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A novel VP2/VP3 recombinant of Senecavirus A isolated in northern China

Yingru Ma[1, a*], Limei Zheng[1, a], Chen Li[2,a] Xiaoyan Wu[2], Jianli Shi[2], Chang Liu[2], Hong Han[2], Shaojian Xu[2], Nataliia Hrabchenko[2], Mengjie Huang[3], Jianhua Qiu[3], Jun Li[1,2*] and Xianjie Han[1*]

[1]College of Veterinary Medicine, Qingdao Agricultural University, Qingdao, China.
[2]Shandong Provincial Key Laboratory of Animal Disease Control & Breeding, Institute of Animal Science and Veterinary Medicine Shandong Academy of Agricultural Sciences, Jinan, China;
[3]College of Animal Science and Veterinary Medicine Shandong Agricultural University, Taian, China.
[a]These authors contributed equally to this work

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Abstract

Senecavirus A (SVA), previously called the Seneca Valley virus, is the only member of the genus Senecavirus within the family *Picornaviridae*. This virus was discovered as a serendipitous finding in 2002 and named Seneca Valley virus 001 (SVV-001). SVA is an emerging pathogen that can cause vesicular lesions and epidemic transient neonatal a sharp decline in swine. In this study, an SVA strain was isolated from a pig herd in Shandong Province in China and identified as SVA-CH-SDFX-2022. The full-length genome was 7282 nucleotides (nt) in length and contained a single open reading frame (ORF), excluding the poly (A) tails of the SVA isolates. Phylogenetic analysis showed that the isolate shares its genomic organization, resembling and sharing high nucleotide identities of 90.5% to 99.6%, with other previously reported SVA isolates. The strain was proved by in vitro characterization and the results demonstrate that the virus has robust growth ability in vitro. The recombination event of the SVA-CH-SDFX-2022 isolate was found and occurred between nts 1836 and 2710, which included the region of the VP2 (partial), and VP3 (partial) genes. It shows the importance of faster vaccine development and a better understanding of virus infection and spread because of increased infection rates and huge economic losses. This novel incursion has substantial implications for the regional control of vesicular transboundary diseases, and will be available for further study of the epidemiology of porcine SVA. Our findings provide useful data for studying SVA in pigs.

Key words: senecavirus A; pigs; virus isolation; IFA; evolutionary analysis; Western blotting

Introduction

The Senecavirus A (SVA) is a reemerging single-stranded RNA virus, and is the only member of the genus Senecavirus within the family Picornaviridae, formerly named Seneca Valley Virus (SVV). The first SVA strain SVV-001 was initially identified in a cell culture of human retinal cells called PER.C6 in the United States (US) in 2002 (HALES et al., 2008; GUO et al., 2016). SVA has some features

*Corresponding author:
Xianjie Han, Department of College of Veterinary Medicine, Qingdao Agricultural University, Qingdao, China.E-mail: Hanxianjie2006@ 163.com; Jun Li, Department of Shandong Provincial Key Laboratory of Animal Disease Control & Breeding, Institute of Animal Science and Veterinary Medicine Shandong Academy of Agricultural Sciences, Jinan, China; junli79@163.com

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that are shared among all picornaviruses, such as a non-enveloped capsid about 25 to 30 nm in diameter with icosahedral symmetry, a linear single-stranded ribonucleic (RNA) genome of positive polarity and approximately 7. 3 kb (VENKATARAMAN et al., 2008). The viral genome is similar to other members of the Picornaviridae family. The SVA genome encodes a single open reading frame expressed as a polyprotein precursor. The 5'end of the polyprotein is flanked by an untranslated region (5'UTR) and a short polyadenylated untranslated 3'end (3'UTR), followed by a poly (A) tail (WILLCOCKS et al., 2011). The SVA polyprotein was cut into 12 different proteins by viral proteases, following a standard L-4-3-4 layout. The viral genome consists of a leader protein (L) and three major protein regions, named polyproteins 1 to 3 (P1, P2, and P3), respectively (GUO et al., 2016).

