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Hemagglutinin–Neuraminidase and fusion genes are determinants of NDV thermostability

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ABSTRACT

Newcastle disease (ND) caused by infections with virulent strains of Newcastle disease virus (NDV) continues to be a threat for poultry industry worldwide. The prospect of developing a thermostable and effective NDV vaccine is still highly desirable. To investigate the determinants of thermostability in NDV, we generated recombinant NDV strains by exchanging viral hemagglutinin–neuraminidase (HN) gene or by mutating the fusion (F) gene. The results showed that the HN and F protein were both determinants of NDV thermostability. With increased thermostability, the HN protein-chimeric virus showed significantly reduced neuraminidase and hemadsorption activities, but its hemolytic activity was retained. We also found that changing the amino acid in the F protein cleavage sites, affected the thermostability as well as the pathogenicity and fusogenic capacity of the virus. Taken together, our results suggest that HN and F proteins both contribute to the thermostability of NDV, and other viral biological activities change as the thermostability of the virus changes. These findings should be of benefit to the development of a thermostable and efficacious NDV vaccine.

1. Introduction

Newcastle disease virus (NDV), which belongs to the genus *Avaluvirus* (*Paramyxovirinae* subfamily, *Paramyxoviridae* family), can cause acute, highly contagious Newcastle disease (ND). As an enveloped virus with a single-stranded, non-segmented RNA genome of negative sense, NDV genome contains six open reading frames (ORFs) that encode nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large polymerase (L) proteins (Ganar et al., 2014). Additionally, two other proteins, V and W, are produced by RNA editing during transcription of the P gene (Motz et al., 2013). HN is an important immunogenicity protein and virulence factor for NDV (Huang et al., 2004; Khattar et al., 2009; Liu et al., 2015a). And the F protein cleavage site is a major determinant of NDV virulence (Connaris et al., 2002; Connolly et al., 2009).

Effective prevention and control of ND usually depends on prophylactic vaccination in many countries that produce poultry on a commercial scale. In the past, ND was effectively controlled by using the traditional vaccines developed decades ago from NDV genotype II strains, like La Sota or B1 strain (Dimitrov et al., 2017). However, the currently circulating NDV strains that cause epidemics are more complex, and mainly comprise genotype VII (Zhang et al., 2012). It was shown that the La Sota vaccine components afforded significantly lower antibody titers than those in the SG10 vaccine against the NDV VII strain (Yang et al., 2017).

Most NDV vaccine strains such as La Sota and B1 are thermolabile, and only a few strains are thermostable (e.g., V4, I-2, K148/08 and TS09-C) (Bensink and Spradbrow, 1999; Boumart et al., 2016; Jeong et al., 2013). Unfortunately, the specific antibody levels generated and the immune effect of the NDV thermostable vaccine are poor against the currently circulating NDV strains (Mahmood et al., 2014). Therefore, studying the thermostable determinants of NDV with the prospect of developing a thermostable and effective NDV vaccine is highly desirable. Recently, researchers found that the HN gene from thermostable NDV genotype I (TS09-C) could be engineered into the thermolabile NDV genotype II (La Sota) for the development of a novel thermostable NDV vaccine (Wen et al., 2016). However, these two genotypes cannot provide antigens matched to the circulating strain. Therefore, new vaccines are still needed.

In this study, we generated a recombinant NDV by exchanging viral genes between the thermostable V4 strain and the thermolabile rSG10 strain using reverse genetics technology. Data showed that the HN protein is an important determinant of NDV thermostability. We also investigated the influence of different amino acid sites of F gene on viral thermostability. Data showed that changing these amino acid sites alone or in combination could affect the thermostability of the virus.

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These results revealed that HN and F protein could both contribute to the thermostability of NDV. Our findings provide a platform for the development of a thermostable and efficient vaccine.

2. Materials and methods

2.1. Animal ethics statement

Specific pathogen-free (SPF) chicken embryos and chickens were purchased from Beijing Boehringer Ingelheim Vital Biotechnology Co., Ltd. (Beijing, China). All birds were fed in isolators in the Animal Facility of the China Agricultural University (CAU). The Animal Facility is approved by the Administration Committee of Laboratory Animals under the auspices of the Beijing Association for Science and Technology (approval ID SYXK [Jing] 2013–0013). This study was carried out in strict accordance with the protocols for the Care and Use of Laboratory Animals formulated by the Ministry of Science and Technology of China. The experimental protocol was approved by the Animal Welfare and Ethical Censor Committee at CAU (approval number 1603–05).

2.2. Cells and viruses

The chicken embryonic fibroblast cell line (DF-1) and Vero cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Thermo, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, Thermo, USA), 1% Penicillin-Streptomycin (100x, Macgene, China) and maintained in DMEM containing 2% FBS. Baby hamster kidney cells expressing T7 RNA polymerase (BSR T7/5) were grown in DMEM containing 10% FBS and 1 mg/ml G418 (Invitrogen, Carlsbad, CA, USA). All cell lines were kept at 37 °C in a 5% CO_2 incubator (Thermo Forma, Marietta, OH, USA).

The virulent NDV strain SG10, which belongs to genotype VIId, was isolated in China from an outbreak in a vaccinated broiler flock. The aSG10 strain is an attenuated mutant virus derived from SG10 using a reverse genetics system (Liu et al., 2015b). The thermostable NDV strain V4, which belongs to genotype I, is kept in our laboratory. The recombinant or mutant viruses, which were generated from a full-length cDNA copy of the SG10 strain (rSG10), were described as below. All viruses were propagated and titrated in 9–10 days old embryonated SPF chicken eggs, and allantoic fluids that exhibited high HA titers were collected and stored at −80 °C until use.

