Research Article

An Inactivated Novel Trivalent Vaccine Provides Complete Protection against FAdV-4 Causing Hepatitis-Hydropericardium Syndrome and FAdV-8b/-11 Causing Inclusion Body Hepatitis

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Received 19 October 2022; Revised 12 November 2022; Accepted 21 November 2022; Published 22 February 2023

Academic Editor: Lin-Zhu Ren

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Outbreaks of hepatitis-hydropericardium syndrome (HHS) caused by fowl adenovirus serotype 4 (FAdV-4) and inclusion body hepatitis (IBH) related to FAdV-8b and FAdV-11 have been increasing in chickens in China since 2015. Clinical concurrent infections of FAdV-4, FAdV-8b, and FAdV-11 are quite common, yet there are no commercially available trivalent vaccines against infection by these three serotypes. In our previous study, a bivalent vaccine based on a recombinant FAdV-4, of which fiber-1 was replaced with the fiber of FAdV-8b, has been developed. In this study, a novel recombinant rFAdV-4-fiber/8b+11 was constructed by inserting FAdV-11 fiber gene into the 1966-bp deletion region of rFAdV-4-fiber/8b genome. The in vitro replication ability of the rFAdV-4-fiber/8b+11 was similar to the parental FAdV-4. One dose immunization with the inactivated rFAdV-4-fiber/8b+11 vaccine generated robust immune responses against FAdV-4, FAdV-8b, and FAdV-11, and provided efficient clinical protection against FAdV-4, FAdV-8b, and FAdV-11 challenge. This study provides a novel strategy for developing potential trivalent vaccines for the prevention and control of HHS and IBH.

1. Introduction

Fowl adenoviruses (FAdV) belong to the genus Aviadenovirus of the family Adenoviridae [1]. FAdV have spread worldwide and are ubiquitous in poultry farms. FAdV are separated into five species (A to E) and subdivided into twelve serotypes (FAdV-1 to -8a and FAdV-8b to -11) [2]. Although most of FAdV infections are subclinical, some FAdV acute infections cause clinical diseases such as inclusion body hepatitis (IBH) and hepatitis-hydropericardium syndrome (HHS) and gizzard erosions (GE) [3]. IBH mainly occurs in 3 to 4-week-old chickens with mortality approaching 10%–30% after 3–4 days [4, 5]. Since the first report of IBH in 1963, the disease has spread worldwide [6]. HHS primarily affects broilers between 3 and 6 weeks of age, causing 20%–80% mortality [7, 8]. Since the first report in Pakistan in 1987, HHS has spread to Asia, Europe, South, and North America. Epidemiological data have confirmed HHS is mainly caused by FAdV-, whereas IBH is associated with FAdV-2, -8a, -8b, and -11 [9–13].

The capsid of FAdV consists of Hexon capsomers, Penton bases, and Fiber proteins. Serotype-specific determinants are located on both the Fiber and the Hexon. FAdV-1, -4, and -10 possess two fiber genes with differences in length, namely fiber-1 and fiber-2, whereas other serotypes including FAdV-8a, -8b, and -11 have only one fiber gene in their genomes [14, 15]. Accumulating data has demonstrated that Fiber plays principal roles in initiating FAdV infection, tissue tropism, virulence, and inducing type-specific neutralization [16–19]. Fiber has been...
repeatedly confirmed as an effective immunogen for developing protective vaccines against different FAdV-induced diseases [20–25].

Since 2015, the outbreaks of HHS and IBH have been spreading widely in China [13, 26]. Epidemiological surveys of FAdV infections in China from 2007 to 2021 indicated that FAdV-4, FAdV-8b, and FAdV-11 were the predominant serotypes [13, 26]. Although several potential vaccines against FAdV-4, FAdV-8b, FAdV-4, and FAdV-8b, or FAdV-8b and FAdV-11, have been developed [20–24, 27–30]; no trivalent vaccine for preventing infection of FAdV-4, FAdV-8b, and FAdV-11 simultaneously has been reported so far. In our previous study, a bivalent vaccine against FAdV-4 and FAdV-8b based on a recombinant rFAdV-4-fiber/8b generated by substituting FAdV-4-fiber with FAdV-8b-fiber, has been developed [29]. In this study, a novel recombinant rFAdV-4-fiber/8b+11 was constructed by inserting FAdV-11 fiber gene into the 1966-bp deletion region of the rFAdV-4-fiber/8b genome. The immunogenicity and efficacy of a potential trivalent vaccine based on rFAdV-4-fiber/8b+11 were investigated.

