Construction and characterization of an infectious molecular clone of novel duck reovirus

Qiaomei Wu, Mingyang Ding, Chuanfeng Li, Guangqing Liu and Zongyan Chen*

Abstract

Novel duck reovirus (NDRV), the prototype strain of the species Avian orthoreovirus (ARV), is currently an infectious agent for ducks. Studies on NDRV replication and pathogenesis have been hampered by the lack of an available reverse-genetics system. In this study, a plasmid-based reverse-genetics system that is free of helper viruses has been developed. In this system, 10 full-length gene segments of wild-type NDRV TH11 strain are transfected into BSR-T7/5 cells that express bacteriophage T7 RNA polymerase. Production of infectious virus was shown by the inoculation of cell lysate derived from transfected cells into 10-day-old duck embryos. The in vivo growth kinetics and infectivity of the recombinant strains were identical to those of the wild-type strain. These viruses grew well and were genetically stable both in vitro and in vivo. Altogether, these results show the successful production of an infectious clone for NDRV. The infectious clone reported will be further used to elucidate the mechanisms of host tropism, viral replication and pathogenesis, as well as immunological changes induced by NDRV.

INTRODUCTION

Duck reovirus is the prototype strain of the species Avian orthoreovirus (ARV), one of five species formally recognized to date in the genus Orthoreovirus (subfamily Spinareovirinae, family Reoviridae), which comprises double-stranded RNA (dsRNA) viruses [1]. The other species are Mammalian orthoreovirus, Baboon orthoreovirus, Nelson Bay orthoreovirus and Reptilian orthoreovirus. The virions are non-enveloped and have a double capsid containing 10 dsRNA segments that can be separated by polyacrylamide gel electrophoresis into 3 size classes: large (L1–L3), medium (M1–M3) and small (S1–S4) [1, 2]. Avian orthoreoviruses (ARVs) cause a range of diseases in chickens, including viral arthritis/tenosynovitis, and are associated with respiratory disease, enteric disease, inclusion body hepatitis, hyperdercardium, running-stunting syndrome, malabsorption syndrome and sudden death [3]. ARVs also have been isolated from the Muscovy duck (Cairina moschata). Muscovy duck reovirus infection caused illness in 30% and death in 20% of ducks on poultry farms in Israel, China and other countries [4, 5]. However, it was nonpathogenic for Peking ducks (Anas platyrhynchos) when inoculated subcutaneously. Since 2011, isolates that can cause death in 40% of ducks of various ages and 35–40% mortality in different flocks have increasingly been reported [6–9]. We isolated one strain, named novel duck reovirus (NDRV-TH11) [7, 8]. It also leads to 100% mortality in SPF chickens. The precise reasons for the expanded host range and increased virulence are currently unknown. More strains have been isolated that are pathogenic for Peking ducks [9–11].

Reverse-genetics systems, which enable the generation of infectious virus from cloned cDNA, have been widely used for the site-directed mutagenesis of viral RNA genomes following various applications in basic and applied virology [12, 13]. Furthermore, as many Reoviridae viruses are significant human and animal pathogens, reverse-genetics methods provide a tool to investigate pathogenesis and transmission, and may aid in the development of new vaccine strains [14]. In the past few years, the development of powerful reverse-genetics techniques for several Reoviridae viruses has afforded new opportunities for the study of these viruses. Some Reoviridae viruses for which reverse-genetics systems have been developed are mammalian orthoreovirus (MRV; genus Orthoreovirus) [13], Nelson Bay orthoreovirus (NBV; genus Orthoreovirus) [14], African horse sickness virus (AHSV;...
genus *Orbivirus* [15], bluetongue virus (BTV; genus *Orbivirus*) [16, 17], Ibaraki virus (IBAV; genus *Orbivirus*) [18], epizootic haemorrhagic disease virus (EHDV; genus *Orbivirus*) [19] and rotavirus (RV; genus *Rotavirus*) [20–22].