2.3. Recombinant and mutant cDNA construction

NDV strain SG10 (rSG10) was used as the backbone upon which to construct a series of recombinant or mutant viruses, as illustrated in Fig. 1. Construction of the full-length antigenomic cDNA from rSG10 has been described previously (Liu et al., 2015b). The ORF for the HN gene from the thermostable V4 strain was inserted into the full-length antigenomic cDNA of rSG10 in place of the original HN ORF via the presence of unique restriction enzyme sites and use of the Seamless Assembly Cloning Kit (Invitrogen, USA). Briefly, the full-length antigenome from rSG10 was digested with *Sal* I and *Mlu*I to generate a single DNA fragment lacking the HN gene. The HN V4 ORF was then engineered by PCR amplification. The DNA fragments with overlapping homologous ends of various lengths were joined together using the above-mentioned cloning kit. The full-length rSG10 cDNA plasmid containing the V4 HN gene was named rSG10-V4HN.

To construct recombinant cDNA clones containing one or two or three mutations within the F gene, the full-length antigenomic cDNA rSG10 was used as the backbone. Briefly, the F cleavage site motif of the rSG10 strain, which was ^{112}RRQKR↓F^{117}, was mutated using overlapping PCR, resulting in the amino acids being changed individually or in combination with that of avirulent strain, which was ^{112}GRQGR↓L^{117}. The fragment containing the mutated site was used to replace the corresponding fragment in the full-length cDNA using two unique restriction enzymes, *Sal*I and *Hin*dIII. The mutated cDNA clones containing either R112 G, K115 G or/and F117 L substitutions were designated rSG10-F-A, rSG10-F-B, rSG10-F-C, rSG10-F-AB, rSG10-F-AC, rSG10-F-BC and aSG10 (rSG10-F-ABC) according to the mutation site (Fig. 1). The sequences of all the primers used to construct the full-length cDNA clones are available upon request. All of the constructs were confirmed to be correct by nucleotide sequencing.

2.4. Virus rescue from cDNA plasmids

The recombinant chimeric viruses were recovered by co-transfecting each chimeric, full-length NDV cDNA plasmid, along with helper plasmids expressing the NP, P and L proteins, into BSR T7/5 cells, as described previously (Liu et al., 2015b). On day 4 post-transfection, the cells were frozen and thawed three times. The supernatant was harvested and then injected into the allantoic sacks of 9–10 days old SPF embryonated chicken eggs to rescue the recombinant viruses. Recovery was deemed successful when the allantoic fluid had a positive HA titer. Then the viruses were passaged five times in the SPF embryonated chicken eggs. And the total RNA from the allantoic fluids were extracted using a total RNA purification kit (GeneMark, China) according to the manufacturer's instructions. All of the recombinant viruses were sequenced, and no adventitious mutations were detected. The allantoic fluids with confirmed sequences were collected and centrifuged to remove cellular debris before they were aliquoted and rapidly frozen for storage at −80 °C until use.

2.5. Thermostability tests

Aliquots of the NDV chimeras were incubated in a metal bath at 56°C, and then transferred to ice to stop the heat treatment at various time points, as described previously (Wen et al., 2016). The HA activities and infectivities of the viruses were measured by HA and EID_{50} assays in 9–10 days old chicken embryos, as described in the OIE Terrestrial Manual 2017 (http://www.oie.int/fileadmin/Home/eng/Health_ standards/tahm/2.03.14 _NEWCASTLE_DIS.pdf.). Briefly, for the HA assay, 25 μl of PBS is dispensed into each well of a microtitre plate with V bottom. 25 μl of the virus suspension is placed in the first well. Twofold serial dilutions of 25 μl volumes of the virus suspension are made across the plate. A further 25 μl of PBS is dispensed to each well. 25 μl of 1% (v/v) chicken RBCs is dispensed to each well. The solution is mixed gently. Then incubate the plate at room temperature for about 30 min. HA is determined by tilting the plate and observing the streaming of the RBCs. The titration should be read to the highest dilution giving complete HA (no streaming); this stands for 1 HA unit and can be calculated from the initial dilution. To determine the EID_{50} of the virus, a series of 10-fold dilutions are prepared for the virus suspension. Five embryonated eggs are inoculated with each dilution. After incubation for 96 h, the HA assay is used to determine whether or not the virus has infected and multiplied in each of the eggs. The EID_{50} is calculated by the Reed and Muench method (Reed and Muench, 1938).

2.6. Mean death time (MDT) and intracerebral pathogenicity index (ICPI) assays

The pathogenicities of the recombinant viruses were determined by the MDT assay in 9–10 day-old SPF chicken embryos and by the ICPI assay in day-old SPF chicks, as described previously (Swayne and David, 1998). Briefly, for the MDT test, 10-fold series diluted allantoic fluids were made in sterile PBS. And 0.1 ml of each dilution was inoculated into five 9–10 days old chicken embryos via the allantoic cavities route then incubated at 37 °C. The embryos were observed every 8 h for 7 days. The time at which each embryo was first observed to die was recorded. The highest dilution causing 100% mortality was

