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Generation by reverse genetics of an effective attenuated Newcastle disease virus vaccine based on a prevalent highly virulent Chinese strain

Meng-Meng Liu · Jin-Long Cheng · Xiao-Hui Yu · Zhuo-Ming Qin · Fu-Lin Tian · Guo-Zhong Zhang

Abstract

Objectives To investigate whether the differences between the circulating Newcastle disease virus (NDV) isolates and the used vaccine might account for the current ND outbreaks in vaccinated poultry flocks.

Results A reverse genetics system using prevalent genotype VIId isolate SG10 was constructed and a mutant virus, named aSG10, was developed by changing the virulent F protein cleavage site motif "^{112}RRQKR↓F^{117}" into an avirulent motif "^{112}GRQ GR↓L^{117}". The attenuated pathogenicity of aSG10 was confirmed from the mean death time and intracerebral pathogenicity index. aSG10 and LaSota both protected vaccinated birds from death after challenge with highly virulent genotype VII NDV, strain SG10. However, aSG10 significantly reduced the challenge virus shedding from the vaccinated birds compared to LaSota vaccine. We also generated a recombinant virus, aSG10–enhanced green fluorescent protein (EGFP), which expresses EGFP. aSG10-EGFP stably expressed EGFP for at least 10 passages.

Conclusions The mutant, aSG10, can be safely used as a vaccine vector and is a potential vaccine candidate in increasing the protective efficacy for the control of current ND epidemic in China.

Keywords China · Efficacy · Genotype · Newcastle disease virus · Reverse genetics · Vector

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Introduction

Newcastle disease (ND) is a highly contagious avian disease, which can cause severe economic losses in the poultry industry. The causative agent of the disease, ND virus (NDV), is an enveloped, nonsegmented single-stranded negative-sense RNA virus belonging to the genus *Avulavirus* within the family *Paramyxoviridae* (Mayo 2002). The genome of the virus is

approx. 15 kb and encodes six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin–neuraminidase (HN), and the large protein (L). Two nonstructural proteins, V and W, are also encoded by the P gene of NDV and are expressed after RNA editing (Mebatsion et al. 2001). The two surface glycoproteins, HN and F, are the viral neutralization antigens and the major protective antigens.

NDV strains are classified into highly virulent (velogenic), intermediate (mesogenic), and non-virulent (lentogenic) pathotypes on the basis of their pathogenicity in chickens (Peeters et al. 1999). The F protein is a major virulence determinant and is required for the initiation of infection (Panda et al. 2004). The F protein is synthesized as a precursor (F0) that is activated by its cleavage by a host cell protease into two disulfide-linked subunits, F1 and F2 (Collins et al. 1993). Cleavage of the F protein is necessary for viral entry and cell-to-cell fusion. The cleavage site in the F protein of virulent NDV strains contains the amino acid motif ^{112}R/K–R–Q–K/R–R↓F^{117} and is recognized by ubiquitous intracellular furin-like proteases, resulting in systemic infection. The avirulent NDVs typically have the motif ^{112}G/E–K/R–Q–G/E–R↓L^{117} at the cleavage site and require a secreted protease (or in cell culture, added trypsin or chicken egg allantoic fluid) for cleavage (Panda et al. 2004).

All NDVs belong to a single serotype and can be divided into two classes based on their genome lengths and the sequences of the F gene (Miller et al. 2010). Classes I and II viruses are further divided into different genotypes (Snoeck et al. 2013). Among these, genotype VII of the class II NDV strains has been associated with the most recent outbreaks in Asia, Europe, Africa, the Middle East, and South America (Miller et al. 2010). Genotype VII can be further divided into seven subgenotypes (VIIa–g) based on amino acid substitutions in the F protein (Tan et al. 2010).

In a previous study, we genotypically characterized the NDV isolates recovered from chickens between 2005 and 2012 to establish the nature of the circulating genotype VII strains in China (Zhang et al. 2014). Our results indicated that genotype VIId strains are continuously persistent in China. These VIId NDV isolates were at least 95.1 % identical at the nucleotide level across the whole genome. However, they showed only 82–83 % nucleotide sequence identity with

vaccine strains such as LaSota and B1. These vaccine strains belong to genotype II in class II and were isolated 60 years ago (Zhang et al. 2014). The outbreaks of this disease in vaccinated poultry flocks in recent years are mainly attributed to the significant differences in the biology, serology, and genetics of the prevailing NDV strains and the current vaccine strains (Rui et al. 2010).

NDV strain SG10 is a highly virulent genotype VIId NDV, which was isolated by our laboratory during an outbreak in a vaccinated broiler flock in 2010. In this study, a reverse genetics system for SG10 was constructed. Using this system, we attenuated the velogenic SG10 strain and assessed the potential utility of this attenuated virus as a vaccine and a viral vector.