Despite extensive efforts in several laboratories, the generation of ARV entirely from cloned cDNAs has not been achieved. Although a common narrative can be applied to virtually all members of the family, some obvious differences exist in the replication mechanisms of members of the *Reoviridae* viruses. Therefore, the main objective of this study was to develop a reverse-genetics system for the novel duck reovirus (NDRV) strain. Each segment was cloned under the control of the T7 RNA polymerase promoter and the generation of infectious NDRV was monitored. The generated virus was characterized by infectivity tests in vivo and in vitro as well as by genome sequence analysis. The results should contribute to the further development of a reliable reverse-genetics system for NDRV, enabling site-directed mutagenesis and subsequent host tropism studies on the virus.

**RESULTS**

**Recovery of rNDRV by cotransfecting 10 plasmids**

To generate recombinant NDRV (rNDRV) from cloned cDNAs, each plasmid containing one full-length gene segment cDNA was subcloned into the reconstructed vector, pSK-Rb (Fig. 1a). The cDNA was under control of the T7 RNA polymerase promoter and appended with the HDV ribozyme (Rb) at the 3’ terminus. These plasmids are anticipated to generate full-length (+)-sense RNAs containing native 5’ and 3’ ends following transcription with T7 RNA polymerase [13]. Ten gene segment plasmids were co-transfected into BSR-T7/5 cells (Fig. 1b). The virus yield in the transfected cells was gathered at 72 h post-transfection for the following experiment. The cell debris from transfection was used to infect 10-day-old embryonated duck eggs and the first passage caused embryo death at 5 days post-infection (p.i.), while the second passage caused embryo death at 4 days p.i. Furthermore, the third passage of both recombinant virus and wild-type NDRV-TH11 caused embryo death at 3 days p.i. After three additional individual passages, the virus in the allantoic fluid was amplified by further passage to generate a stock for future use.

**Characterization of rNDRV generated by plasmid transfection**

A genetic tag was introduced in the cDNA to unambiguously identify the recovered virus: the bases at positions 1189 to 1194 of the M2 segment were modified (AAGCTG to AAGCTT) to create a HindIII restriction site. rNDRV, but not wild-type virus, should encode one unique marker mutation from position 1189 to position 1194 in the M2 segment. Therefore, the rNDRV allantoic fluid was subjected to RNA extraction. RT-PCR was used to generate cDNA amplicons, which were purified and then subjected to restriction enzyme

![Fig. 1. Schematic diagram of the strategies used to assemble the full-length cDNA clone of NDRV. (a) Construction and map of the vector pSK-Rb. pBlueScriptKII(+) vector was digested with BssHII and sequences of BssHII-MCS-Rb-BssHII were introduced into the vector and named pSK-Rb. (b) Reverse-genetics procedure. Ten gene-segment cDNAs were subcloned into vector pSK-Rb. A T7 polymerase promoter was added to each fragment via primer (see Table S1, available in the online version of this article). A red rectangle indicates the location of the 1194 nt mutation in the M2 segment. The 10 cDNA constructs were co-transfected into BSR-T7/5 cells, which constitutively express T7 RNA polymerase. After 4 days of incubation, the transfected cells were lysed by freeze-thaw, and the rNDRV were passaged in 10-day-old duck embryos.](image-url)
digestion with the enzymes HindIII (Fig. 2a). The M2 segment was completely sequenced in order to confirm the presence of the introduced mutations. Subsequently, sequence analysis showed that G at position 1194 in the M2 segment of rNDRV had been changed into T (Fig. 2b). There was no change in the other gene segments. This confirmed that the rNDRV was the recombinant derived from the cloned full-length cDNA. Furthermore, the viral genomic dsRNAs were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The electropherotype of rNDRV was indistinguishable from that of NDRV (Fig. 2c).

