Assessing the efficacy of a recombinant H9N2 avian influenza virus–inactivated vaccine

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ABSTRACT The H9N2 avian influenza virus has been widely spread in poultry around the world. It is proved to the world that the avian influenza virus can directly infect human beings without any intermediate host adaptation in "1997 Hong Kong avian influenza case," which shows that the avian influenza virus not only causes significant losses to the poultry industry but also affects human health. In this study, we aimed to address the problem of low protection of avian H9N2 subtype influenza virus vaccine against H9N2 wild-type virus. We have rescued the H9.4.2.5 branched avian influenza virus isolated in South China by reverse genetics technology. We have recombined these virus (rHA/NA-GD37 and rHA/NA-GD38) which contain hemagglutinin and neuraminidase genes from the H9N2 avian influenza virus (MN064850 or MN064851) and 6 internal genes from the avian influenza virus (KY785906). We compared the biological properties of the virus for example virus proliferation, virus elution, thermostability, and pH stability. Then, we evaluated the immune effects between rHA/NA-GD37 and GD37, which show that the recombinant avian influenza virus–inactivated vaccine can stimulate chickens to produce higher antibody titers and produce little inflammatory response after the challenge. It is noticeable that the recombinant virus-inactivated vaccine had better immune impact than the wild-type inactivated vaccine. Generally speaking, this study provides a new virus strain for the development of a H9N2 vaccine.

Key words: H9N2, reverse genetics system, vaccine efficacy

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INTRODUCTION

Avian influenza viruses (**AIV**) pose a major threat to global biosafety, causing billions of dollars of damage to affected countries (Qi et al., 2014). H9N2 was first isolated in 1994 in China (Li et al., 2019). During 1996 to 2000, more than 90% of chicken flocks were subjected to H9N2 (Gu et al., 2017). What more serious is that the influenza virus is becoming more and more serious risk for humans through genetic drift or genetic recombination. Notwithstanding the classification of H9N2 viruses as being of low pathogenicity, but it induces respiratory signs and reproductive disorders (Qi et al., 2016), in particular the H9N2 virus may increase infection and lead to increased morbidity and mortality when coinfected with other pathogens (Smietanka et al., 2014; Hassan et al., 2017), Moreover, since H9N2 virus has been isolated from 2 children in Hong Kong, indicating that H9N2 AIV may also be transmitted directly from birds to humans (Zhou et al., 2014), the case records of human infections have been found more frequently in Mainland China, which further confirmed that the H9N2 virus can be directly transmitted to humans. In recent years, an increasingly large number of human-diagnosed infections with the H9N2 influenza virus have been reported, which proved it is a destructive power and epidemic (Guan et al., 2000). During surveillance for other zoonotic influenza virus infections, a growing body of serologic evidence shows that

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asymptomatic or mild infections with H9N2 may be fairly common in enzootic areas, especially among poultry workers, hence, the prevention and control of H9N2 become particularly important (Khan et al., 2015).

Influenza viruses belong to the family Orthomyxoviridae, which could be classified into 3 types, A, B, and C. Type (Xu et al., 2018) A influenza viruses were further categorized into different subtypes as per the variant of the surface hemagglutinin (**HA**) and neuraminidase (**NA**) protein's antigenicity (Zhao et al., 2013). There are at least 144 possible combinations between hemagglutinin and 9 NA in the AIV, which have 16 HA and 9 NA subtypes at present research (Alexander, 2000; Fouchier et al., 2005). It caused the H9N2 recombination to be so fast that no vaccine can provide effective protection for chickens against H9N2. The genetic characteristics of HA gene which determined that substitution at key positions may result in different antigenic properties (Sobel et al., 2017). For example, the BJ/94 original HA gene has evolved 13 clades in the past 10 yr, and the contribution of the internal gene to the novel H7N9 virus also leads to an increase in the prevalence of H9N2, which enhances the infectivity and antigenic drift (Pu et al., 2015). Remarkable molecular differences between isolates from 2013 to 2014 were identified in Poland, including mutation in the HA cleavage site leading to conversion which can be responsible for enhanced activity of viral polymerase in mammalian cells (Swieton et al., 2018). Interestingly, the reassortment of HA gene increases the host range of the virus. In the past 30 yr, the host range expanded from the waterfowl to chicken. The H9N2 genotype has been exacerbated owing to the genetic diversity of host small birds and wild birds, whereas the chicken is regarded as most common carriers in the evolution and expansion of the H9N2 virus.