Materials and methods

Virus, plasmids, and cells

NDV strain SG10 was isolated from an outbreak of ND in chickens and identified as velogenic [intracerebral pathogenicity index (ICPI) = 1.79, mean death time (MDT) = 45 h]. The virus was grown in 10-day-old embryonated specific-pathogen-free (SPF) chicken eggs. DF-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum. BSR T7/5 cells, stably expressing the T7 phage RNA polymerase, was a gift from Dr. Li Yu (Harbin Veterinary Institute, China), and were maintained in DMEM. The vector pOK12 was also a gift from Dr. Li Yu. The vector pCI-neo was bought from Promega Corporation (Promega, Madison, USA).

Reverse genetic constructs

RNA was extracted from NDV-positive allantoic fluid with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription-PCR (RT-PCR) was performed as previously described (Zhang et al. 2011). The PCR products were purified with the easy pure quick gel extraction kit (TransGen, Beijing, China) and sequenced at Sunbiotech (Beijing, China). The 3'- and 5'-terminal sequences were confirmed with 3'- and 5'-RACE, respectively. The viral sequences were

analyzed with the software package Lasergene (DNASTAR, Inc., Madison, WI, USA).

To construct the helper plasmids, the ORFs of the NP, P, and L genes were amplified using specific primers, and then cloned into pCI-neo at the *Xba*I and *Sal*I restriction enzyme sites to generate pCI-NP, pCI-P, and pCI-L, respectively. To construct the recombinant SG10 cDNA clone, the T7 promoter sequence was removed from pOK12, generating the pOK12-T7 vector, and six PCR fragments spanning the full-length cDNA of SG10 were generated by RT-PCR. The six fragments were sequentially cloned into the pOK12-T7 vector between the T7 promoter and the HDV antigenomic ribozyme sequence (Fig. 1a). The *Mlu*I and *Hin*dIII restriction sites in the L ORF region of the cDNA were eliminated to act as genetic markers, by mutating two nucleotides at positions

13048 and 14056, which induced no amino acid changes. The constructed plasmid was designated pOK-rSG10.

To rescue the infectious NDV, BSR T7/5 cells stably expressing the T7 phage RNA polymerase were grown to 90 % confluence in six-well plates and then cotransfected with a total of 10 μg DNA consisting of a mixture of pOK-rSG10, pCI-NP, pCI-P, and pCI-L in a ratio of 4:2:1:1 (Zhang et al. 2013) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. At 6 h post-transfection, the cells were washed once with phosphate-buffered saline (PBS) and maintained in DMEM medium containing 2 % (v/v) fetal bovine serum. Three days later, the culture supernatants and cell monolayers were harvested by freeze–thawing the infected cells three times. The allantoic cavities of 10-day-old
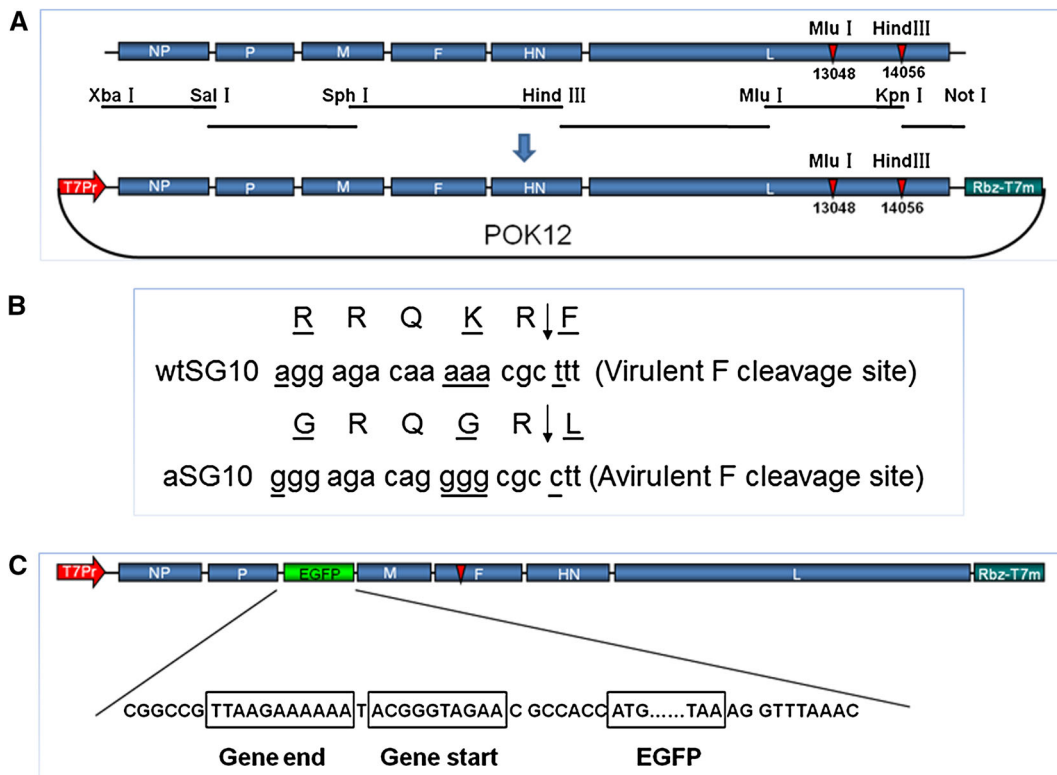
Fig. 1 **a** Construction of a full-length antigenomic cDNA of NDV strain SG10. Six cDNA fragments were generated by RT-PCR from the NDV SG10 genomic RNA. The direction of the T7 promoter is indicated by a *red arrow*. Rbz-T7m represents the site of the hepatitis delta virus ribozyme and the T7 terminator sequence. **b** Modification of the F protein cleavage site. The sequence at the cleavage site of the SG10 virus is shown, and the altered cleavage site in aSG10. The *arrow* indicates where cleavage occurs. **c** Construction of recombinant aSG10 expressing EGFP. The inserted foreign ORF was placed under the control of a set of NDV transcriptional gene-end (GE) and gene-start (GS) signals so that each was expressed as a separate mRNA