2. Materials and Methods

2.1. Viruses, Cells, Plasmids, and Antibodies. The virulent FAdV-4 strain CH/HNJZ/2015 (GenBank No. KU558760), FAdV-8b strain SW2021, and FAdV-11 strain FJSW/2021 (GenBank No. OK336458) were isolated by our group and propagated in leghorn male hepatocellular (LMH) cells (ATCC, CRL-2117). LMH cells were maintained in Dulbecco’s Modified Eagle’s Medium/F-12 (Termo Fisher Scientific, MA, USA) containing 10% fetal bovine serum (FBS, AusgeneX, Australia) in a 5% CO₂ incubator at 37 °C. The concentrated rFAdV-4-fiber/8b+11 vaccine was verified by PCR, sequencing, and Western blot.

2.2. Construction of FAdV-4 Infectious Clone Containing the Fiber Genes of Both FAdV-8b and FAdV-11. A FAdV-4 infectious clone containing the fiber genes of both FAdV-8b and FAdV-11 was generated according to a protocol in our lab [19]. Briefly, the amp-ccdB cassette was amplified using primers 1966-ac-F and 1966-ac-R and pR6K-amp-ccdB as a template, and the FAdV-11 fiber gene was amplified from FAdV-11 strain FJSW2021 using primers 1966-fiber/11-F and 1966-fiber/11-R listed in Table 1. The amp-ccdB fragment was first cloned into the 1966-bp deletion region of the p15A-cm-HNJZ-fiber/8b to get the p15A-cm-HNJZ-fiber/8b-amp-ccdB by using LCHR strategy. Next, the amp-ccdB cassette was replaced by FAdV-11b fiber described previously [25]. The correct p15A-cm-HNJZ-fiber/8b+11 plasmid was identified by restriction enzyme digestion and sequencing.

2.3. Generation and in Vitro Characterization of the Recombinant rFAdV-4-Fiber/8b+11. To rescue the recombinant simultaneously expressing the Fibers of FAdV-8b and FAdV-11, LMH cells were transfected with the Pmel-linearized p15A-cm-HNJZ-fiber/8b+11 using Lipofectamine 3000 (Thermo Fisher Scientific, MA, USA). The transfected cells were monitored until typical cytopathic effects appeared. The rescued chimeric virus rFAdV-4-fiber/8b+11 was verified by PCR, sequencing, and Western blot.

2.4. In vitro Growth Properties and Stability of the rFAdV-4-Fiber/8b+11. The rescued rFAdV-4-fiber/8b+11 was serially passaged continuously in LMH cells. The growth kinetics of the 15th passage of rFAdV-4-fiber/8b+11 and wild-type FAdV-4 was assessed in LMH cells at a multiplicity of infection of 0.001, and the viruses were collected at 24, 48, 60, 72, 96, and 120 h postinfection (hpi), respectively. The median tissue culture infective dose (TCID₅₀) of the collected viruses was determined in three technical replicates and calculated by the Reed-Muench method and presented as the mean ± standard error of mean.

The expression of FAdV-4 Fiber-2, FAdV-8b, and FAdV-11 Fiber proteins was verified by Western blotting. rFAdV-4-fiber/8b+11, FAdV-4, FAdV-8b, and FAdV-11 infected LMH cells were harvested and lysed in RIPA lysis buffer with protease inhibitor (Thermo Fisher Scientific, MA, USA). The extracted proteins were subjected to SDS-PAGE and transferred to a nitrocellulose (NC) membrane. The NC membrane was incubated with primary antibody, and then reacted with HRP-labeled secondary antibody. After washing, the membrane was treated with ECL substrate (Millipore, Germany) and developed on Amersham Imager 600 RGB scanner.

2.5. Preparation of Oil-Adjuvant Inactivated rFAdV-4-Fiber/8b+11 Vaccine. The concentrated rFAdV-4-fiber/8b+11 (≥10⁶ TCID₅₀/100 μl) was inactivated with formaldehyde in 0.2% final concentration. The inactivated virus was emulsified with ADJ 501 (W/O/W) adjuvant (Zhengzhou Adjuvant Biotech CO., LTD, Zhengzhou, China) at a ratio of 1: 1 (v/v). The final dose of rFAdV-4-fiber/8b+11 in the oil-emulsion vaccine was 10⁵ TCID₅₀ in 200 μl per bird.