There are 2 main forms of influenza virus antigenic variation: antigenic shift and antigenic drift (Robertson, 1987). So it is difficult for us to figure out the regular pattern of their changes, which is also the main reason why the influenza virus is still unable to be effectively controlled (Webster et al., 1992). Therefore, it is necessary to continually update the H9N2 clade classification to follow their genetic evolution. The best strategy for controlling an influenza pandemic is to produce vaccines early from the pandemic strain itself or closely matched strains of antigenicity. Fodor built a 12-plasmid system for assembling influenza viruses in 1999; then, to increase efficiency, Robert tried to used pHE2000 plasmid containing pol I to pol II bidirectional promoter building a 8-plasmid system (Karron et al., 2009). Currently, this system is widely used to save the influenza virus and study the molecular mechanism of influenza virus antigen variation.

The present study was undertaken to address this problem that low protection rate of the classical inactivated avian influenza (H9N2) virus vaccine against the wild H9N2 virus. In this study, we used the H9N2 virus isolated (MN064850) from the South China in 2018 preserved in our laboratory; then, we obtained the recombinant AIV by using the 8-plasmid system. Next, we conducted animal experiments to evaluate its immune efficacy compared with its inactivated vaccine. In this study, we performed HA gene sequencing of H9N2 subtype AIV. To understand the pandemic H9N2 subtype strains, phylogenetic analysis of HA genes was carried out among the isolation and represents H9N2 subtype strains. For the selection and development of candidate vaccine, animal experiments were performed to evaluate the effectiveness of the vaccine strains This provides basis for scientific control of H9N2 avian influenza in the future.

MATERIALS AND METHODS

Clinical Samples and Virus Isolation

The samples of chicken swabs were collected from chicken farms in southern China. After being immunized with the commercial H9N2 AIV vaccine, the vaccine cannot protect chickens from infection by epidemic strains. A total of 50 samples were individually inoculated into 9-day-old specific pathogen–free (**SPF**) embryonic chicken eggs via the allantoic cavity, and the allantoic fluid was harvested individually after 48 h inoculation. The hemagglutination assay were performed as per OIE laboratory standards to determine the virus titers.

Cells and Plasmids

HEK 293T cells were preserved in our laboratory and cultured with 10% fetal bovine serum (Invitrogen Gibco Co.). The Hoffmann vector system used to generate recombinant influenza viruses was kindly provided by Dr. Liu Qinfang of the Chinese Academy of Agricultural Sciences.

Phylogenetic and Molecular Analysis

Viral RNA was extracted from allantoic fluids and reverse transcribed using a standard protocol (TaKaRa, Dalian). Subsequently, PCR was performed using the specific primer pairs (Table 1). The amplicons were purified using a QIAquick PCR Purification Kit (QIAGEN), inserted into the pMD19-T vector (TaKaRa, Dalian), and sequenced using synthetic oligonucleotides (Invitrogen). Phylogenetic analysis was conducted based on the neighbor-joining method using the software package MEGA v3.1. Bootstrap values were calculated from 1,000 replicates. All of the nucleotide sequences have been deposited in the GenBank database (Table 2).

Plasmids Construction and Generation of Recombinant Viruses

Reverse transcription-PCR was performed with segment-specific primers as described previously (Table 1) (Hoffmann et al., 2001). Briefly, the viral RNA of A/chicken/China/GD37/2018 and A/chicken/China/GD38/2018 were extracted and used as a

Table 1. Primer set used for RT-PCR amplification of the 8 vRNAs of influenza A viruses.

Gene	Primer pairs(5′–3′)	Product (bp)
PB2	F:TATTGGTCTCAGGGAGCGAAAGCAGGTC R:ATATGGTCTCGTATTAGTAGAAACAAGGTCGTTT	2,370
PB1	F:TATTGGTCTCAGGGAGCGAAAGCAGGCA R:ATATGGTCTCGTATTAGTAGAAACAAGGCATTT	2,370
PA	F:TATTGGTCTCAGGGAGCGAAAGCAGGTAC R:ATATGGTCTCGTATTAGTAGAAACAAGGTACTT	2,262
HA	F:TATTGGTCTCAGGGAGCGAAAGCAGGGG R:ATATGGTCTCGTATTAGTAGAAACAAGGGTGTTTT	1,807
NP	F:TATTGGTCTCAGGGAGCGAAAGCAGGGTA R:TATTGGTCTCAGGGAGCGAAAGCAGGGTATTTTT	1,594
NA	F:TATTGGTCTCAGGGAGCGAAAGCAGGAGT R:TATTGGTCTCAGGGAGCGAAAGCAGGAGTTTTTT	1,442
M	F:TATTGGTCTCAGGGAGCGAAAGCAGGTAG R:TATTGGTCTCAGGGAGCGAAAGCAGGTAGTTTTT	1,056
NS	F:TATTGGTCTCAGGGAGCGAAAGCAGGGTG R:TATTGGTCTCAGGGAGCGAAAGCAGGGTGTTTT	919

template to amplify the HA and NA genes. The amplicons were inserted into the Phw2000 vector, which were used to reconstruct eukaryotic expression vectors. All plasmids were designated Phw2000-HA and Phw2000-NA.