embryonated SPF chicken eggs were then inoculated with 200 µl cells to amplify the recovered viruses. After incubation for 4 days, the allantoic fluid was harvested and the rescued virus was detected with the hemagglutination (HA) test. The genetic markers in the L ORF region were confirmed by nucleotide sequencing.

Attenuation of the SG10 virus

To attenuate the SG10 virus, the F protein cleavage site was mutated with overlapping PCR (Fig. 1b). The protease cleavage site of the F0 protein was altered from ^{112}RRQKR↓F^{117} to the consensus sequence of the avirulent NDV strains, ^{112}GRQGR↓L^{117} (Xiao et al. 2012). The fragment that contained the mutated F0 protein cleavage site was used to replace the corresponding fragment in the full-length cDNA of pOK-rSG10. The resulting cDNA clone with the mutated F gene was designated pOK-aSG10. The cotransfection of pOK-aSG10 with the three helper plasmids and the recovery of the modified virus were performed as described above. At 6 h post-transfection, the cells were washed once with PBS and maintained in DMEM medium containing N-tosyl-phenylalanine chloromethylketone (TPCK)-treated trypsin at 1 µg/ml (Zhang et al. 2013). Four days later, embryonated chicken eggs were inoculated with the cell culture supernatants, and recovery was deemed successful when the allantoic fluid had a positive HA titer. The sequence of the F protein cleavage site was confirmed with nucleotide sequencing.

Construction of a recombinant aSG10 cDNA clone containing the EGFP gene

The full-length aSG10 cDNA clone, pOK-aSG10, was used as the backbone to construct a recombinant cDNA clone containing the enhanced green fluorescent protein (EGFP) gene between the P and M genes in the aSG10 genome, as an additional transcription unit (Fig. 1c). Briefly, two unique restriction enzyme sites, EagI and PmeI, were introduced into the region between the P and M genes (positions 3161–3175) of the infectious pOK-aSG10 clone using overlap extension PCR. The EGFP ORF, amplified with the appropriate primer pair, was engineered to contain the NDV gene-start (GS) and gene-end (GE) signal

sequences, and was then inserted into the EagI and PmeI restriction sites in pOK-aSG10, according to the "rule of six". The resulting cDNA clone containing the EGFP gene was designated pOK-aSG10-EGFP. The cotransfection pOK-aSG10-EGFP with the three helper plasmids and the recovery of the modified virus were performed as described above. The expression of EGFP was examined at 24 h postinfection (hpi) of BSR T7/5 cells with the recovered rSG10-EGFP using an inverted fluorescence microscope.

Biological characterization of the generated viruses

The pathogenicity of the wild-type (wt) and mutant SG10 viruses was evaluated with the standard pathogenicity tests for NDV, ICPI and MDT. Their pathogenicity in cells was determined by assessing the ability of the rescued NDVs to exert a cytopathogenic effect (CPE) on BSR T7/5 cells. The growth dynamics of the mutant viruses were examined in DF-1 cells. Briefly, monolayers of DF-1 cells were infected with the SG10, rSG10, aSG10, or aSG10-EGFP strain at a multiplicity of infection (MOI) of 0.01 in the presence of 5 µg TPCK-treated trypsin/ml, and incubated at 37 °C in 5 % (v/v) CO_2. The culture supernatants were collected at 12 h intervals for 72 h, and the viral titers were determined with the median tissue culture infective dose ($TCID_{50}$) method.