2.6. Animal Experiment. Seventy 7-day-old SPF chickens were equally divided into seven groups designated as vaccinated/challenge FAdV-4, vaccinated/challenge FAdV-8b, vaccinated/challenge FAdV-11, FAdV-4 challenge control, FAdV-8b challenge control, FAdV-11 challenge control group, and the uninfected control group. The vaccinated chickens were inoculated intramuscularly with one dose vaccine. The control chickens
were inoculated with 200 μl of cell culture medium emulsified with ADJ501 adjuvant. Two weeks postimmunization, the vaccinated and challenge control chickens were challenged with either 200 μl (2 × 10^5 TCID_{50}) of the virulent FAdV-4 or 200 μl (2 × 10^6 TCID_{50}) of the FAdV-8b or FAdV-11, correspondingly, and the uninfected control chickens were injected with PBS. Chickens were monitored daily until 7 days postchallenge (dpc).

Serum samples were collected from all the chickens before vaccination and weekly after vaccination for evaluation of FAdV-specific antibody responses by ELISA. Cloacal swabs were collected daily postchallenge. The liver, heart, spleen, kidney, lung,ecal tonsil, pancreas, bursa of Fabricius, proventriculus, and duodenum were collected for viral load measurement. Histopathological lesions were examined in selected organs.

### 2.7. Enzyme-Linked Immunosorbent Assay (ELISA)

The antibody responses against FAdV-4, FAdV-8b, and FAdV-11 induced by the inactivated vaccine were evaluated by a FAdV-specific indirect ELISA with some modifications [33]. Briefly, ELISA plates were coated with *E. coli* expressed Fiber-2 protein of FAdV-4, Fiber protein of FAdV-8b, or FAdV-11, respectively. After washing with PBS containing 0.1% Tween-20 (PBST), diluted sera were added and incubated at 37°C for 1 h. After repeated washing with PBST, the plate was incubated with rabbit antichicken IgY conjugated with horseradish peroxidase (Abcam, Cambridge, UK) at 37°C for 1 h. Then, the plate was washed again and incubated with TMB substrates (Solarbio, Beijing, China) for 10 min at 37°C. Then, 2 M sulphuric acid (Solarbio, Beijing, China) was added to each well to stop the reaction. The OD_{450nm} was measured on the Multiskan GO spectrophotometer (Thermo Fisher Scientific, MA, USA).

### 2.8. Quantification of Viral Loads and Detection of Viral Shedding

To quantify the viral loads in tissues and detect the viral shedding through cloaca, total DNA was extracted from tissue or cloacal swab samples using commercial DNA extraction kit (Tiangen, Beijing, China). The viral loads of challenged viruses were determined by a previously described SYBR Green I quantitative real-time PCR (qRT-PCR) [34]. The final viral DNA concentration was calculated as copy numbers per milligram of the tissue sample.

Viral shedding of challenge viruses was determined by conventional PCR using primers for the fiber-2 gene of FAdV-4, fiber gene of FAdV-8b, and FAdV-11, respectively (Table 1).

### 2.9. Statistical Analysis

All the data were presented as the means ± standard error of mean. Statistical analysis in this study was executed using the Unpaired *t*-test within the GraphPad 6 software. *P* < 0.05 was considered statistically significant.

