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Genomic profiling of three progenies isolated from a swarm of eGFP-tagged canine distemper viruses undergoing 40 serial passages

Shuning Zhou[1#], Ling Wang[2#], Jiahui Lin[1], Ning Wang[1], Yongle Yu[1], Youming Zhang[3], Fuxiao Liu[1*]

[1]College of Veterinary Medicine, Qingdao Agricultural University, Qingdao, 266109, P. R. China; [2]University Hospital, Qingdao Agricultural University, Qingdao, 266109, P. R. China; [3]State Key Laboratory of Microbial Technology, Shandong University, Qingdao, 266237, P. R. China

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Summary. – Canine distemper virus (CDV) is classified into the genus *Morbillivirus* in the family *Paramyxoviridae*. This virus has a single-stranded genomic RNA with negative polarity. The L protein is the viral RNA-dependent RNA polymerase (RdRp), closely associated with the fidelity of genome replication. We previously constructed an enhanced green fluorescence protein (eGFP)-tagged recombinant CDV (rCDV-eGFP). This recombinant underwent a total of 47 passages *in vitro* including 7 blind and 40 ribavirin-treated passages, consequently showing a rich diversity of viral quasispecies by the analysis of next-generation sequencing (NGS). In the present study, through one round of plaque purification, three single-plaque-derived variants (SPDVs) with the brightest fluorescence were independently isolated from a swarm of rCDV-eGFP progenies at passage-47, and then subjected to NGS analysis. The NGS results comprehensively uncovered mutation profiles of these three SPDVs. More importantly, this study aimed to unravel the potential relation between viral replication fidelity and L protein sequence. However, two SPDVs showed no single-amino acid mutation in their own L proteins, implying the rCDV-eGFP progenitor that might be a high-fidelity strain.

Keywords: rCDV-eGFP; SPDV; next-generation sequencing; mutation; L protein; high-fidelity

Introduction

CDV, now renamed canine morbillivirus, causes a highly contagious disease, canine distemper, which affects a variety of domestic and wild carnivores, such as dog, mink, tiger and lion (McCarthy *et al*., 2007). This virus is classified into the genus *Morbillivirus* in the family *Paramyxoviridae*. CDV virion is typically enveloped, pleomorphic particle, into which a viral genome is enclosed. The CDV genome is a single-stranded, linear RNA with negative polarity. The wild-type genome is 15, 690 nt in length, following the "rule of six", necessary for efficient replication between genome and antigenome (Kolakofsky *et al*., 2005). The genome contains six transcriptional units, separately coding for six structural proteins, namely N, P, M, F, H and L proteins. Six ORFs are separated by untranslated regions with uneven lengths. The L protein, also known as RdRp, the largest one of the viral proteins, is assumed to carry all activities necessary for genomic RNA transcription and replication (Barrett *et al*., 2006).

Most RNA viruses show high mutation frequencies in nature, mainly attributed to the lack of proofreading mechanisms in their RdRps (Mandary *et al*., 2019). Owing to the morbillivirus RdRp characterized by low-fidelity

*Corresponding author. E-mail: laudawn@126.com; phone: +86-0532-86080734. #The contribution is equal to this work.

Abbreviations: CDV = canine distemper virus; eGFP = enhanced green fluorescence protein; NGS = next-generation sequencing; ORF = open reading frame; P47 = passage-47; rCDV-eGFP = eGFP-tagged recombinant CDV; RdRp = RNA-dependent RNA polymerase; SAAM = single-amino acid mutation; SNM = single-nucleotide mutation; SPDV = single-plaque-derived variant; SVDS = single-virus-derived strain; TSNS = total single-nucleotide substitution; VDS = Vero-Dog-SLAM

