Veterinary Microbiology 232 (2019) 146–150

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Short communication

Recombinant duck enteritis viruses expressing the Newcastle disease virus (NDV) F gene protects chickens from lethal NDV challenge

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ARTICLE INFO

Keywords:
NDV
F protein
HN protein
Recombinant duck enteritis viruses

ABSTRACT

Newcastle disease virus (NDV) is a major threat to poultry worldwide. Virulent Newcastle disease virus infection can cause 100% morbidity and mortality in chickens. Vaccination is the most effective way to prevent and control NDV outbreaks in poultry. Previously, we demonstrated that a duck enteritis virus (DEV) vaccine strain is a promising vector to generate recombinant vaccines in chickens. Here, we constructed two recombinant DEVs expressing the F protein (rDEV-F) or HN protein (rDEV-HN) of NDV. We then evaluated the protective efficacy of these recombinant DEVs in specific-pathogen-free chickens. rDEV-F induced 100% protection of chickens from lethal NDV challenge after a single dose of 10^4 $TCID_{50}$, whereas rDEV-HN did not induce effective protection. rDEV-F may therefore serve as a promising vaccine candidate for chickens. This is the first report of a DEV-vectored vaccine providing robust protection against lethal NDV infection in chickens.

1. Introduction

Newcastle disease (ND) is caused by virulent strains of ND viruses (NDVs), ND is a highly contagious and pathogenic disease in chickens leading to major economic losses in the poultry industry worldwide, and is classified by the World Organization for Animal Health (OIE) as a notifiable disease (Dimitrov et al., 2016). NDV is a single-stranded, negative-sense, non-segmented RNA virus that belongs to the genus *Avulavirus* of the family *Paramyxoviridae*. The NDV genome is approximately 15.2 kb in length, containing six genes that encode six structural proteins in the order 3'-NP, (nucleocapsid)-P, (phosphoprotein)-M, (matrix)–F, (fusion)-HN (hemagglutinin-neuraminidase)-L, and (large polymerase)-5', and the nonstructural protein V (Chambers and Samson, 1982).

The F and HN proteins of NDV are surface glycoproteins. The F protein mediates the fusion of the viral envelope to the host cell membrane (Nagai et al., 1989). The HN protein possesses both receptor recognition and neuraminidase activities (Morrison et al., 1991). The F and HN proteins play important roles in virus attachment, entry, and release (Yusoff and Tan, 2001). Hence, the F and HN proteins are neutralizing and protective antigens of NDV, which makes them primary targets for anti-viral vaccine development (Boursnell et al., 1990; Ge et al., 2016; Lee et al., 2008; Loke et al., 2005; Park et al., 2014).

Vaccination is the most effective way to prevent and control ND in poultry, especially in regions where ND is endemic (Dimitrov et al., 2017). Live attenuated vaccine, such as that derived from the LaSota strain developed in the 1940's, has been widely used to prevent NDV infection (Miller et al., 2010). Although these kinds of vaccine were very effective, they raised biosafety concerns. More recently, recombinant vaccines have shown promise polyvalent or antigen-delivery vaccines. Recombinant vaccines expressing F or HN of NDV have been developed by using different viral vectors such as Vaccinia virus (Meulemans et al., 1988), Fowlpox virus (Boursnell et al., 1990; Karaca et al., 1998), Pigeonpox virus (Letellier et al., 1991), Herpesvirus of turkeys (Reddy et al., 1996), Marek's disease virus (Sakaguchi et al., 1998) and avian adeno-associated virus (Perozo et al., 2008). Several of these recombinant virus vaccines have been licensed in certain countries for use in poultry.

