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# Rescue of Two Recombinant Canine Distemper Viruses That Separately Express Dabie Bandavirus Gn and Gc in Vitro

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**Short Report**

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# Abstract

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging tick-borne zoonosis with a high mortality rate in humans. Additionally, dogs are frequently reported to be infected with this disease. There has been no commercially available vaccine for humans and animals as yet. The SFTS is caused by Dabie bandavirus (DBV), formerly known as SFTS virus. The DBV is now classified into the genus *Bandavirus* in the family *Phenuiviridae*. DBV Gn and Gc can induce specific immune responses *in vivo*. In this study, we used reverse genetics to construct two recombinant canine distemper viruses (rCDV), rCDV-Gn and -Gc, which could express Dabie bandavirus Gn and Gc *in vitro*, respectively. Two foreign sequences, Gn and Gc open reading frames, were genetically stable during twenty serial viral passages in cells. Growth curve of the rCDV-Gc basically coincided with that of a wild-type CDV, but showed a significant difference from that of the rCDV-Gn. The rCDV-Gn and -Gc were derived from a common parental CDV, the virulence-attenuating QN strain. Therefore, if proven to be efficient in resisting both canine distemper and SFTS in dogs, either or both recombinant CDVs would be potential vaccine candidates.


# Introduction

Severe fever with thrombocytopenia syndrome (SFTS) was an emerging infectious disease, caused by Dabie bandavirus (DBV), formerly known as SFTS virus. This disease was initially reported in Central China in 2009. Its clinical signs included fever, thrombocytopenia, gastrointestinal symptoms and leukocytopenia in patients. There was an unusually high initial case fatality rate of 30% (Yu et al. 2011). In recent years, this disease raised serious public health concerns, especially in China (Miao et al. 2020). The SFTS is a tick-borne zoonosis. The DBV can rapidly evolve by gene mutation, reassortment and homologous recombination in ticks and reservoir hosts (Liu et al. 2014). More recently, it was frequently reported that non-human animals, especially dogs, were infected by DBV, or were diagnosed with DBV antibody-positive (Kang et al. 2019; Lee et al. 2017; Lee et al. 2019; Nam et al. 2020; Niu et al. 2013; Tian et al. 2017). Dog-to-human transmission of DBV can even occur through manual de-ticking of domestic dogs (Chung et al. 2020). No specific treatment of SFTS is available now. Development of veterinary vaccines would be one of the most effective ways to protect companion dogs from SFTS, thereby interrupting a potential route of dog-to-human transmission.

The DBV belongs to the genus *Bandavirus* in the family *Phenuiviridae* of the order *Bunyavirales*. Its genome is segmented into three pieces: L, M and S segments. The M segment encodes a membrane protein precursor (Fig. 1A) that matures into two glycoproteins, Gn and Gc, embedded within the viral envelope (Fig. 1B). Bunyaviral Gn and Gc can induce specific immune responses *in vivo* (Duehr et al. 2020; Said et al. 2017; Wernike et al. 2017; Wright et al. 2020). Different virus-vectored vaccines were reported to be capable of inducing DBV-specific immune responses (Dong et al. 2019; Tian et al. 2021; Yoshikawa et al. 2021; Zhao et al. 2020b). For example, Dong et al (2019) constructed a live-attenuated recombinant vesicular stomatitis virus that could express the DBV Gn/Gc glycoproteins. Single-dose vaccination with it was demonstrated to be able to elicit complete protection in mice against DBV (Dong

et al. 2019). More recently, Tian et al (2021) reported that Gn-expressing recombinant rabies virus conferred protective immune responses in mice (Tian et al. 2021).