**Infectivity of recombinant virus in vitro**

As shown in Fig. 3(a), viable rNDRV was rescued with similar cytopathic effect to the wild-type NDRV-TH11 strain. Duck reovirus plaques were routinely visualized by crystal violet staining, where zones of cell death reflect virus-induced lysis (Fig. 3b). Digital images of these plaques were then obtained, and their sizes (in pixels) were compared. While the average plaque size for rNDRV was 678±12 pixels, NDRV produced plaques with 661±15 pixels. We next assessed the replication kinetics of the rescued viruses in DEF cells. Cells were infected at a multiplicity of infection

---

**Fig. 2.** Identification of genetic markers in the genome of rNDRV. RT-PCR was performed from the genomic RNA extracted from viruses amplified in embryonated duck eggs. The M2 partial segments of rNDRV and the NDRV-TH11 strain were from 757 to 2023 nt. (a) Digestion map to confirm the introduction of a point mutation. The RT-PCR products corresponding to the M2 partial segment of rNDRV and the NDRV-TH11 strain were both digested by the enzymes HindIII. The HindIII site is at 1189–1194 nt in the M2 segment of rNDRV. The PCR products and digested products were run through a 1 % agarose gel in the presence of ethidium bromide. Lane M, DNA marker. Lane 1, rNDRV M2 partial segments digested by the enzymes HindIII. Lane 2, NDRV M2 partial segments digested by the enzymes HindIII. Lane 3, rNDRV M2 partial segments. Lane 4, NDRV M2 partial segments. (b) Chromatograms demonstrating a mutation of NDRV and the NDRV-TH11 strain. The amplified fragments mentioned above were subjected to direct sequence analysis. Base A was changed into T at position 1194 in the M2 segment of rNDRV. (c) The electropherotype of dsRNA of rNDRV and NDRV. Viral dsRNAs were separated using SDS-polyacrylamide gel electrophoresis, and visualized by silver staining. The classes of gene segments based on their sizes (L1–3, M1–3, and S1–4 gene segments) are indicated.
(m.o.i.) of 0.01, and supernatants were collected at various times post-infection (Fig. 3c). No obvious difference was observed in the replication kinetics of NDRV and rNDRV viruses. No plaques were visualized in the case of negative controls, further confirming the specificity of the recovery of rNDRV from cDNA. The recovered virus was designated rNDRV, to distinguish it from the parental NDRV-TH11.

Immunoblotting with rabbit antiserum specific for σC demonstrated equivalent expression levels in cells infected with the NDRV and rNDRV, suggesting a similar level of infection by the NDRV and rNDRV (Fig. 4a). We confirmed by confocal microscopy that the rNDRV’s σC protein expression was comparable to that of the parental NDRV-TH11 viruses (Fig. 4b). No σC protein was expressed in the negative controls.

**Infectivity of recombinant virus in vivo**

Next, we assessed the virulence of rNDRV in ducklings. No major differences were observed in the virulence of NDRV and rNDRV. There were no deaths in each group at 2 days p.i., but at 3 days p.i. the ducklings began to die in the infected group, and all ducklings died by 5 days p.i. Grossly, the lung showed slight congestion and oedema. Pale white foci were observed on the surface of the spleen. The liver exhibited enlargement, congestion and petechial haemorrhages. No gross lesions were found in uninfected ducklings. Microscopically, representative haematoxylin and eosin (H&E) staining and immunohistochemical (IHC) staining of liver tissues are shown in Fig. 5. The hepatocytes showed severe swelling and hyaline degeneration. Some cells were dissolved and necrotic foci were found in the NDRV (Fig. 5a1) and rNDRV (Fig. 5a2) groups.

The presence of σC antigen was detected by IHC testing in the livers from the virus-inoculated ducklings. Generally, IHC staining indicated that widespread σC antigen was observed in the corresponding areas of the liver lesions in the NDRV (Fig. 5b1) and rNDRV (Fig. 5b2) groups. Strongly stained signals with an irregular shape were scattered through the liver tissues. No obviously positive IHC staining or observable pathological change was found in the tissues of the non-infected control ducklings (Fig. 5b3).