HEK 293T cells were seeded to 70 to 90% confluency were used for transfection. Dilute Lipofectamine 3000 Reagent in Opti-MEM Medium (2 tubes) was mixed well. Reparing master mix of 1 μg DNA of each individual plasmid in no more than 25 μL by diluting DNA in Opti-MEM Medium, then adding P3000 Reagent and mixed well, and incubating for 15 min at room temperature. After incubation at 37°C for 36 h, 1 μg/mL TPCK trypsin was then added. After 2 h, the transfection mix was collected. Ten-day-old SPF embryonated chicken eggs were inoculated with 200 μL of transfection mix. After 48 h of incubation, chick embryo allantoic liquid was collected. After purification for 3 generations, rescued viruses were titrated for viral infectivity by 50% embryo infective dose (**EID_{50}**). The virus was divided and stored at −70°C. All SPF chickens were purchased from WENS (Yun Fu, China).

Indirect Immunofluorescence

HEK 293T cells were cultured in a 6-well microplate at a density of 1 × 10^5/well. Once the cell density reached 70%, the constructed recombinant plasmid was transfected into HEK 293T cells as described previously. After 48 h, cells were fixed with 4% paraformaldehyde (PanEra, China), punched with 0.1% Triton X100 (PanEra, China), and blocked with 5% skim milk powder (BD) at room temperature for 30 min; then, the cells were incubated with the corresponding primary antibodies. Finally, the cells were observed under a fluorescence microscope (Nikon, Japan).

Table 2. Reference strain of the avian influenza virus (H9N2).

Virus	GenBank accession numbers
A/chicken/China/GD38/2018	MN064850
A/chicken/China/GD37/2018	MN064851
A/chicken/Shandong/696	DQ064376
A/chicken/Guangdong/SS/94	AF384557
A/guineafowl/HongKong/NT184/03	AY664674
A/chicken/Jiangsu/454/2013	KP693787
A/chicken/Fujian/25/00	DQ064355
A/chicken/Guangdong/LRZ01/2012	KJ769001
A/chicken/Fujian/SL6/2011	JF715052
A/chicken/Hubei/SC122/2013	KM113081
A/chicken/Guangdong/H07/2013	KJ768987
A/chicken/Zhejiang/HE6/2009	GU471873
A/chicken/Jiangsu/WJ57/2012	KJ000710
A/chicken/Beijing/243/2010	KF746876
A/Hebei/218/2010	KC296446
A/chicken/Shandong/HL/2010	KC821004

Virus Proliferation

The EID_{50} of the recombinant virus was detected using SPF chicken embryos. Chicken embryos were inoculated at the same dose, and virus titers were detected at 12, 24, 36, 48, 60, 72, 84, and 96 h. The titers of all samples were determined using hemagglutination assay.

Thermostability, pH Stability, and Virus Elution from Erythrocytes

Recombinant viruses were divided into 60 μL aliquots. All aliquots were exposed to 56°C at 0, 5, 10, 15, 30, 60, 90, 120, 150, 180, and 210 min incubation and then quickly cooled to 4°C. Methanol-inactivated recombinant viruses were incubated at 37°C or 42°C at 2 h intervals for 18 h. In addition, recombinant viruses were mixed with an equal volume of 100 mmol/L acetate buffer (pH = 4.0 and pH = 5.0), 100 mmol/L phosphate buffer (pH = 6.0), or neutral phosphate buffer (pH = 7.0) and incubated at 37°C for 10 min (Wang et al., 2019).

Virus elution assay was performed as per previous reports (Wang et al., 2019). Briefly, the mutant viruses were diluted 2 times in PBS, and 50 μL aliquots were incubated with 50 μL of chicken erythrocytes (0.5% in PBS) in V-bottomed microtiter plates at 4°C for 30 min. Afterward, the plates were transferred to 37°C, and the precipitation of agglutinated erythrocytes was monitored periodically for the next 10 h.

Ethics Statement

This study was approved by the Animal Care Committee of South China Agricultural University (approval ID: SYXK-2019-0136). All study procedures and animal care activities were conducted in 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.