Unfortunately, there has been no report concerning canine distemper virus (CDV)-vectored vaccine against DBV as yet. Canine distemper virus (CDV) causes a highly contagious disease, canine distemper, which affects a wide variety of domestic and wild carnivores (McCarthy et al. 2007). This virus is classified into the genus *Morbillivirus* in the family *Paramyxoviridae*. Virulence-attenuating CDV strains were potential vectors for delivering foreign antigens to induce protective immunities against canine distemper and other diseases (Zhao et al. 2020a), such as leishmaniasis (Miura et al. 2015) and rabies (Wang et al. 2012). We have developed one virulence-attenuating CDV (QN strain) previously. Considering anti-DBV vaccines unavailable for dogs, we rescued two recombinant CDVs (QN strain) in this study. These two recombinant CDVs were demonstrated to able of separately expressing DBV Gn and Gc. If proven to elicit protective immunities against canine distemper and SFTS, both viruses would be potential vaccine candidates for dogs.

# Materials And Methods

### Cells, virus and plasmids

The BSR-T7/5 (Buchholz et al. 1999) and Vero-Dog-SLAM (VDS) cells, kindly provided by the China Animal Health and Epidemiology Center, were cultured at 37 °C with 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, and containing penicillin (100 U/mL), streptomycin (100 μg/mL), amphotericin B (0.25 μg/mL) and G418 (500 μg/mL). The wild-type CDV (wt-CDV), QN strain, was propagated in VDS cells. The QN strain-based platform of reverse genetics had been established previously in our laboratory, mainly containing four plasmids, namely one full-length cDNA clone of recombinant CDV that expressed a foreign protein, and three helper plasmids (pCAGGS-N, pCAGGS-P and pCAGGS-L).

### Construction of rCDV-Gn and -Gc cDNA clones

The rCDV-Gn and -Gc cDNA clones were schematically shown in Fig. 1C and D, respectively. They were separately flanked by the T7 promoter and a hepatitis delta virus ribozyme-T7 terminator fusion sequence at their 5' and 3' ends, respectively. In order to optimize protein expression, Gn and Gc open reading frames (ORFs) (Genbank access No.: MT236316) were subjected to codon optimization based on dogs, and then independently subcloned into the *Not* I/*Pme* I sites of another CDV cDNA clone that was constructed previously in our laboratory. Two recombinant plasmids of cDNA clones were separately purified using the PureLink™ HiPure Plasmid Maxiprep Kit (Thermo Fischer, Carlsbad, USA) according to the manufacturer's instruction.

### Rescue and passaging of rCDV-Gn and -Gc

BSR-T7/5 cells were seeded into a 12-well plate, and cultured at 37 °C with 5% $CO_2$. To rescue recombinant viruses, a cell monolayer at 70% confluency was co-transfected with either of the cDNA clones (2.0 μg/well), pCAGGS-N (1.0 μg/well), pCAGGS-P (0.5 μg/well) and pCAGGS-L (0.5 μg/well) using Lipofectamine 2000 (Thermo Fisher, Carlsbad, USA) according to the manufacturer's instruction. Two co-transfected cell monolayers were digested with trypsin at 72 h post transfection (hpt), and then separately co-cultivated with VDS cells in two T25 flasks. The rescued viruses were subjected to serial blind passages in VDS cells.

## RT-PCR analysis

The rCDV-Gn and-Gc were harvested at passage-10 (P10) for extraction of viral RNAs, which were separately used as templates for RT-PCR analysis using the PrimeScript™ High Fidelity One Step RT-PCR Kit (Takara, Dalian, China). The forward primer (5'-TCAAGAGTATTACTCATGCTTAA-3') targeted the downstream region of P ORF, and the reverse primer (5'-TCGAAGTCGTACACCTCAGTCAT-3') targeted the upstream region of M ORF. The RT-PCR reaction underwent 45 °C for 10 min, 94 °C for 2 min and then 30 cycles at 98 °C (10 s), 55 °C (15 s) and 68 °C (20 s). The extracted RNAs were simultaneously subjected to PCR analysis using the same primers. The PCR reaction contained 2 × PrimeSTAR Max Premix (Takara, Dalian, China) and underwent 30 cycles at 98 °C (10 s), 55 °C (10 s) and 72 °C (10 s). RT-PCR and PCR products were detected by agarose gel electrophoresis, followed by Sanger sequencing for analyzing two RT-PCR products.