SVA has been spreading covertly in pigs in the United States since 1988. In June 2007, SVA infection was detected in trailer load of 187 Canadian market hogs that arrived at a harvest facility in Minnesota from Manitoba. (PASMA et al., 2008). Since then, SVA infections have been reported in China, Vietnam, the United States, Brazil, Colombia, and many other countries (LEME et al., 2015; VANNUCCI et al., 2015; LEME et al., 2016; MONTIEL et al., 2016; LEME et al., 2017; ARZT et al., 2019). Before 2014, little research had been done on the viral characteristics and infection of the disease, including epidemiology, transmission and pathogenesis, pathology, clinical features, and diagnostic techniques. This shows that this disease is indeed a global problem that has not yet been resolved. In this study, an SVA strain SVA-CH-SDFX-2022 was isolated and identified from a pig farm in Feixian County, Shandong Province in 2022, and the full-length genome of the SVA isolate was identified and compared with previously reported isolates. SVA can replicate and proliferate in BHK-21 cells, and produce CPE after infection. The virus one-step growth curve, electron microscopy, and quantitative PCR showed that the strain grew well in BHK-21 cells, and the virus titer reached $10^{7.8}$. An SVA strain was successfully isolated and its biological characteristics were studied in this study.

Materials and methods

Cell reagents and clinical samples. The virus was grown in Baby hamster kidney 21 (BHK-21) cells (maintained in our laboratory) cultured in Dulbecco's modified Eagle's medium (DMEM) (1×) (Gibco) supplemented with 10% fetal bovine serum (Biological Industries, Israel) grown at 37 °C under 5% CO_2.

SVA Virus isolation. The SVA was isolated from a swine serum sample from the Shandong province. Total genomic RNA was extracted from the vesicular lesion sample and cell culture supernatants using a Simply P Total RNA Extraction Kit (BioFlux, Hangzhou, China), and converted to cDNA with HiScript® III RT SuperMix (+gDNA wiper) (Vazyme, Nanjing, China). The RT-PCR assay was performed using the following cycling conditions: 42°C for 2 min for the genome DNA removal, followed by 37 °C for 15 min and 85 °C for 5s for the RT reaction. 1μL of cDNA was used as the template for the subsequent PCR analysis with constructed SVA specific primers (Table 1). The expected size of the PCR product on electrophoresis gel is also shown on Table 1. Each fragment was amplified by PCR with the 2 × Phanta® Flash Master Mix(Dye Plus) (Vazyme, Nanjing, China) and purified PCR using a Gel Extraction Kit (Omega, Guangzhou, China), and sent to the Tsingke Biotechnology company for sequencing. The PCR assay was performed using the following cycling conditions: 98 °C for 30s, followed by 30 cycles at 98 °C for 10s and 58 °C for 5s, 72 °C 4-5s/kb, with a final extension at 72 °C 1min. The virus was grown at 37 °C with 5%CO_2 in DMEM supplemented with 10% fetal bovine serum in Baby hamster kidney (BHK-21) cells. The 2 times supernatants were harvested for further propagation until a cytopathic effect (CPE) was observed. The harvested CPE-positive cell cultures were examined for SVA with qRT–PCR using HiScript® III U+ One Step qRT-PCR Probe Kit. (Vazyme, Nanjing, China) The four fragments were amplified with PCR using 2×Phanta Flash Mix(Dye Plus) (Vazyme, Nanjing, China). The complete genome of the virus was divided into four overlapping fragments with specific primers (Table 1). The whole genome of the newly isolated

SVA was sent them to the Tsingke Biotechnology company for sequencing. The only SVA-positive virus isolated was designated SVA-CH-SDFX-2022 (Genbank no. OP714192).

Table 1. Primers used for the amplification and identification of the SVA isolate.

