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Generation and evaluation of a recombinant genotype VII Newcastle disease virus expressing VP3 protein of Goose parvovirus as a bivalent vaccine in goslings

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ABSTRACT

Newcastle disease virus (NDV) and Goose parvovirus (GPV) are considered to be two of the most important and widespread viruses infecting geese. In this study, we generated a recombinant rmNA-VP3, expressing GPV VP3 using a modified goose-origin NDV NA-1 by changing the multi-basic cleavage site motif RRQKR↓F of the F protein to the dibasic motif GRQGR↓L as that of the avirulent strain LaSota as a vaccine vector. Expression of the VP3 protein in rmNA-VP3 infected cells was detected by immunofluorescence and Western blot assay. The genetic stability was examined by serially passaging 10 times in 10-day-old embryonated SPF chicken eggs. Goslings were inoculated with rmNA-VP3 showed no apparent signs of disease and developed a strong GPV and NDV neutralizing antibodies response. This is the first study demonstrating that recombinant NDV has the potential to serve as bivalent live vaccine against Goose parvovirus and Newcastle disease virus infection in birds.

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1. Introduction

Goose parvovirus (GPV) infection also known as Derzsy's disease, goose hepatitis, or gosling plague is an acute, contagious, and fatal disease to domestic goslings and Muscovy ducklings (Derzsy, 1967; Gough et al., 2005; Schettler, 1971). This is a gastrointestinal disease characterized mainly by diarrhea and fibrous hemorrhagic necrotic enteritis. With high mortality and morbidity, GPV has caused substantial economic losses to goose farming countries of Europe and Asia (Jansson et al., 2007; Takehara et al., 1995). To control the disease caused by GPV, live attenuated vaccines are widely used in breeder geese or goslings in addition to flock management (Gough and Spackman, 1982; Kisary, 1977). Usually, the attenuated viruses for vaccines were obtained by serial passages in cultured goose-embryo fibroblasts (GEF) or embryonated goose eggs to reduce the pathogenicity (Wang et al., 2014). However, the potential risk of reversion to virulence of attenuated live vaccines is always a concern. Additionally, the current available vaccines are prepared with chorioallantoic fluid collected from infected embryos, which is costly and inefficient due to the limited availability of SPF (specific pathogen free) goose and duck embryos (Ju et al., 2011; Lee et al., 2010). Therefore, the development of alternative strategies for producing safe and effective vaccines may be of great significance.

Newcastle disease, caused by Newcastle disease virus (NDV), is one of the most serious infectious diseases of many avian species and has caused substantial losses in the poultry industry worldwide (Aldous and Alexander, 2001; Alexander, 2000). On

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the basis of their pathogenicity for birds, NDV has been classified into three different pathotypes: velogenic (highly virulent), mesogenic (moderately virulent) or lentogenic (low virulence) viruses. The molecular basis for the pathogenicity of NDV is mainly determined by the amino acid sequence of the protease cleavage site located in F protein. Mesogenic and velogenic viruses have a polybasic amino acid motif of ^{112}R/KR-Q-K/R-R-F^{117}, while viruses of low virulence have a monobasic amino acid sequence of ^{112}G/E-K/R-Q-G/E-R-L^{117} at the cleavage site (Choi et al., 2010; Panda et al., 2004; Peeters et al., 1999). Lentogenic and, in some cases, mesogenic strains of NDV are widely used as live attenuated vaccines in poultry. In recent years, they also have been developed as a vector to express foreign protective antigens based on reverse genetic techniques. The use of such live vector vaccines for immunization not only protect birds against NDV and another poultry pathogens, such as highly pathogenic avian influenza virus (HPAIV) (Ge et al., 2007) and infectious bursal disease virus (IBDV) (Huang et al., 2004b), but also has the potential to serve as effective vaccines for human use against SARS-CoV or other emerging viruses (DiNapoli et al., 2007a,b, 2010).

In this study, we generated a recombinant NDV expressing the VP3 protein of GPV using a modified avirulent virus vector derived from NDV NA-1 strain isolated from goose flocks. The safety, stability, and feasibility of this recombinant rmNA-VP3 to serve as a bivalent live vaccine in goslings were evaluated.

2. Materials and methods

2.1. Cells and viruses

BHK-21 (ATCC, CCL-10) and DF-1 (ATCC, CRL-12203) cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and maintained at 37 °C with 5% CO_2. The GPV vaccine strain, GD-01, was obtained from the China Veterinary Culture Collection. The viral stock was inoculated into the chorioallantoic cavity of 12-day-old embryonated goose eggs.

NDV strain NA-1 was a virulent strain isolated by our laboratory from geese stocks in Nong'an county of Jilin province, China in 1999 (GenBank Access No. DQ659677) and recovered from the full-length antigenomic cDNA using a reverse genetics system as describe previously (Wang et al., 2015).