### 3. Results

#### 3.1. Construction and in Vitro Characterization of the Recombinant rFAdV-4-Fiber/8b+11

To construct a recombinant FAdV-4, expressing fiber of both FAdV-8b and FAdV-11, a recombinant infectious clone p15A-cm-HNJZ-fiber/8b+11 was generated by inserting the FAdV-11 fiber expression cassette into the 1966-bp deletion region of previously constructed infectious clone p15A-cm-HNJZ-fiber/8b as indicated in Figure 1(a). LMH cells were transfected with Pmel-linearized p15A-cm-HNJZ-fiber/8b+11, and the recombinant rFAdV-4-fiber/8b+11 was rescued successfully. The *in vitro* stability of the serially passaged rFAdV-4-fiber/8b+11 was verified by PCR and sequencing (data not shown). The efficient expression of FAdV-4 Fiber-2 (~53 kDa), Fibers of FAdV-8b (~55 kDa), and FAdV-11 (~70 kDa) by the recombinant rFAdV-4-fiber/8b+11 was verified by Western blot in Figure 1(b). The growth kinetics of rFAdV-4-fiber/8b+11 and wild-type FAdV-4 were determined and compared. As indicated in Figure 2, rFAdV-4-fiber/8b+11 replicated efficiently in LMH cells with very similar replication kinetics to that of wild-type FAdV-4, and the peak titer of rFAdV-4-fiber/8b+11 could reach to 10^{5.67} TCID_{50}/100 μl.
3.2. Inactivated rFAdV-4-Fiber/8b+11 Vaccine Provided Efficient Cross-Protection against FAdV-4, FAdV-8b, and FAdV-11. The efficacy of the inactivated rFAdV-4-fiber/8b+11 as a trivalent vaccine candidate was evaluated. After immunization with one dosage of the inactivated rFAdV-4-fiber/8b+11 oil emulsion vaccine, the antibody immune responses against FAdV-4, FAdV-8b, and FAdV-11 were assessed by FAdV-specific ELISA, respectively. As indicated in Figure 3, in the immunized groups, the antibodies against Fiber-2 of FAdV-4, Fibers of FAdV-8b, and FAdV-11 Fibers could be efficiently detected as early as week 1 and markedly increased at week 2 postimmunization (Figures 3(a)–3(c)). The relevant antibodies could not be detected in unvaccinated control chickens.

Two weeks postimmunization, chickens in vaccinated/challenge and challenge control groups were challenged with either virulent FAdV-4, FAdV-8b, or FAdV-11, correspondingly. FAdV-4 challenge control chickens developed HHS-indicative clinical signs including depression and adopting a crouching position. The onset of mortality started...
at 48 hpc and there were no survivors at 96 hpc (Figure 4(a)). On the contrary, all vaccinated chickens survived and did not show any HHS-indicative clinical signs. Although all FAdV-8b and FAdV-11 challenged chickens survived throughout the experiment (Figures 4(b) and 4(c)), FAdV-8b or FAdV-11 challenge control chickens showed temporary loss of appetite and green watery excrement from day 2 to 5 postchallenge.

To further evaluate the efficacy of the trivalent vaccine, the gross and microscopic lesions, viral DNA in different tissues and viral shedding through cloaca were examined. As indicated in Figure 5(a), After FAdV-4 challenge, all sick/dead chickens in FAdV-4 challenge control group exhibited typical HHS-indicative gross lesions. In contrast, chickens immunized with the inactivated rFAdV4-fiber/8b + 11 vaccine did not show any obvious lesions (Figure 5(a)). FAdV-8b or FAdV-11 challenge control chickens groups presented typical gross IBH-indicative lesions, characterized by swollen livers with white focal necrosis or petechial hemorrhages (Figures 5(b) and 5(c)). However, no obvious gross lesions in the kidney and heart were observed in unvaccinated and FAdV-8b or FAdV-11 challenged chickens (data not shown).

The typical histopathological lesions presented by the sick/dead chickens in the FAdV-4 challenge control group included widened myocardial cell gap and myocardial fiber rupture; severe degeneration and necrosis of hepatocytes; degeneration and necrosis of renal tubular epithelia; necrosis of mucosal epithelia in duodenums; and necrosis and depletion of lymphocytes in the bursa of Fabricius (Figure 6(a)). Comparing with the massive severe degeneration and necrosis of hepatocytes caused by FAdV-4, the primary histopathological lesions presented by the unvaccinated and FAdV-8b or FAdV-11 challenge groups included multifocal degeneration and necrosis of hepatocytes and mononuclear cell infiltration (Figure 6(b)). However, the inactivated rFAdV4-fiber/8b + 11 vaccine immunized chickens did not show HHS- or IBH-indicative histopathological lesions (Figures 6(a) and 6(b)).

