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Molecular evolution and characterization of novel Seneca Valley virus (SVV) strains in South China

Minmin Wang[a,1], Lulu Chen[a,1], Shuonan Pan[a], Chunxiao Mou[a], Kaichuang Shi[c], Zhenhai Chen[a,b,d,*]

[a] College of Veterinary Medicine, Yangzhou University, Yangzhou, JS, China
[b] Jiangsu Co-Innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou University, China
[c] Guangxi Center for Animal Disease Control and Prevention, Nanning, China
[d] Joint International Research Laboratory of Agriculture and Agri-Product Safety, The Ministry of Education of China, Yangzhou University, China

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SUMMARY

Seneca Valley virus (SVV) is an emerging swine virus associated with porcine vesicular disease. From June to the end of 2017, five SVV strains were isolated from the swine herds in Guangdong, China. Complete genomic sequences of these newly discovered SVV strains were analyzed by genomic and phylogenetic analysis. The results revealed that these SVV strains could be grouped into five genetic branches together with most of other Chinese strains. Interestingly, it is for the first time that a stretch of 11-nucleotide insertion was found in the 5' UTR region of SVV GD04/2017 strain. Additionally, phylogenetic analysis based on the ORF and VP1 genes showed that the SVV GD06/2017 strain is significantly distinct from all previous Chinese SVV strains. Furthermore, the five SVV strains displayed similar growth kinetics in the ST-R cell line, while the SVV GD04/2017 and GD06/2017 strains presented relatively slower rates than the GD01/2017 and GD03/2017 strains. The findings of this study indicate the emergence of novel SVV strains in China, which would increase the knowledge about SVV genetic diversity and reinforce the importance of SVV surveillance.

1. Introduction

Seneca Valley Virus (SVV), also known as Senecavirus A (SVA), is the sole species in the genus Senecavirus of family Picornaviridae. SVV is a relatively new picornavirus member and most closely related to the genus Cardiovirus. The genome of SVV is a nonsegmented (+) ssRNA with about 7310 nucleotides (nt) in length (Hales et al., 2008). It contains a single open reading frame (ORF) encoding a polypeptide, L/1A(VP4)-1B(VP2)-1C(VP3)-1D(VP1)-2A/2B-2C/3A-3B-3C-3D, flanked by a 5′ nontranslated region (NTR) and a 3′ NTR. SVV was initially discovered in cell culture media in 2002, and speculated to be introduced by contamination of porcine trypsin or bovine serum used for cell culture (Hales et al., 2008). Recently, the speculation is strongly supported by the finding that a SVV strain (SVA-715) was detected in the porcine-derived trypsin by Ceva Animal Health, USA (Clampitt et al., 2016). The porcine idiopathic vesicular disease (PIVD) was characterized with vesicular lesions on coronary bands, snout, and oral cavities, as well as lameness in clinical signs. Historically, PIVD has been found in Australia, Europe, and USA (Munday and Ryan, 1982; Sensi et al., 2010; Amass et al., 2004). SVV has been linked to PIVD (Pasma et al., 2008; Singh et al., 2012), but was not well known in the veterinary field until 2015 with the sudden occurrence of vesicular disease in multiple pig herds in Brazil. SVV has been identified as the potential causative agent, as other suspected pathogens were not detected in the clinical samples (Leme et al., 2015; Leme et al., 2016). Clinical signs of vesicular disease caused by SVV infection are comparable to those by foot-and-mouth disease virus, vesicular stomatitis virus, swine vesicular disease virus, and vesicular exanthema of swine virus. However, it should be clarified here that the PIVD refers to a disease of unknown cause, which does not apply to the recently reported porcine vesicular disease associated with SVV infection. Therefore, it was provisionally named as SVV-associated vesicular disease

* Corresponding author at: College of Veterinary Medicine, Yangzhou University, Yangzhou, JS, China.
E-mail address: zhenhai@yzu.edu.cn (Z. Chen).
[1] co-first authors

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Table 1
Primers used in the RT-PCR for detection of SVV and amplification of complete virus genome.