## Indirect immunofluorescence assay (IFA)

Recoveries of rCDV-Gn and-Gc were confirmed by IFA. Briefly, two VDS cell monolayers were independently infected with the P15 rCDV-Gn and -Gc for 24 h, and then fixed in 4% paraformaldehyde at room temperature for 30 min. After fixation, cells were washed four times with PBS, and then permeated with 0.4% Triton X-100 at room temperature for 30 min. After permeation, cells were washed three times with PBS and blocked in blocking solution at 37 °C for 1 h. Subsequently, cells were incubated with the anti-CDV monoclonal antibody (MAb, 1: 400 in blocking solution) at 37 °C for 2 h. After incubation with the primary antibody, cells were washed three times with PBS and incubated with the Alexa Fluor® 555 conjugate (Thermo Fisher, Waltham, MA, USA) (1: 250 in blocking solution) at 37 °C for 1 h. Cells were washed three times with PBS, coated with 90% glycerin, and visualized under the fluorescence microscope. As a control, one non-infected cell monolayer was subjected to the same treatments.

## Mass spectrometry

The expressions of Gn and Gc were separately analyzed by mass spectrometry (MS) at the Shanghai Bioprofile Biotechnology Co., Ltd (Shanghai, China), as described previously (Liu et al. 2021). In brief, culture supernatants of P10 progenies were inactivated by 0.1% formalin at 4 °C for 48 h. Protein digestion was performed with a method of filter-aided sample preparation (Wiśniewski et al. 2009). Liquid chromatography linked to tandem mass spectrometry (LC-MS/MS) was performed on a Q Exactive Plus mass spectrometer that was coupled to Easy nLC (Thermo Fisher, Waltham, MA, USA). The MS data

were analyzed using MaxQuant software v1.6.0.16. The database search results were filtered and exported with <1% false discovery rate at peptide-spectrum-matched level, and protein level, respectively.

### Growth kinetics

The rCDV-Gn and -Gc were compared with each other on their growth kinetics *in vitro*. Briefly, VDS cells were seeded into five 12-well plates ($10^6$ cells/well, and 6 wells/plate) for incubation at 37 °C for 2 h. The P15 rCDV-Gn and -Gc were separately inoculated (MOI = 0.0002) into all plates (3 wells/progeny in each plate) for incubation at 37 °C for 3 h, and then supernatants were replaced with DMEM for further incubation at 37 °C. At 0, 24, 48, 72 and 96 h post inoculation (hpi), any of plates was randomly removed from the incubator, and subjected to two freeze-and-thaw cycles to collect supernatant for viral titration using the Spearman-Kärber equation (Finney 1952). The wt-CDV as a control was subjected to the same treatments. Kinetic curve of virus growth was drawn using the GraphPad Prism software (Version 7.0). Data at each time point were representative of three independent experiments.

### Genetic stabilities of two foreign sequences

Two recombinant viruses were subjected to twenty serial passages (3 d/passage) in VDS cells. Their culture supernatants at P15 and P20 were harvested for RT-PCR analysis, as described in Subheading RT-PCR analysis. Two RT-PCR products were detected by agarose gel electrophoresis, followed by Sanger sequencing.

# Results

# Rescue of rCDV-Gn and -Gc

The co-transfection of four plasmids was carried out in BSR-T7/5 cells that could express the T7 RNA polymerase. Owing to the absence of CDV receptors on it, the BSR-T7/5 cell line was only used for virus recovery, rather than passaging. Alternatively, the VDS cell line, because permissive to CDV infection, was used for serial blind passages of rescued viruses in this study. Typical cytopathic effects (CPEs), such as exacerbated cell-to-cell fusion (Fig. 2A) and syncytium formation (Fig. 2B), appeared on VDS cell monolayers with viral passaging. The CPEs were also visible during serial blind passages.

# RT-PCR analysis of rCDV-Gn and -Gc

The P10 rCDV-Gn and -Gc were simultaneously analyzed by RT-PCR to confirm their identities. Two expected bands, 1887 (Fig. 2C) and 1899 bps (Fig. 2D), were observable only on the RT-PCR lanes by agarose gel electrophoresis. As a control, PCR analysis (Fig. 2C and D, Lane PCR) showed no residue of cDNA clone plasmid affecting the RT-PCR analysis. The identities of rCDV-Gn and -Gc were confirmed by Sanger sequencing.

