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Generation and evaluation of a genetically attenuated Newcastle disease virus rGM-VIIm as a genotype-matched vaccine

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Abstract Despite intensive vaccination campaigns, outbreaks of Newcastle disease (ND) have been frequently reported in China, especially of genotype VII that first emerged in the late 1990s. Given the dire need for vaccines against the circulating genotype VII virus, we developed an alternative method to recover a highly virulent recombinant GM (rGM) virus that involves a T7 system with a hammerhead ribozyme sequence introduced downstream of the T7 promoter. By changing the F_0 polybasic cleavage site RRQKR↓F to the monobasic GRQGR↓L, we generated a mutant virus (rGM-VIIm) that was found to be highly attenuated in chickens. The rGM-VIIm virus not only produced fourfold higher hemagglutination assay (HA) titers than the parental virus, but also exhibited genetic stability after 15 continuous passages in specific-pathogen-free (SPF) embryonated eggs. Whether live or inactivated, rGM-VIIm and LaSota vaccines can protect vaccinated birds from GM challenge infection. However, live and inactivated rGM-VIIm vaccines reduced virus shedding of the homologous challenge virus significantly better than the LaSota virus vaccine did. Altogether, our results suggest that rGM-VIIm vaccines could aid in the control of ND in China.

Keywords Newcastle disease virus · Genotype VII · Reverse genetics · Vaccine

Introduction

As one of the most devastating diseases in poultry, Newcastle disease (ND) can lead to 100 % morbidity and mortality in nonimmune chicken flocks [1, 2]. ND's causative agent, Newcastle disease virus (NDV), is classified into the order of Mononegavirales, family *Paramyxoviridae*, subfamily *Paramyxovirinae*, and genus *Avulavirus* [3]. It is a negative-sense, single-stranded RNA genome of approximately 15.2 kb comprising six genes, 3′-NP-P-M-F-HN-L-5′, which, respectively, encode the nucleoprotein,

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phosphoprotein, matrix protein, fusion protein, hemagglutinin–neuraminidase, and large polymerase protein [2]. The amino acid sequence at the fusion protein cleavage site is the major virulence determinant of NDVs [4–6]. At those sites, virulent NDV strains frequently contain the ^{112}R/K-R-Q/K–K/R–R-F^{117} motif, whereas those of low virulence mostly have the sequence ^{112}G/E-K/R-Q-G/E-R-L^{117} [2, 7].

Since first being documented, ND has resulted in at least four pandemics [2], each caused by a new NDV genotype [8]. Studies have shown that the most predominant genotype VII was responsible for the fourth pandemic [8–11], in which some isolates were able to overcome protection offered by the available vaccines [12, 13]. As research has demonstrated, antigenic differences between those vaccine strains and the genotype VII strains could have caused outbreaks in intensively vaccinated areas during the fourth pandemic [13, 14]. In fact, as several studies have shown, birds that received vaccine with higher antigenic similarity to the challenge virus could reduce virus shedding [14–18]. Consequently, the development and application of vaccines of the same genotype are critically needed to control ND in China.

To date, several attenuated viruses of genotype VII developed by reverse genetics techniques—among them, ZJ1 [15], Banjarmasin/010/10 [16], and SG10 [17]—have proven effective as vaccine candidates. Generally, attenuation has involved polybasic-to-monobasic mutations of the fusion protein cleavage site; however, the proliferation of attenuated viruses in different tissues and their shedding from oropharyngeal and cloacal swabs of inoculated chickens remain poorly documented.

In response, we generated an infectious clone of the GM strain of genotype VII which was isolated from immunized chicken flocks [19]. After attenuating the recombinant GM (rGM) by replacing the polybasic motif ^{112}RRQKRF^{117} with the monobasic motif ^{112}GRQGRL^{117} to generate a vaccine candidate, rGM-VIIm, we evaluated the vaccine potency of both the live attenuated virus and the inactivated virus which was adjuvanted with oil emulsion.

Materials and methods

Viruses, plasmids, and cells

GM (GenBank accession number: DQ486859), a genotype VII isolate with an intracerebral pathogenicity index (ICPI) of 1.98 [20] which derived from a vaccinated broiler flock in 2003, was propagated in 9-day-old specific-pathogen-free (SPF) embryonated eggs. Then, the virus was purified three times by cell plaquing on chicken embryo fibroblast (CEF) monolayers. The virus stocks were stored at −70 °C until use. The vector pCI-neo and pGEM-T easy vector were purchased from Promega Corporation (Promega, Madison, USA). The vector TVT7R (0,0) [21] was a gift from Dr. Hongzhuan Wu (Alabama State University). BSR T7/5 cells [22], stably expressing the T7 phage RNA polymerase, was kindly provided by Dr. Zhigao Bu (Harbin Veterinary Institute, China).