Genome fragment	Primer	Primer Sequence (5′-3′)	Amplified fragment	Sizes(bp)
A	SVA-19F	GCCCTCATGCCCAGTCCTTCC	AB	2284
	SVA-433R	CACGTGGACTCTGTGTCGGAGCTTG		
B	SVV-311F	GCGGAAAGCGCTGTAACCACATGC		
	SVV-2303R	CAAAATGTCAGAGTGATTTGGATACACC		
C	SVV-2211F	GCCTCTCATCTCCTTCCCGATCAC	CD	2989
	SVV-4160R	GAGACTTTGGGCCAAATTAGTCTTCC		
D	SVV-3547F	GATCTAGTCACTCTGGCCTCTCTC		
	SVV-5200R	GACCGCAGCCTCAAAGCCCATGTC		
E	SVV-4918F	TGCGGTCGACGAGGCGCTTGTCG	EF	2328
	SVV-6802R	CCGTAGGCGATGATATCAACCATG		
F	SVA-6661F	GCTTACGGCGAGCGTCGCATCAAG		
	SVA-7246R	GACTGAGTTCTCCCAGAATCGCC		

Note: AB(19F-2303R), CD(2211F-5200R), EF(4918F-7246R).

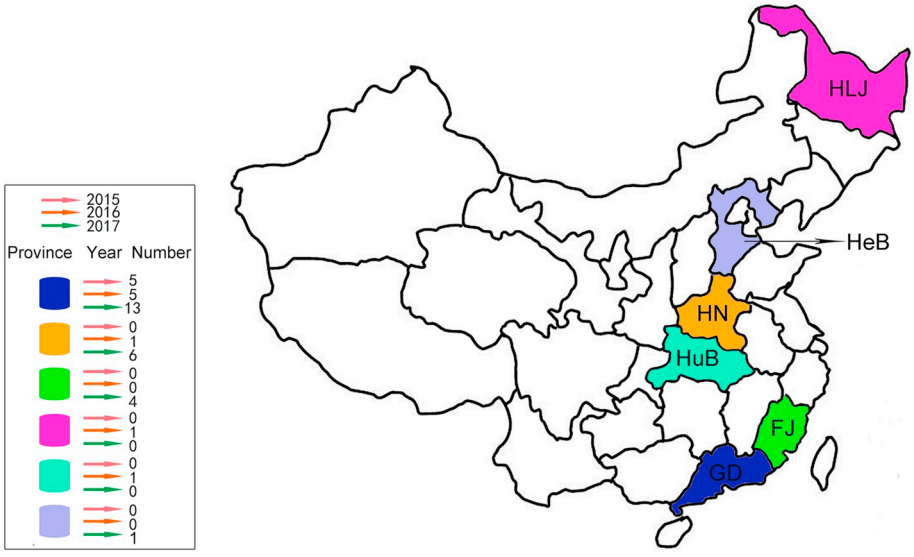

Fig. 1. Geographical distribution of SVV in China. Different intensity of colored areas represent total numbers of SVV strains reported during 2015 to 2017. The number of cases per year was counted with information of SVV sequences deposited in the GenBank. HLJ, Heilongjiang; HN, Henan; HeB, Hebei; HuB, Hubei; FJ, Fujian; GD, Guangdong.

(SAVD) in this study.

Soon after the outbreak of SAVD in Brazil, SVV infection has frequently been reported in pig farms in the U.S. in multiple states, including Iowa, South Dakota, Kansas, North Carolina, Minnesota, Louisiana, Illinois, and California (Gimenez-Lirola et al., 2016; Canning et al., 2016; Bracht et al., 2016; Guo et al., 2016; Hause et al., 2016; Wang et al., 2016). To date, SAVD has been reported in the following countries, China (Wu et al., 2017), Canada (Xu et al., 2017), Colombia (Sun et al., 2017), and Thailand (Saeng-Chuto et al., 2018) during the past two years. The first occurrence of SAVD in China was reported in pig herds in Guangdong province in 2015 (Wu et al.., 2017), and thereafter it was found in Hubei, Heilongjiang, Guangdong, Fujian and Henan provinces during 2016–2017 (Qian et al., 2016; Zhao et al., 2017; Luo et al., 2017; Zhang et al., 2018; Wang et al., 2017; Zhu et al., 2017a).

As a newly emerging viral pathogen in the field of porcine disease, it is crucial to monitor SVV epidemiology, understand the potential role of other species in virus transmission, develop rapid and specific diagnostics, and elucidate the virus pathogenesis. Updated knowledge about SVV will contribute to managing the newly discovered porcine virus. Here, we report five novel SVV strains from Guangdong from June to the end of 2017. Genomic analysis shows that they are distinct from the other Chinese strains, suggesting that novel SVV strains emerged in China. Furthermore, the growth characteristic of the five SVV strains in vitro was also investigated in this study, which will provide valuable information for further research.