Primer	Sequencing (5'-3')	Amplicon size(bp)	Usage
SVA-3D-U	5' CAGAGTACGCTCATCGGGTTTTC 3'	1002	For diagnostics assay
SVA-3D-L	5' AGGAGAGCATGGCTTCCAAATTC 3'		
SVA-A-U	5' CCTGCGTCGCCAAAGGTGTTAGC 3'	1773	Amplification of the gene fragments of SVA isolate
SVA-A-L	5' CCTTACAGAGAATGTAATTTCTG 3'		
SVA-B-U	5' CCTGGACCCTCCTCGTTATGGTG 3'	2108	Amplification of the gene fragments of SVA isolate
SVA-B-L	5' CGGCTATTTGGTTCCAGTCTTTG 3'		
SVA-C-U	5' GGGGGAAGGCTCCATCGCCTTGG 3'	1762	Amplification of the gene fragments of SVA isolate
SVA-C-L	5' AGTAAAATGAACCGTCTGGAAAG 3'		
SVA-D-U	5' AAGTGGTCGTCCCCATTACCTTC 3'	2024	Amplification of the gene fragments of SVA isolate
SVA-D-L	5' TCTCCCAGAATCGCCGGCAGCAC 3'		

*Sequencing and phylogenetic analyses***.** A phylogenetic genomic analysis was performed with the MEGA version 7.0 program using the neighbor-joining method and bootstrap validation with 1,000 replications (KUMAR et al., 2016). To analyze possible recombination signals among global SVA, in this study we used RDP4 software to analyze the reorganization event(s) of SVA-CH-SDFX-2022 using different algorithms (MARTIN et al., 2015) .

Indirect immunofluorescence assay (IFA). BHK-21 cells were infected in 96-well plates with SVA-CH-SDFX-2022 (MOI=0.01). At 18 h post-infection (h.p.i.), the monolayer cells were fixed with ice-cold anhydrous ethanol for 15min at 4°C. After 1 h with SVA VP2 monoclonal antibodies which were prepared in our laboratory by hybridoma technology and incubated with 100μL (diluted 1:50) for 1 h at 37 *°C,* the cell monolayers were washed with PBS three times. After incubating with fluorescein isothiocyanate (FITC)-conjugated goat antibody against mouse IgG (H + L) (1:200) for 1 h, the cells were observed with an inverted fluorescence microscope.

Western blotting analysis. Cell lysates were separated with 12.5% SDS-PAGE and then transferred to nitrocellulose membranes (Pall, 66485),. The membranes were incubated with SVA VP2 monoclonal antibodies (diluted 1:500) and then incubated with horseradish-peroxidase-labeled goat anti-mouse IgG antibody (Sigma, St Louis, MO, USA) (diluted 1:200). Proteins on the membranes were detected using a SuperSignal West Pico PLUS Chemiluminescent Substrate Kit (ZETA) and visualized using chemiluminescence apparatus (ImageQuant LAS500.GE).

Viral growth kinetics curves. BHK-21 cells were cultured in 6-well tissue culture plates and incubated with the SVA-CH-SDFX-2022 at an MOI of 0.01. The infected cells were collected at 6, 12, 18, 24, 36, and 48 h.p.i. The viral titers of the supernatants were determined by a $TCID_{50}$ assay (BROWN , 1964). Mean values and standard deviations were calculated from the results of three independent experiments. Finally, a one-step growth curve was constructed.

Transmission electron microscopy. Negatively stained SVA-CH-SDFX-2022 was examined by transmission electron microscopy (TEM). Briefly, the SVA virus was purified with sucrose density gradient centrifugation. The virus was pelleted from the supernatant, resuspended in 500 μL of 0.01 M PBS (pH 7.2), and adsorbed onto Formvar-coated grids. The grids were then stained with 1% phosphotungstic acid (pH 7.0) and observed under TEM (Hitachi HT7700 TEM, Japan).