As a member of the family Herpesviridae, Duck enteritis virus (DEV) possesses a large genome (approximately 158 kb), composed of a unique long (UL) region and a unique short (US) region, with the US region flanked by inverted repeat sequences (IRS and TRS) (Li et al., 2009). Previously, we successfully constructed a new recombinant duck enteritis virus, rDEV-re6, by inserting the HA gene of H5N1 influenza

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https://doi.org/10.1016/j.vetmic.2019.04.022
Received 16 October 2018; Received in revised form 12 April 2019; Accepted 12 April 2019

virus between the US7 and US8 genes of the DEV vaccine strain. rDEV-re6 is safe for chickens and induced a solid immune response in them after a single dose (Liu et al., 2013). The DEV vaccine strain is therefore a promising vector to generate recombinant vaccines expressing other antigens of infectious disease pathogens of chickens.

In this study, we generated two recombinant DEVs containing the F or HN gene of the NDV LaSota strain inserted between the US7 and US8 genes of the DEV vaccine strain and evaluated their protective efficacy in specific-pathogen-free (SPF) chickens against lethal NDV challenge.

2. Materials and methods

2.1. Ethics statement

The animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences (CAAS). SPF chickens were provided by Harbin Veterinary Research Institute, CAAS.

2.2. Viruses and cells

The DEV vaccine strain was obtained from the China Veterinary Culture Collection and propagated in primary chicken embryo fibroblasts (CEFs). The recombinant DEVs were propagated in CEFs, which were prepared from 10-day-old embryonating SPF chicken eggs and maintained in M199 medium (Gibco) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) plus 100 μg/mL of penicillin/streptomycin (Gibco). The NDV LaSota strain and the highly virulent F48E9 strain were originally obtained from the China Veterinary Culture Collection. The NDVs were grown in 9-day-old SPF embryonating chicken eggs and kept in a −80 °C freezer before RNA extraction or use in the challenge study.

2.3. Construction of recombinant DEVs

Recombinant DEVs were constructed by insertion of NDV genes into the intergenic region of the US7 and US8 genes of the DEV genome as previously described (Liu et al., 2011). Briefly, the F and HN genes were amplified from the NDV LaSota strain with specific primer pairs, and then cloned into Gateway entry vectors that contained the SV40 promoter and polyadenylation signal sequence. The expression cassette of F or HN was transferred into destination fosmid T by using Gateway LR recombination (Fig. 1). Recombinant DEVs were rescued respectively by co-transfection of the resultant fosmids with other four fosmids into CEFs as described previously (Liu et al., 2011).

2.4. Stability of recombinant DEVs and confirmation of the expression of the F or HN gene

To evaluate the genetic stability of the rDEVs, total DNA was extracted from rDEV-F- or rDEV-HN-infected cells after six passages in CEFs and detected with a pair of specific primers, Pdus78f and Pdus78r (Fig. 1E). The PCR results were analyzed by agarose gel electrophoresis and DNA sequencing.

The F or HN gene expression of the rDEVs in the infected CEFs was confirmed by Western blotting and an indirect immunofluorescence assay (IFA). Western blotting was performed as described previously (Ge et al., 2007). Briefly, CEF cells were infected with rDEV-F and rDEV-HN at a multiplicity of infection (MOI) of 0.1. The cells were then harvested at 48 h post-infection (h.p.i), lysed, and analyzed by Western blotting using a monoclonal antibody against the HN protein or chicken antiserum specific for the NDV F protein. For the IFA, primary CEFs in 6-well plates were infected with rDEV-F, rDEV-HN, or DEV at an MOI of 0.1. At 48 h.p.i, the cells were fixed with cold (4 °C) methanol for 15 min at room temperature, and then washed three times with phosphate-buffered saline (PBS). Then, specific chicken anti-NDV polyclonal serum (1:100 dilution, prepared in our laboratory) was added, followed by an incubation for 1 h at 37 °C. After three washes with PBS containing 0.1% Tween-20, the cells were incubated with a fluorescein isothiocyanate (FITC)-conjugated goat anti-chicken IgG (1:500, Sigma, Missouri, USA) for 1 h at 37 °C. The cells were then washed three more times before being observed with a laser-scanning confocal microscope (Leica).