2. Materials and methods

2.1. Virus isolation and genomic sequencing

From June to the end of 2017, a number of FMDV-suspected tissue

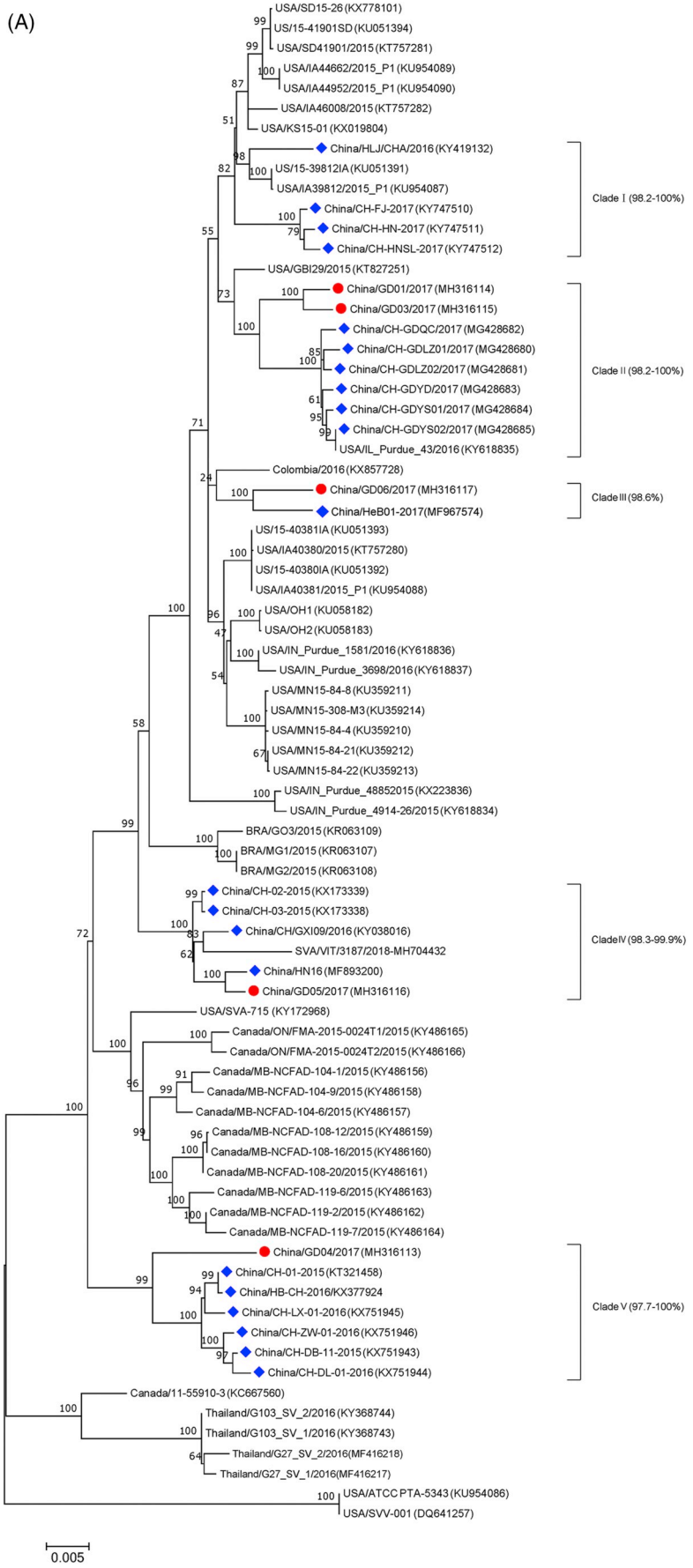

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Fig. 2. Phylogenetic analysis of SVV strains. The genomic sequences of GD01/2017, GD03/2017, GD04/2017, GD05/2017, GD06/2017, and the other SVV strains available in GenBank were included in the alignment. The tree was constructed based on analysis of ORF(A) and VP1(B) gene sequences of SVV strains, following the distance-based maximum likelihood method using MEGA version 7.0 software. The scale bar indicates nucleotide substitutions per site, and bootstrap values are shown at the nodes.

