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Short communication

Protective efficacy in farmed ducks of a duck enteritis virus-vectored vaccine against H5N1, H5N6, and H5N8 avian influenza viruses

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ABSTRACT

Ducks play a key role in the maintenance and spread of avian influenza viruses (AIVs) in nature, and control of AIVs in ducks has important implications for AIV eradication from poultry. We previously constructed a recombinant duck enteritis virus (DEV), rDEVus78HA, that expresses the HA gene of an H5N1 AIV and showed that rDEVus78HA immunization provides complete protection against both DEV and H5N1 AIV challenge in specific-pathogen-free ducks. In this study, we performed a 60-week clinical trial and found that this rDEVus78HA vaccine can function as a bivalent vaccine in farmed ducks against lethal challenge with DEV and H5N1 virus. Moreover, we found that rDEVus78HA-vaccinated ducks were efficiently protected against challenges with recently isolated heterologous H5N6 and H5N8 viruses. Our results demonstrate that rDEVus78HA could be extremely valuable for the control of DEV and H5 AIVs in ducks.

Highly pathogenic avian influenza virus infections always cause huge economic loss to the poultry industry and pose a severe threat to human public health. The H5N1 viruses have been widely spread in poultry and wild bird populations around the world over the last two decades. These viruses have been classified into 10 different clades based on the evolution of their hemagglutinin (HA) genes [1], and the clade 2 viruses have further evolved into different sub-clades [2–4]. Since 2013, clade 2.3.4.4 H5 viruses with N6 and N8 neuraminidase (NA) genes have been isolated in Asia, Europe, and North America (Office International des Epizooties [OIE]; http://www.oie.int) and have caused huge disease outbreaks in domestic poultry [5,6].

Whole virus inactivated vaccines and Newcastle disease virus-vectored vaccines have been widely used to prevent and control the H5 viruses in domestic poultry in China and many other countries [7,8]. However, since many H5 viruses replicate asymptomatically in ducks and do not cause economic loss, duck farmers are not motivated to use the H5 vaccines in their ducks. This huge reservoir of unvaccinated ducks therefore serves as a "propagator" and "spreader" of the H5 viruses when they are trading through the live poultry markets, as recently reported by Li et al. [9].

Lethal duck enteritis virus (DEV) usually causes up to 100% lethality in ducks, and a live attenuated DEV vaccine has been widely used in ducks to control the duck DEV infection. We previously developed a DEV-vectored vaccine, rDEVus78HA, by inserting the HA gene of a clade 2.3.4 virus, A/duck/Anhui/1/2006 (H5N1), between the unique short (US) 7 and 8 genes of the DEV vaccine strain and demonstrated that rDEVus78HA immunization of specific-pathogen-free (SPF) ducks provides complete protection against lethal DEV and H5N1 virus challenge [10]. In the present study, we performed a 60-week clinical trial to investigate the protective efficacy of the recombinant DEV vaccine in farmed ducks. The experiments with live H5 viruses were conducted in the enhanced animal biosafety level 3 (ABSL3+) facility in the Harbin Veterinary Research Institute (HVRI) of the Chinese Academy of Agricultural Sciences (CAAS), which is approved for such use by the Ministry of Agriculture and Rural Affairs of China. All animal studies were approved by the Review Board of the HVRI, CAAS.

Seven hundred 2-week-old Shaoxing ducks in a small-scale farm were used. Of these, 500 were injected intramuscularly (i.m.) with 10^5 50% tissue culture infectious dose ($TCID_{50}$) of rDEVus78HA, and 200 ducks were injected i.m. with PBS as controls. Consistent with the usage of the commercial DEV vaccine, ducks were inoculated three times at the age of 2-, 5- and 15-weeks. Sera were collected weekly from 20 randomly picked ducks in each group for hemagglutinin inhibition (HI) antibody detection. As shown in Fig. 1, a low level of HI antibody (mean HI titer of 2.1

