	0/8	0/8	0/8	0/8	8/8
		Control	8/8 (2.6 ± 0.6)	8/8 (2.6 ± 0.9)	6/7 (1.9 ± 0.8)	6/7 (2.4 ± 0.9)	5/6 (2.7 ± 0.7)	5/6 (2.1 ± 0.7)	6/8

[a]Swabs were collected from all of the available ducks on days 3, 5 and 7 *p*.c. for virus titration in eggs. The titer shown is the mean ± standard deviation for the ducks that shed viruses. "/" indicates that the animals had died by that time point.

[b]Ducks were vaccinated with two doses of 10^5 $TCID_{50}$ of rDEV dH5/H7, at a three-week interval.

Table 3. Protective efficacy of rDEV-dH5/H7 against heterologous H5 virus challenge in ducks.

		Virus isolation from swabs: shedding/total (titer, $log_{10}EID_{50}$/ml) [a]						
		Day 3 *p.c.*		Day 5 *p.c.*		Day 7 *p.c.*		
Challenge virus (HA clade)	Vaccine	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	Survival/total
HuN/S11553/20 (H5N6) (clade 2.3.4.4 h)	Vaccinated	0/8	0/8	0/8	0/8	0/8	0/8	8/8
	Control	5/5 (2.6 ± 0.5)	5/5 (2.4 ± 0.9)	1/1 (2.5)	1/1 (2.5)	/	/	0/5
HuN/2/20 (H5N1) (clade 2.3.2.1d)	Vaccinated	0/8	0/8	0/8	0/8	0/8	0/8	8/8
	Control	5/5 (1.5 ± 0.5)	5/5 (2.6 ± 0.6)	3/5(1.5 ± 0.0)	0/5	0/5	0/5	5/5
GD/S4525/21 (H5N1) (clade 2.3.4.4b)	Vaccinated	0/8	0/8	0/8	0/8	0/8	0/8	8/8
	Control	5/5 (1.5 ± 0.5)	5/5 (2.4 ± 0.9)	0/5	5/5 (1.4 ± 0.3)	0/5	0/5	5/5

[a]Swabs were collected from all of the available ducks on days 3, 5 and 7 *p.c.* for virus titration in eggs. The titer shown is the mean ± standard deviation for the ducks that shed viruses. "/" indicates that the animals had died by that time point.

(Table 1). To evaluate the protective efficacy of this vaccine against recently circulating strains, we selected three viruses that were detected in China in recent years and performed challenge studies in ducks. HuN/S11553/20(H5N6) was a clade 2.3.4.4 h virus isolated in 2020, HuN/2/20 (H5N1) was a clade 2.3.2.1d virus isolated in 2020, and GD/S4525/21 (H5N1) was a clade 2.3.4.4b virus isolated in 2021 [33,48,49]. The HA genes of these three H5 viruses shared 88.1%–98.2% identity at the nucleotide level and 90.5%–98.6% identity at the amino acid level with the HA of GZ/S4184/17(H5N6) virus, and shared 87.8%–93.6% identity at the nucleotide level and 90.3%–95.8% identity at the amino acid level with the HA of LN/SD007/17(H5N1) virus (Table 1). Some of these strains cross-reacted poorly with the antisera induced by the GZ/S4184/17 and LN/SD007/17 viruses (Table 1), with HI titers 64-fold lower than the homologous titers (Table 1).

To evaluate the protective efficacy of rDEV-dH5/H7 against these heterologous viruses, groups of eight 2-week-old ducks were inoculated with one 10^5TCID_{50} dose of rDEV-dH5/H7, and groups of five 2-week-old ducks were inoculated with PBS as a control. All of the ducks were challenged with 10^6EID_{50} of the test virus at two weeks post-vaccination. As shown in Table 3, all ducks vaccinated with rDEV-dH5/H7 were completely protected from the challenge of all three viruses, meaning no virus shed at any timepoint tested, and all ducks survived during the two-week observation period. In the PBS-inoculated control groups, all of the ducks shed viruses through both the oropharynx and cloaca on day 3 post-challenge with all three viruses, and some also shed viruses on day 5 post challenge; however, none of them shed virus on day 7 post-challenge (Table 3). All of the PBS-inoculated control ducks died after the HuN/S11553/20 (H5N6) challenge, but all of the PBS-inoculated control ducks survived during the observation period after the HuN/2/20 (H5N1) or GD/S4525/21 (H5N1) challenge (Table 3). These results indicate that rDEV-dH5/H7 can prevent the replication of heterologous antigenically drifted H5 viruses in ducks, although clear antigenic differences were observed between the H5 HA donor viruses and the challenge viruses.