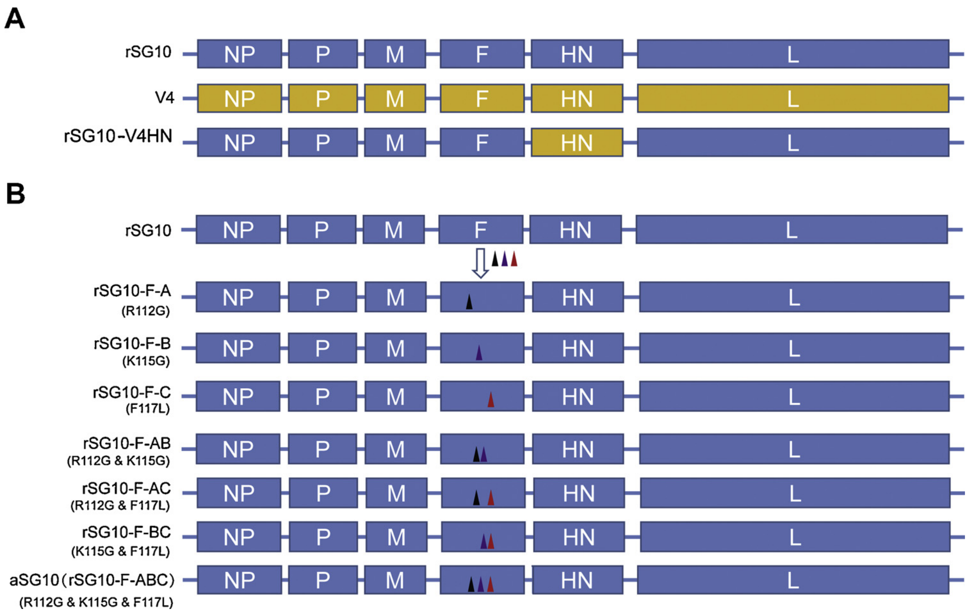

Fig. 1. Chimeric virus construction. (A) Genes from the rSG10 and V4 strains are displayed as blue or yellow bars, respectively. Exchange of the rSG10 HN gene for the V4 HN gene produced rSG10-V4HN. (B) The full-length antigenomic rSG10 cDNA was used as the backbone to construct recombinant cDNA clone mutations within the F gene. The mutated cDNA clone containing the R112 G, K115 G or/and F117 L substitution(s) were designated rSG10-F-A, rSG10-F-B, rSG10-F-C, rSG10-F-AB, rSG10-F-AC, rSG10-F-BC and aSG10 (rSG10-F-ABC) according to the mutation site (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

considered to be the minimum lethal dose. The MDT was determined as the mean time (hours) for the minimum lethal dose of the virus to kill all the inoculated embryos. The MDT has been used to classify NDVs: velogenic strain (taking less than 60 h to kill), mesogenic strain (taking 60–90 h to kill) or lentogenic strain (taking more than 90 h to kill). For the ICPI assay, 0.05 ml of 1:10 diluted fresh allantoic fluid with a HA titer $> 2^4$ was injected intracerebrally into each of ten 1-day-old SPF chicks. The birds were monitored every 24 h for 8 days. At each observation, the birds were scored: 0 if normal, 1 if sick, and 2 if dead. The ICPI is the mean score per bird per observation over the 8-day period. The pathotype definitions by the ICPI are: virulent strains, 1.50–2.00; moderately virulent strains, 0.70–1.50; avirulent strains, 0.00–0.70.

2.7. Viral growth kinetics

The growth kinetics of rSG10 and the chimeric viruses were determined under multiple-cycle growth conditions in DF-1 cells. Triplicates of 12-well plates seeded with 10^5 DF-1 cells per well were infected at a multiplicity of infection (MOI) of 0.01. After a 1 h of adsorption, the cells were washed with PBS, covered with DMEM containing 2% FBS, and then placed at 37 °C under 5% CO_2. The maintained medium of the infected cells was supplemented with 5 μg/ml TPCK-treated trypsin (T1426; Sigma, USA). The supernatant was collected and replaced with an equal volume of fresh medium every 12 h until 72 hpi. The 50% tissue culture infective dose ($TCID_{50}$) of the supernatants was quantified using the endpoint method of Reed and Muench (Reed and Muench, 1938).

2.8. Hemadsorption (HAd) activity assays

HAd activity was assayed as the adsorption ability of the viruses to chicken red blood cells (CRBCs), as described previously (Jin et al., 2016). Briefly, Vero cells seeded in 24-well plates were infected with virus at a MOI of 0.1. At 24 hpi, the supernatant was discarded and the cells were washed with cold PBS and then incubated at 4 °C for 30 min with a 2% (vol/vol) suspension of CRBCs. After incubation, the unbound CRBCs were washed with cold PBS and the CRBCs bound to the infected cells were lysed with red blood cell lysis buffer (Macgene, China). The lysate was centrifuged and transferred to a 96-well plate, and absorbance at 549 nm was measured with a Spectramax M5 ELISA reader (Molecular Devices, USA).

2.9. Neuraminidase assays

Neuraminidase activity was determined with a Neuraminidase Assay Kit (Beyotime, China) according to the manufacturer's instructions, and as described previously (Jin et al., 2016). Briefly, 10^5 Vero cells per well were seeded onto 96-well plates one day before the infections commenced. The cells were inoculated with recombinant viruses at 0.1 MOI. At 24 hpi, the cells were washed with cold PBS and covered with 30 μl of substrate mix comprising 325 mM 2-*N*-morpholinoethanesulfonic acid (pH 6.4), 0.5 mM 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUN; Sigma, USA), and 10 mM calcium chloride, in the ratio of 1:2:3 per well, to achieve a final concentration of 100 μM MUN in the assay. The cells were incubated at 37 °C for 30 min with shaking, and the reaction was terminated by adding 0.014 M sodium hydroxide in 83% (vol/vol) ethanol. The fluorescence intensity was measured at an emission wavelength of 450 nm and an excitation wavelength of 360 nm with a Spectramax M5 ELISA reader (Molecular Devices).

2.10. Hemolytic activity titrations

The hemolytic activities of the recombinant viruses were assessed as described previously (Jin et al., 2016). Briefly, the rescued viruses were centrifuged for 20 min at 500 × g at 4 °C. Supernatants were diluted to the same HA unit/ml (HAU/ml) based on the HA titer. Samples (0.5 ml) of the diluted viruses were each mixed with 1 ml of 1% CRBCs and then incubated on ice for 20 min with occasional shaking. The CRBCs were centrifuged at 500 × g for 3 min, washed with phosphate-buffered saline (PBS), and then resuspended in 0.5 ml of PBS followed by incubation at 37 °C for 60 min. After incubation, the samples were centrifuged at 200 × g for 5 min, and the supernatants were transferred to 96-well plates. The hemoglobin contents were measured (absorbance, 549 nm) using a Spectramax M5 ELISA reader (Molecular Devices). NH_4OH (0.03 M) was added to the assay as the positive control and PBS was used as the negative control. The hemolytic values for all the viruses were each displayed as a percentage of the values obtained for rSG10 at 2^8 HAU/ml, which was considered to be 100%.