Results

SVA Isolation, genomic sequence and polygenetic analyses. In the current study, we used a pair of SVA-specific primers for RT-PCR (Table 1), and the SVA isolates were designated as SVA-CH-SDFX-2022. The complete genome sequence of SVA-CH-SDFX-2022 was determined by overlapping PCR and rapid amplification of cDNA ends. Our results showed that the complete genomic sequence of SVA-CH-SDFX-2022 was 7282 nt in length, with a 5'UTR of 668nt and 3'UTR of 66 nt. (BankIt 2635898 OP714192). It had the standard picornavirus L-4-3-4 genome constitution (Table 2) , without insertions or deletions.

Table 2. The genome organization of the SVA isolates.

Gene	Nucleotides location	Amino acids number
5'UTR	1-668	-
L	669-905	79
VP4	906-1118	71
VP2	1119-1970	284
VP3	1971-2687	239
VP1	2688-3479	264
2A	3480-3506	9
2B	3507-3890	128
2C	3891-4856	322
3A	4857-5125	89
3B	5126-5192	22
3C	5193-5825	211
3D	5826-7214	463
3'UTR	7216-7282	-

The nucleotide sequences of the SVA-CH-SDFX-2022 were compared to the other 106 strains available in the GenBank. Phylogenetic analysis showed that the SVA-CH-SDFX-2022 shares high nucleotide identities, 90.5% to 99.6%, with other SVA strains at the complete genome level. Phylogenetic analysis based on complete genomic sequences indicated that SVA-CH-SDFX-2022 was clustered within the USA SVA branch (Fig. 1). Notably, compared to the USA strains, the SVA-CH-SDFX-2022 has the highest homology (98.2%) with US-2015-MN164664.1. The nucleotide homology with the Thai strain was 94.8%~95.0%, the nucleotide homology with the Colombian strain was 97.5%, the nucleotide homology with the Brazilian strain was 96.9%, and the nucleotide homology with the Canadian strain was 95.2%~95.6%, Among them, the proportion of SVA-CH-SDFX-2022 isolate to Chinese isolate SVA-CH-SDGT-2017 was 99.6%. And the proportion of SVA-CH-SDFX-2022 isolate to Chinese isolate SVA/HLJ/CHA/2016-KY419132.1 was 99.5%.

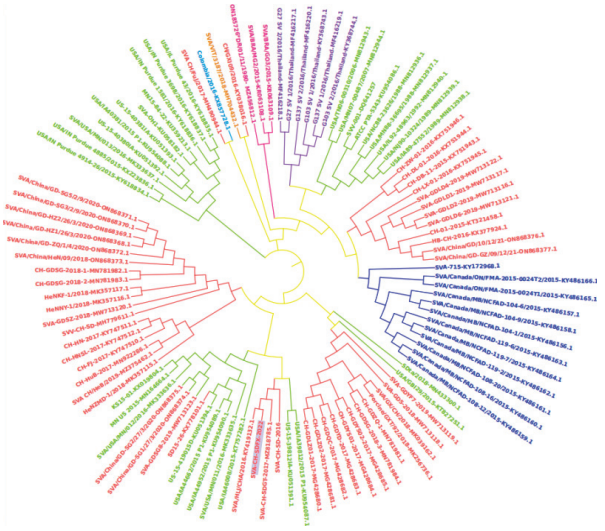

Fig 1. Phylogenetic analysis of SVA strains.
The tree was constructed on the basis of the analysis of the complete genome, following the neighbor-joining method and bootstrap validation with 1,000 replications using the MEGA version 7.0.

All recombinant analytical methods with all automated runs available in RDP4 were employed and 33 recombination events were detected in total (MARTIN et al., 2015). Among them, the SVA isolates SVA-CH-SDFX-2022 were predicted to be recombinant and indicated that the SVA/USA/MN/011/2016-MK333635 strain, which was isolated in the United States in 2016, was suspected as the major parent, whereas the minor parent was the SVA-GX-CH-2018 which was most closely related to the SVA/USA/MN/011/2016-MK333635 strain. The recombination event of the SVA-CH-SDFX-2022 isolate occurred between nts 1836 and 2710, which included the region of the VP2 (partial), and VP3 (partial) genes (Fig. 2).