samples from pigs with clinical appearance of vesicles on snouts and hooves were collected in Guangdong and Guangxi provinces. Viral RNA was extracted from the samples using TaKaRa MiniBEST Viral RNA/DNA Extraction Kit (Takara, Beijing, China) according to the manufacturer's instructions, and reverse transcribed to cDNA for detection of SVV, FMDV, VSV, all of which can cause vesicular diseases, by RT-PCR using specific primers described previously (Lung et al., 2011). An IFN-α/β receptor knockout ST-R cell line (ST-R; originally named IFNAR2–6) was developed by gRNA-guided CRISPR/Cas9 technology and used to isolate SVV strains in this study (Zhu et al., 2017a,b). The virus strains were harvested 2–3 days post infection with appearance of significant cytopathic effects (CPE).

The SVV RNAs from cell culture were extracted and reverse transcribed to cDNAs by RT-PCR. A panel of primers was designed based on the relatively conserved regions of the SVV genome (Table 1). Three to six overlapping genomic fragments covering nearly complete genomes of SVV strains were amplified by PCR with PrimeSTAR DNA polymerase (TaKaRa, Beijing, China). Generally, each PCR reaction was set up with 20 μL water, 25 μL PrimeSTAR Max Premix, 10 pmol each primer, and 3 μL sample cDNA. PCR was performed as follows: 98 °C, 3 min; 33 cycles of (98 °C, 10s; 55 °C, 10s; 72 °C, 3 min); 72 °C, 5 min. The resulting PCR amplicons were cloned into pMD19-T vector (TaKaRa, Beijing, China). Positive plasmid clones were sent for sequencing by Sanger sequencing technology. Thereafter, the genomic sequences of five SVV strains were assembled in the Lasergene SeqMan Program (DNASTAR, USA), and then deposited in GenBank.

2.2. Sequence alignment and phylogenetic analysis

The complete genomic sequences of five newly identified SVV strains and 67 SVV strains from GenBank were aligned using Clustal W (http://www.clustal.org) and Clone Manager 9.0 (http://www.scied.com/pr_cmpro.htm). Phylogenetic trees were constructed using MEGA version 7.0 based on the maximum likelihood method with 1000 bootstrap replicates to calculate pairwise distances to strengthen the robustness of the results.

2.3. Virus growth kinetics in vitro

Growth kinetics were examined by infecting ST-R cells with SVV strains at a multiplicity of infection (MOI) of 0.01. Infected cells were collected at 6, 12, 24, 36, 48, and 60 hours post infection (hpi). Virus titers were determined by CPE observation and quantified as 50% tissue culture infective dose ($TCID_{50}$) mL^{-1}. Plaque morphology of the five SVV strains was compared by plaque assay on ST-R cells. Confluent cell monolayers were infected with virus at 0.01–0.0001 MOIs. After 1–2 h, the cell culture supernatant was removed and an agar overlay was applied. Plaques were identified after 2 days incubation at 37 °C by staining with 0.5% crystal violet.

2.4. Indirect immunofluorescence assay (IFA)

Virus-infected ST-R cells were fixed with 4% formaldehyde in PBS (pH 7.4) for 8–10 min, and then permeabilized with 0.1% Triton X-100 and 2% BSA in PBS for 30 min at room temperature. Fixed cells were incubated for 1 h with the anti-SVV VP1 mouse monoclonal antibody (made in our lab) at dilution of 1:400 at 37 °C. DyLight 488-conjugated goat anti-mouse monoclonal antibody (1:1000 dilution; Abbkine, Inc.) was used as the secondary antibody. Cell nuclei was stained with 4, 6-diamidino-2-phenylindole-dihydrochloride (DAPI) performed as suggested by the manufacturer (Molecular Probes). Cell preparations were imaged under an confocal microscopy (Leica). Images were acquired with an objective magnification (100 ×) and then processed with Adobe Photoshop 6.0 software.