As indicated in Figure 7, viral DNA copy numbers in different tissues from vaccinated chickens were significantly lower than those of challenge control chickens. Only
A background level of viral loads was detected in most tissues from vaccine immunized and uninfected control chickens. The efficacy of inactivated rFAdV4-fiber/8b + 11 vaccine protected chickens from shedding challenge virus was also assessed. As shown in Table 2, vaccinated chickens stopped excreting challenged FAdV-4, FAdV-8b, and FAdV-11 after 3, 4, and 5 dpc, respectively. All the challenge control chickens excreted the challenge viruses until the end of experiment.

4. Discussion

FAdV have been related with numerous disease conditions in chickens. FAdV-4 causing HHS and FAdV-8b or FAdV-11 causing IBH have spread worldwide and resulted in significant economic losses to the world poultry industry. Vaccination has been demonstrated as one of the most effective and cost-efficient infectious disease interventions that there is. However, the control of HHS and IBH turns out to be more complicated by the involvement of multiple FAdV serotypes [3]. Most HHS are caused by FAdV-4, whereas FAdV-2, FAdV-8a, FAdV-8b, and FAdV-11 are related to IBH. The outbreaks of HHS and IBH have been increased since 2015 [13, 26, 35]. There is an urgent need for developing multivalent vaccines against various FAdV serotypes causing HHS and IBH.

The fiber protein of FAdV has been proven as an efficacious antigen for developing vaccine candidates [21, 36]. However, the intrinsic serotype-specific neutralizing activity of Fiber limits the application and efficacy of fiber-based vaccines [36]. For controlling HHS, vaccine candidates based on inactivated FAdV-4 or Fiber-2 protein have been...
Figure 5: Continued.
generated and proven to be efficient [20, 22, 24, 37–42]. In order to develop potential vaccines providing broad cross-protection against HHS and IBH caused by various serotypes of FAAdV, several bivalent vaccine candidates against both FAAdV-4 and FAAdV-8b, FAAdV-8a and FAAdV-8b, or FAAdV-4 and FAAdV-11, have been generated [23, 27–29]. De Luca et al. and Schachner et al. have developed vaccines based on chimeric fibers crecFib8b/8a or crecFib-4/11 containing epitopes from fibers of FAAdV-8a and FAAdV-8b, Fiber-2 of FAAdV-4, and Fiber of FAAdV-11, respectively [23, 27]. The chimeric Fibers induced cross-neutralizing antibodies against the corresponding both serotypes and provided protection against FAAdV-8a or FAAdV-8b challenge and FAAdV-4 or FAAdV-11 challenge. These efforts provide new ideas for the development of multivalent subunit vaccines against FAAdV. Lu et al. have generated a chimeric FAAdV-4, FA4-F8b by inserting the fiber gene of FAAdV-8b between fiber-1 and fiber-2 of FAAdV-4, and proven the potential application of the inactivated FA4-F8b as a vaccine candidate against FAAdV-4 and FAAdV-8b [28].

FAAdV-4 possesses two fiber genes that encode Fiber-1 and Fiber-2 proteins. Fiber-1 plays crucial role in mediating FAAdV-4 infection and Fiber-2 plays important roles in inducing protective immune responses [37, 43, 44]. FAAdV-8b and FAAdV-11 only have one fiber gene in their genomes. Based on the above facts, we speculate that the Fiber of FAAdV-8b or FAAdV-11 plays dual roles in mediating viral infection and inducing effective antiviral immune responses. In our previous study, a recombinant FAAdV-4, rFAAdV-4-fiber/8b was successfully constructed by replacing fiber-1 of FAAdV-4 with the fiber of FAAdV-8b [29]. The recombinant rFAAdV-4-fiber/8b maintained efficient replication capacity and pathogenicity as the parent FAAdV-4. The inactivated bivalent vaccine based on the rFAAdV-4-fiber/8b also provided efficient protection against FAAdV-4 causing HHS and FAAdV-8b causing IBH. As the 1966-bp natural deletion region and more than 20 genes in the FAAdV-4 genome have been identified as potential insertion sites for foreign genes [19, 45], thus using wild type or modified FAAdV-4 as a vector to express fiber genes from other serotypes of FAAdV provides another strategy for developing multivalent vaccines for simultaneous control of HHS and IBH.

