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Rescue of recombinant canine distemper virus that expresses S1 subunit of SARS-CoV-2 spike protein *in vitro*

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ABSTRACT

The coronavirus disease 2019 (COVID-19), as an unprecedented pandemic, has rapidly spread around the globe. Its etiological agent, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), belongs to the genus *Betacoronavirus* in the family *Coronaviridae*. The viral S1 subunit has been demonstrated to have a powerful potential in inducing protective immune responses *in vivo*. Since April 2020, farmed minks were frequently reported to be infected with the SARS-CoV-2 in different countries. Unfortunately, there has been no available veterinary vaccine as yet. In this study, we used reverse genetics to rescue a recombinant canine distemper virus (CDV) that could express the SARS-CoV-2 S1 subunit *in vitro*. The S1 subunit sequence was demonstrated to be relatively stable in the genome of recombinant CDV during twenty serial viral passages in cells. However, due to introduction of the S1 subunit sequence into CDV genome, this recombinant CDV grew more slowly than the wild-type strain did. The genomic backbone of recombinant CDV was derived from a virulence-attenuating strain (QN strain). Therefore, if able to induce immune protections in minks from canine distemper and COVID-19 infections, this recombinant would be a potential vaccine candidate for veterinary use.

1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has rapidly spread worldwide, overburdening health systems, and causing numerous economic and social impacts. The latest update on COVID-19 epidemiology is available online in the situation reports [1], World Health Organization. In addition to countless cases of human infection, reverse zoonotic transmission has been reported in different settings where SARS-CoV-2-infected humans have close contact with domestic or wild animals [2,3]. In April 2020, outbreak of SARS-CoV-2 infection was reported in farmed minks in the Netherlands [4]. Since then, COVID-19 has reached numerous mink farms in several countries, including Spain, Denmark, Italy, Sweden, the USA, Canada and so forth. The American mink is the most important species in fur-farming operations. Nowadays, the main fur-producing countries are Denmark, the Netherlands, Poland and China [5]. To date, anti-SARS-CoV-2 vaccines are available only for humans.

SARS-CoV-2 is classified into the genus *Betacoronavirus* in the family

Coronaviridae. The virion includes four structural elements, namely the spike (S), envelope, membrane, and nucleocapsid proteins. The S protein forms a homotrimer on the virion surface (Fig. 1A), and has the ability to induce protective immunity [6,7]. The nascent S protein is composed of the S1 and S2 subunits (Fig. 1B). Its activation requires two proteolytic cleavage events: the full-length S protein is firstly cleaved into S1 and S2 subunits, and then the S2 subunit is further cleaved at the S2' site [8]. The S1 subunit contains the viral receptor-binding domain (RBD). A recombinant vaccine, comprising residues 319–545 of the RBD, was demonstrated to induce a potent functional antibody response in immunized mice, rabbits and non-human primates [9]. Therefore, the S1 subunit is an ideal antigen for development of new generation vaccines against the SARS-CoV-2.

Canine distemper is a highly contagious disease, affecting a wide variety of domestic and wild carnivores, such as dogs and minks [10]. Its etiological agent is canine distemper virus (CDV), belonging to the genus *Morbillivirus* in the family *Paramyxoviridae*. Live-attenuated vaccines have been widely used for vaccination of animals against canine distemper. Recent advances have revealed that virulence-attenuating CDV

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strains are potential vectors for delivering foreign antigens to induce protective immunities against canine distemper and other diseases [11]. A virulence-attenuating strain, CDV QN strain, had been previously developed in our laboratory, and more recently was subjected to next-generation sequencing, uncovering the full-length sequence of viral antigenome. In the present study, considering no available veterinary vaccine against the SARS-CoV-2, we constructed a recombinant CDV QN strain that was demonstrated to be able of expressing the S1 subunit *in vitro*. If proven to elicit protective immunities against both diseases in minks, this recombinant virus (rCDV-S1) would be a potential candidate of bivalent vaccine.

2. Materials and methods

2.1. Cells, virus and plasmids

The BSR-T7/5 [12] and Vero-Dog-SLAM (VDS) cells, kindly provided by the China Animal Health and Epidemiology Center, were cultured at 37 °C with 5 % CO₂ 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. Three plasmids, pCAGGS-N, pCAGGS-P and pCAGGS-L, had been constructed previously in our laboratory [13], and would be used as helpers for virus rescue.