Stability of the generated viruses

To further check the stability of the foreign gene in the viral genome, SG10, rSG10, aSG10, or aSG10-EGFP four 4-day intervals, and RT-PCR was performed to confirm the genetic stability of the mutated region. BSR T7/5 cells were infected with the 10th passage of the SG10-EGFP virus, and the expression of EGFP was examined.

Challenge study in 1-week-old chickens

To evaluate the protective efficacy induced by aSG10 against ND, forty 1-week-old SPF chickens were randomly divided into four groups, 10 for each. Chicks in groups 1 and 2 received 100 µl of PBS (pH 7.4) via intranasal (IN) and eye drop (ED) routes. Chicks in groups 3 and 4 received 10^6 EID50 of aSG10 or LaSota viruses via ED/IN route, respectively. Sera

were collected on 4, 14 and 21 day post-immunization (dpi) and subjected to HI assays against both of two strains: aSG10 and LaSota. All birds except group 1 were challenged through ED/IN route with 10^5 ELD50 of virulent NDV strain SG10 at 21 dpi. After challenge, birds were monitored daily for clinical signs of ND during 10 day post-challenge (dpc). Oropharyngeal and cloacal swabs were collected on 3 dpc for virus isolation.

All chickens were kept in isolators throughout the experiment and the rearing facilities were approved by Beijing Administration Committee of Laboratory Animals under the leadership of the Beijing Association for Science and Technology [the approval ID is XYXK (Jing) 2013-0013]. The protocol (including the possibility of animal death without euthanasia) was specifically considered and approved by the Animal Welfare and Ethical Censor Committee at China Agricultural University.

Results

Sequence alignment and analysis

The genome of the SG10 strain consists of 15,192 nt. A phylogenetic analysis indicated that the SG10 strain belongs to subgenotype VIId in genotype VII of class II NVD, and is genetically distinct and phylogenetically distant from the vaccine strains. The F protein cleavage site is ^{112}RRQKR↓F^{117}, which is consistent with a virulent phenotype. The protective antigenic proteins F and HN of the SG10 strain displayed 88.3 and 87.9 % amino acid sequence identity with the F and HN proteins of LaSota. In contrast, the F and HN proteins of the SG10 strain displayed 96.2–99.8 and 96.7–99.7 % amino acid sequence identity with F and HN, respectively, of the NDV isolates recovered between 2005 and 2012 from chickens in China.

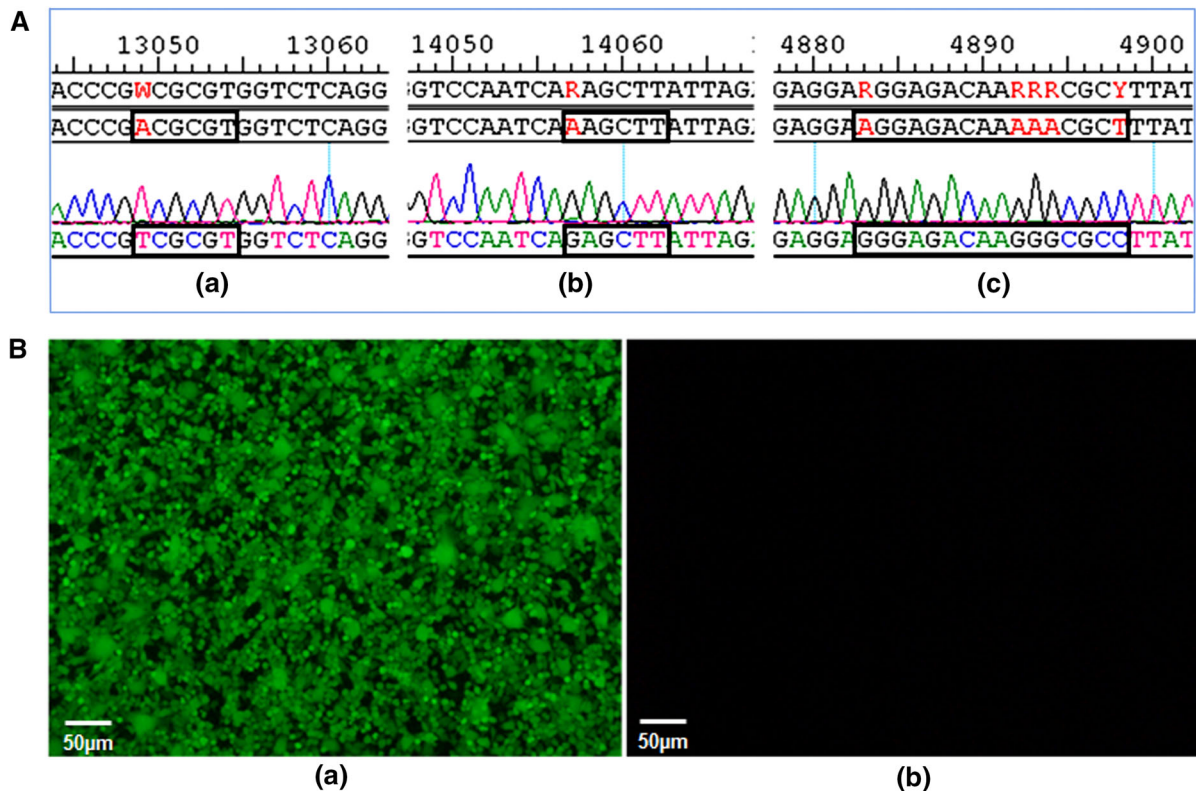### Generation of recombinant SG10 viruses