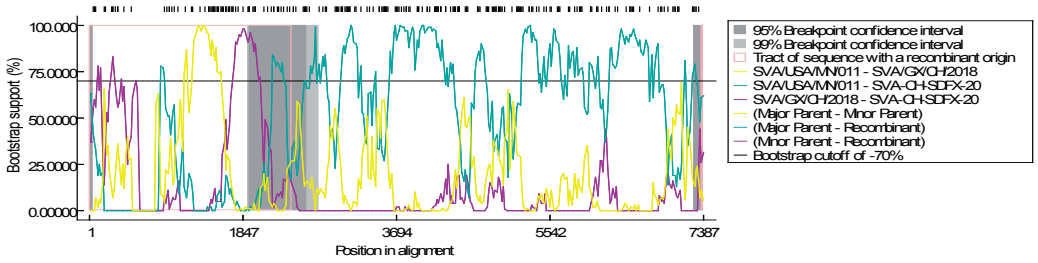

Fig 2. Recombination analysis of strain SVA-CH-SDFX-2022.
The x-axis shows the position in the alignment, and the y-axis shows the bootstrap support (%).

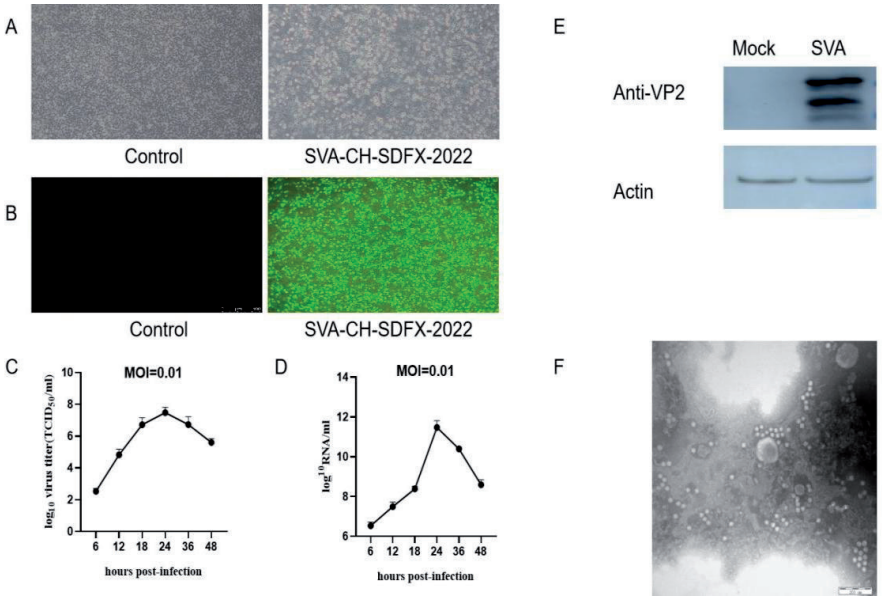

Fig 3. Isolation and identification of SVA strain SVA-CH-SDFX-2022.
(A)Cytopathic effects (CPE) of the SVA-CH-SDFX-2022 isolates in infected BHK-21 cells. CPE effects, such as rounding, shrinkage, and nonadherence, were observed in BHK-21 cells infected with SVV-CH-SD at 18hpi. No CPE was observed in uninfected cells. (B)BHK-21 cells were fixed and analyzed with immunofluorescence, using an anti-VP2 antibody. Fluorescent signals were detected in the SVA-infected BHK-21 cells, but only nonspecific fluorescence was observed in uninfected-BHK-21 cells. (C)One-step growth curves for the viruses and viral titers were determined with a $TCID_{50}$ assay. Values are presented as the means ± SD of three independent experiments. (D)qRT-PCR was used to detect genomic copy numbers in the same sample. The standard deviation of the mean viral titer is shown (n = 3). (E)Viral protein VP2 was detected with western blotting, using an anti-VP2 monoclonal antibody. (F)SVA-CH-SDFX-2022 particles were observed with TEM, with a diameter of approximately 27–30 nm.The SVA virus was purified with sucrose density gradient centrifugation and the particles were stained with 1% phosphotungstic acid.