Construction of reverse genetics system for NDV strain GM

To construct a cDNA clone of GM, five pairs of primers were designed (Table 1). Following previous research [23], to generate authentic 5′ ends of full-length viral antigenomic RNA transcripts, we introduced an overhanging

Table 1 Primers used for full-length cDNA clone construction

Primer[a]	Nucleotide sequence[b]	Order of cloning[c]
P1-Ham1U	CGTCTCGTATAtgttaagcgtctgatgagtccgtgaggacgaaactataggaaaggaattcctatagtc ACCAAACAGAGAATCTGTGAG	I
P1-2928L	ACTAGTGACAGTGTCCTTCTCCACTCCCATGTCAGG	
P2-2879U	TTGACCGGTGCAGCACCCCTCTGAAT	V
P2-6767L	GTT**GGCGCGCC**ACATTCGCTATTGTTCTCAG	
P3-6774U	TAGACCGGTCAATAGCGAATGT**GGCGCGCC**TGTTCATGAC	IV
P3-8081L	TGGACTAGTAAGGGAACGATCCTAAATT	
P4-8100U	CGGACTAGTTGAGATCCTCAAGGATG	III
P4-13030L	AAAACGCGTCGAGTGCAAGAGACTAAC	
P5-12995U	CGGACTAGTGCAGATATAGACTTCGTCGAGG	II
P5-15192L	CGGACTAGTCGTCTCTACCCACCAAACAGAGATTTGGTGAATGAC	

[a] The sequences of five sets of primers P1–P5 are in accordance with the sequences of GM (GenBank accession number DQ486859)

[b] The hammerhead ribozyme sequence is shown in lowercase, restriction enzyme sites are underlined, and the biomarker is bolded

[c] The products generated by specific primers are cloned in the order of I to V

hammerhead ribozyme (HamRz) sequence instead of two or three additional guanidine residues (Gs) at the 5′ terminal into the cDNA clone of GM. To establish a biomarker, an *Asc* I restriction enzyme site was generated by specific mutagenesis. The schematic cloning strategy is shown in Fig. 1a.

Briefly, after preparing viral cDNA using superscript III transcriptase (Invitrogen, USA) according to the manufacturer's instructions, we amplified five gene fragments using Q5 DNA polymerase (New England Biolabs, USA). The first two fragments, in the order of I and II, were sequentially cloned into pGEM-T easy vector by the shared *Spe* I restriction site; the resulting plasmids were then digested by *Bsm* B I, and subcloned into the TVT7R (0,0) vector digested by *Bbs* I restriction enzyme. Then, the three remaining fragments were sequentially inserted into the backbones by the unique restriction enzyme sites (*Age* I, *Spe* I, and *Mlu* I) they shared to generate the full-length cDNA clone, TVT-rGM (Fig. 1a).

The helper plasmids, pCI-NP and pCI-P, were constructed by amplifying the open reading frames (ORF) of NP, P genes, and cloning the PCR products into the pCI-neo vector with the help of the *Eco*R I and *Sal* I restriction enzyme sites. Similarly, the pCI-L plasmid was constructed using the *Nhe* I and *Sal* I restriction enzyme sites. To rescue the infectious virus, BSR T7/5 cells with 80–90 % confluence were washed three times with PBS free of Ca^{2+} and Mg^{2+} and co-transfected with a total of 3 μg DNA including TVT-rGM, pCI-NP, pCI-P, and pCI-L in a ratio of 2:2:1:1 using Lipofectamine LTX (Invitrogen, USA), according to the manufacturer's instructions. Three days later, the culture supernatants and cell monolayers were harvested by freeze–thawing three times, and the mixture was inoculated into the allantoic cavities of 9-day-old embryonated SPF chicken eggs (500 μL/each egg) for virus recovery. At 3 days post-inoculation, the allantoic fluid was harvested and titrated with hemagglutination assay (HA). To detect deviations from the parental GM virus other than the biomarker, the full-length genomic sequence of the rGM virus was determined.