Animal Experiments

A total of 120 1-day-old SPF chicks were randomly divided into 6 groups (20 chicks per group). The SPF chicks in group I and II were vaccinated at 10 D with recombinant inactivated vaccine (rHA/NA-GD37) at $10^{7.8}$ EID_{50}/0.35 mL via the hypodermic route; the SPF chicks in group III and IV were vaccinated with inactivated vaccine (GD37) $10^{7.8}$ EID_{50}/0.35 mL via the hypodermic route. The SPF chicks in group V were vaccinated with PBS as a negative control, and subsequent immunization of SPF chickens at 20 D once again was carried out. Finally, the group II (r-HA/NA-△GD37), IV (△GD37), and VI (△GD37/2018) were inoculated with A/chicken/China/GD37/2018 at 10^6 EID_{50}/0.1 mL through the respiratory tract at 34 D.

The serum antibody levels against wild H9N2 (A/chicken/China/GD37/2018) were determined using a hemagglutination inhibition assay For hemagglutination inhibition assays, at 5 and 7 D postchallenge (**dpc**), 3 chicks in II, IV, and VI groups were humanely euthanized to collect the lungs. Each piece was fixed in 10% neutral buffered formalin for 24 h and embedded in paraffin; then, sections of 4 mm thickness were stained with hematoxylin and eosin stain. The pulmonary tissue slice was observed using a microscope. Five and 7 D after challenge, throat swabs and cloacal swabs in II, IV and VI groups were collected for virus isolation.

Statistical Analysis

Data analysis was conducted using GraphPad Prism (version 5.0; La Jolla, CA) and were expressed as the mean ± SE. Unless otherwise noted, the differences between treatment groups were analyzed using a Student 2-tailed t test and 1-way ANOVA. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Phylogenetic Analysis

The whole genome sequences of 2 isolated H9N2 AIV were successfully sequenced. The coding sequence of HA of 2 H9N2 AIV strain was 1,683 bp in length. The sequences were deposited into the GenBank database under the accession numbers MN064850 and MN064851. The phylogenetic relationships of HA are shown in Figure 1. Both isolates belonged to the H9.4.2.5.

Construction and Characterization PR8-Derived H9N2 Recombinant Virus

To rescue the recombinant H9N2 AIV (rHA/NA-GD37 and rHA/NA-GD38), we first reconstructed the expression vectors Phw2000-HA and Phw2000-NA and then transfected 8 vectors into 293 cells. The recombinant viruses were determined using an immunofluorescence assay. As a result, HA expression was detected (Figure 2). Cell supernatants were inoculated into 9-day-old SPF chicken embryos. Allantoic fluid was collected and passaged serially to 20 generations. The

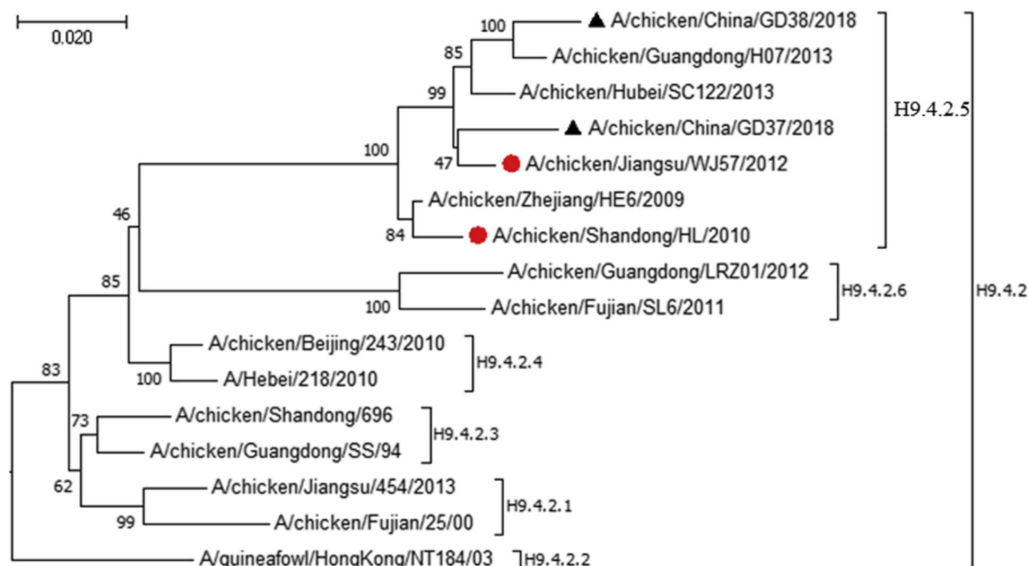

Figure 1. Phylogenetic tree of the representative viruses strains based on the HA gene. Clade numbers are indicated on the right panel. Trees were constructed with MEGA 7.0 software using the neighbor-joining method. Bootstrap analysis was performed with 1,000 replications. "•" are vaccine strain sequences; "▲" are Isolated virus strains sequences determined in this study. All sequences obtained from GenBank.