2.2. Plasmid construction and virus rescue

To attenuate the NDV NA-1 strain the sequence encoding the protease cleavage site of the F protein was modified by means of PCR mutagenesis. Thus, a fragment between *Pme*I and *Sac*II restriction sites (nt 3165–4900) was amplified from the full-length cDNA clone of NA-1, and subcloned into pBluescript II SK+ (Stratagene) at the EcoRV site. Then, the fragment *Pme*I-*Sac*II of pCI-NA-1 was PCR amplified using the primers Fmut-PF (5′-GCTTGTTTAAACAA-AACAACAGCCCTCTCTCACCC-3′, with *Pme*I site underlined) and Fmut-PR1 (5′-CCAAGAGCTACACTGCCAATAACGGCACCTATAA-**G**GCG**CCCC**TGTCTCC**C**TCCTCCAGACGTGGACACAGAC-3′, with modified sequence bolded). The PCR products were subjected to a second round PCR with the primes Fmut-PF and Fmut-PR2 (5′-GGGCCGCGGCTGCTGTTATCTGTGCCGCTGTTGCAACCCCAAGAGCT-ACACTGCCAATAACGGC-3′, with *Sac*II site underlined) resulting in a new fragment encoding an avirulent protease cleavage site GRQGR↓L of LaSota mutated from RRQKR↓F of NA-1. Finally, the new fragment was excised from pBluescript II SK+ using the restriction enzymes *Pme*I and *Sac*II and was used to replace the corresponding fragment in the full-length cDNA clone of the pCI-NA-1. The resultant plasmid was designated as pCI-mNA-1.

The open reading frame (ORF) of VP3 gene of GPV strain GD-01 was amplified from the genome DNA by using the primer pair of 5′-GCGCGTTTAAAC**TTAAGAAAAAAT**ACGGGTAGAA*GCCGCCACC*AT-GGCAGAGGGAGGAGGCGG-3′ and 5′-GCGCGTTTAAACTTACAGAT-TTTGAGTTAGATATC-3′, in which the gene end and gene start sequences of NDV (bold), the optimal Kozak sequence (italic), and the *Pme*I restriction sites (underlined) were included. The amplified product was ligated into EcoRV-cut pBluescript II SK+ vector, sequenced, and inserted into the NDV full-length cDNA clone contained in pCI-mNA-1 through the introduced *Pme*I site in the P–M noncoding region at nucleotide position 3165 of the NDV genome, as described previously(Wang et al., 2015). The new full-length plasmid was designated as pCI-NA-VP3. Rescue of the infectious viruses were performed by transfecting the full-length cDNA clone and supporting plasmids into BHK-21 cells as described previously (Wang et al., 2015).The rescued viruses were named rmNA-1 and rmNA-VP3, respectively.

2.3. Transmission electron microscopy (TEM)

A 2% solution of phosphotungstic acid (pH 6.8) was added to the virus samples for negative staining. Then the mixtures were transferred to carbon-coated grids, and the specimens were photographed with an electron microscope H-7650 (Hitachi Ltd., Hitachi, Japan).

2.4. Immunofluorescence assays

For immunofluorescence analysis, BHK-21 cells were plated on coverslips in 35-mm-diameter dishes and infected with NDV rmNA-1 or rmNA-VP3. After 24 h, the cells were fixed in ice-cold 3% paraformaldehyde in phosphate buffered saline (PBS) for 15 min at room temperature and then washed with PBS 3 times. Cells were blocked in PBS containing 1% (w/v) bovine serum albumin (BSA) at 4 °C for 1 h. Cells were then incubated with chicken serum anti-NDV or horse serum against GPV for 1 h at room temperature and were washed 3 times with PBS containing 0.05% Tween 20. Then, the cells were stained with FITC (fluorescein isothiocyanate)-conjugated goat anti-chicken antibody (Sigma) or an Alexa Fluor 555-conjugated rabbit Anti-horse IgG (bioss) for 30 min. Finally, cells were washed 3 times with PBS, and DAPI (4′,6′-diamidino-2-phenylindole) was used to stain the cell nucleus. Cells were analyzed with a confocal laser microscope (Olympus Corp, Tokyo, Japan).

2.5. Western blotting

DF1 cells were infected with rescued virus rmNA-1 or rmNA-VP3 at a multiplicity of infection (MOI) of 5 and incubated for 48 h. The expression of cell-associated proteins was analyzed by Western blotting. Proteins from the lysates of infected cells were separated using SDS-PAGE under denaturing conditions and analyzed with chicken serum anti-NDV or rabbit anti-VP3 polyclonal antibody (bioss), or mono-clonal antibody against β-actin (Cell Signaling). Immunostained proteins were visualized using a 3,3′-diaminobenzidine (DAB) reagent. Mock-infected DF1 cells were used as the negative control.