Generation of rGM-VIIm from cloned cDNA

To attenuate rGM, the nucleotide sequences of the cleavage site of the TVT-rGM plasmid were modified from 5′-AGGAGACAAAAACGCTTT-3′ to 5′-GGGAGACAG GGGCGCCTT-3′ by PCR mutagenesis, which correspondingly change the motif from ^{112}RRQKRF^{117} to ^{112}GRQGRL^{117} (Fig. 1b). The full-length plasmid TVT-rGM-VIIm was used for virus recovery as described above,

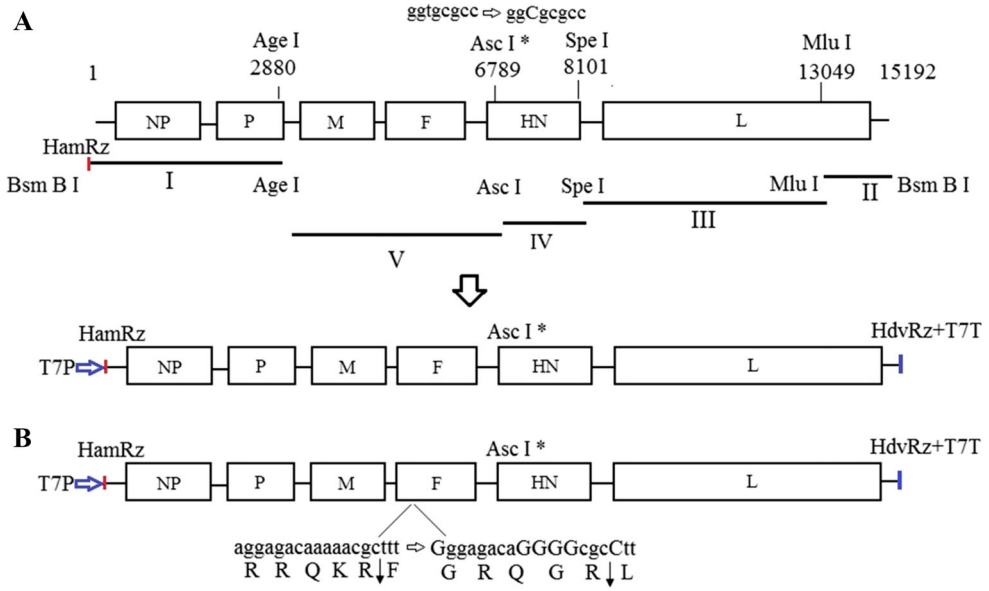

Fig. 1 Simplified scheme for the construction of full-length cDNA clones. **a** The HamRz sequence (*red vertical line*) and *Bsm* B I sequences were introduced by primer design. The *Asc* I restriction enzyme site (*labeled with asterisk*) was generated as a biomarker; the mutated nucleotides was *bolded*. The order of GM fragments cloning, shown by heights, were marked as I–V. Briefly, the first two fragments, in the order of I and II, were sequentially cloned into pGEM-T easy vector by the shared *Spe* I restriction enzyme site and then digested by *Bsm* B I; the products were subcloned into TVT7R(0,0) vector that digested by *Bbs* I restriction enzyme. Then, the rest fragments were sequentially inserted into the backbones by the unique restriction enzyme cutting sites (*Age* I, *Spe* I, *Mlu* I) they shared to generate the full-length cDNA clone, TVT-rGM. *Blue arrow* indicates the T7 promoter (T7P) in the vector; HdvRz and T7 terminator (T7T) of the vector are shown by *blue vertical line*. **b** Nucleotides modification of the F protein cleavage site. Six nucleotides were modified (capitalized) in order to generate an avirulent virus, rGM-VIIm. The *arrow* indicates where cleavage occurs (Color figure online)

except an additional TPCK-treated trypsin (1 μg/mL) has been added at 24 h post-transfection. After sequencing the recombinant virus to confirm the mutations, we examined the genetic stability of rGM-VIIm with three passages in the respiratory tract of 1-day-old chicks [16].

Pathogenicity indices, in vitro growth kinetics, and western blot analysis of NDV GM constructs

The GM, rGM, and rGM-VIIm fresh allantoic fluids were examined for pathogenicity by mean death time (MDT) and ICPI [18]. The EID_{50} of viruses was determined on SPF embryonated eggs by tenfold serial dilution.