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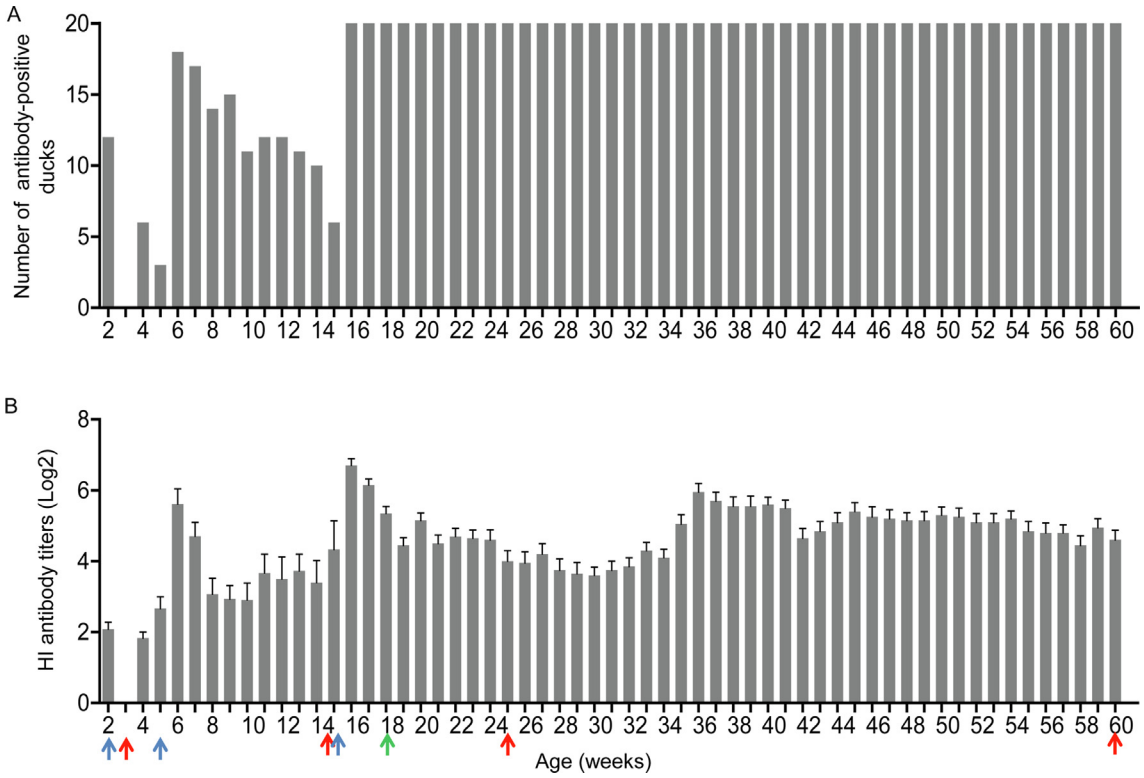

Fig. 1. Antibody detection in farmed ducks inoculated with rDEVus78HA vaccine. Ducks were injected intramuscularly with 3 doses of 10^5 50% tissue culture infectious dose ($TCID_{50}$) of rDEVus78HA at the age of 2-, 5-, and 15-week-old. Sera were collected randomly from 20 ducks weekly for HI antibody detection. (A) Numbers of HI antibody-positive ducks; (B) mean HI antibody titers. The mean titers of the HI antibody were calculated based on the number of ducks that had detectable HI antibody titers. The blue arrows indicate the vaccination time, the red arrows indicate the challenge time with the DK/HB/49/05 virus, and the green arrow indicates the challenge time with the H5N6 and H5N8 viruses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

log2) was detected in 12 of 20 ducks before vaccine inoculation, indicating that some ducks had maternal antibodies that lasted for two weeks. The HI antibody was detected in six and three ducks at 2 and 3 weeks after the first vaccination, with mean titers of 1.8 and 2.7 log2, respectively (Fig. 1). One week after the second vaccination, 18 of the 20 tested ducks had HI antibodies, with a mean titer of 5.6 log2. After the third vaccination, all tested ducks had detectable HI antibodies that lasted for more than 45 weeks (Fig. 1A), with mean HI antibody titers ranging from 3.6 to 6.7 log2 (Fig. 1B). In the PBS-inoculated ducks, HI antibody was detected from 11 of the 20 tested ducks, with a mean titer of 2 log2 when the ducks were two weeks old, but was not detected from any ducks at any other timepoint tested thereafter.