2.11. Fusion index assays

The fusogenic abilities of the recombinant viruses were examined in Vero cells, as described previously (Jin et al., 2016). Briefly, 2×10^6

Vero cells were seeded in 6-well plates and infected with the recombinant viruses at an MOI of 0.1. Then the infected cells were kept in the maintained medium, which was supplemented with 5 μg/ml TPCK-treated trypsin. After the cytopathic effect was observed at 36 h post-infection, the supernatant was discarded, and the cells were washed and then incubated with 1 ml EDTA for 2 min at room temperature. The cells were then washed with PBS and fixed with methanol for 20 min at room temperature and stained with hematoxylin-eosin. Fusion was calculated from the fusion index, which involved calculating the ratio of the total number of nuclei to the number of cells in which these nuclei were observed. The values obtained from the HAd and NA assays, hemolytic activity and fusion index for all the chimeras were expressed as the percentages of the values for the parental virus rSG10, which was considered to be 100%.

2.12. Statistical analyses

All statistical analyses were performed in GraphPad Prism Software Version 6.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical differences between experimental groups were determined using the analysis of variance method. Statistical significance was set at $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****).

3. Results

3.1. Successful rescue of recombinant viruses

A cDNA clone encoding the antigenome of the SG10 strain (rSG10) was constructed from six cDNA segments that were synthesized by RT-PCR from virion-derived genomic RNA, as described previously (Liu et al., 2015b). To investigate the role of the HN gene in NDV thermostability, the complete HN gene ORF from the thermolabile genotype VII NDV rSG10 strain was replaced with the full-length HN gene from the thermostable genotype IV4 strain using reverse genetics technology. The recombinant virus recovered was named rSG10-V4HN. To identify the thermostable determinant of the F gene in NDV, six chimeric viruses, which are rSG10 strain derivatives in which three amino acid substitutions at positions 112(R112), 115(K115), and 117 (F117) relative to the cleavage site genes, were replaced individually or in combination with those from aSG10, which was achieved by changing the virulent F protein cleavage site motif ^{112}RRQKR↓F^{117} into the avirulent ^{112}GRQGR↓L^{117} motif. The six recombinant viruses were named rSG10-F-A, rSG10-F-B, rSG10-F-C, rSG10-F-AB, rSG10-F-AC and rSG10-F-BC according to the mutation site involved (Fig. 1). All chimeric viruses were passaged in SPF chicken embryos for five times before sequenced and each was confirmed to have the correct sequence for each amino acid exchange and lack any undesired mutations.

3.2. Thermostability in NDV is associated with HN and F genes

Thermostability in NDV refers mainly to the remaining hemagglutinin activity and infectivity of the virus to the host after its treatment at a particular temperature (56°C) and time (30 min) (Lomniczi, 1975). To investigate the roles of the HN and F genes in NDV thermostability, all the chimeric or mutant viruses were treated at 56°C for the time points indicated in Fig. 2. The HA activities (Fig. 2A) and 50% embryo infectious dose (EID_{50}) (Fig. 2B) were then examined in the context of thermostability. When the ORF from the rSG10 HN gene was replaced with that from the thermostable V4 strain, the rSG10-V4HN recombinant virus showed significantly decreased thermostability comparing to the V4 virus. When the mutations were present at specific amino acid sites in the F gene, the mutant viruses with the thermolabile background of the rSG10 strain showed marked decreased thermostability comparing to the V4 strain. Among the mutant viruses, aSG10, which contains three mutated amino acid sites, displayed statistically significant increased thermostability. Moreover, we also measured the HA activities of all the chimeric viruses after incubation at 25°C for 36 days or at 37°C for 20 days, and the HA titers only reduced slightly compared with the initial data. Therefore, the thermostable determinants of NDV are associated with the HN gene as well as the F gene.

3.3. Some mutations in F gene have reduced viral growth kinetics

To investigate whether the changes in the HN or F gene from the thermolabile velogenic strain to the thermostable lentogenic strain would affect viral replication in cell culture, the growth kinetics of all the recombinant or mutant viruses were further compared using multicycle growth curves in DF-1 cells, as shown in Fig. 3. The replication kinetics of the HN gene-replaced rSG10-V4HN virus was similar to those of rSG10. In contrast, the infectious viral titer of the mutant (F gene) rSG10-F-A virus was significantly lower than that of the rSG10 virus at 24 h post-infection (hpi). Additionally, the rSG10-F-C and rSG10-F-BC viruses both showed delayed growth and significantly lower infectious viral titers than those of rSG10 at 48, 60 and 72 hpi. These results indicate that replacement of the HN gene in a thermolabile strain with that from a thermostable strain can increase the thermostability of a thermolabile strain without a concomitant reduction in viral replication in the cells. Furthermore, when the velogenic rSG10 strain was engineered to contain the F117L (rSG10-F-C) or K115 G & F117L (rSG10-F-BC) mutations in the F cleavage site from the avirulent aSG10 strain, the thermostability of the two mutants increased with a concomitant reduction in viral replication efficiency in the cells.