replication, SNMs unavoidably arise at random with replication of viral genome (Liu *et al*., 2016). The measles virus was proven to have a mutation rate of 9×10^{-5}/nt/replication, and a genomic mutation rate of 1.43/replication (Schrag *et al*., 1999). Under nonselective conditions, the measles virus was recently reported to show a spontaneous mutation rate of 1.8×10^{-6}/nt/replication (Zhang *et al*., 2013). Viruses, especially RNA viruses, if subjected to serial passages, would form a swarm of complex mutants, named viral quasispecies (Andino and Domingo, 2015). Ribavirin is a broad-spectrum antiviral reagent, and a mutagen of RNA viruses (Crotty *et al*., 2000). It can quicken the occurrence rate of SNMs in viral genomes (Liu *et al*., 2021c). If cultured in ribavirin-treated cells, RNA viruses may evolve to generate ribavirin-resistant variants, characterized by increased fidelity in their RdRps (Griesemer *et al*., 2017; Li *et al*., 2019; Pfeiffer and Kirkegaard, 2003; Tian and Meng, 2016; Zeng *et al*., 2013).

Using the reverse genetics technique, we previously rescued a recombinant CDV that could efficiently express the eGFP in cells. The passage-7 rCDV-eGFP underwent 40 extra passages separately in ribavirin- and non-treated cells. More recently, we comparatively analyzed mutation profiles between two progenies at P47. Compared with the non-treated control, the ribavirin-screened progeny showed a rich diversity of viral quasispecies *via* the NGS analysis (Liu *et al*., 2021b). The excellent coverage offered by NGS technique has facilitated the assessment of the population complexity of RNA viruses at an exceptional level of detail (Knyazev *et al*., 2021). In the present study, three SPDVs were isolated from a swarm of ribavirin-screened rCDV-eGFP at P47, and then subjected to NGS analysis for uncovering their individual mutation profiles.

Materials and Methods

Cell line and virus. The VDS cell line was cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (VivaCell, Shanghai, China), penicillin (100 U/ml), streptomycin (100 μg/mL), amphotericin B (0.25 μg/ml) and G418 (500 μg/ml). The replication-competent rCDV-eGFP was rescued previously in our laboratory. Fig. 1a schematically shows all ORFs and untranslated regions in proportion to their actual distributions in the viral antigenome. This recombinant underwent a total of 47 passages, including 7 blind and 40 ribavirin-treated passages, in VDS cells (Liu *et al*., 2021b).

RT-PCR analysis of rCDV-eGFP progenies. The rCDV-eGFP progenies at P8, 17, 27, 37 and 47 were harvested for extracting total RNAs and subsequently used as templates for RT-PCR analysis of the genetic stability of the foreign sequence, as described previously (Liu *et al*., 2021b). The forward (5'-gatcaaaagtatcacacatgcttaa-3') and reverse (5'-gatcgaagtcgtacaccctcagtcat-3') primers were used for RT-PCR detection, which 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 (10 s), followed by agarose gel electrophoresis.

Isolation of three SPDVs. The P47 rCDV-eGFP was subjected to the plaque purification. Briefly, VDS cell monolayers in a 6-well plate were inoculated with the P47 supernatant in 4-fold serial dilutions. After 2 h adsorption, the inocula were removed, and virus-infected cell monolayers were overlaid with medium containing 1% low-melting-point agarose. After three days of incubation at 37°C, three single plaques with the brightest fluorescence were independently picked out from the 6-well plate into growth medium, followed by two freeze-and-thaw cycles to collect supernatants for inoculating VDS cells in T25 flasks.

NGS of three SPDVs. The cell cultures of SPDVs (SPDV-1, -2 and -3) were harvested at 72 h post inoculation, followed by one freeze-and-thaw cycle for collecting their individual supernatants. A total of three RNA samples were extracted for reverse transcription by random hexamers using the 1st Strand cDNA Synthesis Kit (Takara, Dalian, China), according to the manufacturer's instruction. The Illumina sequencing, library construction and data processing were carried out in the Shanghai Tanpu Biotechnology Co., Ltd (Shanghai, China), as described in our previous report (Liu *et al*., 2021a).