To investigate the protective efficacy of rDEVus78HA against lethal DEV and H5N1 virus, 20 ducks from each group were transferred to the ABSL3+ facility and challenged with 100 50% lethal dose (LD_{50}) of lethal DEV or 10^5 50% embryo lethal dose (ELD_{50}) of A/duck/Hubei/49/2005 (H5N1) (DK/HB/49/05) (clade 2.3.4) virus at four timepoints: 1 week after the first dose (timepoint 1), 10 weeks after the second dose (timepoint 2, before the third dose), 10 weeks after the third dose (timepoint 3, the peak time that ducks lay eggs), and 45 weeks after the third dose (timepoint 4, the end of the observation period), respectively. Oropharyngeal and cloacal swabs were collected from DK/HB/49/05 virus-challenged ducks on days 3, 5, and 7 post-challenge (p.c.) for virus titration. Ducks were observed for disease and death for 2 weeks p.c.

As shown in Fig. 2, after challenge with the DK/HB/49/05 virus, all of the PBS-inoculated control ducks shed virus through both the oropharynx (Fig. 2A–D) and the cloaca (Fig. 2E–H), but virus shedding was not detected in any of the rDEVus78HA-vaccinated ducks (Fig. 2A–H). All of the rDEVus78HA-vaccinated ducks survived from the DK/HB/49/05 virus or lethal DEV challenge at all four challenge timepoints. In the PBS-inoculated groups, ten, seven, eight, and six ducks died at challenge timepoints 1, 2, 3, and 4, respectively, after challenge with DK/HB/49/05 virus (Fig. 2I–L); all ducks died at all four timepoints after challenge with the lethal DEV virus (Fig. 2M–P). These results indicate that rDEVus78HA is immunogenic and can provide solid protection against lethal DEV and H5N1 virus challenge in farmed ducks.

The rDEVus78HA vaccine bears the HA gene of A/duck/Anhui/1/2006 virus, an early clade 2.3.4 virus [11]. H5N6 and H5N8 viruses bearing the HA of clade 2.3.4.4 have been detected in several countries [12]. The HA genes of H5N6 viruses detected in domestic poultry and the H5N8 viruses detected in wild birds in China in 2016 share 93.2–93.5% identity at the nucleotide level and 93.7–94.7% identity at the amino acid level with that of A/duck/Anhui/1/2006 virus (Table 1). Antisera generated in chickens against the H5N1 virus A/duck/Anhui/1/2006 cross-reacted with the H5N6 and H5N8 viruses with titers 16-fold to 32-fold lower than that to the homologous virus (Table 1). These data indicate that the clade 2.3.4 viruses have not only undergone genetic changes but have also undergone considerable antigenic drift during their 10 years of circulation in nature.

To investigate the protective efficacy of the rDEVus78HA vaccine against these recent heterologous H5N6 and H5N8 viruses, we transferred 40 18-week-old ducks (3-weeks after their third vaccination) from each group to our ABSL3+ facility and challenged them with 10^5 ELD_{50} of two H5N6 viruses and 10^5 ELD_{50} two H5N8 viruses (ten ducks in each group for one challenge virus).