2.5. Growth properties of rDEVs

To investigate the growth properties of the rDEVs, the titers of rDEV-F, rDEV-HN, and the DEV vaccine strain were analyzed and compared by using the 50% tissue culture infectious dose ($TCID_{50}$) assay on CEFs. Briefly, cells were infected with different viruses at an MOI of 0.01 in 6-well plates. At 24, 48, 72, and 96 h.p.i., the cells and supernatants were harvested and frozen at −80 °C. Then, one-step growth analyses were performed as described previously (Liu et al., 2011).

2.6. Efficacy of recombinant DEVs in chickens

A total of 40 two-week-old SPF chickens were randomly divided into four groups of 10 chickens. Each group was immunized by intramuscular injection with either $10^{5}$$_{TCID_{50}}$ of rDEV-F, rDEV-HN and $10^{6}$$_{EID_{50}}$of LaSota, or PBS as control in a 100-μl volume. Serum samples were collected for evaluation of neutralization antibody response each week post-vaccination. The virus neutralization test was carried out in 96-well tissue culture plates by using a reporter NDV virus that expressing green fluorescent protein, as described previously (Ge et al., 2007, 2006). Briefly, 2-fold serial dilutions of serum samples were incubated for 1 h with 10^2 $TCID_{50}$ of NDV in DMEM. Then, BHK21 cells were infected with the virus-serum mixture. The neutralizing antibody titer (NT) was obtained by confirmation of the presence of GFP in the wells with the highest dilution of serum. Fourteen days post-vaccination (d.p.v.), chickens were challenged with 10^3 50% chicken lethal dose (CLD_{50}) of the highly virulent NDV strain F48E9 (volume, 100 μl; intramuscular injection) and observed for 14 days for signs and symptoms of NDV disease and death. Oropharyngeal and cloacal swabs were collected on days 3, 5, and 7 days post-challenge (d.p.c.) for virus titration.

2.7. The minimum dose of rDEV-F needed for an immune response

Forty two-week-old SPF chickens were randomly divided into four groups of 10 chickens. Three groups of chickens were intramuscularly immunized (100 μl per chicken) with 10^3, 10^4, and 10^5 $TCID_{50}$ of the rDEV-F vaccine, respectively; the fourth group of chickens was injected with 100 μl of PBS as a control. Two weeks p.v., the chickens were challenged with 10^3 CLD_{50} of NDV strain F48E9 (volume, 100 μl; intramuscular injection). The challenged chickens were monitored daily for 14 days for clinical signs of NDV disease and death.

3. Results

3.1. Rescue of recombinant DEVs

To confirm insertion of the expression cassettes in the rDEVs, we amplified the insertion genes by using the primers Pdus78f and Pdus78r (Fig. 1E). The amplified fragments obtained were 4356 bp and 4428 bp in length from rDEV-F and rDEV-HN, respectively (Fig. 2A). The foreign gene cassettes were further confirmed by sequence analysis (data not shown). These data indicate that we successfully rescued two recombinant viruses rDEV-F and rDEV-HN, containing sv40-F-polyA and