3. Results and discussion

The current study reported that five novel SVV strains were identified by RT-PCR in pig farms in Guangdong, but not in Guangxi. However, other pathogens including FMDV and VSV were not detected in the clinical samples. Virus isolation was performed in ST-R cell line and the five SVV strains from the samples of different farms were obtained with no more than 3 passages in cell culture. Nearly complete genomes of the five SVV strains were sequenced. Genomic sequences of GD01/2017, GD03/2017, GD04/2017, GD05/2017, and GD06/2017, were then submitted to NCBI (GenBank accession numbers: MH316114, MH316115, MH316113, MH316116, MH316117). Nucleotide identities between the genomes of these five SVV strains ranged from 96.4 to 99.4%, and the amino acid identities of polypeptides from 98.1% to 99.4%. These results also now update the information about epidemiology of the SVV strains in China (Fig. 1).

Genetic analysis based on the ORF revealed that the GD01/2017 and GD03/2017 strains shared the highest homology of genomic identity with each other (99.4%), and were closely related to USA/GBI29/2015 (98.6%) and most of the GD strains including CH-GDLZ01-2017, CH-GDQC-2017, CH-GDYS01-2017 (98.2%–98.4%). Also, the GD04/2017, GD05/2017 and GD06/2017 strains show the highest similarity in genomic identity to the CH-01-2015, HN16, and Columbia-2016 respectively (98.1%, 99.5%, 98.3%). When the five SVV strains were compared with the other Chinese SVV strains, GD06/2017 displayed the lowest homology of genomic identity to the others (96.2%–98.1%).

In addition, phylogenetic analysis based on the ORF (Fig. 2A) and VP1 (Fig. 2B) genes show that the Chinese SVV strains can be mainly grouped into five genetic branches, provisionally named clade-I (HLJ-CHA-2016, CH-FJ2017, US-1539812A), clade-II (CH-GDLZ01-2017, CH-GDYD-2017, USA-IL_Purdue), clade-III (HeB01–2017), clade-IV (CH-GXI09-2016, HN16), and clade-V (CH-01-2015, CH-DL-01-2016). The GD01/2017 and GD03/2017 belong to clade-II, while the GD04/2017, GD05/2017, and GD06/2017 are within clade-V, IV, III respectively. The results indicate that these Chinese SVV strains could not be simply clustered together according to country, but they could be assigned to the five genetic clades differentiating from most of SVV strains from the other countries. It also suggests the SVV strains currently circulating in China may have different origins.

Interestingly, sequence analysis based on available 5′UTR region revealed that there were separately 1-nucleotide and 11-nucleotide insertions in the 5′UTR region of GD04/2017 (Fig. 3). As we know, this is the first report showing the existence of a stretch of nucleotide insertion in the 5′UTR region of SVV. Further studies are required to