**DISCUSSION**

Reverse-genetics technology permits the testing of tightly focused, rational hypotheses about complex questions of
virus structure, virus–cell interactions and viral pathogenesis through direct engineering of the viral genome without needing to devise complicated selection strategies for the isolation of viral mutants [12, 13]. Furthermore, the reverse genetics of RNA viruses have the potential to develop vaccines and to use recombinant viruses as gene delivery vehicles [13, 23, 24]. Ten plasmids were used in this system and recombinant viruses were generated without helper virus being required and free of any selection. By using this approach, we can now easily manipulate the genomes of NDRV in vitro using the infectious cDNA clones and then directly test for the effect of various genetic manipulations (such as mutations or deletions) on virus virulence, pathogenicity and replication in animals. The introduction of a genetic marker into the M2 segment identified the recombinant virus as being different from the wild-type virus.

A reverse-genetics 10-plasmid system for MRV was first developed in 2007 [13]. To increase the efficiency of virus recovery, a four-plasmid system was developed in 2010 [25]. The system is driven by bacteriophage T7 RNA polymerase, which can be supplied transiently by recombinant vaccinia virus. To eliminate recombinant vaccinia virus, plaque purification was performed on the initial virus stock obtained following transfection before further propagation.

In the present study, BSR T7/5 cells were used for the transfection experiments. To increase transfection efficiency, we optimized the quantity of different segments for transfection. It is a well-established, time-saving and cost-effective method.

Plaque experiments suggested that viable rNDRV was rescued with an efficiency that was similar to that for the wild-type viruses. These sequences were genetically stable and had little effect on the growth characteristics of the virus in vitro during serial passage. IHC staining indicated that widespread σC antigen was observed in the corresponding areas of obvious pathological change. Similar to the former reports [19–21], the fact that rNDRV grows efficiently and remains genetically stable even upon the introduction of heterologous sequences supports the concept that rNDRV can be developed as a vector for the expression of foreign proteins.

In summary, a field isolate of NDRV that is highly pathogenic to ducks has been recovered from cDNA, and a plasmid-only reverse-genetics technique has been developed. It is convenient to use this 10-plasmid system to engineer changes in both structural and nonstructural proteins for studies of NDRV replication and pathogenesis. The system
is also suitable to create reassortants to order. Thus, this technique provides a means to directly and precisely engineer the viral genome in the context of infectious virus.

**METHODS**

**Construction of plasmids**

The genomic viral dsRNA was extracted from purified virions using the RNeasy Mini kit (Qiagen, USA). cDNA corresponding to each NDRV-TH11 gene segment was amplified from viral dsRNA via the full-length amplification of cDNA as previously described [8]. The NDRV-TH11 strain is isolated in 2011 and stored in our laboratory [7, 8]. Each full-length viral segment cDNA was ligated into the pMD18-T vector (TaKaRa, Dalian, People’s Republic of China). A genetic tag was introduced in the cDNA to identify the recovered virus: base G at position of 1194 nt in the M2 segment was mutated into T to create a HindIII restriction site at positions 1189–1194nt. pMD18-L1, pMD18-L2, pMD18-L3, pMD18-M1, pMD18-M2, pMD18-M3, pMD18-S1, pMD18-S2, pMD18-S3 and pMD18-S4 encoding the full-length cDNA of each gene segment derived from NDRV were separately subcloned into pSK-Rb, named pSK-L1, pSK-L2, pSK-L3, pSK-M1, pSK-M2, pSK-M3, pSK-S1, pSK-S2, pSK-S3 and pSK-S4. The plasmids were sequenced and compared to the wild-type NDRV-TH11 strain.