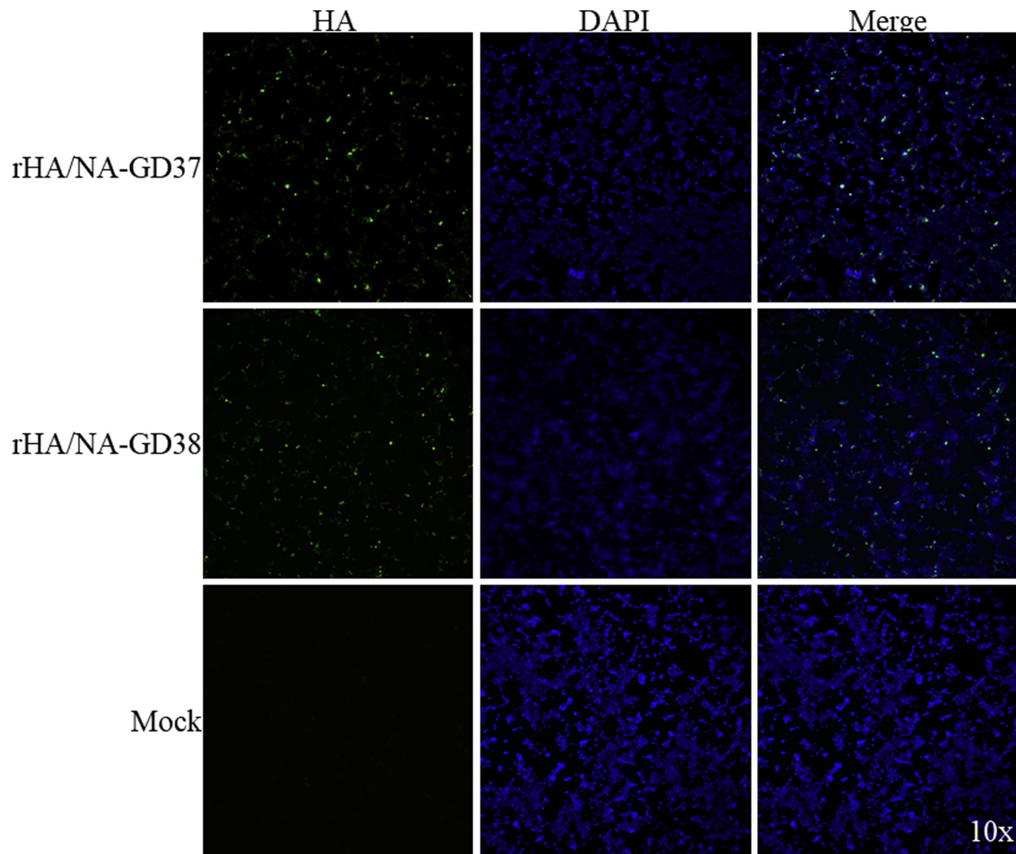

Figure 2. HA gene expression detection. Accommodation of HA genes in virus and HA expression in HEK 293T virus–infected cells. Expression of HA was examined by fluorescence microscopy.

virus titer reached log2^6 indicating that the recombinant virus was successfully rescued.

Virus Proliferation

To evaluate the stability of the 20th generation recombinant virus, the hemagglutination titer was determined. GD-38 began to proliferate significantly at 36 h, and the virus titer reached the highest after 72 h. Finally, the virus titer was maintained at 2^8. rHA/NA-GD38 began to proliferate significantly at 48 h, and the virus titer reached the highest after 72 h. Finally, the virus titer was maintained at 2^6 (Figure 3A). Both rHA/NA-GD37 and GD-37 began to proliferate significantly at 36 h. The virus titer reached the highest after 72 h, but the highest rHA/NA-GD37 titer is 2^8 and the highest GD-37 titer is 2^7 (Figure 3B).

Characterization of Recombinant Virus

We determined the hemagglutination titer to evaluate the stability of recombinant virus elution and thermostability and pH stability. As a result, the elution time of 2 H9N2 AIV strains ranged from 8 h to 10 h (Figure 4A). The recombinant virus rHA/NA-GD37 eluted from erythrocytes at 8 h, which was faster than wild-type GD-37 (Figure 4B). Two recombinant viruses were