# IFA and mass spectrometry

In order to confirm recovery of rCDV-Gn and -Gc, the IFA was performed using the anti-CDV MAb as the primary antibody. Bright red syncytia were visible on the rCDV-Gn- and rCDV-Gc-infected cell monolayers. As a control, non-infected cells did not show a similar phenotype (Fig. 2E). The IFA result confirmed that two competent recombinant CDVs had been recovered from their cDNA clones. Expressions of Gn and Gc were demonstrated by mass spectrometry, which exhibited that Gn- and Gc-specific peptide sequences matched to the MS/MS spectra. Two representative MS/MS spectra were shown in Fig. 3A and B.

# Growth kinetics of rCDV-Gn and -Gc

To determine growth curves of both viruses *in vitro*, VDS cell monolayers were independently inoculated with the P15 rCDV-Gn and -Gc. Typical syncytia were visible at 24 hpi, and exacerbated over time to cause intercellular hyperfusogenicity at 48 hpi (Fig. 3C and D). The growth curves of both viruses were compared with each other and with that of the wt-CDV (Fig. 3E). These two recombinants exhibited different growth kinetics: the rCDV-Gn replicated more slowly during 0 and 24 hpi, but maintained a higher titer level than the rCDV-Gc did during 48 to 96 hpi. The rCDV-Gc showed the similar growth kinetics to that of the wt-CDV.

# Genetic stability of foreign sequences

To test genetic stability of two foreign sequences, both the rCDV-Gn and -Gc were serially passaged in VDS cells for a total of twenty passages. The RT-PCR showed that individual specific products were separately amplified from RNA samples of P15 and P20 progenies (Fig. 3F). The P20 RT-PCR products were collected for Sanger sequencing, indicating no point mutation appearing in the foreign sequence of rCDV-Gn. The rCDV-Gc showed a nonsynonymous mutation (A157C) in the Gc ORF, correspondingly resulted in a mutation of amino acid (T53P) in Gc.

# Discussion

In recent years, the SFTS was frequently reported in China, Japan, and the Republic of Korea. This disease, characterized by a high case-fatality rate in humans, is primarily transmitted *via* tick bite, and can also be transmitted from person to person through contacting patient's blood (Liu et al. 2012). Domesticated animals, like companion dogs, should be considered as a source of animal-to-human transmission, as evidenced by recent case reports (Chung et al. 2020; Han et al. 2020; Kang et al. 2019; Nam et al. 2020). Unfortunately, there has been no commercially available vaccine against SFTS for dogs as yet. CDVs are efficient vectors for expressing heterologous proteins (Liu et al. 2020; Ludlow et al. 2012; Parks et al. 2002; Plattet et al. 2004), or antigens that can confer specific immune responses in animals (Li et al. 2015; Miura et al. 2015; Wang et al. 2012). This prompted us to develop new generation CDV vaccines using reverse genetics for delivering DBV antigens to induce protective immunity in dogs.

We have recently constructed a platform of CDV (5804P strain) reverse genetics for rescue of recombinant virus (Liu et al. 2020). In the present study, we rescued two recombinant virulence-attenuating CDVs (QN strain), independently coding for DBV Gn and Gc in cells. The reason why the DBV glycoprotein precursor (Gn-Gc) was not used for construction of recombinant CDV was that the full-length

sequence of Gn-Gc was theoretically too long (3222 nt) to be accommodated in a CDV genome. Even if a Gn-Gc-inserting CDV can be rescued from its recombinant cDNA clone, both viral replication and protein expression would be affected by the excessive load of heterologous sequence in a CDV genome to some extent. Therefore, we independently rescued Gn- and Gc-expressing CDVs, in order to maintain the viral propagation that was not significantly affected by foreign sequences.