A cDNA clone encoding the antigenome of strain SG10 was constructed from six cDNA segments that were synthesized by RT-PCR from virion-derived genomic RNA (Fig. 1a). The NDV cDNA was a faithful copy of the SG10 consensus sequence except for two nucleotide changes that were introduced to

eliminate two restriction sites (*Mlu*I and the *Hin*dIII) to facilitate detection. To increase the rescue efficiency of the infectious virus from cDNA, three extra G residues were introduced behind the T7 promoter. We then constructed a mutant, designated aSG10, in which the wt SG10 F protein cleavage site (^{112}RRQKR↓F^{117}) was replaced with the cleavage site that is common in avirulent strains, including B1 and LaSota, ^{112}GRQGR↓L^{117} (Fig. 1b). This involved three amino acid substitutions, at positions 112, 115, and 117 relative to the cleavage site. Using the full-length aSG10 cDNA clone as the backbone, we constructed a third recombinant cDNA clone, aSG10-EGFP, containing the *EGFP* gene between the P and M genes in the LaSota genome, as an additional transcription unit (Fig. 1c).

The recombinant viruses SG10, aSG10, and aSG10-EGFP were recovered the transfection of BSR T7/5 cells with the corresponding antigenomic cDNA plasmids and three helper plasmids and the subsequent inoculation of embryonated chicken eggs with the cell culture supernatants. The HA-positive allantoic fluids was processed to isolate the viral genomic RNA, which was subjected to RT-PCR and sequence analyses. The sequencing results confirmed the presence of the mutations introduced into the genetic markers and the cleavage site of the F0 protein (Fig. 2A). The expression of EGFP was observed in BSR T7/5 cells infected with the recovered aSG10-EGFP virus (Fig. 2B-a). No EGFP expression was seen in control cells (Fig. 2B-b).

Pathogenicity of the recombinant viruses

The pathogenicity of wt SG10, recombinant SG10, aSG10, and aSG10-EGFP was evaluated with the MDT test in 10-day-old embryonated SPF chicken eggs and the ICPI test in 1-day-old SPF chicks. The ICPI values of the parental and recombinant SG10 viruses were 1.79 and 1.89, respectively (Table 1). The MDT values of the parental and recombinant SG10 viruses were 42 and 45 h, respectively. The ICPI values of aSG10 and aSG10-EGFP were 0.25 and 0.00, respectively. The MDT values of aSG10 and aSG10-EGFP were both >120 h. When the recombinant viruses were used to inoculate BSR T7/5 cells without TPCK-treated trypsin, CPEs were observed in the SG10- and rSG10-infected cells, whereas the cells infected with aSG10 and aSG10-EGFP displayed the

Fig. 2 **A** Genetic markers of the recovered viruses. The genetic markers, the eliminated *Mlu*I (*a*) and *Kpn*I (*b*) restriction sites, are *boxed*. (*c*) Modification of the F protein cleavage site. The mutated nucleotides are *boxed*. **B** EGFP expression in vivo. BSR T7/5 cells were infected with aSG10-EGFP. (*a*) Twenty-four hours later, the expression of EGFP in BSR T7/5 cells was detected with an inverted fluorescence microscope. (*b*) BSR T7/5 cells were used as the negative control