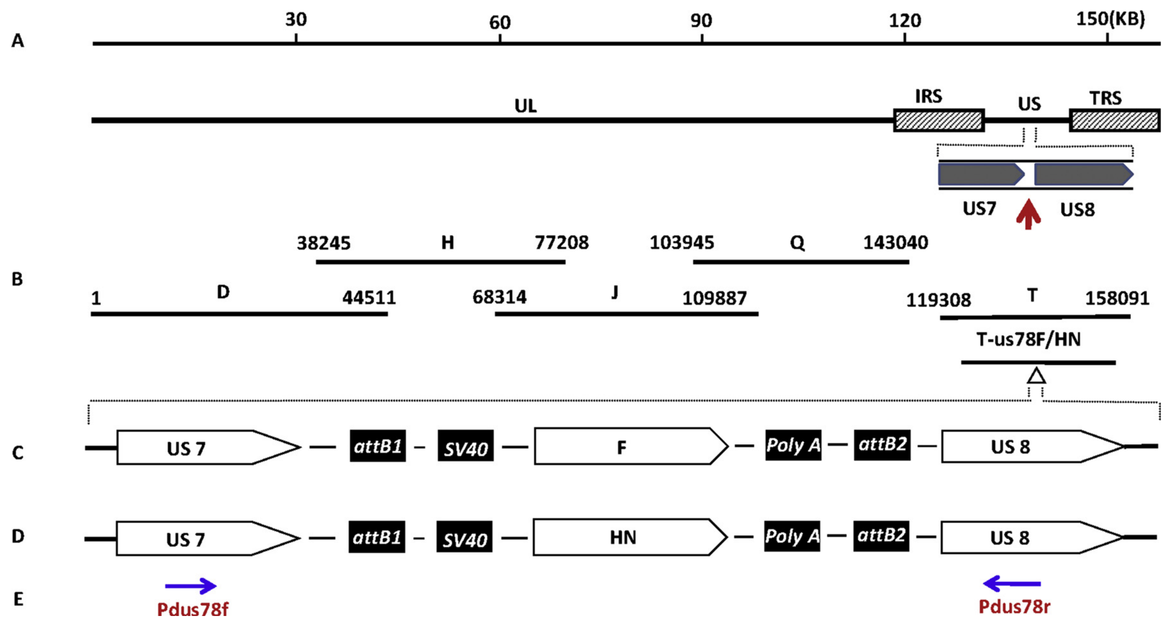

Fig. 1. Insertion of foreign genes into the DEV genome. (A) Genomic structure of DEV. (B) the five fosmid DNAs used for DEV regeneration. Numbers show the location of each fosmid fragment in the DEV genome. (C) Construction of the fosmid with the F gene inserted within the US7 and US8 genes of the DEV genome. (D) Construction of the fosmid with the HN gene inserted within the US7 and US8 genes of the DEV genome. (E) PCR primer design and identification for the assessment of the stability of the recombinant DEVs.

sv40-HN-polyA gene cassettes inserted between the US7 and US8 genes of the DEV genome, respectively (Fig. 1C, D).

3.2. Confirmation of foreign gene expression and growth properties of rDEVs in infected CEFs

Expression of the F and HN genes was confirmed by Western blotting and IFA analysis. All of the rDEV-infected cells showed specific protein bands and green plaques, neither of which were observed in the DEV control group (Fig. 2B, C). These results indicate that the F an HN genes were expressed during rDEV replication in CEFs.

The growth kinetics of rDEV-F and rDEV-HN were examined in vitro by using a $TCID_{50}$ titration assay. As shown in Fig. 3, rDEV-F and rDEV-HN showed comparable growth kinetics to those of the parental virus (DEV vaccine strain). These results demonstrate that insertion of the foreign F or HN gene into the US7 and US8 genes of the DEV genome did not affect virus replication in vitro.

3.3. Protective efficacy against lethal NDV challenge in chickens

To evaluate the efficacy of the rDEVs against lethal NDV challenge in chickens, four groups of SPF chickens were vaccinated with rDEV-F, rDEV-HN, LaSota, or PBS as control, respectively. Neutralizing antibody titers were detected at 7 and 14 d.p.v. Neutralizing antibodies were detectable from most of the chickens in the rDEV-F group, with mean titers of 1.0 and 3.8 $\log_2$, respectively, at 7 and 14 d.p.v.; however, in the rDEV-HN group, neutralizing antibody were detectable from only some of the chickens, and the titers were clearly lower than those for