2.2. Construction of rCDV-S1 cDNA clone

The pBR322 plasmid was used to construct rCDV-S1 cDNA clone (Fig. 1C), in which the full-length sequence of CDV (QN strain) antigenome served as a backbone sequence. This cDNA clone was flanked by the T7 promoter and a hepatitis delta virus ribozyme-T7 terminator fusion sequence at its 5' and 3' ends, respectively. In order to improve its expression, the 2055-bp-long open reading frame (ORF) of SARS-CoV-2 (Genbank access No.: MT385483.1) S1 subunit was subjected to codon

optimization based on human cells using an online tool of codon optimization (<https://www.vectorbuilder.cn/tool/codon-optimization.html>), and then flanked by the Kozak sequence [14] at its 5' end. The Kozak sequence-S1 ORF fusion fragment was flanked by *Not* I and *Pme* I recognition sites at its 5' and 3' ends, respectively. The modified S1 ORF was regulated by M gene start (GS) and P gene end (GE) sequences at its 5' and 3' ends, respectively. The rCDV-S1 cDNA clone was purified using the PureLink™ HiPure Plasmid Maxiprep Kit (Thermo Fischer, Carlsbad, USA) according to the manufacturer's instruction.

2.3. Rescue and passaging of rCDV-S1

BSR-T7/5 cells were seeded into a 12-well plate, and cultured at 37 °C with 5 % CO₂. A cell monolayer at 70 % confluency was co-transfected with the rCDV-S1 cDNA clone (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. The co-transfected cell monolayer was digested with trypsin at 72 h post transfection (hpt), and then co-cultivated with VDS cells in a T25 flask. The rCDV-S1 would be recovered, released from the BSR-T7/5 cells, and further infect the VDS cells. The rescued rCDV-S1 was subjected to serial blind passages in VDS cells.

2.4. RT-PCR analysis of rCDV-S1

The rCDV-S1 was harvested at passage-10 (P10) for extraction of viral RNA, which was used as template 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 (25 s). The extracted RNA was simultaneously subjected to PCR analysis as a control using the same primers. The PCR reaction contained 2 × PrimeSTAR