2.6. Stability and pathogenicity of the recombinant viruses

To evaluate the genetic stability of the recombinant viruses, rmNA-1 or rmNA-VP3 was serially passaged by 10 times in 10-day-old embryonated eggs, respectively. The viral genome RNA was isolated from allantoic fluids of each passage and subjected to RT-PCR to check the modified region in the F gene cleavage site of

rmNA-1 or rmNA-VP3 and confirm the presence of the VP3 gene in the genome of rmNA-VP3 by DNA sequencing.

The pathogenicity of the modified virus was evaluated by the mean death time (MDT) and the intracerebral pathogenicity index (ICPI) according to the standard pathogenicity assay (Alexander, 2008).

2.7. Multistep growth kinetics of the recombinant viruses

Ten-day-old embryonated chicken eggs were inoculated with rmNA-1, rmNA-VP3 and rNA-1, at 100$TCID_{50}$ per egg. Viral growth was analyzed at different time points after inoculation. A 50% tissue culture infective dose ($TCID_{50}$) of each virus was determined by immunofluorescence assay. Ninety-six well plates of 80%-confluent DF1 cells were infected with serial 10-fold dilutions of virus (four wells per dilution). Cells were incubated for 3 days and fixed with 80% cold acetone. Viral antigens were detected with chicken serum anti-NDV and FITC-conjugated goat anti-chicken antibody.

2.8. Immunization studies in goslings

Goslings at 4 days of age were randomized into three groups of 10 birds each. Group 1 and Group 2 were injected with 10^6 EID_{50} of the rescued rmNA-1 or rmNA-VP3 in a 0.2-ml volume by subcutaneous administration and a booster injection was performed 2 weeks later. The third group of goslings was injected with PBS used as negative controls. All animals were housed in a specific pathogen-free facility with free access to water and food. Blood samples were collected at 3, 6, 9, and 12 weeks post-immunization and the sera were separated by low speed centrifugation and stored at −20 °C.

2.9. Serologic tests

The neutralizing antibodies (NAs) of individual goose serum samples against GPV and NDV were tested in the primary goose embryo fibroblasts (GEFs) and DF1 cells respectively. For the GPV NAs assay, GEFs were prepared from 13-day-old goose embryos and double dilutions of serum samples were pre-incubated with 100 $TCID_{50}$ GPV GD-01 at 37 °C for 1 h and then added to 10^5 GEF cells in 96-well plates. For the NDV NAs assay, 100 $TCID_{50}$ NDV NA-1 was incubated for 1 h with serial dilutions of the test sera and then were added to DF1 cells in 96-well plates. Cells were observed microscopically for cytopathic effects (CPE) starting at 48 h post-infection and the titers were calculated by using the method detailed by Reed and Muench (1938).

2.10. Statistical analysis

Statistical analysis was performed using of the analysis of variance (ANOVA) method and considered significant when $p \leq 0.05$. Data are shown as means ± standard deviation (SD).

3. Results

3.1. Generation and identification of the modified NDV NA-1 strain

To generate an attenuated NDV NA-1 strain, modification were made to the full-length cDNA clone of NA-1 by changing the amino acid sequence of the F protein cleavage site from the naturally occurring RRQKR↓F to an avirulent motif GRQGR↓L, resulting in a new cDNA clone pCI-mNA-1 (Fig. 1A). The recovery of the modified virus rmNA-1 was confirmed by RT-PCR and sequence analysis of the F gene from the HA-positive allantoic fluid (Fig. 1B). Pathogenicity of the recombinant viruses was assessed by performing MDT and ICPI tests. The results showed that the MDT of rmNA-1 was 132 h, while that of rNA-1 was 59 h. Additionally, the ICPI of rmNA-1 was 0, while that of rNA-1 was 1.9. These results suggest that rmNA-1 is highly attenuated.

To investigate the capability of CPE and the trypsin-dependent infectivity in cell culture, DF1 cells were infected with rNA-1, rmNA-1, or LaSota viruses at a MOI of 0.01 in the presence or absence of 10% normal allantoic fluid as exogenous protease supplementation(Fig. 1C). The results showed that syncytium formation could be observed in cells infected by parental virus rNA-1 with or without exogenous protease. LaSota only induced the formation of syncytia in the presence of added protease. In contrast, rmNA-1, which has exactly the same F protein cleavage site as LaSota, did not cause any apparent syncytia even in the presence of exogenous protease.