In initial blind passages after co-transfection, both recombinants revealed a weak adaptability in VDS cells, as evidenced by slow appearance of virus-induced CPE foci (data not shown). Such a weak adaptability was gradually improved with serial passaging in VDS cells. Each viral progeny is theoretically better than its previous one in growth kinetics during initial blind passages (Liu et al. 2016). We speculated that both recombinants had been basically adapted to the VDS cell line at P15. Thus, the P15 progenies were used for determining the growth curves of two recombinant viruses. The rCDV-Gc had a similar growth curve to that of the wt-CDV. The rCDV-Gn showed totally different growth kinetics from those of the rCDV-Gc and wt-CDV, implying that the Gn sequence exerted an impact on viral replication *in vitro*. Nevertheless, Tian et al (2021) recently revealed that the insertion of DBV Gn did not affect replication of a recombinant rabies virus *in vitro*, compared with that of its parental strain (Tian et al. 2021). We recently rescued another recombinant CDV QN strain that expressed a SARS-CoV-2 S1 subunit (686 aa) in VDS cells. The rCDV-Gn was measured to have a similar growth curve to that of the S1 subunit-expressing CDV (data not shown).

To enhance expression levels of Gn and Gc, their full-length ORFs were subjected to codon optimization based on dogs. Owing to neither Gn- nor Gc-specific antibody available in our laboratory, their expressions were qualitatively analyzed by mass spectrometry, demonstrating that the rCDV-Gn and -Gc were able of encoding the Gn and Gc in VDS cells, respectively. Unfortunately, the protein expression was not quantitatively analyzed by Western blot or other methods, due to absence of Gn- and Gc-specific antibodies. Because virus-vectored vaccines are actually live recombinant viruses, over-expression of foreign antigens is not always advantageous for vaccines to elicit protective immunity in animals. A given foreign antigen should be expressed at a proper, but not high, level in a host (Liu et al. 2019). Our present study revealed that both recombinant CDVs could independently express Gn and Gc *in vitro*. If demonstrated to be efficient against both canine distemper and SFTS in dogs in future, either or both recombinants would be potential vaccine candidates.

# Declarations


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## Authors' contributions

FL conducted experiments and wrote the manuscript. JL and QW performed the experimental works. HS provided the funding, and supervised the project.

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### Data availability

All data generated or analyzed during this study are included in this published article.

### Ethics approval

Not applicable.

### Consent to participate and consent for publication

All authors agreed to participate in this work. They read and approved the final manuscript.

### Conflict of interest

The authors declare no conflict of interest.

### Disclosure statement

No competing financial interest exists.