3.4. Decreased pathogenicity of the recombinant viruses

To investigate whether the changes in the HN or F gene from the thermolabile velogenic strain to the thermostable lentogenic strain could affect the pathogenicity of NDV in vivo, we performed a mean death time (MDT) experiment with 9 day-old SPF chicken embryos and an intracerebral pathogenicity index (ICPI) assay in day-old SPF chicks. The data from these experiments (Table 1), show that the MDT and ICPI scores for rSG10-V4HN indicate that compared with rSG10, rSG10-V4HN had a slight decrease in virulence but can still be classified as a velogenic strain. The ICPI values for the six mutant viruses were all above 1.60, indicating that they can also be classified as velogenic strains. However, the MDT values for the viruses with single-point mutations were less than 60 h, while those of the viruses with two-point mutations ranged from 65 to 75 h, which means that these viruses can be classified as moderately virulent strains. Among these viruses, the virulence of the rSG10-F-BC virus decreased the most. The aSG10 strain that contains three mutant amino acid sites was classifiable as an avirulent strain. These results indicate that when the HN or F gene of the thermolabile velogenic strain is replaced by a thermostable lentogenic strain, the recombinant virus has decreased pathogenicity.

3.5. Reduced neuraminidase and hemadsorption activities of rSG10-V4HN

In NDV, the HN gene has multiple functions that are responsible for the attachment of the virus to its sialic acid-containing receptors, while NA activity is needed to hydrolyze the sialic-acid molecules from progeny viral particles to prevent viral self-aggregation. To investigate whether the changes in the HN or F gene from the thermolabile velogenic strain to the thermostable lentogenic strain could affect other viral biological functions that are associated with HN protein in NDV, we performed neuraminidase, hemadsorption, and hemolytic activity assays. As shown in Fig. 4, with increased thermostability, the chimeric rSG10-V4HN virus showed reduced neuraminidase activity, hemadsorption activity and hemolytic activity compared with rSG10 as well as V4. However, the reduction in hemolytic activity compared with rSG10 was not statistically significant.

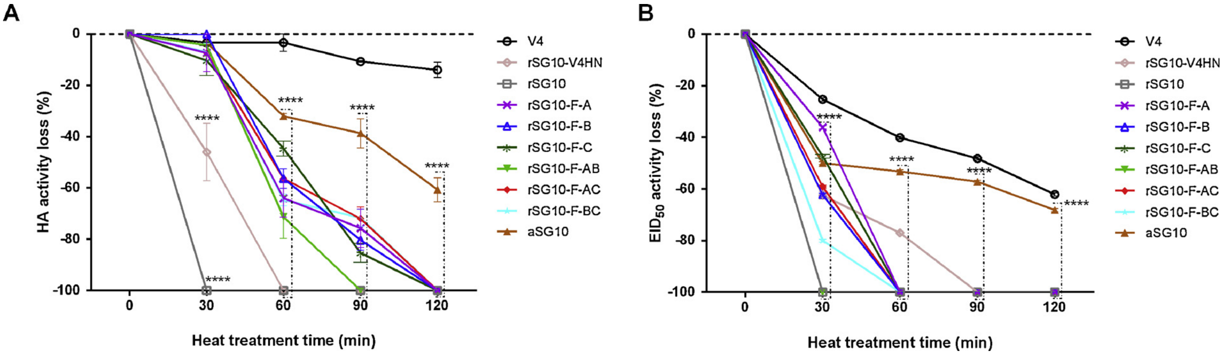

Fig. 2. Thermostability of the chimeric viruses. Chimeric viruses were heat treated at 56℃ at the time points indicated. The HA activity (A) and infectivity (B) of rSG10-V4HN and those of the mutant viruses were measured by performing the HA assay and EID_{50} assay. The decreased HA activity and infectivity of these viruses are expressed as percentages of the values for the untreated viruses, which was considered to be 100%, as the heating time increased. Mean values and their standard deviations are shown for three independent experiments. Asterisks indicate the significance of the difference from the recombinant viruses comparing to the V4 virus. P values are calculated by two-way ANOVA. Statistical significance was set at $P < 0.0001$ (****).

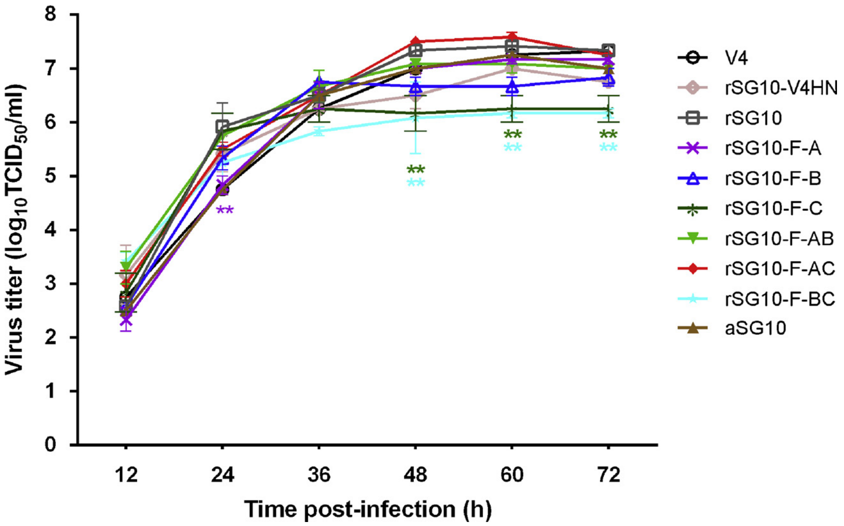

Fig. 3. Growth kinetics of the chimeric viruses. Multiple-cycle growth kinetic experiments were used to assess the growth differences in these viruses. DF-1 cells were infected with rSG10, rSG10-V4HN, rSG10-F-A, rSG10-F-B, rSG10-F-C, rSG10-F-AB, rSG10-F-AC, rSG10-F-BC, aSG10 or V4 at a MOI of 0.01, and the $TCID_{50}$ values were determined at the time points indicated. Mean values and their standard deviations are shown for three independent experiments. Asterisks indicate the significance of the difference from the recombinant viral titer comparing to the rSG10 viral titer. P values were calculated by a two-way ANOVA. Statistical significance was set at $P < 0.01$ (**).