Results and Discussion

The virulence-attenuated Rockborn strain of CDV was historically used to produce commercially available vaccines (Schultz, 1976). Unfortunately, it was proven to be able of reverting back to the high-virulence status after consecutive passages *in vitro* and *in vivo* (Appel, 1978). Therefore, the Rockborn strain was withdrawn from several markets after the mid-1990s (Martella *et al*., 2011). Due to potential risk factors causing the reversion to virulence, it is necessary for screening high-fidelity CDV strains to explore their own mechanisms of high-fidelity replication. Ribavirin has been broadly used to screen for viral variants with mutagen resistance (Cheung *et al*., 2014; Griesemer *et al*., 2017; Pfeiffer and Kirkegaard, 2003; Zeng *et al*., 2014). The ribavirin-resistant variants, especially RNA viruses, are generally characterized by high-fidelity replication, since the ribavirin is a useful mutagen that can increase the error rate of viral genome replication. The ribavirin-resistant variants exhibit crucial molecular mechanisms in viral RdRps, which are involved in the high-fidelity replication of viral genomes (Griesemer *et al*., 2017; Li *et al*., 2019; Pfeiffer and Kirkegaard, 2003; Tian and Meng, 2016; Zeng *et al*., 2013).

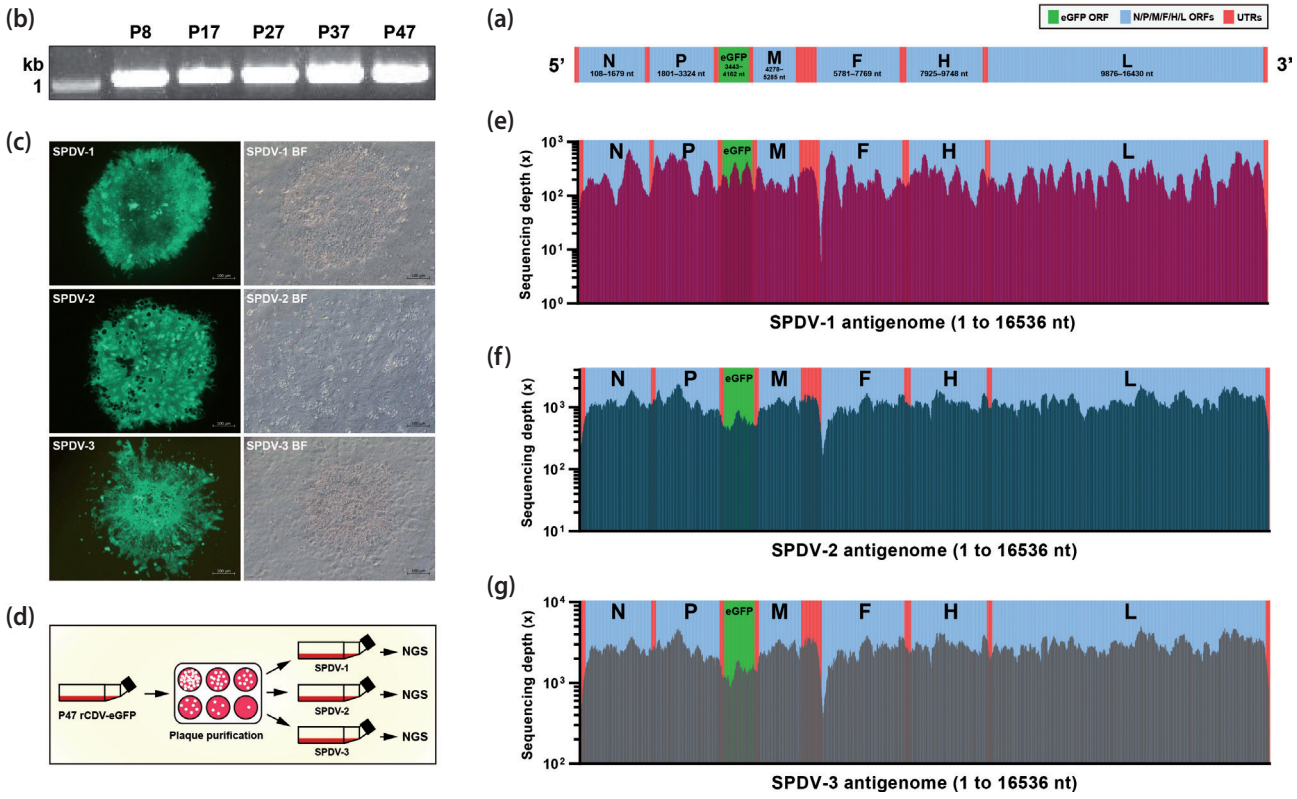

Fig. 1

Isolation of three single-plaque-derived variants for analysis of full-length antigenome by next-generation sequencing
Schematic representation of rCDV-eGFP antigenome **(a)**. All elements proportionally match their actual lengths in viral antigenome. UTRs: untranslated regions. RT-PCR analysis of the P8, P17, P27, P37 and P47 rCDV-eGFPs **(b)**. The expected band of amplicon size is 1001 bp. Single plaques with the brightest fluorescence separately induced by SPDV-1, -2 and -3 **(c)**. Schematic representation of plaque purification to isolate SPDV-1, -2 and -3 for NGS analysis **(d)**. Sequencing depth and coverage across the SPDV-1 **(e)**, -2 **(f)** and -3 **(g)** antigenomes. All elements proportionally match their actual lengths in viral antigenome.