The genetic stability of the modified sites of rmNA-1 was examined by serially passaging them 10 times in 10-day-old embryonated chicken eggs and sequencing the F gene from each passage. The results indicate the cleavage sites were preserved and stably maintained after 10 passages in chicken embryos (data not shown).

3.2. Generation of recombinant NDV virus expressing the VP3 gene of GPV

To obtain the cDNA clone pCI-mNA-VP3, an artificial transcription cassette coding for the VP3 protein of GPV was inserted into pCI-mNA-1 between the P and M genes of the genome (Fig. 2A). The resultant recombinant virus, rmNA-VP3, was recovered entirely from this cDNA by using our established reverse genetics procedures (Wang et al., 2015). The presence of the VP3 gene in the genome was confirmed by RT-PCR (data not show). The virions of rmNA-VP3 were examined by observation with TEM after negative staining, and compared with rNA-1 and rmNA-1, the VP3 gene inserted into the genome did not affect viral morphology (Fig. 2B).

3.3. Expression of the VP3 protein by rmNA-VP3

Expression of the VP3 protein by rmNA-VP3 was examined by indirect confocal immunofluorescence staining infected BHK-21 cells. As expected, cells infected with rmNA-1 were not stained by horse serum against GPV, but they were positive for immunostaining using chicken serum against NDV (Fig. 3A). Cells infected with rmNA-VP3 were stained by horse serum against GPV as well as chicken serum against NDV (Fig. 3A). The VP3 expression by the recombinant virus was further confirmed by Western blotting analysis of infected DF1 cell lysates with rabbit anti-VP3 polyclonal antibody (Fig. 3B), whereas no band was detected in purified rmNA-VP3 virus (data not shown). These results suggested that the recombinant rmNA-VP3 stably expressed theVP3 protein and that the VP3 protein was probably not incorporated into the virions.

3.4. Biological characterization of the recombinant viruses

The biological properties of the recombinant viruses were compared to parental rNA-1 by determination of growth characteristics in tissue culture and in embryonated chicken eggs. As the cleavage site of F protein was modified to a monobasic motif from the dibasic amino acid motif, both the rmNA-1 and rmNA-VP3 cannot be propagated as well as rNA-1 in tissue culture (data not shown). The growth kinetics of the recombinant viruses were determined and compared in embryonated chicken eggs at different time points after inoculation. A 50% tissue culture infective dose ($TCID_{50}$) of each virus was determined in DF-1 cells by immunofluorescence assay. The results showed that both rmNA-VP3 and rmNA-1 have