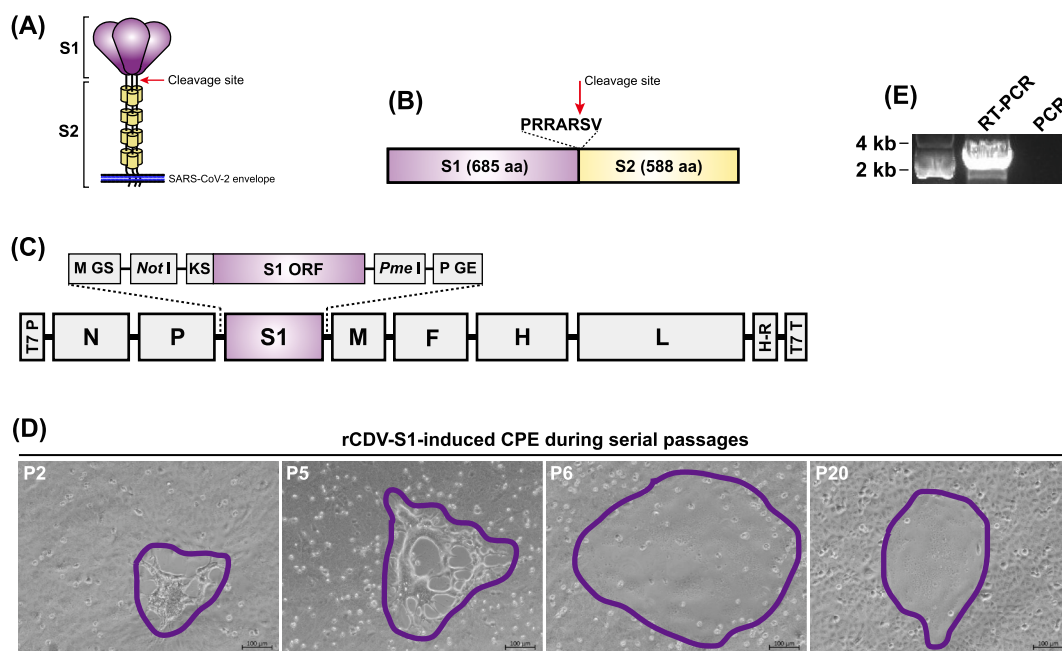


Fig. 1. Rescue and identification of rCDV-S1. Schematic representations of SARS-CoV-2 spike trimer (A) and monomer (B). Each spike monomer comprises one S1 subunit and one S2 subunit, and their cleavage site is marked with red arrow. Schematic representation of rCDV-S1 cDNA clone (C). T7 P: T7 promoter; GS: gene start; GE: gene end; KS: Kozak sequence; H-R: hepatitis delta virus ribozyme; T7 T: T7 terminator. Cell-to-cell fusion and syncytium formation on VDS cell monolayers during serial viral passaging (D). CPEs are enclosed by purple lines. RT-PCR and PCR analyses of the P10 rCDV-S1 (E). An expected band (2343 bp) is observable only on the RT-PCR lane.

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 the RT-PCR product.

2.5. Indirect immunofluorescence assay (IFA) of rCDV-S1

A VDS cell monolayer was infected with the P15 rCDV-S1 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, the non-infected cell monolayer was subjected to the same treatments.

2.6. Mass spectrometry of S1 subunit expression

The culture supernatant of P10 rCDV-S1 was inactivated by 0.1 % formalin at 4 °C for 48 h, and subsequently subjected to mass spectrometry (MS) at the Shanghai Bioprofile Biotechnology Co., Ltd (Shanghai, China), as described previously [15]. Briefly, protein digestion was performed with a method of filter-aided sample preparation [16]. 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.

2.7. Growth kinetics of rCDV-S1

VDS cells were seeded into five 12-well plates (10^6 cells/well, and 3 wells/plate) for incubation at 37 °C for 2 h. The P15 rCDV-S1 was inoculated (MOI = 0.0002) into all plates 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 infection (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 [17]. The wt-CDV as a control was subjected to the same treatments. Kinetic curves of virus growth were drawn using the GraphPad Prism software (Version 8.0). Data at each time point were representative of three independent experiments.

2.8. Genetic stability of foreign sequence

The rCDV-S1 was subjected to twenty serial passages (3 d/passage) in VDS cells. The culture supernatants at P15 and P20 were harvested for RT-PCR analysis, as described in Subheading 2.4. Two RT-PCR products were detected by agarose gel electrophoresis.

3. Results

3.1. rCDV-S1 is rescued from its cDNA clone

The T7 RNA polymerase-expressing BSR-T7/5 cell line was used for co-transfection to recover the competent rCDV-S1 from its cDNA clone. The CDV infection-permissive VDS cell line was used for blind passages of rescued viruses. The cytopathic effect (CPE), local cell-to-cell fusion, appeared on the VDS cell monolayer at P2 (Fig. 1D, P2). The typical

syncytium formation was also visible during serial blind passages (Fig. 1D, P6 and P20).

3.2. Recovery of rCDV-S1 is demonstrated by RT-PCR

The P10 rCDV-S1 was analyzed by RT-PCR to confirm its identity. An expected band of amplicon size (2343 bp) was observed only on the RT-PCR lane by agarose gel electrophoresis (Fig. 1E, Lane RT-PCR). As a control, PCR analysis (Fig. 1E, Lane PCR) showed no contamination of cDNA clone affecting RT-PCR detection. The identity of rCDV-S1 was confirmed by Sanger sequencing.

3.3. Recovery of rCDV-S1 is confirmed by IFA

The IFA was carried out to confirm further the identity of rCDV-S1 using the anti-CDV MAb and the fluorescein-conjugated antibody. Red syncytium formation was visible on the rCDV-S1-infected cell monolayer, and however non-infected cells did not show a similar phenotype (Fig. 2A). Therefore, the IFA result confirmed that the competent virus had been recovered from its cDNA clone.