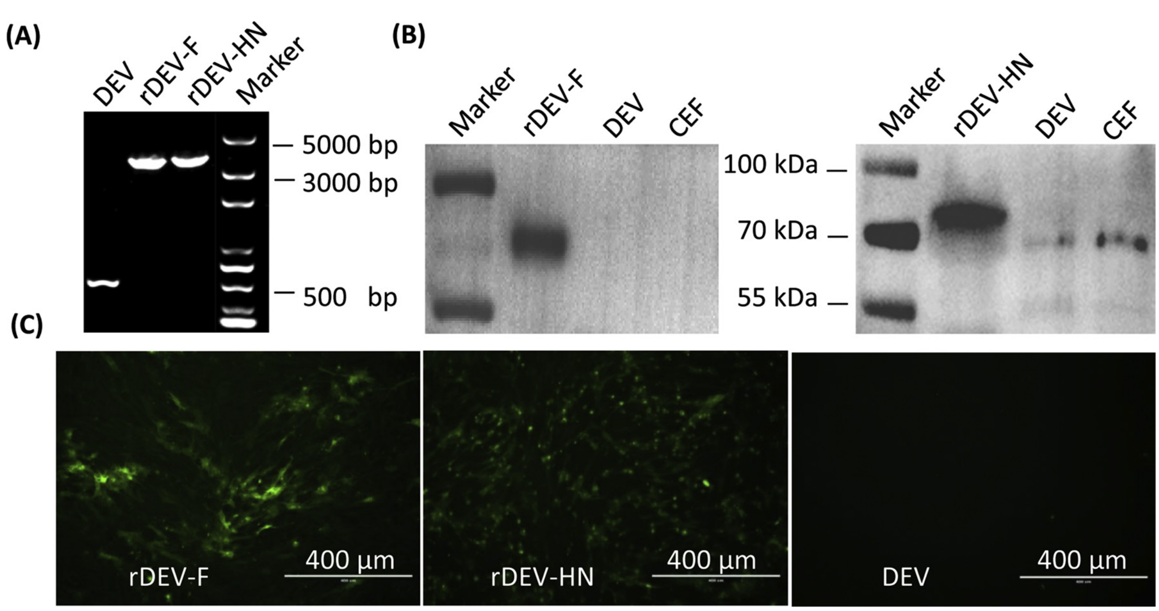

Fig. 2. Stability of the recombinant DEVs. (A) Detection of F/HN gene insertion in rDEV-F or rDEV-HN by use of PCR; (B) Confirmation of F and HN expression in CEFs infected with the rDEVs; (C) Detection of F or HN expression in rDEV-F- or rDEV-HN-infected CEFs by use of immunofluorescence.

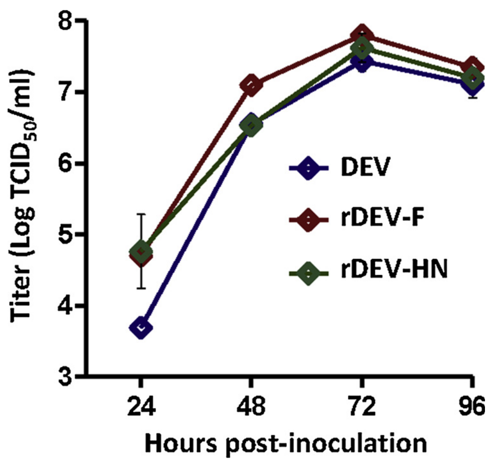

Fig. 3. One-step growth curves of the recombinant virus and its parental virus in CEFs. Infected cells and supernatants were collected, and viral titers were determined at the indicated time points post-inoculation.

the chickens in the rDEV-F group (Table 1). We then challenged four groups of chickens with the NDV F48E9 strain at 14 d.p.v. The chickens in the rDEV-F vaccination group were completely protected from lethal NDV challenge, and showed no signs of disease for the two-week observation period; only one chicken shed virus at 3 and 5 d.p.v. (Fig. 4A, Table 1). However, all of the chickens in the rDEV-HN group showed severe clinical signs from 3 d.p.c., including listlessness, ruffled feathers, and anorexia. All of these chicken shed virus and died within 5 days. All of the chickens in the LaSota group survival and showed no signs of disease for the two-week observation period. The chickens in the control group also showed severe clinical signs from the 3 d.p.c.; they all shed virus and died within 4 days (Fig. 4A, Table 1). These results indicate that rDEV-F can induce 100% protection against lethal NDV challenge in chickens, but rDEV-HN does not induce effective protective immunity in chickens.