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# Figures

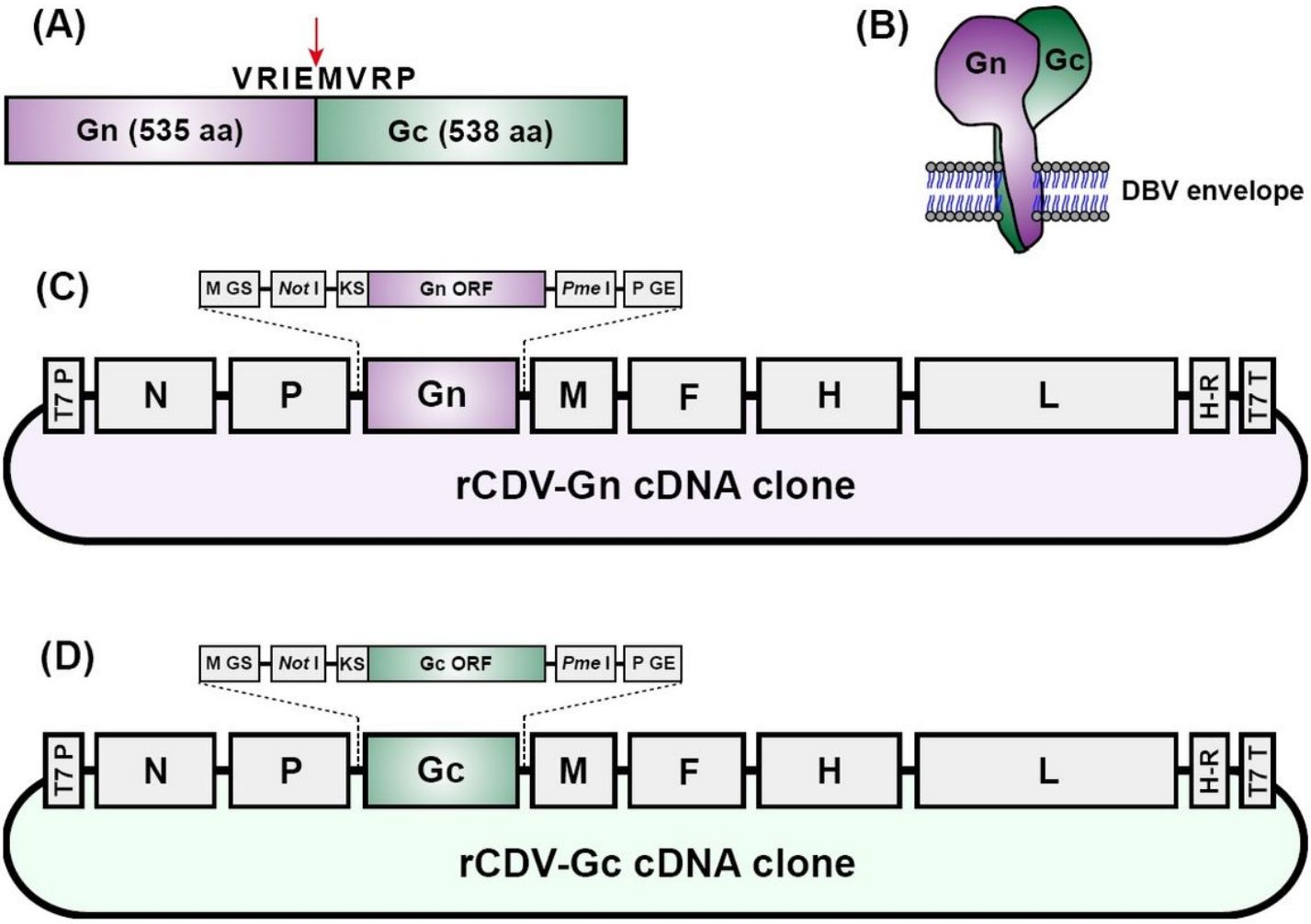
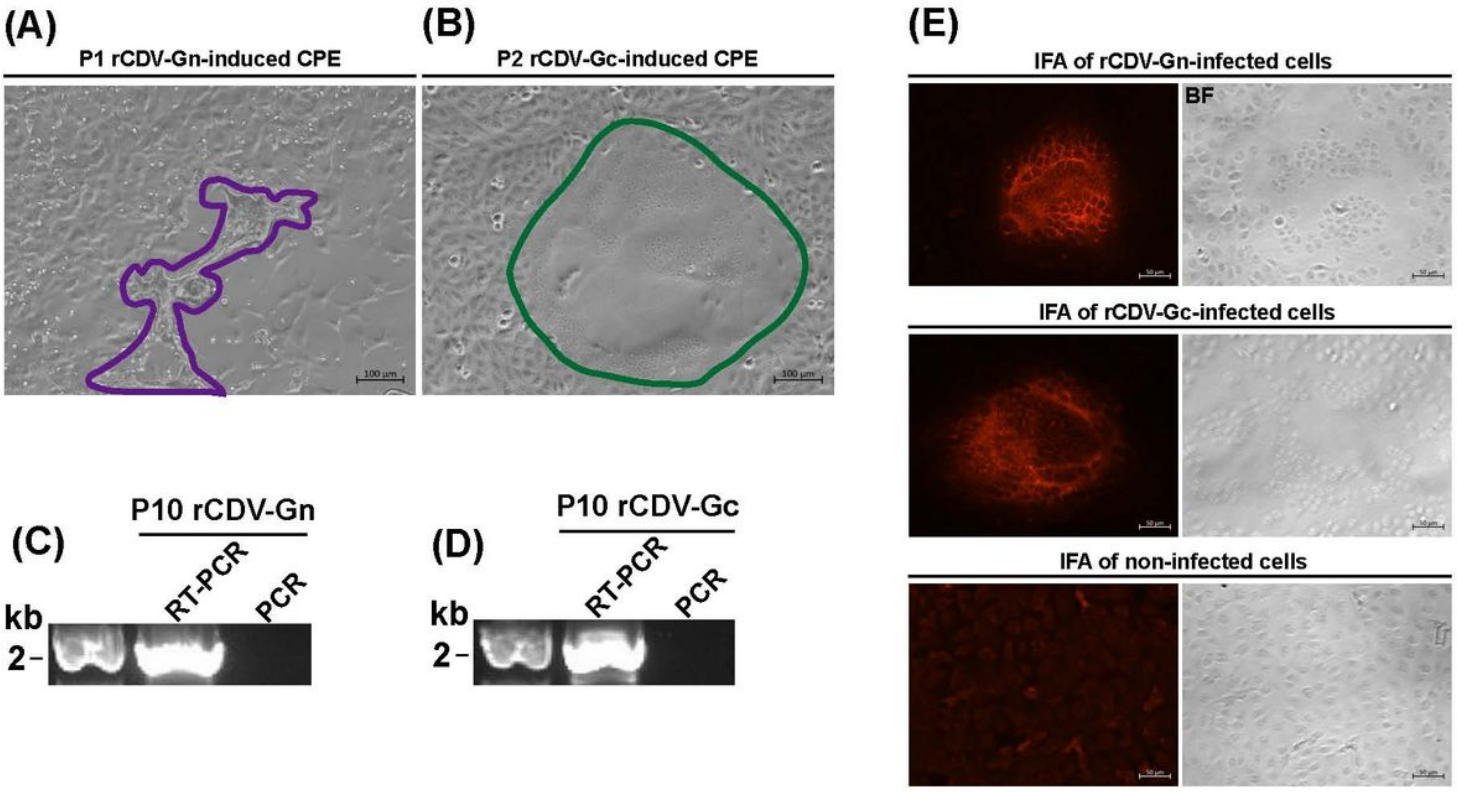