In order to screen for ribavirin-resistant CDV progenies, the passage-7 rCDV-eGFP was subjected to 40 serial passages in ribavirin-treated cells. The foreign sequence, containing eGFP ORF and other elements, was not deleted from the rCDV-eGFP genome with passaging, as evidenced by the RT-PCR detection that showed five expected bands of amplicon size (1001 bp) (Fig. 1b). The RT-PCR result suggested that the foreign eGFP sequence was genetically stable during serial passages. Moreover, our previous study indicated that the P47 progeny harbored only two low-frequency SNMs in the eGFP ORF (Liu *et al*., 2021b). This result was opposite to our prior assumption that the eGFP sequence had the highest of mutation frequencies in the rCDV-eGFP antigenome during serial passaging, owing to the eGFP as a foreign protein theoretically uninvolved in virus-associated events. Interestingly, the L ORF had the highest of mutation frequencies among seven (N, P, eGFP, M, F, H and L) ORFs in the rCDV-eGFP antigenome (Liu *et al*., 2021b). Therefore, we postulated that there would be key single or complex SNMs involved in ribavirin-resistant characteristics of the P47 progeny. This prompted us to isolate three single variants to reveal their own SNMs in the L ORF.

The P47 progeny was subjected to one round of plaque purification to isolate three SPDVs with the brightest fluorescence (Fig. 1c) for further NGS analysis, as schematically shown in Fig. 1d. Here, to avoid the possible impact of Muller's ratchet on viral sequence (Clarke *et al*., 1993; Duarte *et al*., 1992), we performed only one round of single-plaque purification, followed by viral culturing in T25 flasks for NGS analysis, resultantly yielding acceptable data that were deposited in the NCBI SRA repository, accession number PRJNA786279. Landscapes of sequencing depths for these three samples were displayed in Fig. 1e, f and g. The average depths were 248×, 1172× and 2765× for the SPDV-1, -2 and -3 antigenomes, respectively. The rCDV-eGFP had a 16536-nt-long antigenome. Three SPDVs were determined to have an approximately 99.9%

of coverage range across the full-length antigenome. The SPDV-1, -2 and -3 showed 25, 31 and 38 SNMs in their antigenomes, respectively. Figure 2a to c and 2d to f revealed absolute and relative sequencing depths for these SNMs, respectively.