3.4. The minimum dose of rDEV-F needed for an immune response

To determine the minimum immune dosage of rDEV-F in chickens, four groups of SPF chickens were vaccinated with either 10^3, 10^4, or 10^5 $TCID_{50}$ of rDEV-F or with PBS. Two weeks later, they were all challenged with the NDV F48E9 strain. As shown in Fig. 4B, two chickens in the 10^3 $TCID_{50}$-vaccinated group showed clinical signs from 4 d.p.c., of excessive sleepiness and anorexia, one died at 6 dpi, and the other chickens died on the last day of the experiment. All of 10^4 and 10^5 $TCID_{50}$-vaccinated chickens showed no clinical signs and survived the duration of the experiment. The control chickens experienced severe clinical signs and all of them died within 4 d.p.c. These results indicate that 10^4 $TCID_{50}$ of rDEV-F can provide 100% protection against lethal NDV challenge in chickens.

4. Discussion

In the present study, we constructed two recombinant DEVs (rDEV-F and rDEV-HN) expressing the major surface glycoproteins of NDV (LaSota), the F protein or the HN protein. Compared with the parental DEV vaccine strain, one-step growth dynamics analyses indicated that insertion of the foreign genes did not affect virus replication in CEFs; IFA analysis confirmed that the F or HN gene was expressed during recombinant virus replication.

The animal studies revealed that rDEV-F provided 100% protection from lethal NDV challenge in chickens after a single dose of 10^4 $TCID_{50}$. However, rDEV-HN did not provide any effective protection. This result is consistent with previous studies, such as that of Kumar S. et al. (2011), who reported that a recombinant avian paramyxovirus (APMV3) type 3 vector expressing the F protein of LaSota (rAPMV3-F) could induce 100% protection against a virulent NDV strain in chickens, but that the recombinant APMV3 expressing the HN protein (rAPMV3-HN) only provided partial protection (Kumar et al., 2011). Similarly, Yang X. et al. (2016) generated a recombinant infectious bronchitis virus (IBV) H120 vaccine strain expressing the HN of LaSota (IBV R-H120-HN/5a) ans showed that it provided 80% protection against NDV F48E9 in chickens (Yang et al., 2016). Sun et al. (2008) constructed a recombinant Fowlpox virus co-expressing the F and HN genes of LaSota and the gB gene of infectious Laryngotracheitis virus (rFPV), which provided 70% protection in chickens from NDV F48E9 challenge (Sun et al., 2008). The reason for these different levels of protective efficacy among the different vector viruses is unknown; one possible explanation maybe that the differences stem from the different antigen-delivering platforms.

Compared with other vector vaccines, the DEV-vectored vaccine has several advantages: it induces rapid protective immunity within a few hours, it is not affected by maternal antibodies, and it grows to high titers in CEFs. Previously, recombinant DEVs expressing the HA protein of H5N1 virus (Liu et al., 2011), the PrM/E protein of duck Tembusu virus (Chen et al., 2014), and the N, S, or S1 protein of infectious bronchitis virus (IBV) (Li et al., 2016) have been reported. These studies indicate that the DEV vaccine strain is an excellent vector for recombinant vaccine development. In the present study, rDEV-F provided 100% protection against a virulent NDV challenge in chickens despite low levels of neutralization antibody, whereas rDEV-HN provided no effective protection. These findings suggest that the humoral response may not be the only factor that helps birds develop protective immunity. The F protein may induce the cell-mediated immune response against NDV infection, thereby protecting birds against the intravenous challenge, which was not the case when HN was used as the antigen. The contribution of each glycoprotein in protection and immunity

Table 1
Protective efficacy of recombinant DEV vaccines against virulent NDV in chickens.