Epidemiological data have indicated that FAAdV-4, FAAdV-8b, and FAAdV-11 are the predominant serotypes in China [25, 46, 47]. Clinical concurrent infections of various serotypes of FAAdV are quite common, yet there are no trivalent vaccines for the prevention and control of HHS and IBH. In the present study, a novel recombinant rFAAdV-4-fiber/8b + 11 coexpressing Fibers of FAAdV-8b and FAAdV-11 was constructed by inserting the fiber gene of FAAdV-11 into the 1966-bp deletion region of the rFAAdV-4-fiber/8b genome. The rFAAdV-4-fiber/8b + 11 showed similar growth kinetics in vitro with the wild-type FAAdV-4. A single immunization with the inactivated rFAAdV-4-fiber/8b + 11 vaccine generated specific immune responses against FAAdV-4, FAAdV-8b, and FAAdV-11. All immunized chickens developed full resistance to clinical disease and did not present HHS- and IBH-indicative gross and histopathological lesions. Compared with unvaccinated and challenged chickens, vaccinated chickens exhibited significantly lower viral loads in various tissues. This study confirmed Fiber as a critical immunogen for developing vaccines against diseases caused by various FAAdV. Since both the recombinant rFAAdV-4-fiber/8b in our previous study and the recombinant rFAAdV-4-fiber/8b + 11 in the present study can replicate efficiently as the parent FAAdV-4, it implies that the Fiber of FAAdV-8b and Fiber-1 of FAAdV-4 might play the similar role in initiating viral infection of target cells. In order to

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**Figure 5:** The representative gross lesion in the heart, liver, and kidney from chickens in each group after challenging with FAAdV-4 (a), FAAdV-8b (b), and FAAdV-11 (c). (a) Chickens in the unvaccinated and FAAdV-4 challenge group showed accumulation of clear straw-colored fluid in the pericardial sac, swollen and discolored liver, and enlarged kidneys with distended tubules. (b) and (c) Chickens in the FAAdV-8b or FAAdV-11 challenge control group exhibited IBH-indicative gross lesions, characterized by swollen livers with small white focal necrosis and petechial hemorrhages.
verify whether the Fiber of FAdV-11 may also play a similar role as Fiber-1 of FAdV-4, we are making another chimeric FAdV-4 in which fiber-1 of FAdV-4 will be seamlessly replaced with the fiber of FAdV-11.

In summary, a novel chimeric FAdV-4 coexpressing Fibers of FAdV-8b and FAdV-11 was generated for the first time. The inactivated rFAdV-4-fiber/8b + 11 could be used as a trivalent vaccine for simultaneous prevention of the concurrent infection of FAdV-4, FAdV-8b, and FAdV-11. This study provides a novel strategy for developing potential multivalent vaccines for the prevention of IBH and HHS.

Figure 6: Representative histopathological lesions in tissues from chickens in each group after challenging with FAdV-4, FAdV-8b, and FAdV-11. (a) Histopathological lesions including widened myocardial cell gap and myocardial fiber rupture; severe degeneration and necrosis of hepatocytes; degeneration and necrosis of renal tubular epithelium; mucosal epithelial cell nuclear fragmentation and necrosis in duodenum; and severe depletion and necrosis of lymphocytes in the bursa of Fabricius were observed in the chickens from the unvaccinated and FAdV-4 challenge group. (b) Compared with the massive severe degeneration and necrosis of hepatocytes caused by FAdV-4, the primary histopathological lesions presented by the unvaccinated and FAdV-8b or FAdV-11 challenge groups included multifocal degeneration and necrosis of hepatocytes and mononuclear cell infiltration. However, chickens from the inactivated rFAdV4-fiber/8b + 11 vaccinated group did not present histopathological lesions of HHS and IBH mentioned above (HE staining, original magnification ×400).
**Figure 7: Continued.**

(a) **log_{10} (Copy number/mg)**

(b) **log_{10} (Copy number/mg)**

- Unvaccinated
- Vaccinated
- Uninfected Control
Data Availability
The data used to support the findings of this study are included within the article.

Conflicts of Interest
The authors declare that there are no conflicts of interest regarding the publication of this article.

Authors’ Contributions
Congcong Song, Shiyi Zhao, and Mingzhen Song contributed equally to this work.

Acknowledgments
This work was supported by the National Natural Science Foundation of China (Grant no. 31772771). Prof. Hailong Wang and Prof.Jun Fu from State Key Laboratory of Microbial Technology, Shandong University, Qingdao, China, provided valuable assistance in generating the recombinant virus.

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