Cell culture of the virus. In the current study, we isolated and identified an SVA strain SVA-CH-SDFX-2022 with BHK-21 cells. After the third blind passage, CPEs were observed at 18h.p.i., including rounding, shrinkage, and the loss of adherence (Fig. 3A).

In vitro characterization of the SVA-CH-SDFX-2022. The IFA showed fluorescence, reflecting the expression of the viral VP2 in the cytoplasm of the infected cells (Fig. 3B). The infected cells were collected at 6, 12, 18, 24, 36, and 48 h.p.i.. The results showed that the maximum titer of the virus was $10^{7.8}$ $TCID_{50}$/mL at 24h.p.i. (Figure. 3C). Then the virus titer began to decrease until 48h when it was $10^{5.8}$ $TCID_{50}$/mL (Figure. 3D). Western blotting also confirmed the expression of SVA VP2 in the infected cells, and the molecular weight of VP2 protein was about 34-37 kDa (Figure. 3E). The virions of the isolate had icosahedral symmetry and a diameter of 27-30 nm (Figure. 3F). All these results demonstrate that the SVA-CH-SDFX-2022 strain has robust growth ability *in vitro*.

Discussion

Sudden outbreaks and the spread of swine infectious diseases can lead to severe economic losses in the pig industry. The SVA is an important clinical and economic infection affecting farm animals. In 2015, the first case of an SVA-infected pig was reported in Guangzhou, China (WU et al., 2017). The clinical manifestations were infected pigs with nasal ulcers, anorexia, lameness, and the acute death of newborn piglets. In recent years, clinical cases of SVA infection have been increasing in many regions of China, which has brought great harm to the pig industry. Although some progress has been made in the study of SVA, the specific characteristics of the SVA virus, including its biological and molecular evolution characteristics, remain to be further studied.

In this study, we isolated and identified an SVA strain SVA-CH-SDFX-2022 with BHK-21 cells. Molecular phylogenetic analyses are important in revealing the origins of viruses. Here, our results showed that the complete genomic sequence of SVA-CH-SDFX-2022 was 7282 nt in length (GenBank OP714192). It had the typical picornavirus L–4–3–4 genome layout. Phylogenetic analysis showed that it has homology with 8 strains of 94.4% -97.1% reported in Canada (SVA/Canada/MB/NCFAD-104-1/2015-KY486156.1), (SVA/Canada/ON/FMA-2015-0024T2/2015-KY486166.1), Brazil (SVA/BRA/GO3/2015-KR063109.1), (SVA/BRA/MG2/2015-KR063108.1) and the United States (USA/TN06-00310/2006-MN812943.1), (USA/MN07-00487/2007-MN812944.1), (USA/IN_Purdue_4914-26/2015-KY618834.1), (USA/IN_Purdue_1581/2016-KY618836.1). Our research identified the SVA isolate SVA-CH-SDFX-2022 in 2022 which shared 90.5 % - 99.6 % with Chinese isolates (Figure. 1) (CH/GXI09/2016-KY038016.1), (CH-DB-11-2015-KX751943.1), (CH-FJ-2017-KY747510.1), (SD15 -26-KX778101.1), (SVA/HLJ/CHA/2016-KY419132.1), (SVA-CH-SD-2016), (SVA-CH-SDGT-2017-MZ818785.1). SVA-CH-SDFX-2022 shared the highest homology (99.6%) with strain SVA-CH-SDGT-2017 (MZ818785). Both SVA-CH-SDGT-2017 and SVA-CH-SDFX-2022 strains were isolated in the same laboratory and were from Shandong province. The recombination analysis showed that SVA-CH-SDFX-2022 was probably the major parent of recombinant virus USA/MN/011/2016 (MK333635), and SVA-GX-CH-2018 might be the minor parent. The SVA isolates SVA-CH-SDGT-2017 were predicted to be recombinant, and indicated that the USA/IA44952/2015-P1strain, which was isolated in the United States in 2015, was suspected as the major parent, whereas the minor parent was the CH-GDLZ01-2017, which was most closely related to the USA/ IA39812/2015-P1 strain.