The growth kinetics was examined in CEF cells. Briefly, CEF cells were infected with the GM, rGM, and rGM-VIIm with 1 μg/mL TPCK-treated trypsin (trypsin+) and rGM-VIIm without trypsin (trypsin−) at a multiplicity of infection (MOI) of 0.01. The infected cultures were harvested at 6, 12, 24, 36, 48 and 72 h post-infection (hpi); the viral titers of GM and rGM were determined in triplicates with the standard median tissue culture infective dose ($TCID_{50}$) assay, and those of rGM-VIIm (trypsin+) and (trypsin−) were determined both with an additional 1 μg/mL TPCK-treated trypsin. Viral titers were presented using GraphPad Prism 5 (GraphPad, USA).

The effect of exogenous trypsin on the F protein cleavage of the rGM and rGM-VIIm virus was examined in CEF cells in the presence and absence of 1 μg/mL TPCK-treated trypsin, and the Western blot was performed with a polyclonal anti-NDV serum.

In vivo replication of rGM-VIIm

Three groups of 7-day-old SPF chickens ($n = 15$) were inoculated intranasally with 100 μL of either $10^{6.0}$ EID_{50} of live rGM-VIIm virus, $10^{6.0}$ EID_{50} of commercial live LaSota vaccine (Guangdong Winsun, China), or 100 μL of PBS. Tissue samples of each three birds including brain, trachea, lung, thymus, heart, liver, spleen, kidney, bursa of Fabricius, and oropharyngeal and cloacal swabs were harvested at 1, 3, 5, 7 and 9 days post-infection (dpi) for virus isolation.

Immunization and challenge experiments

Five groups of 7-day-old SPF chickens ($n = 10$) were immunized intranasally, either with 100 μL of PBS, $10^{6.0}$ EID_{50} of fresh rGM-VIIm allantoic fluid or $10^{6.0}$ EID_{50} commercial live LaSota vaccine, or 300 μL of inactivated oil emulsion rGM-VIIm (O-rGM-VIIm) and commercial oil emulsion LaSota (O-LaSota) (Guangdong Winsun, China) subcutaneously. Serum samples were collected at 21 days post-immunization and measured with HI test using LaSota virus as antigen. Meanwhile, birds were challenged intranasally with $10^{5.0}$ ELD_{50} of GM viruses and monitored daily for clinical signs of ND for 14 days post-challenge (dpc). Oropharyngeal and cloacal swabs were collected at 1, 3, 5, 7 and 9 dpc for virus isolation. All animal experiments in the study were approved by the South China Agricultural University Experimental Animal Welfare Ethics Committee (contract number 2015-10).

Results

Stability of the rescued viruses, rGM, and rGM-VIIm

We compared the genomic sequence of infectious cloned-derived virus rGM with that of the parental wild-type GM virus. Other than the biomarker at nt 6789, there were five nucleotides mutations, G430T, C4584A, A6927C, G7091A, and A7644G, which resulted in the D103E substitution of the NP protein, the P12H substitution of the F protein, and the R225H substitution of the HN protein. The rescued virus rGM-VIIm demonstrated the expected six nucleotide differences from rGM, which shifted the cleavage site from ^{112}RRQKRF^{117} to ^{112}GRQGRL^{117}. For the stability test, both rGM and rGM-VIIm viruses were passaged for 15 times on SPF embryonated eggs. The HA titer of the attenuated virus was at least fourfold greater than that of the virulent parental virus. Moreover, the biomarkers of the rGM and rGM-VIIm viruses were stable after 15 passages (Fig. 2a, b), and the cleavage site of the rGM-VIIm virus was the unchanged ^{112}GRQGRL^{117} (Fig. 2c). Three passages in the respiratory tract of 1-day-old chicks did not revert the rGM-VIIm virus to its original state of virulence.

Virulence and in vitro growth of rGM-VIIm

As shown in Table 2, the ICPI values of the parental GM, rGM, and rGM-VIIm viruses were 1.98, 2.0 and 0.4, respectively, while their MDT values were 59, 59 and 99 h. The rGM was thus as virulent as the parental GM virus, yet far more pathogenic than the rGM-VIIm virus. The titers of the GM, rGM, and rGM-VIIm viruses were $10^{9.0}$ EID_{50}/mL, $10^{10.8}$ EID_{50}/mL, and $10^{11.0}$ EID_{50}/mL, respectively. Growth kinetics of the recombinant and parental viruses on CEF cells showed that the titers of rGM and rGM-VIIm (trypsin+) were comparable to those of the parental GM virus (Fig. 3), although rGM had slightly higher titers. However, the viral titer of rGM-VIIm in the absence of exogenous trypsin (trypsin−) was approximately 100–1000-fold less than those of rGM-VIIm in the presence of exogenous trypsin (trypsin+) (Fig. 3). Retardant