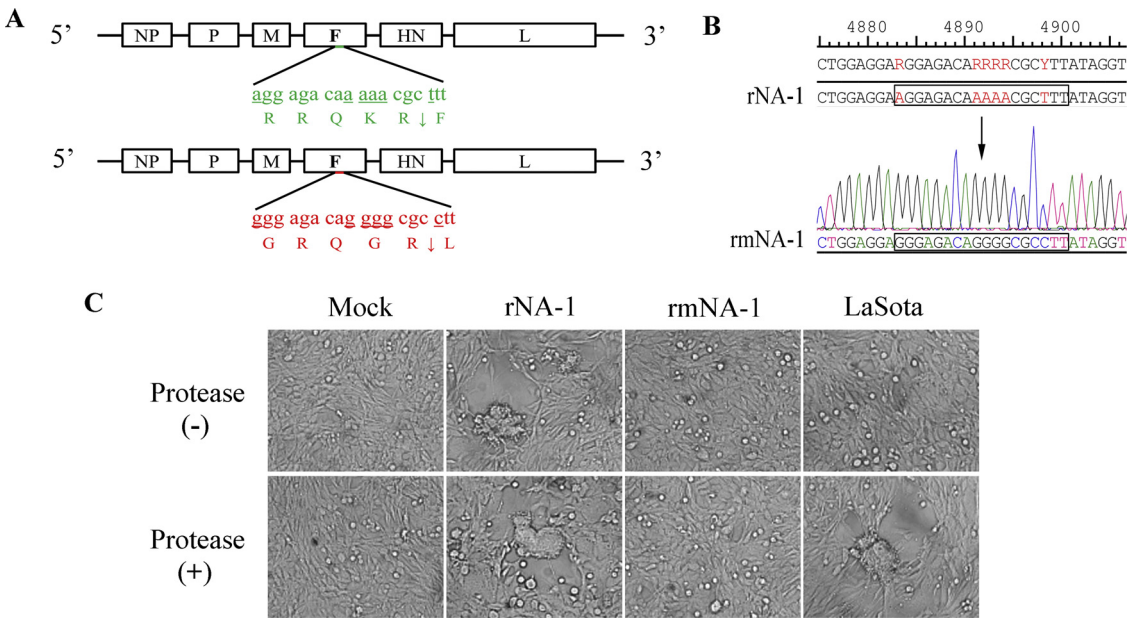

Fig. 1. Generation of the modified NDV NA-1 strain. (A) Schematic representation of the modification of full-length cDNA clone of NA-1 strain at F protein cleavage site. The regions of the F gene encoding the cleavage site were mutated to change the amino acid sequence from the virulent motif RRQKR↓F of NA-1 to an avirulent motif GRQGR↓L of LaSota. The mutated nucleotides are underlined in red (below), and original sequences were underlined in green (above). (B) The modified nucleotides of the recovered rmNA-1 were confirmed by sequencing. F gene from the recovered viruses was amplified by RT-PCR and subjected to sequence. The regions encoding the cleavage site were boxed. (C) Investigate the capability of CPE and the trypsin-dependent infectivity in cell culture. DF1 monolayers were infected with NDV strains, LaSota, rmNA-1 and rNA-1 at an MOI of 0.01. The infected cells were cultured in the presence or absence of 10% normal allantoic fluids as exogenous protease. The cells were photographed at 36 hpi with an inverted microscope at 200× magnifications (Olympus Corp, Tokyo, Japan). (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.)

similar growth patterns compared to parental rNA-1. At 60 h post-inoculation, rNA-1 and rmNA-1 reached the maximum titers of $10^{8.25}$ $TCID_{50}$/ml and 10^{8} $TCID_{50}$/ml respectively, whereas the maximal viral titer of rmNA-VP3 achieved at 72 h post-inoculation and was approximately 20-fold lower (Fig. 4A).

The genetic stability of rmNA-VP3 was also examined by serial passage 10 times in 10-day-old embryonated SPF chicken eggs as described previously. RT-PCR confirmed the presence of the VP3 gene in each passage, and sequence analysis showed that the VP3 gene remains unchanged during 10 passages. Also the mutant sequence at the cleavage sites in the F gene were proved to be preserved and stably maintained after serial passage through chicken embryos by DNA sequencing (data not shown).

The pathogenicity of recombinant NDV expressing the VP3 protein was evaluated by the MDT and ICPI assays (Fig. 4B). The value of MDT exceeds 168 h and the ICPI was 0. These results show that rmNA-VP3 continues to keep the characteristics of the vector virus rmNA-1.

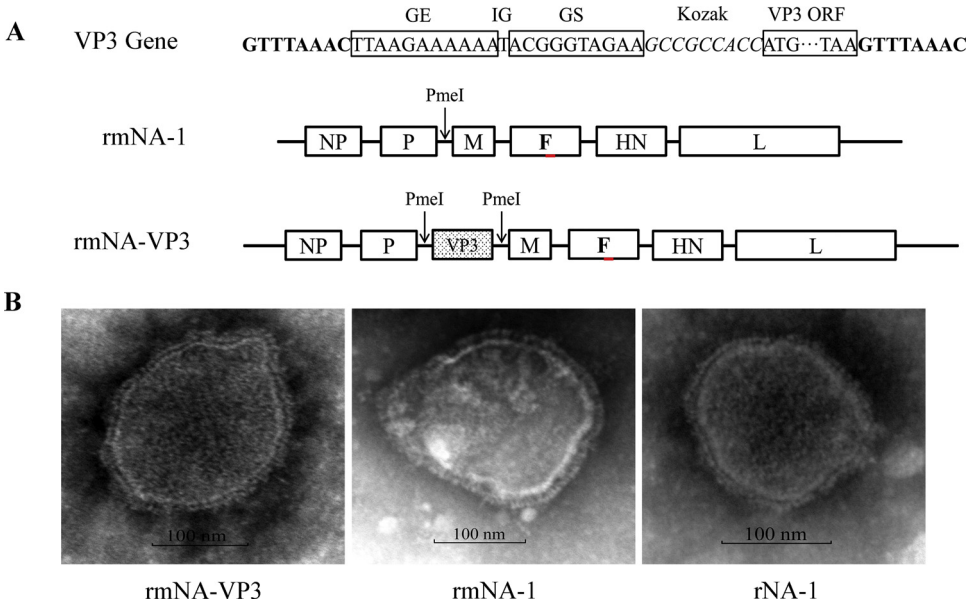

Fig. 2. Construction and generation of recombinant NDV expressing the VP3 gene of GPV. (A) The VP3 gene with conserved gene end (GE), intergenic sequence (IG), gene start (GS) signals and the optimum Kozak sequence was amplified from the genome DNA (top) and introduced into the *Pme*I site between the P and M genes (bottom). (B) Analysis of the recovered viruses by electron microscopy. The rmNA-VP3, rmNA-1, and rNA-1 virions were negatively stained and observed by electron microscopy.