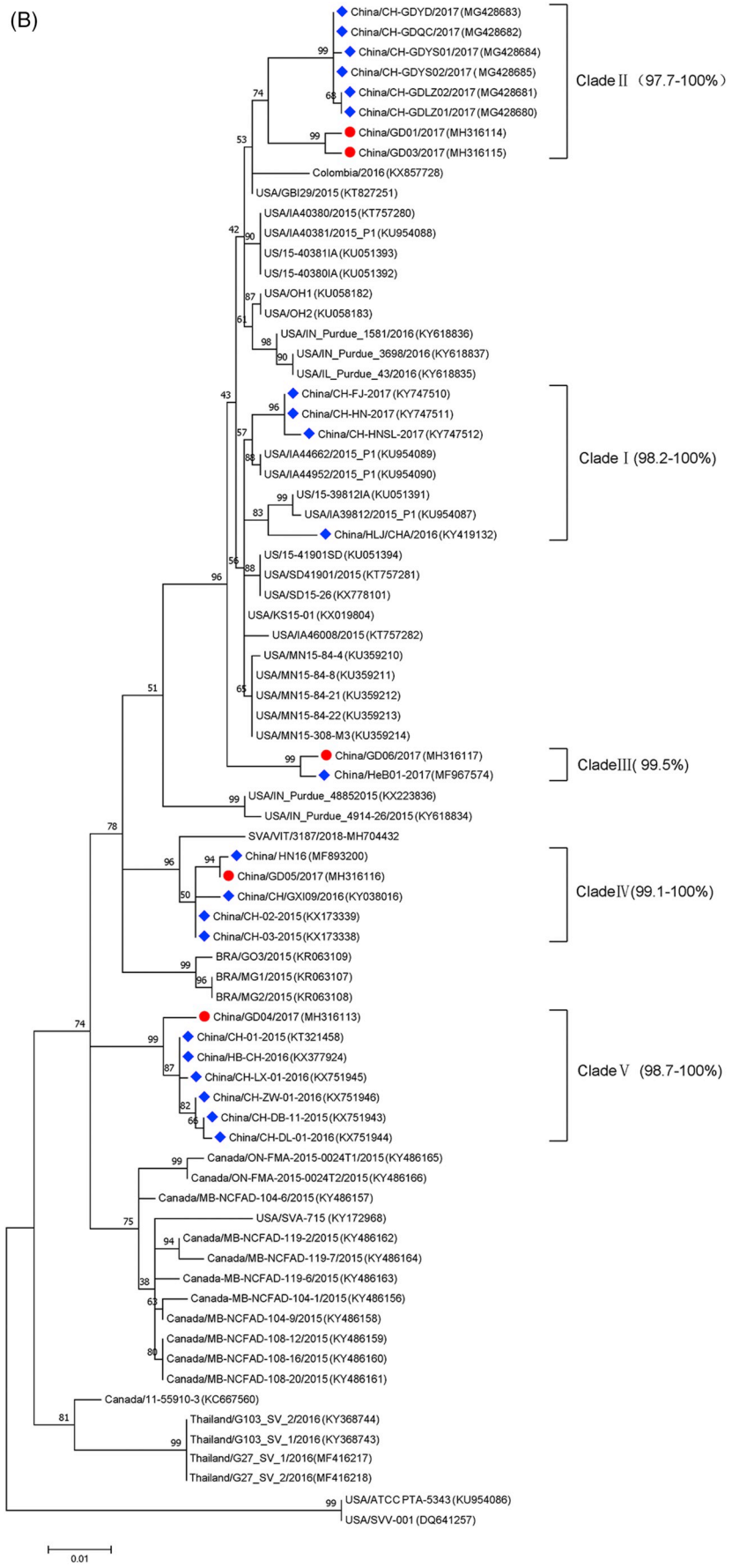

Fig. 2. (*continued*)

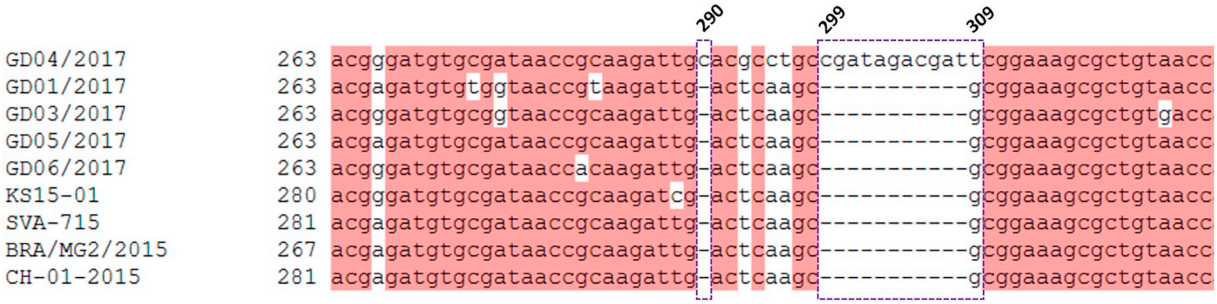

Fig. 3. Sequence alignment of partial SVV 5′ UTR region. The 5′UTR regions of GD01/2017, GD03/2017, GD04/2017, GD05/2017, GD06/2017, and the other four SVV strains were aligned. A series of numbers on the left indicate the positions of bases at the start of the partial 5′UTR regions of SVV strains respectively. The boxed regions denote the inserted nucleotides in the 5′UTR region of GD04/2017.