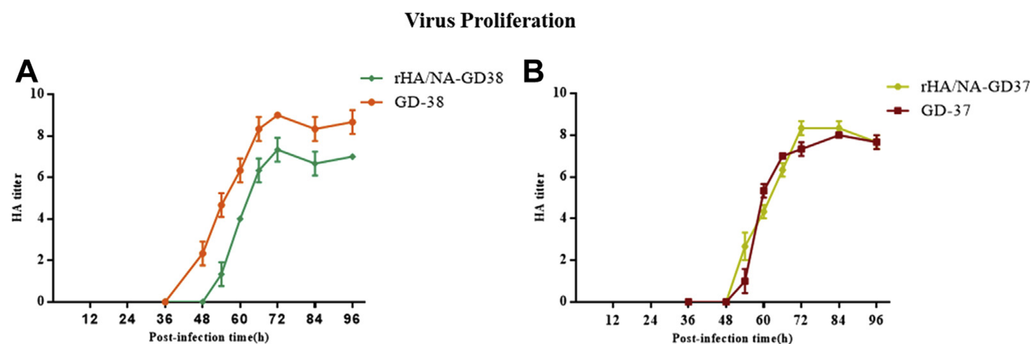

Figure 3. Virus proliferation of wild-type strains (A) and the recombinants (B).

considered stable at 56°C as the hemagglutination titer decreased by $2log^2$ after 30 min (Figure 4C). The inactivated recombinant viruses were thermostable at 37°C (Figur 4D) and 42°C (Figure 4E). The hemagglutination titer of recombinant viruses showed no obvious loss when pH dropped from 6.0 to 5.0, compared with the PBS-treated control at pH 7.0 (Figure 4F). The hemagglutination titers of all strains decreased to 0 at pH 4.0. In summary, we selected rHA/NA-GD37 as a vaccine candidate.