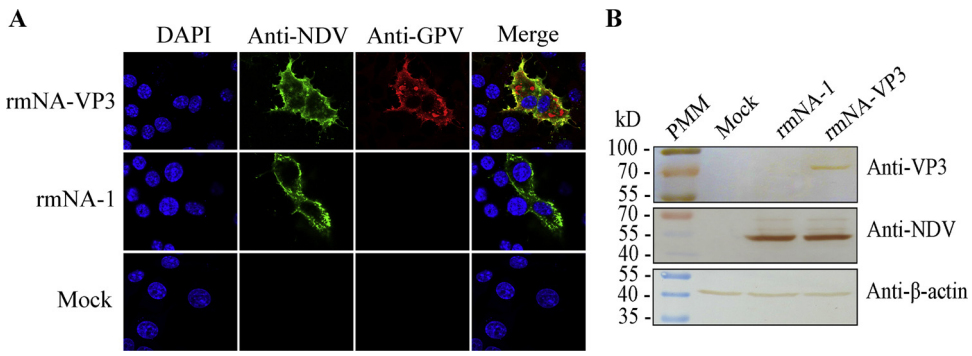

Fig. 3. Analysis of expression of the VP3 protein by rmNA-VP3. (A) Immunofluorescence analysis of VP3 protein expression. BHK-21 cells were infected with rmNA-1 or rmNA-VP3 at an MOI of 0.01. The infected cells were fixed and probed with chicken serum against NDV and horse serum against GPV and then incubated with a FITC-conjugated goat anti-chicken antibody and an Alexa Fluor 555-conjugated rabbit Anti-horse IgG. DAPI (Sigma) was used for cell nucleus staining. Cells were analyzed by using a confocal laser microscope. (B) Western blot analyses of VP3 expression. Lysates of DF1 cells infected with rmNA-VP3 or rmNA-1 were incubated with chicken serum anti-NDV, rabbit anti-VP3 polyclonal antibody or mono-clonal antibody against β-actin. Binding was visualized with 3, 3-diaminobenzidine reagent after incubation with peroxidase-conjugated secondary antibodies. The locations of marker proteins are indicated on the left and the antiserum or antibody used is indicated on the right.

3.5. *Immunogenicity of recombinant viruses in goslings*

To investigate the immunogenicity of the recombinant rmNA-VP3, groups of 10 4-day-old goslings were inoculated with rmNA-1, rmNA-VP3, or PBS. During the experimental period, the birds inoculated with the recombinant virus appeared healthy without any signs of vaccine side-effects. Serum samples of the birds were collected before and after vaccination. The antibody titres against GPV were determined by virus neutralization test. At 3 weeks after the first dose, the mean titer of GPV NA in the blood of goslings inoculated with rmNA-VP3 was $3.25\log_2$ (Fig. 5A). At 3 weeks after the second dose, the mean titer reached up to $6\log_2$. Then the mean titer of $5.25\log_2$ was detected at 9 weeks after first immunization and still maintained a titer of $5.25\log_2$ until 12 weeks. No GPV-specific antibodies were detected from goslings in the rmNA-1 or PBS vaccine control group after either initial or boost vaccination.

Meanwhile, NDV NAs were detected in all serum samples (Fig. 5B). The two groups of goslings inoculated with rmNA-1 or rmNA-VP3 displayed no significant differences in NDV VNAs antibody titers ($p > 0.05$). The mean titers of $4.5\log_2$ in rmNA-VP3 group and $4.75\log_2$ in rmNA-1 group were detected at 3 weeks after the first dose. Three weeks after the second dose, the mean titer for the two groups reached $10.5\log_2$ and $10\log_2$, respectively, showing substantial boost responses. After 9 weeks, the antibody titers are maintained at $7.25\log_2$ in rmNA-VP3 group and at $7.75\log_2$ in rmNA-1 group, and at 12 weeks after vaccination the mean titers is still $6.25\log_2$ and $6.75\log_2$ respectively.

A

Viruses	Pathogenicity in eggs and chickens	
	MDT(h)	ICPI
rNA-1	59	1.9
rmNA-1	132	0
rmNA-VP3	>168	0

B

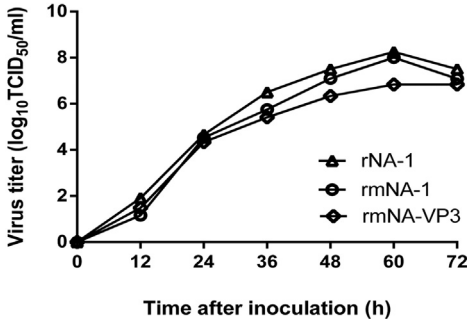

Fig. 4. Biological characterization of the recombinant viruses. (A) Multistep growth kinetics of recombinant viruses in 10-day-old SPF chicken embryos. Ten-day-old embryonated chicken eggs were inoculated with 100TCID_{50} of each virus, and allantoic fluids were harvested at 12 h intervals. The viral titers (TCID_{50}) in DF1 cells were determined by immunofluorescence assay using chicken serum anti-NDV and a FITC-conjugated goat anti-chicken antibody. Mean values from triplicate samples are shown. (B) Pathogenicity assay in SPF eggs and chickens. The MDT and ICPI were determined as described in Section 2.