Table 1
Pathogenicities and titers of the chimeric viruses.

Virus	MDT(h)[a]	ICPI[b]	HA titer (log2)	EID_{50}/ml (log10)
V4	≥120h	0.09	10	9
rSG10-V4HN	50	1.75	8	8
rSG10	42	1.93	9	7
rSG10-F-A	48	1.69	9	8.63
rSG10-F-B	48	1.74	8	8.50
rSG10-F-C	48	1.66	8	8.63
rSG10-F-AB	72	1.63	8	8.63
rSG10-F-AC	67	1.75	8	8.63
rSG10-F-BC	72	1.60	9	7.50
aSG10	≥120h	0.25	10	9

[a] Mean death time (MDT): virulent strains, < 60 h; moderately virulent strains, 60–90 h; avirulent strains, > 90 h.

[b] Intracerebral pathogenicity index (ICPI): virulent strains, 1.50–2.00; moderately virulent strains, 0.70–1.50; avirulent strains, 0.00–0.70.

3.6. Reduced the fusogenic capacities of the recombinant viruses

To determine whether the changes in the HN or F gene from the thermolabile velogenic strain to the thermostable lentogenic strain could affect viral syncytium formation ability, a fusion index assay was performed. As shown in Fig. 5A, the rSG10 caused the most severe cellular membrane fusion. And the syncytia induced by the two-point mutant viruses were obviously smaller than those induced by the rSG10 parental virus. Quantification of the fusogenic abilities of the mutants (fusion index compared with rSG10) showed that rSG10-F-B, rSG10-F-AB, rSG10-F-AC and rSG10-F-BC had significantly decreased fusogenic activities (91%, 60%, 69% and 48%, respectively) (Fig. 5B), which means that changing multiple F protein cleavage sites can significantly affect syncytium formation in Vero cells. When there were three mutations, the virus lost its syncytium formation ability even with the presence of TPCK-treated trypsin and became a NDV lentogenic strain (aSG10). Additionally, compared with the parental strain rSG10, the fusion ability of rSG10-V4HN was reduced significantly, which confirmed that the HN protein could participate in membrane fusion with the F protein. The V4 strain, which was a lentogenic strain, could not cause syncytium formation in Vero cells under the same infection conditions with the presence of TPCK-treated trypsin. These results indicate that when the HN or F gene of the thermolabile velogenic strain is replaced by a thermostable lentogenic strain, the fusogenic capacity of the chimeric viruses have been reduced.

4. Discussion

Thermostability in NDV strains is characterized by retained infectivity and hemagglutinin activities after treatment at 56 °C for 30 min. The first known thermostable NDV strain, V4, was isolated from the proventriculus of an 8 week-old chicken in Queensland, Australia. The hemagglutinin activity and infectivity of V4 was not affected when it was heat treated at 56 °C for 30 min, and it retained good

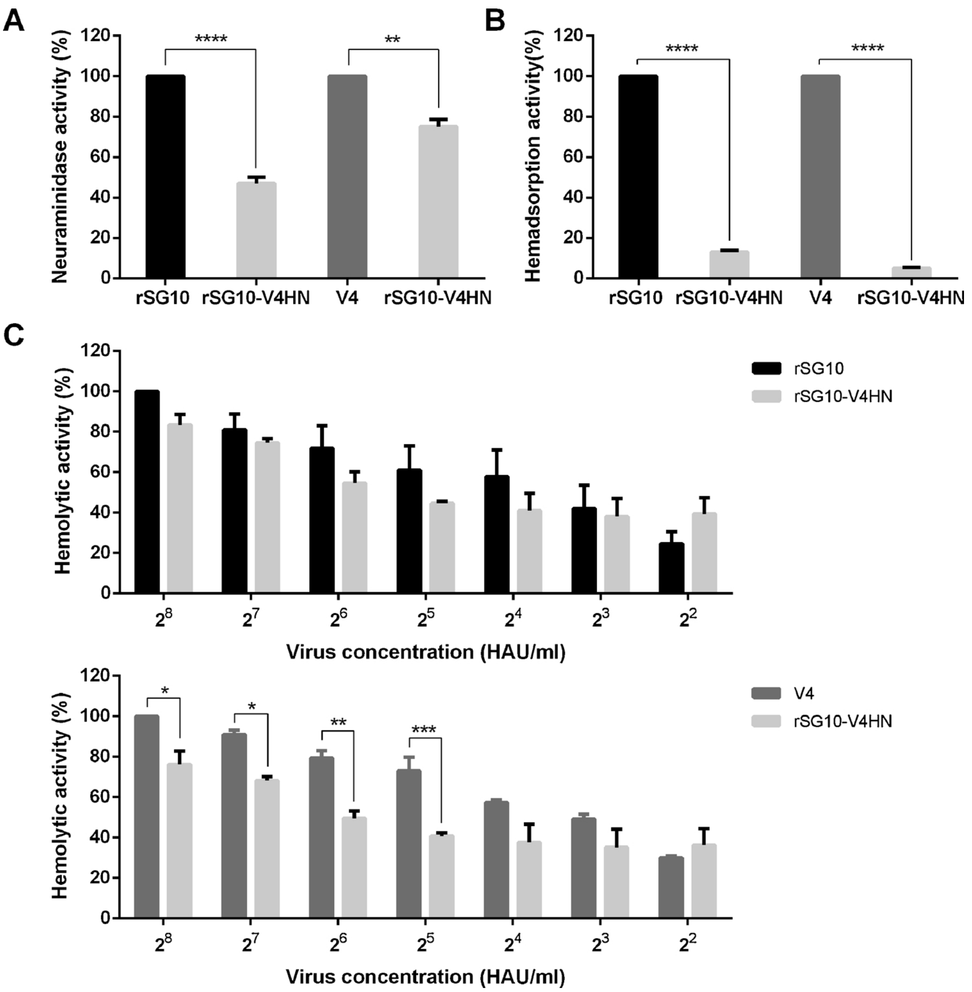

Fig. 4. Biological activities of rSG10, V4 and rSG10-V4HN. Neuraminidase activity (A), hemolytic activity (B) and hemolytic activity (C) were compared between rSG10 with rSG10-V4HN and V4 with rSG10-V4HN. All values are expressed relative to the value for rSG10 or V4 (100%), respectively. Mean values and their standard deviations are shown for three independent experiments. P values were calculated by *t*-test or two-way ANOVA. Statistical significance was set at $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****).