**Recovery of recombinant viruses from cloned cDNA**

For the transfection experiments, six-well tissue culture plates (Corning) were used. BSR-T7/5 cells were propagated in the presence of selective agent G418 (Geneticin; Invitrogen) in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated foetal serum once every other passage. The cells were cultured at 37°C in a humidified atmosphere of 5% CO2 in air. The BSR-T7/5 cells, which constitutively express T7 RNA polymerase, were a generous gift from X. S. Qiu (Shanghai Veterinary Institute, Shanghai, People’s Republic of China). The day before transfection, BSR-T7/5 cells (1 x 10^6 cells per well each) were washed with Opti-MEM I reduced serum medium and used for the transfection experiments.

Ten gene segments of strain NDRV-TH11 (pSK-L1, 0.66 µg; pSK-L2, 0.66 µg; pSK-L3, 0.66 µg; pSK-M1, 0.58 µg; pSK-M2, 0.58 µg; pSK-M3, 0.58 µg; pSK-S1, 0.5 µg; pSK-S2,
0.5 µg; pSK-S3, 0.5 µg; and pSK-S4, 0.5 µg) were diluted in Opti-MEMI reduced serum medium and then mixed with Lipofectamine 2000 diluted in Opti-MEMI reduced serum medium. After 25 min of incubation at room temperature, the mixture was added to the cells. Five hours post-infection, the cells were washed and 2 ml Dulbecco’s modified Eagle’s medium with 5% FBS was added. The supernatant and cells were harvested at 72 h post-infection. The cell debris was pelleted by low-speed centrifugation, and then the supernatant was used to infect 10-day-old embryonated duck eggs. More virus was produced by additional passages in embryonated eggs to establish the working seed stocks for future use.

**RE M2 gene and sequencing recovery of infectious NDRV**

RT-PCR was performed on the RNA extracted from recombinant virus to generate partial M2 cDNA amplicons, which were purified and then subjected to HindIII restriction digestion, resolved on 1% agarose gels, and visualized, excised and purified using a QIAquick Gel Extraction kit (Qiagen). The forward primer was from 757 to 780 bp of the M2 segment – gagacgcagagctcccttctc – and the reverse primer was from 2012 to 2031 bp of the M2 reverse-complement sequences – tccccggtcagctggtgaaacag (Table S1). A parallel experiment was conducted on the parental strain of NDRV-TH11 as a control. There is one HindIII site in this fragment of the M2 cDNA of the recombinant virus, while there is no HindIII site in the corresponding M2 cDNA fragment of the parent virus. The sequences of the different M2 cDNAs were confirmed by full-length sequencing. The viral sequences were determined using the ABI 3130 genetic analyzer (Life Technologies). The primers used in this study are listed in Table S1.

**Electrophoretic analysis of viral RNAs**

Viral dsRNAs were extracted separately from rNDRV and NDRV using the Triozol (TaKaRa, Dalian, People’s Republic of China) and precipitated with 5M LiCl to remove the ssRNA. The dsRNAs were analysed by SDS-PAGE on vertical gels (7.5% polyacrylamide gel) and segments were visualized by silver staining [6].

**Cytopathogenic effect and plaque formation**

Duck embryonic fibroblast (DEF) cells were obtained from the American Type Culture Collection and grown in Dulbecco’s modified Eagle’s medium (Hyclone, Beijing, People’s Republic of China) supplemented with 10% heat-inactivated foetal serum (Invitrogen, Carlsbad, CA, USA). The cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air. Monolayers of DEF cells (6x10⁶ cells) in six-well plates were infected with the virus at a m.o.i. of 0.01. After 1 h of incubation, the cells were washed twice using PBS and incubated with maintenance medium. The cytopathogenic effect (CPE) was examined under a microscope daily for 48 h post-infection. A plaque assay formation was performed on the DEF cells to analyse the virulence. Cells were grown in six-well culture dishes and infected with NDRV or rNDRV. After being cultured with 3.8% methyl cellulose (Sigma, MO, USA) in Dulbecco’s modified Eagle’s medium, the cells were fixed with 4% paraformaldehyde and stained with 1% (w/v) crystal violet.