In the H5N6 virus A/chicken/Gansu/8-3/2016-challenged groups, four of the ten PBS-inoculated control ducks shed virus

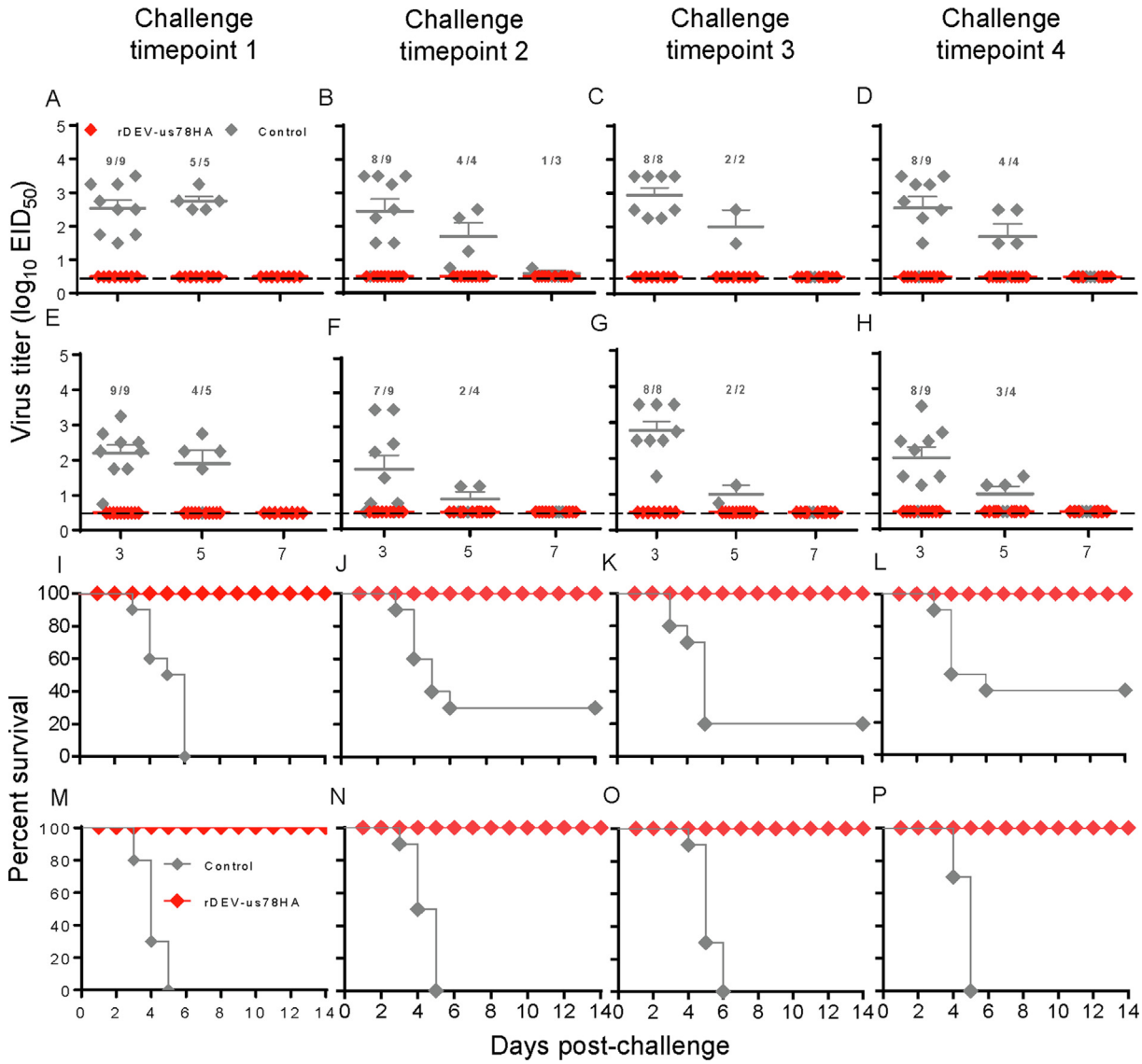

Fig. 2. Protective efficacy of rDEVus78HA vaccine against lethal DEV and H5N1 virus in farmed ducks. Ducks were challenged at four different timepoints as described in the text. Oropharyngeal and cloacal swabs were collected on days 3, 5, and 7 after challenge with the H5N1 virus to test for virus shedding. The viral titers in the oropharyngeal and cloacal swabs are shown in panels A–D and panels E–H, respectively. The numbers on the top of the panels show how many birds among the total live birds shed virus at the indicated time point. The numbers in red show the data in the vaccinated groups, and the numbers in grey show the data in the control groups. The dashed lines show the limits of detection. A value of 0.5 was assigned to virus shedding-negative birds for statistical purpose. The virus shedding data were analyzed by the method of 2-way ANOVA using Graph Pad Prism software. The virus shedding in the vaccinated groups were significantly lower than that in the control groups at all time points tested. The percentage of surviving ducks challenged with H5N1 virus A/duck/Hubei/49/2006 are shown in panels I–L, and the percentage of surviving ducks challenged with DEV are shown in panels M–P. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Genetic and antigenic relationships between the A/duck/Anhui/1/2006 (AH/1) (H5N1) virus and the H5N6/N8 viruses used in this study.

Virus	Gene identity (%) of hemagglutinin		Cross-reactive antibody titers of chicken antisera induced by AH/1 virus
	Nucleotide level	Amino acid level	
A/duck/Anhui/1/2006 (H5N1)	100	100	256
A/chicken/Gansu/8-3/2016 (H5N6)	93.2	93.8	8
A/duck/Jiangxi/S1003/2016 (H5N6)	93.5	93.7	8
A/bar-headed goose/Qinghai/2/2016 (H5N8)	93.4	94.5	16
A/bar-headed goose/Tibet/3/2016 (H5N8)	93.5	94.7	16

through both the oropharynx and cloaca, but none of the rDEVus78HA-vaccinated ducks shed virus (Fig. 3A and B); all ducks survived during the two-week observation period (Fig. 3C). In the H5N6 virus A/duck/Jiangxi/S1003/2016-challenged groups, all ten of the PBS-inoculated control ducks shed virus through both the oropharynx and cloaca and two of them died on day 9 post-challenge (Fig. 3D–F). In contrast, only two of the ten rDEVus78HA-vaccinated ducks shed virus through the oropharynx