Table 1 Biological characteristics of the parental and recovered viruses

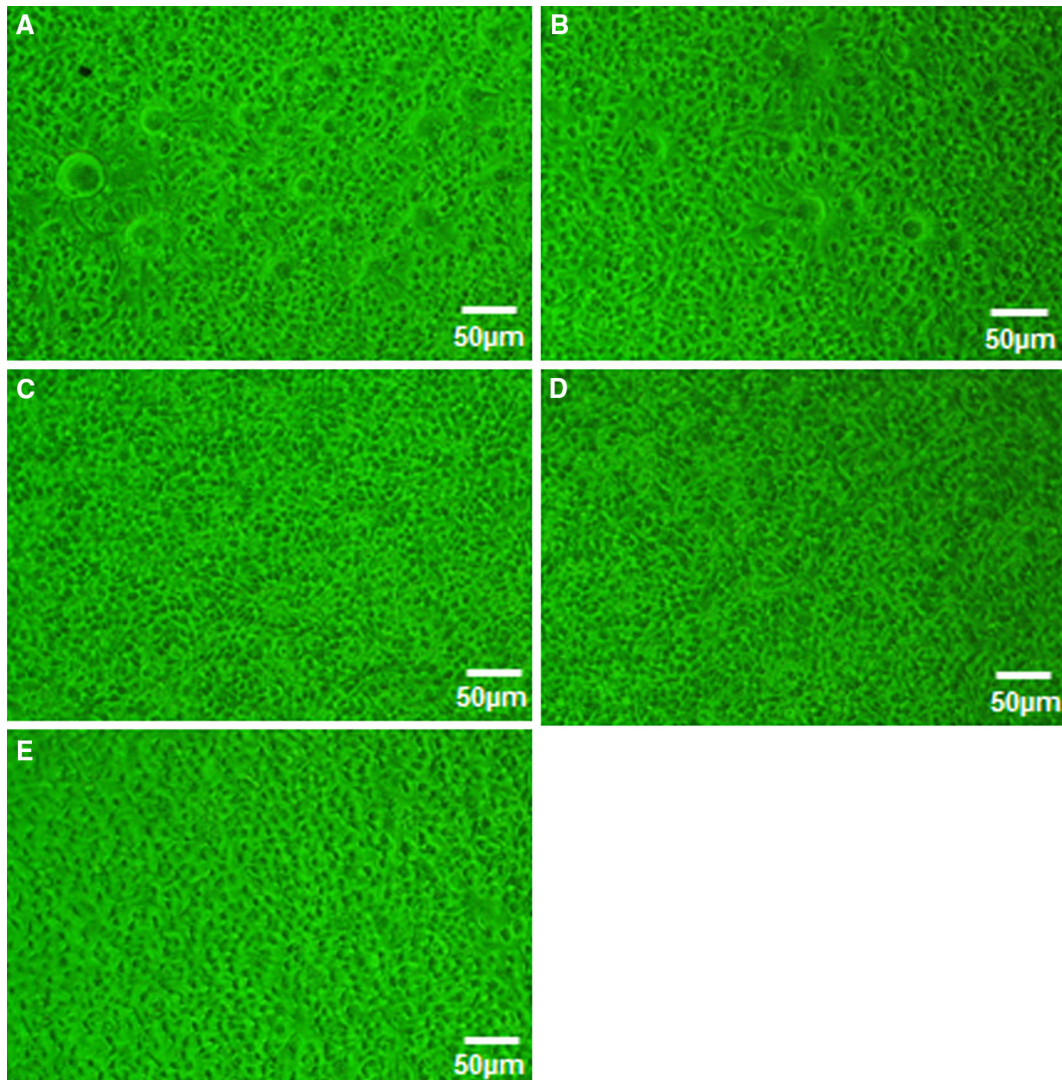| Pathogenicity	Virus			
	SG10	rSG10	aSG10	aSG10-EGFP
ICPI[a]	1.79	1.89	0.25	0.00
MDT[b] (h)	42	45	>120	>120

[a] Intracerebral pathogenicity index in day-old chickens. The velogenic strains have values close to 2.0 and lentogenic strains have values close to 0.00

[b] Mean death time (h) in embryonated chicken eggs. MDT values categorize the NDVs as velogenic (less than 60 h), mesogenic (60–90 h), and lentogenic pathotypes (more than 120 h)

normal morphology (Fig. 3). These results show that the pathogenicity of rSG10 was similar to that of the parental virus strain SG10, whereas the pathogenicity of aSG10 and aSG10-EGFP was highly attenuated.

Growth properties of the parental wt and rescued viruses

The pathogenicity of wt SG10, recombinant SG10, aSG10, and aSG10-EGFP was evaluated with the MDT test in 10-day-old embryonated SPF chicken eggs and the ICPI test in 1-day-old SPF chicks. The ICPI values of the parental and recombinant SG10 viruses were 1.79 and 1.89, respectively (Table 1). The MDT values of the parental and recombinant SG10 viruses were 42 and 45 h, respectively. The ICPI values of aSG10 and aSG10-EGFP were 0.25 and 0.00, respectively. The MDT values of aSG10 and aSG10-EGFP were both >120 h. When the recombinant viruses were used to inoculate BSR T7/5 cells without TPCK-treated trypsin, CPEs were observed in the SG10- and rSG10-infected cells, whereas the cells infected with aSG10 and aSG10-EGFP displayed the normal morphology (Fig. 3). These results show that

Fig. 3 CPE of NDV strains in BSR T7/5 cells. BSR T7/5 monolayers were infected with NDV strains SG10 (**a**), rSG10 (**b**), aSG10 (**c**), and aSG10-EGFP (**d**) at an MOI of 0.01. The cells were photographed at 36 hpi. Negative control cells (**e**) are also shown

the pathogenicity of rSG10 was similar to that of the parental virus strain SG10, whereas the pathogenicity of aSG10 and aSG10-EGFP was highly attenuated.