3.4. S1 subunit expression is demonstrated by mass spectrometry

The S1 ORF underwent codon optimization for improving expression of the S1 subunit. The culture supernatant of P10 rCDV-S1 was analyzed by mass spectrometry, exhibiting six S1 subunit-specific peptide sequences matching to the MS/MS spectra (Fig. 2B): ¹⁸⁹NLREFVFK¹⁹⁶, ²⁰⁸HTPINLVR²¹⁵, ³²¹VQPTESIVR³²⁹, ⁴⁴⁶VGGNYNYLYR⁴⁵⁵, ⁵⁶⁹DIADTTDAVR⁵⁷⁸ and ⁶³⁶VYSTGNSVNFQTR⁶⁴⁷ (Fig. 2C), all of which demonstrated that the S1 subunit could be expressed by the recombinant virus *in vitro*.

3.5. rCDV-S1 replicates more slowly than wild-type strain does

To examine multi-step growth curve of the rCDV-S1 *in vitro*, VDS cells were infected with the P15 rCDV-S1 at MOI of 0.0002. The rCDV-S1-induced syncytia were observable at 24 hpi, and exacerbated over time to cause intercellular hyperfusogenicity at 48 hpi (Fig. 3A). At each indicated time point, virus-infected supernatant was separately collected and titrated in VDS cells. The growth curve of rCDV-S1 was compared with that of the wt-CDV (Fig. 3B). Both viruses exhibited different growth kinetics: the rCDV-S1 replicated more slowly within 24 hpi, but maintained a higher titer level than the wt-CDV did from 48 to 96 hpi. The rCDV-S1 reached its peak titer (4.4×10^4 TCID₅₀/mL) at 48 hpi.

3.6. Foreign sequence is genetically stable during twenty viral passages

To test genetic stability of the S1 subunit, the rCDV-S1 was serially passaged in VDS cells for a total of twenty times. The RT-PCR showed that 2343-bp-specific products were separately amplified from RNA samples of P15 and P20 progenies (Fig. 3C). Both RT-PCR products were confirmed by Sanger sequencing, indicating no fragment deleted from the sequence of S1 subunit.

4. Discussion

SARS-CoV-2-infected minks were identified in April 2020 in the Netherlands [4], and later in other European countries, such as Spain, Denmark, Italy, Sweden, Greece, France and Lithuania [18]. Mink-infesting cases were also identified in several states in the USA [18]. The more recent one was reported in March 2021 in Poland [19]. Besides mink, dog, cat and some wild animals (puma, lion, tiger and so on) were also reported to be infected with SARS-CoV-2 in many countries or regions, including the USA, Argentina, Japan, Brazil, Canada, Estonia, Sweden, Croatia, Germany and Hong Kong. To date,