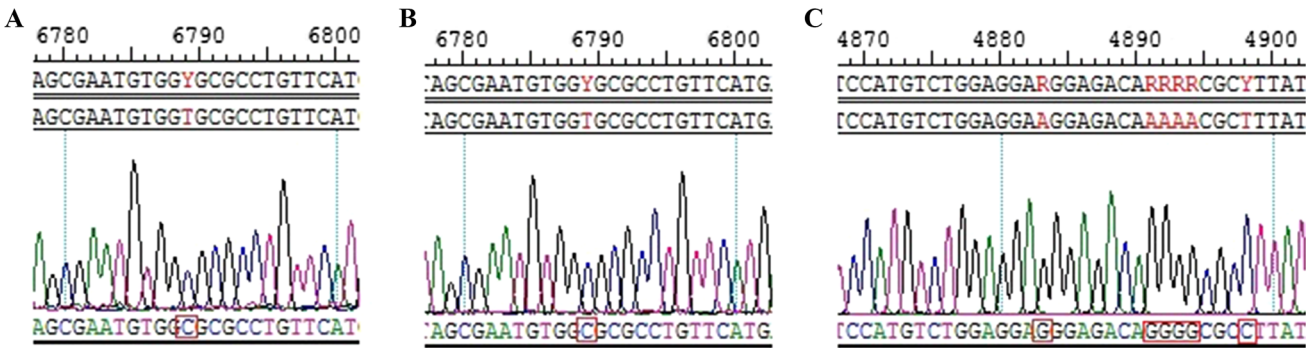

Fig. 2 Mutated nucleotides of the recovered viruses. **a** The biomarker of rGM, *Asc* I restriction enzyme site, generated by T6789C mutation is shown as *red box*. **b** The T6789C mutation for generation of biomarker of rGM-VIIm is shown as *red box*. **c** Modification of the nucleotides of F protein cleavage site. The mutated nucleotides are shown as *red box* (Color figure online)

Table 2 Biological characteristics of the parental and recovered viruses

Virus	Pathogenicity		Allantoic fluids titer	
	MDT (h)[a]	ICPI[b]	EID_{50}/mL	HA (log2)
GM	59	1.98	$10^{9.0}$	8
rGM	59	2.0	$10^{10.8}$	8
rGM-VIIm	99	0.4	$10^{11.0}$	10

[a] Mean death time (MDT) in embryonated chicken eggs. The MDT has been used to classify ND virus strains into the following groups: velogenic (less than 60 h), mesogenic (60–90 h), and lentogenic (more than 90 h)

[b] Intracerebral pathogenicity index in 1-day-old chickens. The most virulent viruses approach the maximum score of 2.0, whereas the score of lentogenic strains closes to 0.0

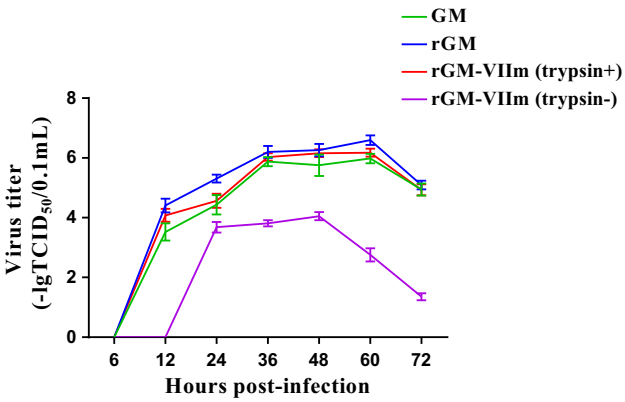

Fig. 3 Multistep growth curves of GM, rGM, and rGM-VIIm (with or without trypsin) in CEF cells. The cells were infected at a MOI of 0.01. Samples were collected at 12 h intervals for 72 h, and the virus titers were determined with $TCID_{50}$ assay in CEF cells

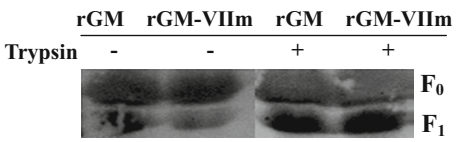

Fig. 4 The effect of exogenous trypsin on the F protein cleavage of the rGM and rGM-VIIm virus. CEF cells were infected with the rGM and rGM-VIIm virus at a MOI of 1; TPCK-treated trypsin was added at 12 h post-infection. The cell lysates were collected at 24 h post-infection and the western blot was performed with a polyclonal anti-NDV serum

replication kinetics of rGM-VIIm (trypsin−) showed that the propagation of rGM-VIIm greatly depended on exogenously added trypsin. Further, the Western blot analysis also showed that the exogenous trypsin effectively cleaved the F_0 protein of rGM-VIIm (Fig. 4).