The results showed that the SVA strains in China had genetic diversity. The results of the phylogenetic tree and recombination analysis show that SVA in Shandong Province is constantly evolving and recombining, indicating that genetic evolution research still has very important indicative significance. Currently, the research on the SVA vaccine is mainly focused on oil adjuvant inactivated and attenuated live vaccines, and there is no commercial vaccine to prevent the disease. An SVA-inactivated vaccine has a good immune

effect, but incomplete inactivation during vaccine production can lead to biosafety problems. At the same time, live attenuated vaccines are also dangerous for toxic resurrectionism. In addition, pigs in China lack an immune barrier against SVA, with a large number of subclinical infections and no commercial vaccine, which increases the difficulty of prevention, control, and purification of the disease in China. Therefore, we need to pay more attention to the SVA virus and develop vaccines faster.

In this study, we found that SVA-CH-SDFX-2022 can be effectively replicated in vitro. Therefore, we used SVA isolates for the study. The results show that effective prevention and control of SVA still face many complex challenges. At the same time, a better understanding of the virus's ability to spread is crucial to controlling SVA.

In summary, in this study an SVA strain was isolated, and the genetic variation of the strain was clarified through identification and pathogenicity analysis of the virus. It shares high sequence homology with US isolates. This knowledge extended significantly our understanding of the SVA. So, more attention should be paid to the infection and transmission of SVA. This work would be useful for studying the pathogenesis of SVA infection and controlling this disease in the future.

Authors contribution

Yingru Ma, Limei Zheng and Chen Li contributed equally to this work

Conflicts of interest

The authors declare that there are no conflicts of interest

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SAŽETAK

Senekavirus A (SVA), prije nazivan virusom doline Seneca Valley, jedini je pripadnik roda senekavirusa u porodici Picornaviridae. Virus je slučajno otkriven 2002. i nazvan virusom doline Seneca 001 (SVV-001). SVA je novi patogen koji može uzrokovati vezikularne lezije i prolaznu epidemiju novorođene prasadi s naglim gubicima u proizvodnji. U ovom je istraživanju soj SVA izoliran u populaciji svinja iz provincije Shandong u Kini i identificiran kao SVA-CH-SDFX-2022. Kompletni genom izolata SVA imao je 7282 nukleotida (nt) u dužini i sadržavao je jedan otvoreni okvir za očitavanje (ORF), bez poli-A repova. Filogenetska je analiza pokazala da izolat u velikoj mjeri sadržava genomsku organizaciju i nukleotidne identitete, od 90,5 % do 99,6 %, s drugim poznatim SVA izolatima. Karakterizacija virusa je pokazala da ima veliku sposobnost rasta *in vitro*. Pronađena je rekombinacija izolata SVA-CH-SDFX-između nukleotida 1836 i 2710 što je uključilo regiju gena VP2 (parcijalno) i gena VP3 (parcijalno). Zbog visoke stope infektivnosti i golemih ekonomskih gubitaka važan je brži razvoj cjepiva i bolje razumijevanje zaraze. Rezultati ovog istraživanja pružaju korisne podatke za proučavanje SVA virusa, posebno s obzirom na njegovu epidemiologiju u svinja i regionalnu prekograničnu kontrolu vezikularnih bolesti.

Ključne riječi: senekavirus A; svinje; izolacija virusa; IFA; evolucijska analiza; Western blotting

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