immunogenicity (Simmons, 1967). By observing the thermostability of the V4 strain, researchers found that the EID_{50} of V4 decreased from $10^{10.4}$ to $10^{9.3}$ after storage at 27–32 °C for 14 days (Nguyen Ba, 1992), indicating that this strain has good thermal stability. And V4 vaccine had been recommended by the Food and Agriculture Organisation (FAO) for chickens in tropical and developing countries.

However, certain problems with thermostable vaccines currently exist. For example, one study has shown that the immunogenicity of the La Sota strain were superior to those of the V4 vaccine (Cao, 2001). Sun et al. found that the NDV-specific antibody levels in chicken serum inoculated with the La Sota-based vaccine were higher than those of the V4 vaccine, and immunological memory was significantly better than that of the V4 vaccine group (Sun, 2001). The HI antibody levels against the V4 strain are low or appear late are the main drawbacks of this vaccine and most farms mainly monitor the serum HI antibody levels to determine the immunity levels in their chicken flocks.

Therefore, to identification of new thermostable vaccine strategies is still urgent. Yusoff et al. analyzed the whole sequences from thermostable strains and other NDV strains and found that thermal stability may be associated with HN gene mutations, but no further biological characteristics were examined (Tan et al., 1995; Yusoff et al., 1996). With the development of reverse genetics, genetic manipulation, viral genome cloning and generating recombinant or chimeric viruses are all now possible. Reverse genetics techniques make it possible to apply genetic approaches for determining the role of a viral gene in viral replication and virulence via the development of infectious cDNA clones from NDV (Cardenas-Garcia and Afonso, 2017; Liu et al., 2015b; Zhang et al., 2013). Wen et al. reported that an infectious clone of avirulent NDV strain TS09-C could be used as a vaccine vector to develop thermostable vaccines against Newcastle disease (Wen et al., 2015). Then their group replaced the HN segment of the thermolabile La Sota strain with the HN segment of the thermostable TS09-C strain using reverse genetics, and found that the thermal stability of this strain was significantly enhanced. Conversely, when the HN segment of the thermostable TS09-C strain was replaced by the HN segment of the thermolabile La Sota strain, the thermostability of TS09-C was reduced. These findings show that thermostability of lentogenic NDV strains is closely associated with the HN gene (Wen et al., 2016).

Previously we reported that the HN sequence of NDV was highly conserved among strains and the HN protein length diversity could affect the viral biological activities (Jin et al., 2016). For the lentogenic strains, such as the Ulster, V4 and TS09-C strains, they have 616 amino acids in HN length. Most other NDV isolates, such as La Sota strain, have 577 amino acids of HN protein, whereas the HN protein of SG10 strain have 571 amino acids which can only be detected in highly virulent strains. The 616 amino acids of Ulster NDV HN protein require cleavage from the C-terminus to recover the activation (Yuan et al., 2012). Proteolytic cleavage of the C-terminal leads to the increases in neuraminidase and hemadsorption activities (Nagai et al., 1976). In the present study, we used the thermolabile genotype VII rSG10 strain as