The SPDV-1 bore 25 SNMs, most of which underwent TSNSs (marked with "*" in Fig. 2d), and the others also had extremely high mutation frequencies, suggesting that the SPDV-1 was not only a SPDV, but also one SVDS for high-fidelity eGFP expression. There were four SPDV-1-specific SNMs (marked with "@" in Fig. 3a), unidentified in the other two SPDVs. Eight SNMs (marked with "#" in Fig. 3a) were recognized within the SPDV-1 to cause SAAMs in P, M, F and H proteins (Fig. 3d). There was no SPDV-1-specific SAAM in the seven proteins. In other words, all SAAMs within the SPDV-1 were also identified in the SPDV-2 and -3

Several obvious SNPs, such as C2331T (Fig. 2b,e) and G2257A (Fig. 2c,f), were found in the SPDV-2 and -3 antigenomes, implying that both progenies, albeit independently derived from two single plaques, were not SVDSs. The SPDV-2 had eight TSNSs (marked with "*" in Fig. 2e), and ten SPDV-2-specific SNMs (marked with "@"

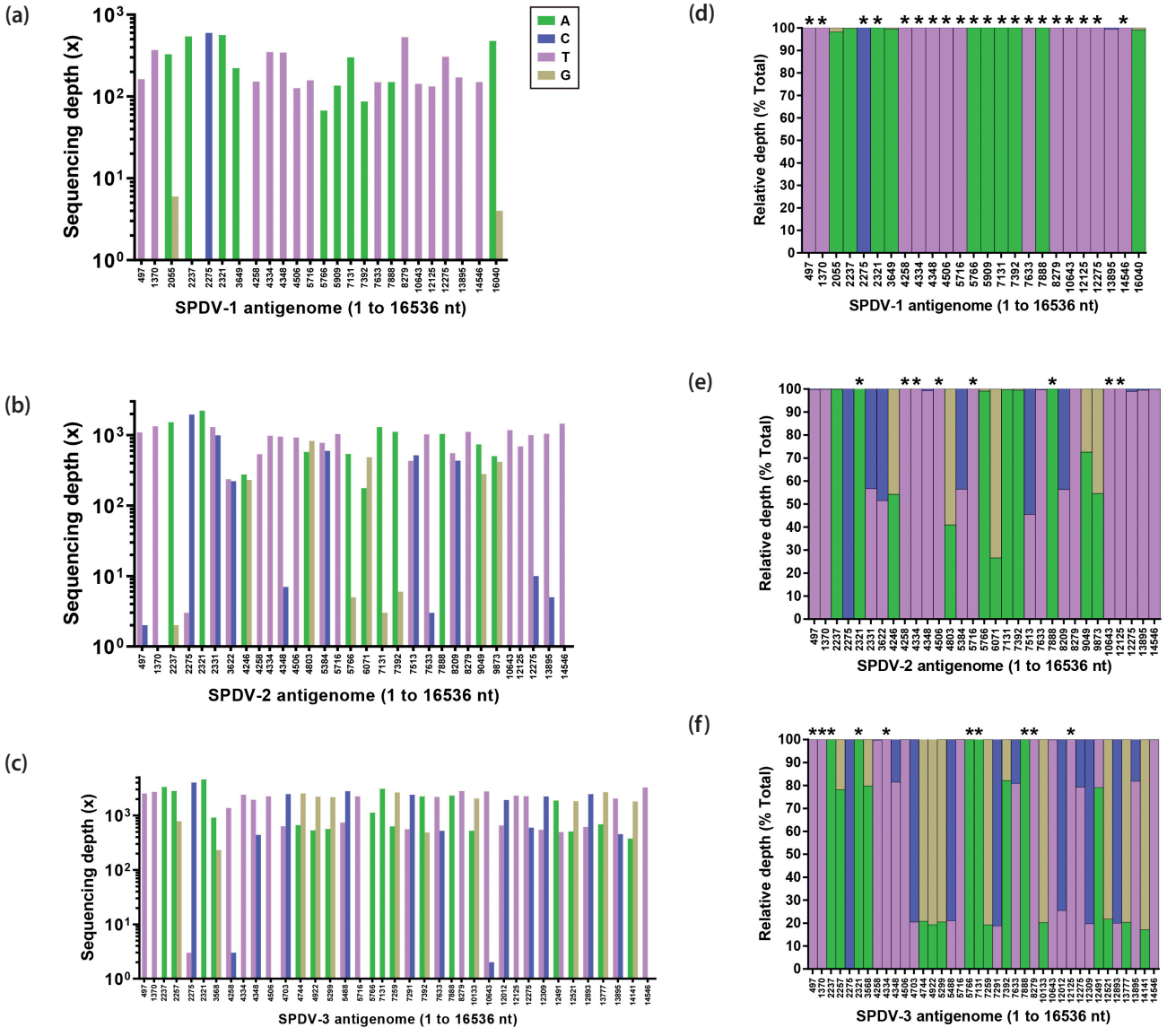

Fig. 2

Depths of next-generation sequencing for detectable positions with SNM in SPDV-1, -2 and -3 antigenomes

Absolute sequencing depths of SPDV-1 (**a**), -2 (**b**) and -3 (**c**) antigenomes. Relative sequencing depths of SPDV-1 (**d**), -2 (**e**) and -3 (**f**) antigenomes. *Relative depth of 100% at a given site.