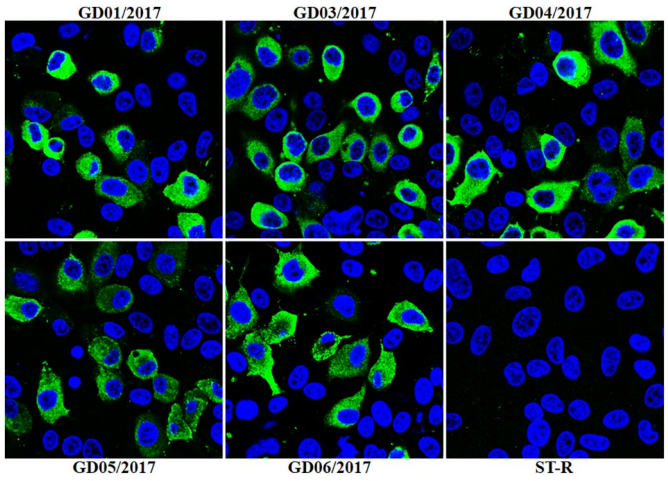

Fig. 4. Detection of VP1 protein expression in the SVV-infected cells by indirect immunofluorescence assay. ST-R cells were infected with one of five SVV strains and fixed at 12 hpi. Cells were stained with anti-SVV VP1 mouse monoclonal antibody. DyLight 488-conjugated goat anti-mouse antibody was used as secondary antibody. Images were obtained by confocal microscopy using a 100× objective.

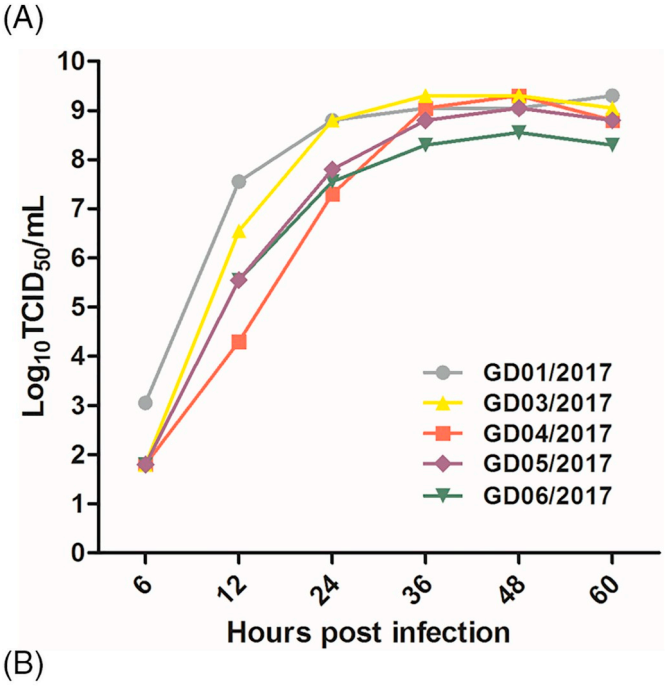

Fig. 5. In vitro characterization of the five SVV strains. (A) Growth kinetics of the five SVV strains. ST-R cells were infected at the MOI of 0.01 with SVV strains. At 6, 12, 24, 36, 48, and 60 hpi, cell supernatants were harvested and virus titers were determined by CPE method, which scores each well as positive or negative depending on the presence of SVV-induced CPE. The results shown are mean values from three replications of the experiment, and viral titers are expressed as $TCID_{50}$ mL^{-1}. (B) Plaque morphology of five SVV strains. Confluent cell culture monolayers were infected with the SVV strains at 0.01–0.0001 MOIs. After 2 h, the cell culture supernatant was removed and an agar overlay was applied. Plaques were pictured after two days incubation at 37 °C and stained with 0.5% crystal violet.

determine whether the nucleotide insertions in the 5′UTR region affect biological function of the virus. Furthermore, indirect immunofluorescence assay confirmed the five SVV strains grew well in the ST-R cells (Fig. 4). Growth kinetics analysis show that the GD01/2017 and GD03/2017 grew more rapidly than the GD04/2017, GD05/2017, and GD06/2017 in the ST-R cells at early time points. However, mean peak viral titers of the GD01/2017, GD03/2017, GD04/2017 and GD05/2017 were comparable with each other during 36–60 hpi, whereas the GD06/2017 has a slightly lower peak titer of about 0.5–1 log (Fig. 5A), which was also confirmed by virus plaque assay (Fig. 5B). The results show that the GD04/2017 and GD06/2017 have weakened growth abilities in ST-R cells, suggesting both strains may be attenuated in virulence. Trials on pigs are necessary to investigate pathogenicity of the SVV strains.

In conclusion, five novel SVV strains were isolated and characterized in this study. The results will contribute to increasing the knowledge about SVV epidemiology, reinforce the importance of SVV surveillance in China, as well as develop strategies to prevent the spread of SAVD.

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Conflict of interest

All authors have declared no conflict of interest.

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