**Growth kinetics of recombinant viruses**

The growth curves for rNDRV used in this study were derived from DEF cells infected at an m.o.i. of 0.1 and tested for the presence of infectious virus in supernatants collected at 12 h intervals until 96 h post-infection. The cell supernatants were centrifuged for 5 min at 1000 g in order to pellet cell debris and the virus infectivity was subsequently titrated by endpoint dilution analysis of DEF cells. The viral titers were calculated using Reed and Muench’s method [26] and expressed as log₁₀ TCID₅₀ ml⁻¹. The experiments were repeated a minimum of three times.

**Immunoblotting and immunofluorescence for the detection of virus infection**

For Western blot analyses, 36 h post-infection in DEF cells, wild-type NDRV and rNDRV were washed in 1xPBS and lysed in RIPA buffer (50 mM Tris–HCl buffer, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail), and the cell lysates were subjected to SDS-PAGE. Viral proteins were detected using rabbit antiserum specific for αC as a primary antibody (1 µg ml⁻¹) (produced and stored in our laboratory) [27] and HRP-conjugated goat-anti-rabbit IgG as a secondary antibody. The proteins recognized by the antibodies were visualized with ECL (Thermo Scientific, IL, USA).

DEF cells were plated on a chamber and infected at an m.o.i. of 0.1 rNDRV at 80% confluence. Following incubation at 37°C, the cells were fixed at 24 h post-infection and washed with phosphate-buffered saline (PBS), permeabilized with 0.05% Triton X-100 for 5 min, washed again, and blocked in 1% BSA in PBS for 1 h. The cells were then incubated with the rabbit antiserum specific for αC [27] diluted in 1% BSA as a primary antibody for 2 h at room temperature, followed by washing in PBS. The cells were then incubated with the secondary antibody goat anti-rabbit IgG conjugated to FITC for 1 h. After incubation with the secondary antibody, the cells were washed with PBS and coverslips were then mounted onto slides with DAPI. Images were acquired using a Zeiss LSM 510 META inverted confocal system (Zeiss, German) with a Zeiss inverted Axiovert 200M microscope. The images were processed using Image-Pro Plus image analysis software (Nikon Devices).

**In vivo pathogenicity studies**

Animal experiments were carried out at the Chinese Academy of Agricultural Sciences following local and national approved protocols regulating animal experimental use. For each virus, two groups (n=10) of 3-day-old ducklings were inoculated orally with 10⁶ p.f.u. ml⁻¹ of either NDRV or rNDRV. Mock-infected controls included litters inoculated with tissue culture media. Ducklings were euthanized at 2 weeks p.i., or earlier if showing advanced clinical signs of depressed health. The pathological symptoms and mortality
were monitored throughout the study period. Formalin-fixed and paraffin-embedded liver tissue sections from inoculated (and mock-inoculated) ducklings were used in H&E staining and IHC staining. Sections (4–6 µm) were examined for the presence of NDRV antigen using rabbit-antiserum-specific for αC and the EnVision (DAKO) detection system.

RNA was extracted from liver tissues in rNDRV group and PCR amplicons were generated by RT-PCR. The PCR amplicons were then purified and sequenced.

**Statistical analysis**
The growth kinetics for two groups were analysed using correlation of XY pairs (Pearson) and P value (two tailed). P values below 0.05 were considered statistically significant. The significance of differences was determined via one-way analysis of variance (ANOVA) using Prism software (GraphPad Software, Inc.).

**Funding information**
This study was supported by grants from the National Key Research and Development Plan for Controlling of Major Diseases of Livestock and Poultry (2016YFD0500802, 2017YFD0500802), the National Nature Science Foundation of China (no. 31502068) and the Shanghai Science and Technology Innovation Action Plan (no. 13391901602).

**Conflicts of interest**
The authors declare that there are no conflicts of interest.

**References**