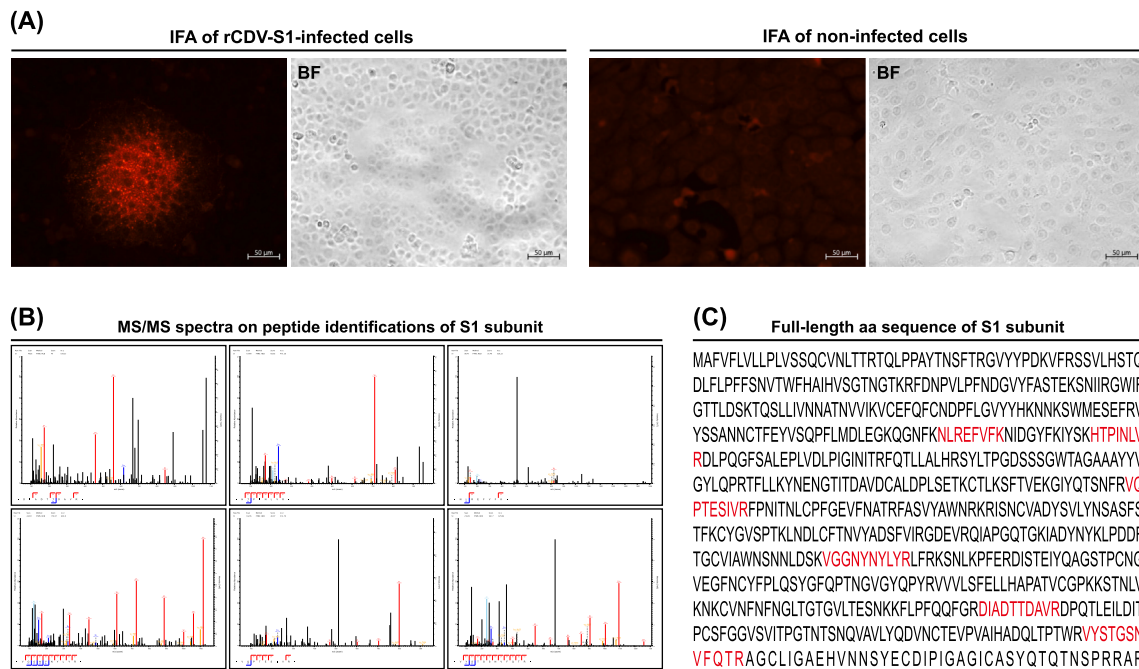


Fig. 2. Confirmation of rCDV-S1 recovery and identification of S1 subunit expression. Indirect immunofluorescence assay of rCDV-S1- and non-infected VDS cell monolayers (A). The primary and secondary antibodies are anti-CDV MAb and Alexa Fluor® 555 conjugate, respectively. Six S1 subunit-specific MS/MS spectra on peptide identifications (B). Amino acid (aa) sequence of full-length S1 subunit (C). Six red letter-marked sequences specifically match to the MS/MS spectra.

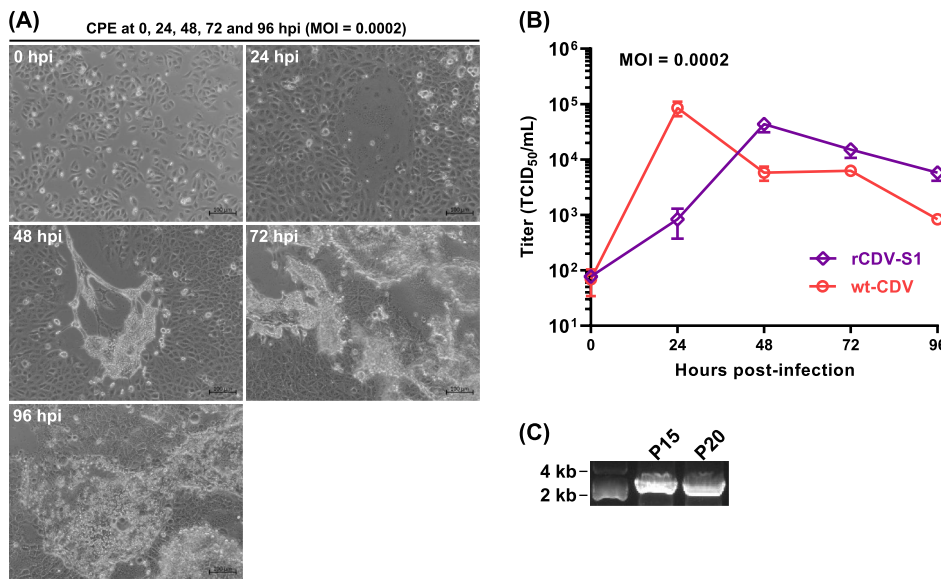


Fig. 3. Growth kinetics of rCDV-S1 and genetic stability of foreign sequence. CPEs on VDS cell monolayers infected (MOI = 0.0002) with the P15 rCDV-S1 at 0, 24, 48, 72 and 96 hpi (A). Multi-step growth curves of the P15 rCDV-S1 and the wild-type strain (B). Viral titers are measured using the Spearman-Kärber equation. Kinetic curves of virus growth are drawn using the GraphPad Prism software. Data at each time point are representative of three independent experiments. RT-PCR analyses of the P15 and P20 progenies (C). Two expected bands (2343 bp) are observable on the gel.

SARS-CoV-2 vaccines have been commercially available for humans, but unavailable for animals. This prompted us to develop a CDV-vectored candidate against SARS-CoV-2 infection in animals. CDV is an ideal vector to encode foreign proteins, such as fluorescence proteins [20,21], luciferases [13,22] and interleukins [23,24]. CDV additionally exhibits specific properties with potential applications in developing multivalent vaccines against canine distemper and other diseases [25–27]. In this study, we attempted to generate a bivalent vaccine candidate against CDV and SARS-CoV-2 in animals. One major issue for developing such a vaccine was selection of a proper antigen for recovery of recombinant CDV.