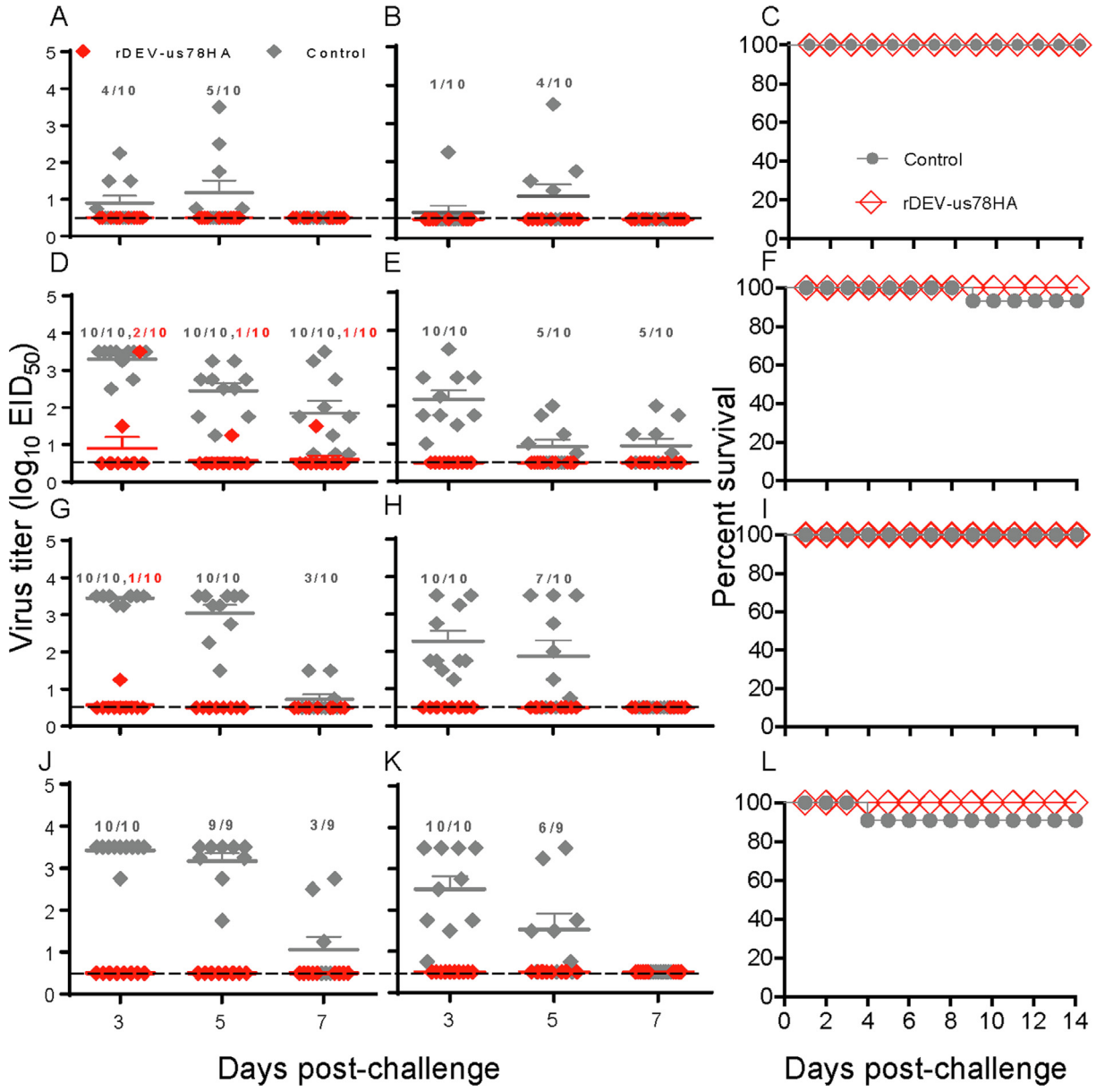

Fig. 3. Protective efficacy of rDEVus78HA vaccine against heterologous H5N6 and H5N8 viruses in farmed ducks. Oropharyngeal and cloacal swabs were collected on days 3, 5, and 7 after challenge to test for virus shedding, and birds were observed for two weeks for disease signs and death. The viral titers in the oropharyngeal samples are shown in panels A, D, G, and J, and the viral titers in the cloacal swabs are shown in panels B, E, H, and K. The numbers on the top of the panels show how many birds among the total live birds shed virus at the indicated time point. The numbers in red show the data in the vaccinated groups, and the numbers in grey show the data in the control groups. The dashed lines show the limits of detection. A value of 0.5 was assigned to virus shedding-negative birds for statistical purpose. The virus shedding data were analyzed by the method of 2-way ANOVA using Graph Pad Prism software. The virus shedding in the vaccinated groups were significantly lower than that in the control groups at all time points tested. The survival patterns of the ducks are shown in panels C, F, I, and L. A, B, and C: A/chicken/Gansu/8-3/2016 (H5N6)-challenged ducks. D, E, and F: A/duck/Jiangxi/S1003/2016 (H5N6)-challenged ducks. G, H, and I: A/bar-headed goose/Qinghai/2/2016 (H5N8)-challenged ducks. J, K, and L: A/bar-headed goose/Tibet/3/2016 (H5N8)-challenged ducks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 3D) and all of these ducks survived during the two-week observation period (Fig. 3F). In the H5N8 virus A/bar-headed goose/Qinghai/2/2016-challenged groups, all ten of the PBS-inoculated control ducks shed virus through both the oropharynx and cloaca, but only one of the ten rDEVus78HA-vaccinated ducks shed virus through the oropharynx (Fig. 3G and H), and all of the ducks survived during the two-week observation period (Fig. 3I). In the H5N8 virus A/bar-headed goose/Tibet/3/2016-challenged groups, all ten of the PBS-inoculated control ducks shed virus through both the oropharynx and cloaca and one of them died on day 4 post-challenge (Fig. 3J–L); none of the ten rDEVus78HA-vaccinated ducks shed virus and all of them survived during the two-week observation period (Fig. 3J–L). These results indicate that although the H5N6 and H5N8 viruses are not highly lethal in ducks, they replicate efficiently in this host and could be shed by ducks for several days. These data also show that the rDEVus78HA vaccine successfully prevents the replication of these heterologous, antigenically drifted H5N6 and H5N8 viruses in ducks.