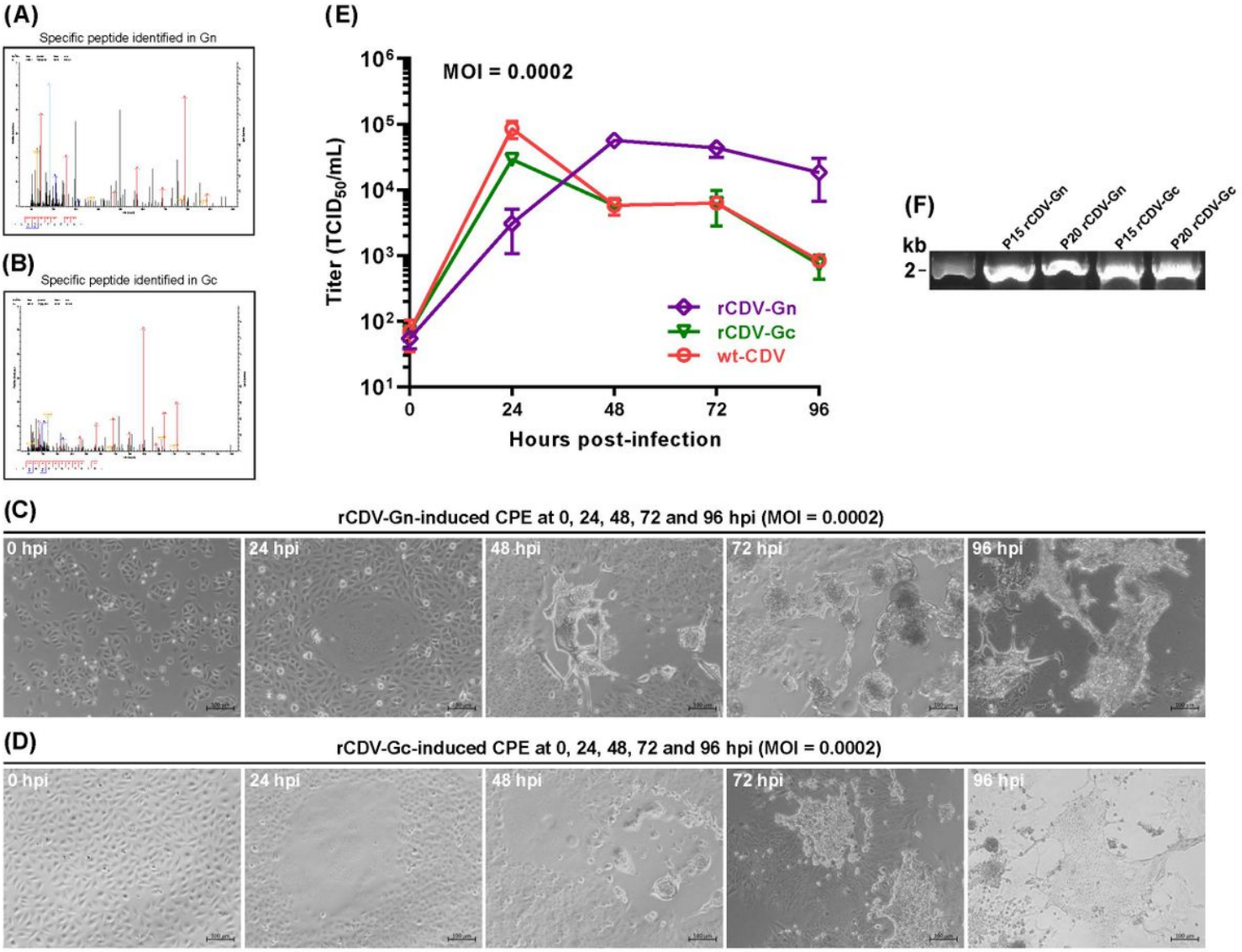

**Figure 1**

Schematic representations of DBV glycoprotein precursor, Gn-Gc heterodimer, rCDV-Gn cDNA clone and rCDV-Gc cDNA clone. DBV glycoprotein precursor composed of Gn and Gc (A). Their cleavage site is marked with red arrow. DBV spike composed of Gn-Gc heterodimer that is embedded within viral envelope (B). rCDV-Gn cDNA clone (C) and rCDV-Gc cDNA clone (D). T7 P: T7 promoter; GS: gene start; GE: gene end; KS: Kozak sequence; H-R: hepatitis delta virus ribozyme; T7 T: T7 terminator.




## Figure 2

Rescue and identification of rCDV-Gn and -Gc. rCDV-Gn-induced cell-to-cell fusion (A, enclosed by purple line) and rCDV-Gc-induced syncytium formation (B, enclosed by green line) on VDS cell monolayers during viral passaging. RT-PCR detection of the P10 rCDV-Gn (C) and -Gc (D) using the forward (5'-TCAAGAGTATTACTCATGCTTAA-3') and reverse (5'-TCGAAGTCGTACACCTCAGTCAT-3') primers. The Gn- and Gc-specific bands are 1887 and 1899 bps, respectively. Indirect immunofluorescence assay of VDS cell monolayers separately inoculated with rCDV-Gn and -Gc (E). The primary and secondary antibodies are anti-CDV MAb and Alexa Fluor® 555 antibody, respectively.




## Figure 3

Characterization of rCDV-Gn and -Gc. Representative Gn- (A) and Gc (B)-specific MS/MS spectra on peptide identifications. CPEs on VDS cell monolayers separately inoculated (MOI = 0.0002) with the P15 rCDV-Gn (C) and -Gc (D) at 0, 24, 48, 72 and 96 hpi. Multi-step growth curves of the P15 rCDV-Gn and -Gc (E). Growth curves are drawn using the GraphPad Prism software (Version 7.0). Data at each time point are representative of three independent experiments. RT-PCR analysis on P15 and P20 progenies of rCDV-Gn and -Gc using the forward (5'-TCAAGAGTATTACTCATGCTTAA-3') and reverse (5'-TCGAAGTCGTACACCTCAGTCAT-3') primers (F).