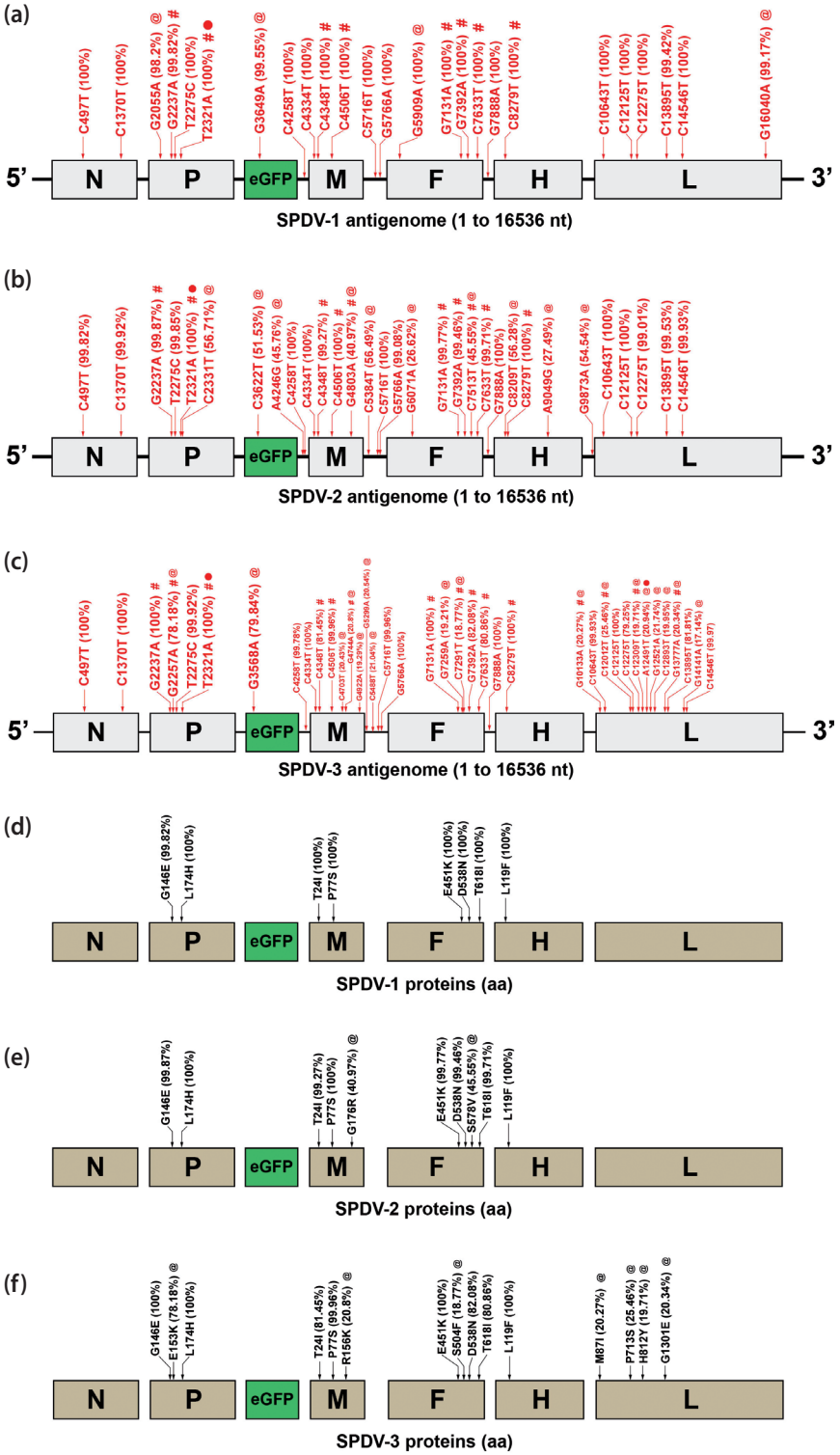

Fig. 3

Mutation profiles of SPDV-1, -2 and -3

Detectable SNMs in SPDV-1 **(a)**, -2 **(b)** and -3 **(c)** antigenomes. The proportion of elements does not exactly match them. Mutation frequencies are enclosed in brackets. •Transversion; #Nonsynonymous mutation; @SPDV-1, -2 or -3-specific SNM unidentified in the other two SPDVs. Detectable SAAMs in SPDV-1 **(d)**, -2 **(e)** and -3 **(f)** proteins. The proportion of elements does not exactly match them. Mutation frequencies are enclosed in brackets. @SPDV-2 or -3-specific SAAM unidentified in the other two SPDVs.

in Fig. 3b). The SPDV-3 had ten TSNSs (marked with "*" in Fig. 2f), and seventeen SPDV-3-specific SNMs (marked with "@" in Fig. 3c). Ten SAAMs were distributed in the P, M, F and H proteins of SPDV-2 (Fig. 3e). The SPDV-2 and -3 exhibited two and seven self-specific SAAMs (marked with "@" in Fig. 3e, f), respectively. Neither the SPDV-1 nor -2 showed any SAAMs in their own L proteins (Fig. 3d,e), whereas the SPDV-3 harbored four low-frequency SAAMs in its L protein (Fig. 3f).

The reason why the SPDVs were collected by plaque purification was that we attempted to use the eGFP as a screening marker to purify one or more SVDSs with potential in high-fidelity replication. Only the SPDV-1 was speculated to be an SVDS, and the other two should be separately a mixture of multiple SVDSs. Error-prone replication caused by viral RdRp is a primary driver forcing the evolution of RNA virus populations (Borderia *et al*., 2016). In the present study, we aimed to map a mutated L protein in the rCDV-eGFP for exploring a molecular mechanism in viral high-fidelity replication under selection pressure. Although four low-frequency SAAMs were recognized in the SPDV-3 L protein (Fig. 3f), we found no SAAM in L proteins of the SPDV-1 and -2 (Fig. 3d, e). If both SPDV-1 and -2 are high-fidelity progenies, no SAAM-related mechanism would be elucidated in their L proteins. In other words, it is their progenitor (CDV 5804P strain) that may be a high-fidelity strain. This is indeed a plausible conclusion, as evidenced by our recent report that has indicated the effect of Muller's ratchet unable to fix deleterious mutations in a single rCDV-eGFP population undergoing 38 plaque-to-plaque transfers (Lin *et al*., 2022).

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