Discussion

In this study, we constructed a recombinant DEV virus rDEV-dH5/H7 that expresses the HA genes of two different H5 viruses and the HA gene of an H7 virus, and we found that these three HA genes could be stably inherited in the recombinant virus and could be properly expressed in virus-infected cells. The rDEV-dH5/H7 vaccine virus induced solid antibody responses against DEV, H5, and H7 viruses, and provided complete protection against lethal DEV and different H5 and H7 viruses in ducks. Our study thus shows that rDEV can tolerate the insertion of multiple foreign genes and is therefore a suitable vector for constructing multivalent vaccines.

Unvaccinated ducks raised in open fields serve as "Trojan horses" for transferring avian influenza viruses from wild birds to domestic poultry. Previous studies have shown that one dose of the commercial inactivated vaccine completely protects ducks against challenges with heterologous H5 virus or H7 virus [33,53,54]. In our present study, we showed that one dose of rDEV-dH5/H7 also provides solid protection against heterologous H5 viruses in ducks [46], suggesting that both the inactivated vaccine and the live DEV-vectored vaccine are highly immunogenic and can induce broad protection in ducks. Given that different H5 and H7 viruses circulating in nature could be spread across countries or continents by migratory birds, vaccinating ducks with the rDEV-dH5/H7 vaccine will not only prevent ducks from lethal DEV infection, but will also largely prevent the H5 and H7 viruses that are carried by migratory wild birds from entering poultry.

Inactivated influenza vaccines have been widely used in poultry, and the HI antibody titer is used as an indicator of the immune status of vaccinated poultry. Usually, a minimal HI antibody titer of $4log_2$ is thought to be required to secure solid protection. Cui et al. previously reported that the ducks vaccinated with an

inactivated vaccine were completely protected against the newly emerged H5N8 virus bearing the clade 2.3.4.4b HA gene at 31 days post-vaccination, even though some ducks did not have any detectable HI antibodies against the challenge virus [55]. Cui et al. further revealed that those ducks had high levels of NT antibodies against the challenge virus [55]. In the present study and our previous studies [45,46], we found that a DEV-based vaccine could induce solid protection against lethal DEV and avian influenza viruses as early as one week post-vaccination. Since NT antibodies against DEV were not detected in any ducks at this timepoint, we speculate that neutralizing antibodies against influenza viruses may not have been induced either. The solid protection offered by rDEV-dH5/H7 at the early timepoint could be attributed to some other type of immunity induced by the vaccine, which requires further study.

Vaccination has been an important strategy for controlling highly pathogenic avian influenza in China since 2004, and studies have shown that inactivated vaccines are highly immunogenic and provide solid protection in ducks against H5 virus challenge [31,33,55–57]. However, because many avian influenza viruses that are highly pathogenic to chickens are very mild to ducks, duck farmers have no incentive to vaccinate their ducks with inactivated avian influenza vaccines. As a result, vaccination coverage in ducks has been below the requirements to prevent the entry and spread of avian influenza viruses [26,31,45]. Since ducks require DEV live vaccine to prevent infection with highly pathogenic DEV, replacing the DEV live vaccine with rDEV-dH5/H7 in the duck vaccination schedule would be a cost-effective strategy that would provide ducks with immunity to lethal DEV and also different H5 and H7 avian influenza viruses. Given that both deadly DEV and highly pathogenic avian influenza viruses are circulating in duck populations, the use of a rDEV-dH5/H7-like vaccine in duck breeding countries is strongly recommended.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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