Group	Dose	No. of antibody positive/total (log_2)[a]		No. of swabs showing virus shedding /total no. (titer in $log_{10}EID_{50}$/ml)[b]						No. surviving/ total
		7 dpv	14 dpv	3 dpc		5 dpc		7 dpc		
				Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	
rDEV-F	10^5TCID_{50}	8/10(1.0 ± 0.7)	10/10(3.8 ± 0.4)	1/10(1.3)	1/10(1.8)	1/10(1.5)	1/10(1.3)	0/10	0/10	10/10
rDEV-HN	10^5TCID_{50}	2/10(0.2 ± 0.4)	6/10(1.1 ± 1.3)	10/10(3.7 ± 0.9)	10/10(4.0 ± 1.0)	/	/	/	/	0/10
La Sota	10^6EID_{50}	7/10(0.9 ± 0.6)	10/10(4.1 ± 0.8)	0/10	0/10	0/10	0/10	0/10	0/10	10/10
Control	–	0/10	0/10	6/6(4.7 ± 0.6)	6/6(4.3 ± 0.3)	/	/	/	/	0/10

[a] Sera were collected from all chickens at 7 and 14 d.p.v., and NT antibody titers were determined for each chicken. The titers shown are the means ± standard deviations.

[b] Swabs were collected from all of the available chickens on days 3, 5, and 7 d.p.c. for virus titration in eggs. The titers shown are the means ± standard deviations for the chickens that shed virus. A backslash indicates that the animals had died by that time point.

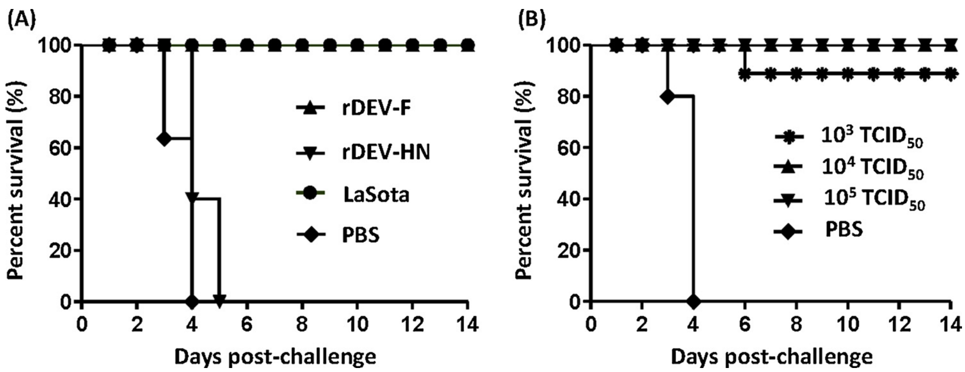

Fig. 4. Protective efficacy of the recombinant virus against lethal NDV challenge and the minimum immune dosage of rDEV-F. (a) Groups of chickens (n = 10 per group) were vaccinated via intramuscular injection with 10^5 $TCID_{50}$ of recombinant DEV viruses (rDEV-F and rDEV-HN), and challenged with lethal NDV at 14 days post-vaccination. Chickens inoculated with PBS served as a negative control. Chickens were monitored daily for 14 days after challenge. (b) Four groups of 10 2-week-old SPF chickens were immunized via intramuscular injection with 10^3, 10^4, or 10^5 $TCID_{50}$ of rDEV-F, or with PBS as a negative control. Fourteen days post-vaccination, the chickens were challenged with a lethal dose of NDV, and observed for symptoms of disease or death for 14 days.

remains unclear and warrants further study. In conclusion, here, we developed a recombinant DEV vaccine expressing the F protein of NDV (LaSota), which was a highly immunogenic and fully protected chicken against lethal NDV challenge. rDEV-F could serve as a promising candidate vaccine in broilers, and could be used in combination with other vaccines in novel immunization strategies.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This work was supported by the National Key Research and Development Program of China (2017YFD0500805), by the National Natural Science Foundation of China (No. 31302064), and by the Harbin Applied Technology Research and Development Project (2013AA6BN001).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.04.022.

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