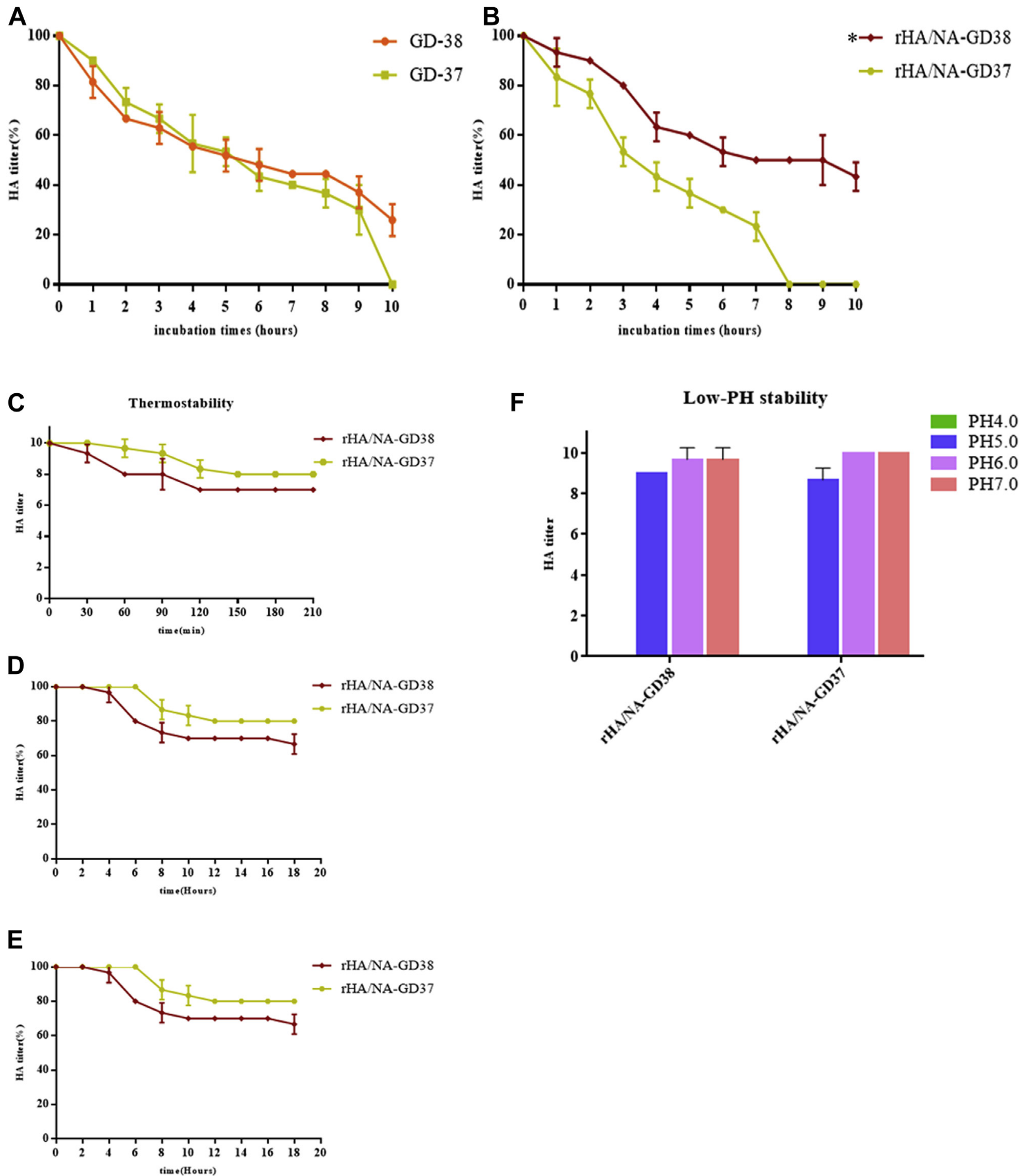

Figure 4. Viral physicochemical properties. Viral elution from chicken erythrocytes with wild-type strains (A) Viral elution from chicken erythrocytes with the recombinants strains (B). Two-fold dilutions of viruses were incubated with equal volumes of chicken erythrocytes at 4°C for 30 min, and the HA titer at 37°C representing virus elution from chicken erythrocytes was monitored each hour. Results were presented as the percentage of the initial HA titer at 4°C. Thermal stability of the recombinant viruses (C). Aliquots of 60 μL of recombinant viruses were exposed to 56°C for 150 min. Then, inactivated viruses were exposed to 37°C (D) or 42°C (E) for 18 h, and aliquots were collected every 2 h. pH stability of the recombinant viruses (F). The titers of all aliquots were determined using hemagglutination assays.

The Titers of the Antibodies of the Sera

There was no significant difference in the titer of antibodies produced by inactivated-vaccine groups I (rHA/NA-GD37) and III (GD37) after 7 D after immunization) (Figure 5). Inactivated-vaccine groups I(rHA/NA-GD37) after 7 D after second-time immunization can stimulate chickens to produce higher antibody titer and eventually reach 2^{14}. Inactivated-vaccine group III (GD37) stimulates chickens to produce antibody titer that have been maintained at 2^{10}.

H9N2 AIV Infection Causes Lung Structure Injury

To test whether the chickens were infected with H9N2,we collected throat swab and the cloaca swab for the detection. As a result, chickens in groups II and IV terminated virus excretion at 5 dpc and 7 dpc, respectively, indicating that the recombinant inactivated vaccines can protect chickens from epidemic H9N2 AIV infection. In addition, we examined the inflammation of chicken in groups II, IV, and VI at 5 dpc and 7 dpc (Figure 6). As a result, no inflammatory reaction were observed in chickens in groups II at 5 dpc and 7 dpc (Figures 6A, 6D), whereas lymphocytic infiltration was observed in chickens in groups Ⅳ and Ⅵ at 5 dpc and 7 dpc (Figures 6B, 6C, 6E, 6F).

DISCUSSION

Owing to the extensive reassortment of H9N2 viruses among chickens, ducks, minor poultry, and wild-bird species, the internal genes of H9N2 viruses are more diversified than are the surface genes (Sun et al., 2010; Smietanka et al., 2014). In fact, the H9N2 virus has not only served as the "donor" but also the "receiver" of internal genes, which has also intrasubtypic reassortment between different lineages of H9N2 (Smietanka et al., 2014). It was also reported that the reassortment is one of the factors that increases virus fitness and shapes epidemiologic and pathobiological dynamics of H9N2 infections (Pu et al., 2017). In addition, it can also provide internal genes for the reassortment of other popular viruses.

In 2013, a new reassortant H7N9 virus avian virus carrying 6 internal genes of the H9N2 influenza virus caused great harm in humans (Lam et al., 2013; Liu et al., 2013). The PB2, M, and NP genes are key virulence genes in human cases of H7N9 virus infection, which are also regarded as H9N2 virus-origin genes (Bi et al., 2015), on the other hand, reassortant H5N1 AIV containing PA or NP gene from an H9N2 virus significantly increased the pathogenicity in mice (Dong et al., 2011). Experimentally, H9N2 viruses have been proved to have extensive reassortment compatibility with pandemic H1N1 and human H3N2 (Sorrell et al., 2009; Kimble et al., 2011; Sun et al., 2011). All these urge us to closely monitor the evolution of the H9N2 virus and take seriously the threat to human health from the H9N2 virus. In this study, we focused on the prevalence and variation of the H9N2 influenza virus in cultured poultry, isolating the H9N2 epidemic strain in South China, and saving a new reassortant with epidemic potential.