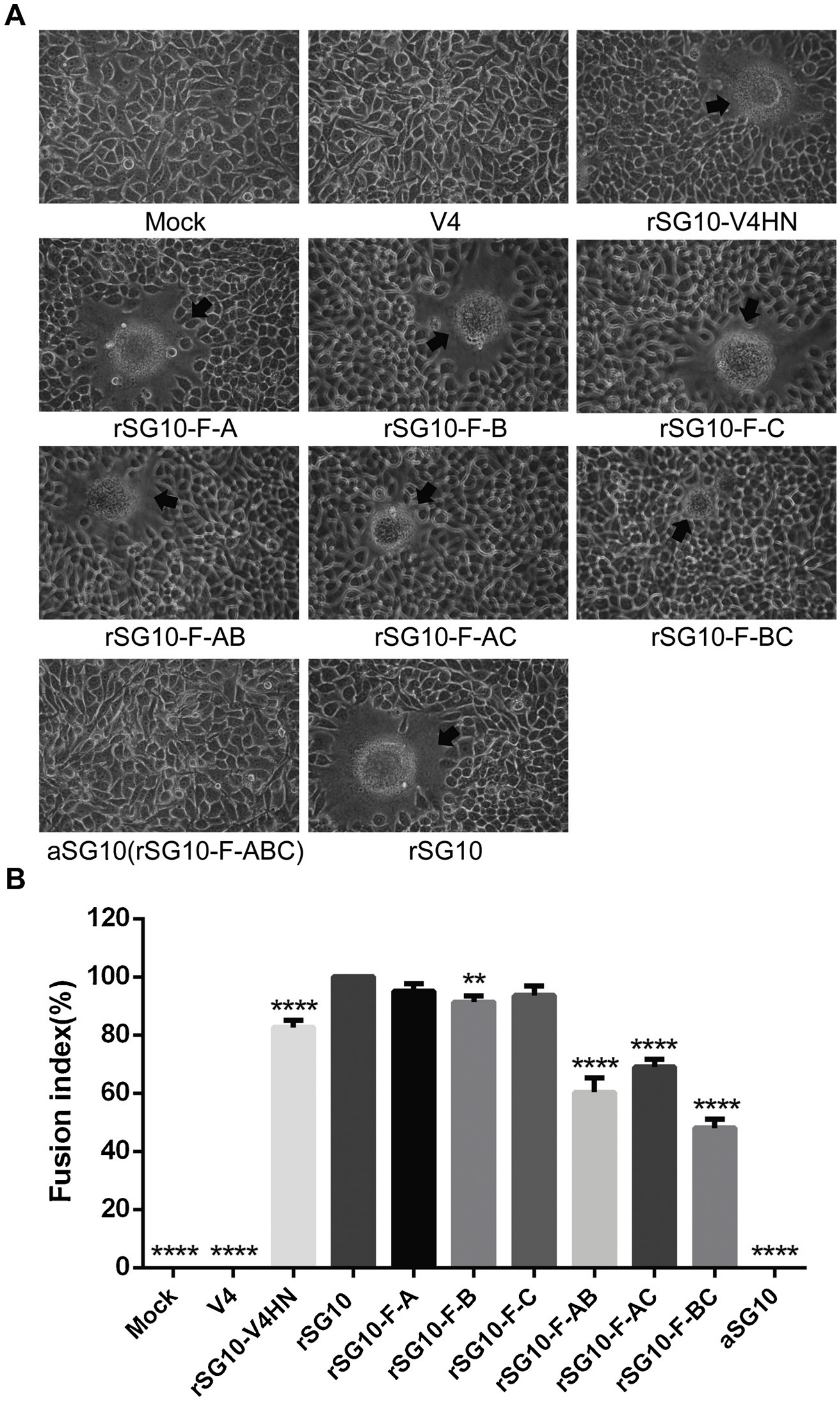

Fig. 5. Syncytium formation induced by chimeric viruses. (A) Vero cell monolayers were infected with chimeric viruses, each at a MOI of 0.1 in 6-well plates. All the groups were digitally photographed under an inverted microscope (450D; Canon) at × 400 magnification at 36 h post-infection. Black arrows indicate syncytia. (B) The fusion index values for the recombinant or mutated viruses were calculated as the ratio of the total number of nuclei to the number of cells in which the nuclei were observed. All values are expressed relative to the value for rSG10 (100%). Mean values and their standard deviations are shown for three independent experiments. P values were calculated by one-way ANOVA. Statistical significance was set at $P < 0.01$ (**) and $P < 0.0001$ (****).

the backbone with the HN gene replaced with that of the thermostable genotype I V4 strain using reverse genetics. The HN length of the rSG10 strain was 571 amino acids and that of the V4 strain was 616 amino acids. The homology of rSG10 HN to sequence of V4 was 89.70% in the amino acid level in this study. We successfully rescued the recombinant rSG10-V4HN NDV. After heat treatment, the rSG10-V4HN recombinant virus showed increased thermostability compared with the parental strain rSG10. However, the HN gene replacement did not affect viral replication or pathogenicity. It is notable that our results reveal that with increasing thermostability, the biological activities of the chimeric virus rSG10-V4HN, such as its neuraminidase and hemadsorption activities, became reduced. These findings expand the research base on the thermostable determinants of NDV. Interestingly, compared with V4, the chimeric virus rSG10-V4HN also showed reduced neuraminidase and hemadsorption activities as well as hemolytic activity. Considering the two viruses only shared the same HN gene, we assume these biological activities of NDV is not only related to HN gene. This indicates that other proteins can also affect the NDV thermostability.

The effect of HN in preventing F protein's thermal activation has been shown for human parainfluenza virus type 3 (Porotto et al., 2012). Therefore, we further investigated the effect of F protein on thermostability of NDV. Velogenic and mesogenic NDV strains have a multibasic amino acid motif in the F cleavage site (^{112}R/G/K-R-Q/K-K/R-R↓F^{117}), which can be cleaved by furin-like proteases intracellularly. In contrast, lentogenic strains have a monobasic amino acid motif at the F cleavage site (^{112}G-R/K-Q-G-R↓L^{117}), which can be cleaved by trypsin-like proteases and is found in the respiratory and intestinal tracts, extracellularly. In the present study, three different amino acids in F gene between the velogenic strain and lentogenic strain were mutated individually or in combination. Compared with the velogenic strain rSG10, the F gene mutations have increased the viral thermostability but reduced the viral pathogenicity, neuraminidase activity, hemadsorption activity, hemolytic activity and fusogenic capacity. And the degree of variation in these viral biological activities varies, depending on the amino acid position and the number of mutations. It has not been reported previously that the NDV F gene is associated with the viral thermostability.

Moreover, for the NDV vaccine development, changes in the viral biological activities need to be considered comprehensively. Although rSG10-V4HN strain is thermostable, the MDT and ICPI assays grouped this virus into the virulent strain. And HN is an important immunogenicity protein for NDV. The HN protein of V4 does not have a good immune effect against the circulating genotype VII NDVs. The MDT and ICPI assays grouped rSG10 with 1 or 2 mutations to the moderate virulence strains. Their safety is worth considering when apply to the vaccine development. Therefore, rSG10-V4HN and rSG10 with 1 or 2 F mutations are not good vaccine candidates due to the safety and efficacy. Our results showed the aSG10 with three specific amino acid site mutations in the F gene had the most increased thermostability and reduced viral pathogenicity in all the mutant viruses. Efficacy experiments previously confirmed that aSG10 has good safety and can protect chickens against infection with a lethal dose of a velogenic strain. The protective efficacy of aSG10 is significantly higher than the commercial La Sota vaccine (Yang et al., 2017). These results together provide a new candidate vaccine strain for the prevention and control of NDV.

5. Conclusion

In conclusion, our research illustrates that thermostability is a complex trait determined by multiple genetic factors in NDV, and that a change in thermostability can influence the biological activities of this virus. These findings augment our understanding of the biology of NDV and should be of benefit to the development of efficacious, thermostable NDV vaccines.

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