In vivo replication of rGM-VIIm

Commercial and live NDV vaccines replicated poorly in their hosts because the F_0 protein could be cleaved only by trypsin-like enzymes found in a limited variety of tissues [24]. As a result, no clinical signs after vaccination appeared. The replication of the attenuated live vaccine candidate rGM-VIIm in vivo, however, remains unclear. In terms of tissue tropism and shedding, we could isolate the rGM-VIIm virus only from the kidney and spleen, whereas the LaSota virus was detectable in the lung, bursa of Fabricius, and spleen at 1 dpi (Table 3). At 3 dpi, samples of trachea, lung, liver, spleen, bursa of Fabricius, and oropharyngeal and cloacal swabs were positive for rGM-VIIm virus isolation, whereas positive samples for the LaSota virus emerged in the lung, thymus, spleen, kidney, and oropharyngeal swab. At 5 dpi, the LaSota virus was isolated only in the thymus, kidney, and cloacal swab, whereas the rGM-VIIm was detectable in the trachea, lung, spleen, kidney, bursa of Fabricius, and oropharyngeal and cloacal swabs. These results indicated that the rGM-VIIm virus replicated more efficiently than the LaSota stain. At 7 dpi, the rGM-VIIm virus was detectable only in the liver

Table 3 Tissues distribution and virus shedding of rGM-VIIm and LaSota in 7-day-old SPF chickens

Sample[a]	rGM-VIIm					LaSota				
	1dpi	3dpi	5dpi	7dpi	9dpi	1dpi	3dpi	5dpi	7dpi	9dpi
Brain	–	–	–	–	–	–	–	–	–	–
Trachea	–	+	+	–	–	–	–	–	–	–
Lung	–	+	+	–	–	+	+	–	–	–
Thymus	–	–	–	+	–	–	+	–	–	–
Heart	–	–	–	–	–	–	–	–	–	–
Liver	–	+	–	–	–	–	–	+	–	–
Spleen	+	+	+	–	–	+	+	–	–	–
Kidney	+	–	+	+	–	–	+	–	–	–
Bursa of fabricius	–	+	–	–	–	+	–	–	+	–
Oropharyngeal swab	–	+	+	–	–	–	+	–	–	–
Cloacal swab	–	+	+	+	–	–	–	+	–	–

– hemagglutination test negative; + hemagglutination test positive

[a] Tissue samples were collected and balanced mixed to a final weight of 0.1 g, then homogenized with 1 ml PBS (with 2000 IU penicillin and 2000 μg Streptomycin). All swabs were treated with 1 ml PBS (with 5000 IU penicillin and 5000 μg Streptomycin). Samples were incubated at 4 °C overnight; then, each 100 μl supernatant was inoculated into three 9-day-old embryonated SPF chicken eggs for virus isolation

and cloacal swab, whereas the LaSota virus was apparent in the bursa of Fabricius. There was no detectable virus at 9 dpi for either group, and all samples of the PBS mock-immunized group were negative in terms of virus detection (data not shown).

Protective efficacy of rGM-VIIm vaccine

Regarding the potency of the vaccines, all chickens that immunized with the live or inactivated rGM-VIIm and LaSota vaccines demonstrated complete protection against the virulent GM virus challenge and did not show clinical signs of disease. By contrast, all birds in the PBS group showed typical signs and symptoms of the virulent NDV infection and died at 4 dpc. The HI titers of the live vaccines administered nasally at 21 days post-immunization were only 5.4 log2 (rGM-VIIm) and 5.2 log2 (LaSota), while those of inactivated vaccines injected subcutaneously were 8.0 log2 (O-rGM-VIIm) and 6.8 log2 (O-LaSota), as shown in Table 4.