As we all know, the current poultry vaccine against avian influenza is mainly an inactivated virus vaccine. To prevent chicken H9N2 AIV infection, Chinese farms have adopted commercial inactivated vaccines such as A/chicken/Shandong/6/1996 and A/chicken/Shanghai/F/1998 and so on, which can induce immunity against clinical diseases and reduce relative mortality in poultry (Swayne et al., 2011). However, H9N2 influenza virus replication allows antigenic drift in subsequent viral populations to continue,which can induce sporadic disease outbreaks in vaccinated flocks (Huang et al., 2010; Sun et al., 2012; Pu et al., 2015), so the use of inactivated vaccines may not fully protect poultry from viral infections (Lee et al., 2004; Choi et al., 2008). What is more? Vaccination is a very labor-intensive job that

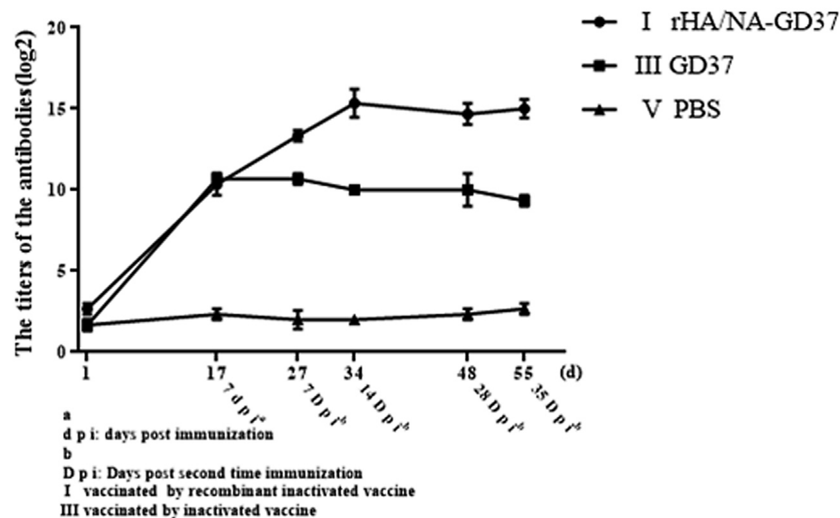

Figure 5. The titers of the antibodies of the sera.

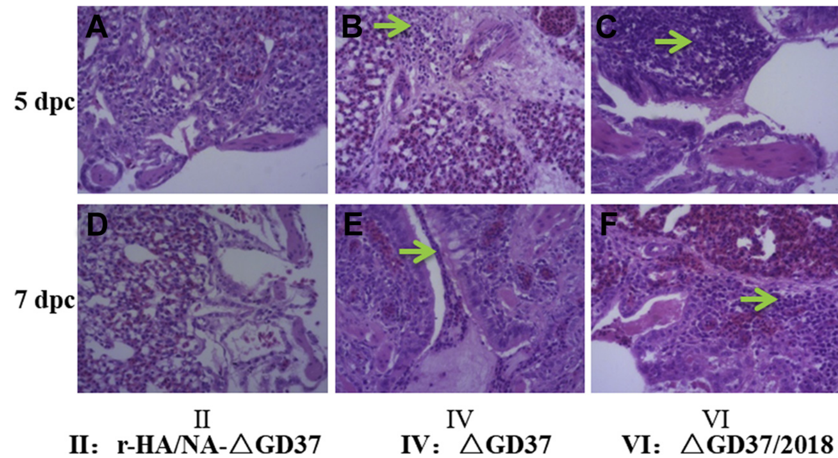

Figure 6. Representative histology images from the lungs. Histopathologic changes in the lung tissue. Histologic features in the II (A, D); Ⅳ (B, E); and Ⅵ (C, F) groups are shown with hematoxylin and eosin staining at 5 dpc and 7 dpc. Lymphocytic infiltration is indicated with the green arrow. Abbreviation: dpc, d postchallenge.

may produce faster antigenic drift between the human and AIV (Suarez et al., 2006). In addition to vaccine stress, the innate immune stress caused by endemic infections in unvaccinated flocks is not negligible for genetic drift in the evolution of influenza viruses, although the antigenic drift of H9N2 chicken influenza viruses from vaccine strains was fully addressed, but the antigen evolution of the H9N2 pandemic virus has not been smooth, which has made the H9N2 vaccine less effective. The H9N2 influenza virus continues to circulate in the vaccinated flocks, causing sporadic outbreaks of disease (Bi et al., 2010). Owing to the commercial vaccine that cannot provide targeted complete or cross-protection of these emerging H9N2 virus infections, updating the vaccine strains regularly is an effective way to avoid H9N2 virus infection causing great harm to poultry and people (Dong et al., 2011). Nowadays, it is a trend to construct a recombinant influenza virus by using various recombined systems. In this study, the H9.4.2.5 branched avian influenza strain isolated in South China was rescued with the reverse genetics system. The recombinant virus will be used to carry out immunoassay experiments to evaluate its immune effects compared with wild-type inactivated vaccines. The purpose of this study is to provide candidate vaccines for the development of novel vaccines.

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Conflict of Interest Statement: The authors declare that there are no conflicts of interest.

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