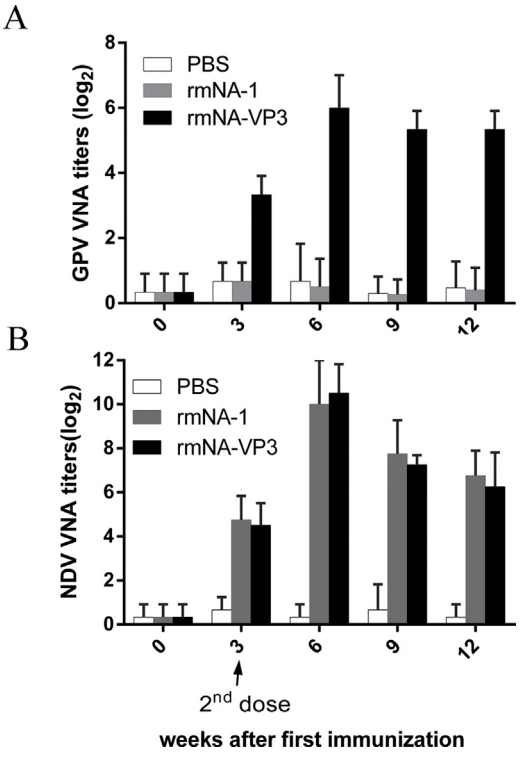

Fig. 5. Immunogenicity evaluation in goslings. Groups of 10 goslings were inoculated subcutaneously with 10^6 EID_{50} of rmNA-1 or rmNA-VP3 or with PBS as a control in a 0.2-ml volume respectively. At 3 weeks after initial vaccination, the goslings received a second dose. The serum samples from goslings were collected prior to vaccination and at different times after vaccination and the VNA titers against GPV (A) and NDV (B) were detected.

4. Discussion

In this study, we generated a recombinant rmNA-VP3, expressing GPV VP3 using a modified goose-origin NDV NA-1 as an avirulent, safe, and effective vaccine vector, and evaluated its potential as a bivalent vaccine against gosling plague and ND in goslings. The results of the gosling immunizing experiment show that rmNA-VP3 could induce strong NA responses to GPV and NDV simultaneously. This is the first study to demonstrate that a highly virulent NDV could serve as vaccine vector expressing exogenous protein after modification by changing the multi-basic cleavage sites of the F protein to the dibasic sequence of avirulent strain LaSota. The recombinant rmNA-VP3 has the potential to serve as a bivalent live vaccine against gosling plague and ND.

GPV is a pathogen of major economic importance to the goose industry worldwide. Currently, prophylactic vaccination is used as protection against GPV infection. Two types of immunization strategies were adopted in field: vaccination of breeder geese before laying, by which the offspring was protected depending on maternally derived antibodies in the egg yolks, and vaccination of susceptible goslings, where the virulence of the vaccine strains needs to be much lower than that of strains used to immunize parent flocks. Preparation of vaccines with varying levels of virulence makes the production very costly and labor-effective. Therefore, the development of a safe, effective and low-cost vaccine is necessary. Among the possible strategies, one of the most promising is the live viral vectored vaccines.

The genomes of GPV encode non-structural and structural proteins, including three capsid proteins of VP1, VP2, and VP3. The VP3 protein is the most abundant of the three core proteins in purified virions and can induce neutralizing antibodies with high immunogenicity (Le Gall-Reculé et al., 1996; Le Gall-Recule and Jestin, 1994). Therefore, VP3 may be a suitable candidate antigen for developing a vaccine against GPV.

As a viral vector, NDV has outstanding advantages. Firstly, NDV grows to very high titers in many cell lines and embryonated eggs, which allows cost-effective and easy manufacture of the vaccine. Secondly, NDV has a simple genome encoding only a few proteins than other viruses such as fowl pox virus and adenovirus. Thirdly, the live NDV vaccines can induce not only humoral immunity and cellular immunity simultaneously, but also a strong mucosal immunity, which makes this expression vector more attractive (Huang et al., 2003). NDV lentogenic vaccine strains such as LaSota can be developed in vectors directly; while mesogenic strains should be modified by changing the multi-basic cleavage site sequence of the F protein to the dibasic sequence of avirulent strains because these strains might cause disease in poultry and most strains are classified as Select Agents (Kim et al., 2014). Recently, it has been demonstrated that the virulent isolates also can be attenuated by modification in the same way to serve as a live-attenuated vaccine candidate (Hu et al., 2009, 2011; Xiao et al., 2012).