The shedding of the challenged virus for each immunized group was examined from oropharyngeal and cloacal swabs at 1, 3, 5, 7 and 9 dpc. As shown in Table 4, the live LaSota vaccine group had 10–30 % positive samples in the oropharyngeal and cloacal swabs at 1 and 3 dpc. However, only 10 % of the cloacal swabs were positive for live rGM-VIIm vaccine group at 5 dpc. Although a high level of HI titers was observed in groups immunized with inactivated vaccines, more virus shedding was detected compared to groups immunized with live vaccines. About 50 % chickens of the O-LaSota groups shed virus at 1 and 3 dpc, whereas the positives for the O-rGM-VIIm group were not greater than 30 % at the same time points. At 5 dpc, the oropharyngeal and cloacal swabs from O-LaSota group were both 20 % positive, whereas the O-rGM-VIIm group showed only 10 % positive virus shedding in cloacal swabs. No virus was detectable at 7 and 9 dpc for the O-rGM-VIIm group, compared with being 10 % positive in cloacal swabs for the O-LaSota group at 7 dpc.

Discussion

To date, the T7—[6, 15–18, 21–23, 25] and Pol II-based reverse genetic systems [26–28] are commonly used for rescuing NDV. In the T7 system, three extra Gs downstream of the T7 promoter and the hepatitis delta virus ribozyme (HdvRz) sequence downstream of the antigenome sequence are highly suitable for efficient Sendai virus recovery [29]. Therefore, the three extra Gs and HdvRz sequence seem to be optimal for the T7 RNA polymerase-dependent NDV rescuing system.

By contrast, in the Pol II-based reverse genetic system, it is vital to generate authentic 3′ and 5′ terminals of the primary viral genomic RNA transcript. Accordingly, the hammerhead ribozyme (HamRz) and HdvRz sequence were usually introduced to maximize the efficiency of virus recovery in Pol II system. To assess whether the HamRz sequence was also applicable in the T7 system, we constructed a cDNA clone of the GM virus that flanked the HamRz sequence downstream of the T7 promoter. The recovery of the rGM virus indicated that the HamRz sequence is also compatible in the T7 system for NDV rescue, although previous studies showed that three Gs

Table 4 Frequency of isolation of challenge virus in different vaccination groups

Group	HI titer (log2)	Numbers of viral shedding chickens (positive/total)									
		1 dpc		3 dpc		5 dpc		7 dpc		9 dpc	
		O	C	O	C	O	C	O	C	O	C
rGM-VIIm	5.4	0/10	0/10	0/10	0/10	0/10	1/10	0/10	0/10	0/10	0/10
LaSota	5.2	2/10	0/10	1/10	3/10	0/10	0/10	0/10	0/10	0/10	0/10
PBS	0	10/10	10/10	10/10	10/10	NS	NS	NS	NS	NS	NS
O-rGM-VIIm	8.0	2/10	0/10	3/10	0/10	0/10	1/10	0/10	0/10	0/10	0/10
O-LaSota	6.8	4/10	0/10	5/10	3/10	2/10	2/10	0/10	1/10	0/10	0/10

HI titer means geometric mean HI titer measured by LaSota virus; *O* oropharyngeal swabs; *C* cloacal swabs; *NS* no survivors

added to the HamRz sequence could increase T7 transcription efficacy and improve the recovery efficacy of rabies virus [23, 30]. Overall, our results indicated that the HamRz sequence without extra Gs is effective in the T7 system and thus could be an alternative method for NDV recovery.

To generate an attenuated live vaccine candidate that genetically matches the pandemic genotype VII in China, we orchestrated the mutation of the cleavage site of F_0 protein from polybasic to monobasic as described in earlier studies [16, 17]. As previously reported, changing the amino acid sequence of the protease cleavage site in the F_0 protein from virulent to avirulent can afford significant attenuation of the NDVs [5, 6]. In our study, the MDT value of rGM-VIIm was 99 h and the IPCI value was 0.4, thereby indicating significant attenuation. It was found the ICPI values of attenuated viruses that derived from different isolates of genotype VII could slightly differ. For example, the difference of ICPI values of the attenuated viruses NDV/ZJ1FM [15] and aSG10 [17] was less than 0.3. The discrepancy could be due to different characteristics of strains and related to other proteins associated with virulence [31–33].