Since the S protein is a glycoprotein that is critical to elicit immune responses, some groups use the S protein as antigen for designing novel SARS-CoV-2 vaccines. More recently, An et al. (2021) optimized a virus

candidate, parainfluenza virus 5 expressing the SARS-CoV-2 S protein, and then demonstrated that a single-dose intranasal immunization protected K18-hACE2 mice from lethal SARS-CoV-2 challenge. Moreover, this novel vaccine also prevented virus infection of ferrets and blocked contact transmission [28]. On the basis of our previous experience, we speculate that the full-length ORF of S protein is too long (3822 nt) to be accommodated in a CDV genome. Even if one S gene-inserting CDV can be rescued from its cDNA clone, there would be impacts on viral replication and on S protein expression to some extent.

The S1 subunit is responsible for virus binding to the host cell receptor. After proteolytic virus activation, the S1 subunit offers additional epitopes with antibody exposure. Therefore, S1 subunit-based vaccines would be more efficacious than the existing ones [29]. Indeed, recent studies indicated that the S1 subunit could also induce robust

immune responses *in vivo* [30,31], and furthermore elicit much higher IgG and IgA antibody levels than the RBD did in immunized mice [32]. Therefore, the S1 subunit was selected as antigen sequence for constructing the recombinant CDV in this study.

The wild-type CDV QN strain was a virulence-attenuating vaccine candidate that had been developed in our laboratory previously. In order to construct a safe and effective recombinant CDV, the antigenome sequence of QN strain was used as a backbone for constructing the rCDV-S1 cDNA clone. To enhance protein expression, the full-length ORF of S1 subunit was subjected to codon optimization based on human cells, and flanked by the Kozak sequence at its 5' end. The P/M intergenic region served as an insertion site for introduction of the foreign sequence into the CDV cDNA clone. The rCDV-S1 was rescued from BSR-T7/5 cells and passaged in VDS cells. Generally, wt-CDV infection induces typical syncytium formation on VDS cells. Such a phenotype was unobservable on or slight cell-to-cell fusion sporadically appeared on rCDV-S1-infected VDS cell monolayers during initial blind passages (Fig. 1D, P2 and P5). The typical syncytium formation was invisible until the sixth passaging (Fig. 1D, P6). Even so, the RT-PCR assay was necessary for detecting the rCDV-S1 sequence, and the PCR assay was simultaneously carried out to eliminate the possibility of potential interference caused by residual cDNA clone plasmids. Besides the RT-PCR detection, the IFA confirmed successful recovery of the competent rCDV-S1.

The foreign sequence was demonstrated to be relatively stable during twenty viral passages *in vitro*, while a few point mutations were identified in the rCDV-S1 genome by Sanger sequencing (data not shown). Due to the absence of S1 subunit-specific antibody in our laboratory, the expression of S1 subunit was qualitatively analyzed by mass spectrometry. Eliminating non-specific peptide sequences of S1 subunit that were identical to those of the Vero cell line, a total of six specific peptide sequences separately matched to their MS/MS spectra (Fig. 2B and C), demonstrating that the rCDV-S1 was able to express the S1 subunit *in vitro*. Unfortunately, the expression of S1 subunit was not subjected to semi-quantitative analysis by Western blot in this study, owing to the lack of S1 subunit-specific antibody.

Generally, a high expression level of foreign antigen is not always advantageous for a virus-vectored vaccine to induce immune responses *in vivo*. In other words, a foreign antigen should be expressed at a proper, but not high, level in hosts [33]. The S1 subunit ORF is a foreign sequence, or a nonstructural gene, for the recombinant virus. Its introduction theoretically reduces the efficiency of viral replication. Indeed, the rCDV-S1 was demonstrated not only to grow more slowly, but also to reach a lower peak titer than the wt-CDV to do (Fig. 3B). This will be one of major challenges on large-scale production of rCDV-S1. This problem may be alleviated or even be solved by production of virus in suspension cells cultured in a particular bioreactor [34]. If proven to be efficient in resisting both CDV and SARS-CoV-2 *in vivo*, the rCDV-S1 would be a potential vaccine candidate for clinical tests in minks, which are highly susceptible to CDV and SARS-CoV-2 infections.

Declaration of competing interest

The authors declare no conflict of interest.

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