To compare the properties of the parental wt and three rescued viruses, we analyzed the kinetics and the final viral yields under multistep growth conditions on DF-1 cells. The results revealed that the kinetics and magnitude of replication of rSG10 were very similar to those of SG10 (Fig. 4), indicating that rSG10 retained the growth characteristics of the parental virus.

However, the kinetics of replication of the attenuated viruses (aSG10 and aSG10-EGFP) differed from those of the parental virus. All the viruses reached their highest titers at 48 hpi, and their titers decreased moderately to the end of the experiment at 72 hpi. However, the highest titers of SG10 and rSG10 were about $10^{6.5}$ TCID$_{50}$/ml lower than those of aSG10 ($10^{7.5}$ TCID$_{50}$/ml) and aSG10-EGFP ($10^{7.75}$ TCID$_{50}$/ml). These results indicate that the attenuated viruses grew more efficiently than the virulent viruses on DF-1 cells.

Fig. 4 Multistep growth curves of SG10, rSG10, aSG10, and aSG10-EGFP in DF-1 cells. The cells were infected with the indicated NDV at an MOI of 0.01. Samples were taken at 12 h intervals for 72 h, and the viral titers were determined with $TCID_{50}$ in DF-1 cells
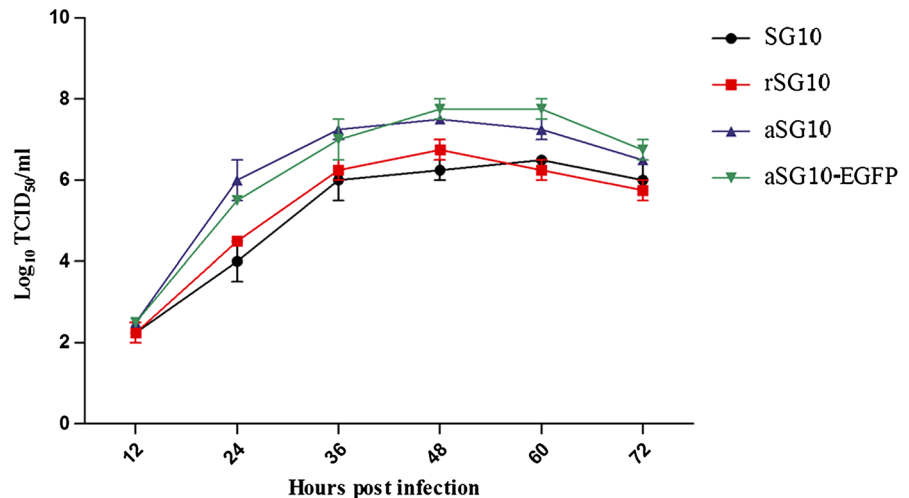
Stability of the rescued viruses

After the three rescued viruses were successively passaged 10 times through SPF eggs, we performed an RT-PCR analysis with the allantoic fluid. The sequencing results confirmed the presence of the mutations introduced into the genetic markers and the mutated cleavage site in the F0 protein. When BSR T7/5 cells were infected with the 10th passage of the rSG10-EGFP virus, the expression of EGFP was observed after 36 h and no autofluorescence was observed in the uninfected cells (data not shown).

Protective efficacy of aSG10 in chickens

All chicks that had been immunized with the LaSota or aSG10 were completely protected against highly virulent NDV challenge without showing any clinical sign of disease. In contrast, all of the birds inoculated with PBS displayed disease signs with severe depression from 3 to 4 dpc and 100 % mortality at 4 dpc (Table 2).

The shedding of challenge viruses was examined from oral and cloaca swabs on 3 dpc. Chicks that had been immunized with aSG10, no oral or cloacal shedding of challenge virus was observed (Table 2). However, in the case of LaSota-vaccinated, both challenge viruses were detected on 3 dpc in the oral and cloaca, with the protection against viral shedding was 40 and 70 %, respectively. The results indicated that the aSG10 vaccine effectively prevented viral shedding in vaccinated chickens against genotype-matched virulent viruses, but the LaSota vaccine failed to prevent mismatched challenge virus shedding.