Not surprisingly, the biological characteristics of the recombinant and the parental viruses were similar [16, 17]. However, the rGM virus showed a little higher EID_{50} titer than the parental GM virus, albeit without any changes in MDT and ICPI values. Meanwhile, growth curves of both viruses revealed that the replication of the rGM virus was slightly more efficient than GM at 12–60 hpi. The discrepancy between GM and rGM viruses was possibly because of the three amino acid substitutions since the single amino acid mutation could facilitate virus replication [34]. In the presence of exogenous protease, attenuated viruses were tended to replicate more efficiently than parental viruses [16, 17]; however, the growth curves of the rGM-VIIm (trypsin+) virus initially (at 24 hpi) resembled those of the GM virus. Later, the titers became comparable to those of rGM. As expected, the rGM-VIIm virus depends on exogenously added trypsin for propagation (Figs. 3, 4). Altogether, those results demonstrated that the viral titers of the rGM-VIIm virus were similar to those of the parental rGM virus in the presence of trypsin.

Successful vaccine candidates should exhibit mutations that are genetically stable during serial propagation. In our study, the rGM-VIIm virus was propagated in SPF embryonated eggs during 15 passages, and no reverse mutation occurred during passaging. Moreover, the chances of three mutations converting from avirulent type back to virulent type could be quite low, even performing intracerebral passage [16]. Accordingly, the attenuation strategy was sufficient to generate a safe vaccine candidate for chickens. In this study, we found the rGM-VIIm virus was safe, and no clinical signs were shown in 1-day-old chicks, inoculating the rGM-VIIm virus (data not shown). In vivo replication results of a previous report revealed that the attenuated and natural avirulent vaccines were detectable in the lung, trachea, spleen, and pancreas of 1-day-old chicks at 4 dpi, but not in the brain [16]. However, for 14-day-old chickens, only the attenuated Ban/AF virus was positive in all four of those tissues [16]. Our results also illustrated that the rGM-VIIm virus was detectable in the trachea, lung, spleen, and bursa of Fabricius in 7-day-old chickens at 3 and 5 dpi, thereby indicating that the trachea, lung, and spleen, but not the brain, frequently harbored the attenuated virus of genotype VII. Furthermore, the period of rGM-VIIm virus shedding from oropharyngeal and cloacal swabs was longer than that of the LaSota vaccine. Interestingly, the virus can be detected in the oropharyngeal swab even at 7 dpi. Xiao et al. [16] also observed a longer virus shedding period of the attenuated Ban/AF virus, even though the age of chickens, sampling time points, and virus strains were different from those in our study. The rGM-VIIm and Ban/AF viruses that retained virulent backbone probably facilitated vigorous replication in tissues and were tended to shed in swabs compared to the LaSota vaccine. Taking both studies into consideration, it seems that the virus

distribution and shedding are inversely correlated with the age of the chicken, but positively related to the pathogenicity index and the replication ability of the attenuated viruses. In our study, the rGM-VIIm virus provided complete protection nearly with the 5 log2 HI titers, which closely resembled results with aSG-10 [17]. Nevertheless, the virus shedding of the live vaccines should not be overlooked. An obvious lesson was that LaSota-like viruses can be frequently isolated in poultry flocks and in wild birds [34–37]. Hu et al. [15] also showed concerns with the safety of using the attenuated virus. We cannot rule out the possibility that the vaccine eventually revert to be virulent; therefore, further attenuation or modification toward reducing the virus shedding of rGM-VIIm is urgently needed in the future studies.

Although the rGM-VIIm virus replicated more efficiently than LaSota in vivo, the geometric mean HI titers of the rGM-VIIm and LaSota live vaccines administered nasally were comparable (higher than 5 log2), suggesting both live vaccines elicited protective antibody responses. While for the inactivated vaccine-immunized groups, the HI titers were greater than those of the live vaccine-immunized groups, which was possibly due to the higher antigen load of inactivated vaccines [38].

Live ND vaccines are easy to produce, convenient to administer, and reportedly superior in terms of protective efficacy, but vaccine shedding raised safety concerns. Inactivated vaccines offer enhanced safety, longer lasting antibody responses, and ability to induce higher HI titers. Despite the many differences between the live and inactivated vaccines, chickens administered nasally with rGM-VIIm and injected subcutaneously with O-rGM-VIIm have consistently shed fewer viruses, which stress the importance of genotypic matching between vaccines and endemic viruses [14–18].

To sum, we constructed a reverse genetics system to rapidly develop genotype-matched vaccines and demonstrated that the attenuated virus, either live or inactivated, can provide better protection than the commercial LaSota vaccines. Our results thus showed that rGM-VIIm vaccines can aid in controlling of ND in China.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of South China Agricultural University Experimental Animal Welfare Ethics Committee.

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