NDV has a single serotype, but many genotypes. To date, NDV had caused four major ND panzootics and different genotypes contributed to each panzootic. Epidemiological studies revealed that genotype VII NDV strains were circulating predominantly worldwide after the fourth panzootic (Huang et al., 2004a; Miller et al., 2009, 2010) and found to be responsible for the ND outbreaks in Asia, Africa, Middle East, and South America (Munir et al., 2012; Perozo et al., 2012; Wang et al., 2006). These strains also caused many outbreaks of ND in domestic geese with high mortality and morbidity in Southern and Eastern China since the late 1990s, which was referred as goose paramyxovirus (GPMV) in earlier reports (Cai et al., 2011; Jinding et al., 2005). However, the genotype II vaccine strains such as LaSota or Hitcher B1 remain in current and widespread use, and the differences in their antigenicity and genotypes between the presently circulating strains and commercial vaccine strains are considered the main reasons for the ND outbreaks in vaccinated poultry flocks (Liu et al., 2003; Miller et al., 2007). Recent work has demonstrated that genotype-matched vaccine strains provide better protection than conventional vaccines in terms of reducing virus shedding and transmission following challenge with NDV virulent virus (Hu et al., 2009, 2011; Xiao et al., 2012). In 1999, a highly virulent genotype VII NDV strain NA-1 was isolated from outbreaks in goose flocks in our lab (Xu et al., 2008). To evaluate whether this genotype VII isolate could be used as a vaccine vector for geese we generated a recombinant virus rmNA-VP3 expressing VP3 protein of GPV after modifying the polybasic F cleavage site of NA-1 to the dibasic motif of LaSota. It is interesting that the modified NA-1 which has exactly the same F protein cleavage site as avirulent LaSota did not form syncytium in virus-infected cells as LaSota did. Even in the presence of exogenous protease no syncytium was observed (Fig. 1C). These results are repeatedly confirmed and consistent with previous research on the mutation of a highly virulent Indonesian strain (Xiao et al., 2012). It is demonstrated that the syncytium formation is not determined by the motif of the F protein cleavage site alone; some other factors may also be involved. Anyway, the inability to induce the formation of syncytia, may be indicative of a highly attenuated of the modified viruses comparing with LaSota.

As a feasibility study, one concern that requires investigation is that whether the mutated F protein cleavage site of vector virus might revert to a wt or wt-like sequence after serial passage. To evaluate its heredity stability, the recombinant virus rmNA-1 and rmNA-VP3 were passaged continuously in chicken embryos and the sequence of F protein cleavage site was determined every generation. The results demonstrated that the mutated F protein cleavage site always remained stable after 10 passages in chicken embryos. The safety issue of the recombinant viruses should be further evaluated through its serial passage in geese prior to its use in field.

Immunogenicity of the recombinant virus rmNA-VP3 was evaluated in goslings. A detectable immune response against GPV was evidenced after the first immunization dose, and after the second immunization administered 3 weeks later, significant booster responses to VNA of GPV were induced. Moreover, the antibody level did not drop markedly during at least the first 12 weeks. Meanwhile, the neutralizing antibodies against NDV vaccine vector were also detected. The mean titer induced by rmNA-VP3 was comparable to that induced by rmNA-1, indicating that this recombinant virus holds its characteristic in stimulating effective immune response against NDV infection.

In summary, as a new type of engineered vaccine the recombinant virus rmNA-VP3 has several advantages over the existing GPV vaccines. Firstly, the recombinant rmNA-VP3 will be highly economical for the poultry industry, because rmNA-VP3 can be cultured in many cell lines and embryonated eggs. Secondly, the recombinant virus can potentially overcome maternal antibody interference. Therefore, it can be used for vaccination with or without maternal antibody. Even in the presence of the maternal antibody, it can be used to enhance protection in the early danger period of gosling life. Finally, this recombinant virus would be developed an in-ovo vaccine against GPV after further modified as describe in in-ovo vaccination against IBDV and NDV elsewhere (Ge et al., 2014).

Conflict of interest

(i) All the authors have agreed to its submission and are responsible for its contents; (ii) All the authors have agreed that Zhuang Ding may act on their behalf regarding any subsequent processing of the paper; (iii) The authors have declared that no competing interests exist.

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