The live attenuated DEV vaccine has been used to control the lethal DEV since the 1960s, and the ideal vaccination time and doses have been well established in ducks [13]. When we followed the vaccine inoculation program used for the commercial DEV vaccine, we found that the ducks were completely protected against

both lethal DEV and H5N1 virus challenge at four key timepoints, indicating that the DEV vaccine inoculation program can be applied to the recombinant rDEVus78HA vaccine. We previously reported that rDEVus78HA induced fast and complete protection against H5N1 avian influenza virus in SPF ducks [10]. In this study, one week after the first vaccination dose, the ducks were completely protected against the lethal DEV and H5N1 virus challenge, indicating that this vaccine can also induce fast protection in farmed ducks.

HI antibody titers have been used as an important parameter to evaluate the efficacy of inactivated vaccines in farmed poultry. A previous study by Tian et al. reported that two doses of inactivated vaccine induced detectable HI antibodies and protection in ducks for over 52 weeks [14]. In the present study, we showed that three doses of the DEV-vectored vaccine induced over 60-weeks of protection in ducks. Although not all of the ducks had detectable HI antibodies after the first two doses of vaccine (Fig. 1A, from week 3 to week 15), after the third dose, all of the tested ducks had detectable HI antibodies, which lasted for over 45 weeks (Fig. 1A, from week 16 to week 60). Since the rDEVus78HA-vaccinated birds were protected even though not all of them had detectable HI antibodies at challenge timepoints 1 and 2, it was not necessary to test the HI antibody titers in the DEV-vectored vaccine-inoculated ducks.

The influenza viruses easily undergo antigenic variation during their circulation in nature. It is important to note that the HA gene of the vaccine strains used in China for H5 avian influenza control have been updated several times since 2004 to ensure an antigenic match between the vaccines and the prevalent strains [8]. The first inactivated vaccine targeted to the clade 2.3.4 viruses was the Re-5 vaccine that carries the HA gene the A/duck/Anhui/1/2006 (H5N1) virus [11] and was used from 2008 to 2012. Challenge studies indicated that the Re-5 inactivated vaccine only provided 70–90% protection in chickens against the clade 2.3.4.4 viruses detected in China after 2015 (unpublished data), and, therefore, a new inactivated vaccine (Re-8) that carries the HA gene of a clade 2.3.4.4 virus [A/chicken/Guizhou/4/2013 (H5N1)] was developed and started to be used for clade 2.3.4.4 virus prevention in 2015 [15]. In the present study, we found that ducks vaccinated with rDEVus78HA, which carries an HA gene similar to that of the Re-5 vaccine, were well protected from challenges from H5N6 and H5N8 viruses that were isolated in 2016. These findings suggest that the DEV-vectored vaccine rDEVus78HA may be able to induce broader protection against different H5 influenza viruses, and therefore may not need to be updated as frequently as the inactivated vaccine.

In conclusion, our extensive clinical trial in farmed ducks demonstrated that rDEVus78HA can function as an excellent bivalent live vaccine against lethal DEV and H5 virus infection. We hope that the application of this new recombinant DEV vaccine will serve the dual function of protecting against lethal DEV infection and also filling the H5 vaccination gap in ducks through its routine use as part of DEV vaccination practices.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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