The serum samples were detected for NDV-specific antibody response by HI assay at 4, 14 and 21 dpc. As the results shown in Table 3, all vaccination sera at 4 dpc were negative to NDV by the HI test. At 14 and 21 dpc the vaccines aSG10 and LaSota were both able to induce protective antibodies against SG10 and LaSota strains, but HI titers were slightly higher against the genotype-matched antigen virus than against genotype-mismatched antigen virus.

Discussion

In China, virulent NDV genotype VII first emerged in the 2000s (Hu et al. 2009), and most isolates recently

Table 2 Frequency of isolation of challenge virus in chickens and survival of chickens after challenge

Groups	Number of viral shedding chickens after challenge		Survivors
	Oral	Cloaca	
PBS	10/10	10/10	0/10
LaSota	6/10	3/10	10/10
aSG10	0/10	0/10	10/10

Table 3 Serum antibody response against NDV following vaccination by HI test

Groups	Antibody response against SG10 (HI titer[a])			Antibody response against LaSota (HI titer[a])		
	Day 4	Day 14	Day 21	Day 4	Day 14	Day 21
PBS	0	0	0	0	0	0
LaSota	0	4.2 ± 1.47	4.8 ± 1.68	0	5.1 ± 0.73	5.8 ± 0.63
aSG10	0	5.8 ± 1.03	5.7 ± 1.56	0	4.6 ± 0.96	4.6 ± 1.17

[a] Hemagglutination inhibition (HI) titer was expressed in log2 of the mean $\pm$ standard deviation

derived from chickens belong to genotype VIId. Recent studies and our present results show that the prevalent genotype VIId isolates differ from the widely used vaccine strains in the immune responses they evoke (Tsai et al. 2004; Rui et al. 2010). Therefore, the antigenic differences between the prevalent genotype and vaccine strains might account for the current ND outbreaks in vaccinated poultry flocks (Qin et al. 2008). To solve this problem, new vaccines that better match the predominant viruses have been developed (Hu et al. 2009; Xiao et al. 2012).

Strain SG10 is a highly virulent genotype VIId NDV, which was isolated from an outbreak in a vaccinated broiler flock. Its F and HN proteins share 96.2–99.8 and 96.7–99.7 % amino acid sequence identity with those of the genotype VIId NDV isolates recovered between 2005 and 2012 from chickens in China (Zhang et al. 2014). In this study, we created a reverse genetics system for strain SG10, used as the parent virus. Because the amino acid sequence at the F protein cleavage site has been postulated to be a major determinant of NDV virulence (Collins et al. 1993), the cleavage site of virulent NDV SG10 was modified to the consensus sequence of avirulent NDV. As expected, the newly generated mutated NDVs were highly attenuated. The ICPI values for aSG10 and aSG10-EGFP were 0.25 and 0, respectively, and their MDT values were both >120 h.

The attenuated NDVs had similar growth patterns but differed from those of the virulent viruses. The attenuated viruses grew more efficiently than the virulent viruses. The highest titers of aSG10 and aSG10-EGFP were about $10^{7.5}$ $TCID_{50}$/ml, higher than those of SG10 and rSG10 ($10^{6.5}$ $TCID_{50}$/ml). Therefore, the attenuated viruses produce a CPE more slowly than the virulent viruses, and the attenuated viruses have more time to replicate. For these reasons, the attenuated viruses produced higher titers in embryonated SPF chicken eggs. The HA titers of

aSG10 and aSG10-EGFP in allantoic fluid reached 9log2, and the titers of SG10 and rSG10 were often 7log2 (data not shown).

The construction of a reverse genetics system for NDV should allow the production of a vaccine vector for both vaccination and gene therapy (Ge et al. 2007; Hu et al. 2011). We inserted the *EGFP* gene between the P and M genes and successfully rescued the recombinant NDV aSG10-EGFP. EGFP expression was observed in BSR T7/5 cells infected with aSG10-EGFP (Fig. 2B), and the virus carried the gene through at least 10 passages. Therefore, the attenuated aSG10 virus has potential utility as a viral vector.

Efficacy studies in 1-week-old chicks showed that strain aSG10 more effectively protected the vaccinated birds from morbidity and mortality against highly virulent genotype VII NDV virus challenge compared to vaccine LaSota. Though the currently available vaccine LaSota could prevent disease it did not stop viral shedding, whereas aSG10 not only prevented disease but also significantly reduced virus shedding, indicating aSG10 is a promising vaccine candidate against NDV strains circulating in China.

Conclusions

A reverse genetic system was constructed and used to rapidly develop a live attenuated vaccine based on a virus currently circulating in China. The attenuated aSG10 strain better matched the predominant viruses than the commercial vaccine strains, and as a vaccine, may provide better protection against genotype-matched isolates in China. Our results also show that aSG10 is a potentially useful viral vector.

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