A virulent and pathogenic infectious clone of Senecavirus A

Maureen H. V. Fernandes^{1,2}, Marcelo de Lima^{2,3}, Lok R. Joshi^{1,2} and Diego G. Diel^{1,2,*}

Abstract

Senecavirus A (SVA) is a picornavirus that circulates in swine populations worldwide causing vesicular disease (VD) in affected animals. Here we developed a reverse genetics system for SVA based on the well-characterized wild-type SVA strain SD15-26 (wt SVA SD15-26). The full-length cDNA genome of SVA was cloned into a plasmid under a T7 RNA polymerase promoter. Following *in vitro* transcription, the genomic viral RNA was transfected into BHK-21 cells and rescue of infectious virus (rSVA SD15-26) was shown by inoculation of highly susceptible H1299 cells. *In vitro* characterization of the rSVA SD15-26 showed similar replication properties and protein expression levels as the wt SVA SD15-26. A pathogenesis study was conducted in 15-week-old finishing pigs to evaluate the pathogenicity and infection dynamics of the rSVA SD15-26 virus in comparison to the wt SVA SD15-26. Animals from both rSVA- and wt SVA SD15-26-inoculated groups presented characteristic SVA clinical signs (lethargy and lameness) followed by the development of vesicular lesions on the snout and/or feet. The clinical outcome of infection, including disease onset, severity and duration was similar in rSVA- and the wt SVA SD15-26-inoculated animals. All animals inoculated with rSVA or with wt SVA SD15-26 presented a short-term viremia, and animals from both groups shed similar amounts of virus in oral and nasal secretion, and faeces. Our data demonstrates that the rSVA SD15-26 clone is fully virulent and pathogenic in pigs, presenting comparable pathogenesis and infection dynamics to the wt SVA SD15-26 strain. The infectious clone generated here is a useful platform to study virulence determinants of SVA, and to dissect other aspects of SVA infection biology, pathogenesis and persistence.

INTRODUCTION

Senecavirus A (SVA) is an emerging picornavirus that has been reported globally as the cause of vesicular disease (VD) outbreaks in swine [1–7]. SVA-induced VD is clinically indistinguishable from other high consequence vesicular diseases (VD) of swine, including foot-and-mouth-disease (FMD), vesicular stomatitis (VS), swine vesicular disease (SVD) and vesicular exanthema of swine (VES) [8, 9]. Vesicular diseases are among the most economically important diseases of livestock and could have catastrophic economic consequences due to international trade restrictions [10]. Endemic circulation of SVA and associated VD outbreaks in countries that are free of other VD causing agents poses a constant threat to the livestock industry. Thus, a better understanding of SVA infection biology and the viral mechanisms underlying disease pathogenesis is critical to

establish effective control strategies for the virus and the associated disease.

Senecavirus A-induced VD starts following a short incubation period [3–5 days post-infection (p.i.)]. Initially, infected animals present mild clinical signs characterized by lethargy and lameness [11–13], which are usually followed by the development of vesicles on the snout and/or feet (dewclaw, coronary band, sole). The vesicles rupture within 2–4 days, leaving ulcerated lesions on the skin [11–13]. These lesions eventually heal and resolve by days 14–16 p.i. [11–13]. A short-term viremia is detected between days 3–10 p.i., with decreasing levels of viremia paralleling the appearance of neutralizing antibodies in serum and increased frequency of CD4⁺ T-cell in peripheral blood mononuclear cells (PBMCs) [12]. Affected animals may shed virus in oral and nasal secretions and in faeces for up to 21–28 days p.i. [11, 14]. Later

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Author affiliations: ¹Department of Population Medicine and Diagnostic Sciences, Animal Health Diagnostic Center, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA; ²Department of Veterinary and Biomedical Sciences, South Dakota State University, Brookings, SD, USA; ³Laboratório de Virologia e Imunologia, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, RS, Brazil.

*Correspondence: Diego G. Diel, dgdiel@cornell.edu

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Abbreviations: CPE, cytopathic effect; dsRNA, double-stranded viral RNA; eGFP, enhanced green fluorescent protein; FMD, foot-and-mouth-disease; IFA, indirect immunofluorescence assay; NA, neutralizing antibody; PBMCs, peripheral blood mononuclear cells; p.i., post-infection; rRT-PCR, real-time reverse transcriptase PCR; SVA, Senecavirus A; SVD, swine vesicular disease; UTRs, untranslated regions; VD, vesicular disease; VES, vesicular exanthema of swine; VS, vesicular stomatitis; wt, wild-type.

events in the course of infection involve the development of SVA-specific CD8⁺ T cells, culminating in the resolution of the disease [12].

Senecavirus A is a non-enveloped virus with an icosahedral-shaped capsid [15, 16]. Its genome consists of a positive-sense single-stranded RNA of approximately 7.2 kb in length, which encodes a single ORF [15, 16]. This ORF is flanked by untranslated regions (UTRs) in the 5' and 3' ends, with the 3' end being followed by a poly A tail [15, 16]. Both 5' and 3' UTRs contain secondary RNA structures, including the internal ribosomal entry site (IRES) in the 5' end [17] and stem-loops forming a 'kissing-loop' structure in the 3' end [18]. These structures play crucial roles on virus replication and translation of picornavirus genomes [19, 20]. The ORF is translated in a unique polyprotein that comprises a leader (L) protein followed by three regions: P1, P2 and P3 [15, 16]. These regions are further processed by a virus-encoded cysteine protease (3C^{pro}) to form mature and functional viral proteins [15]. The structural proteins VP1, VP2, VP3 and VP4 are encoded in the P1 region and form the virus capsid [21]. The non-structural proteins 2A, 2B and 2C, and 3A, 3B, 3C and 3D, are encoded in the P2 and P3 regions, respectively, and they function on virus replication [21] and may play important roles on virus virulence, innate immune evasion and pathogenesis [22, 23]. The development of reverse genetic systems for RNA viruses has provided critical platforms to study the effects of targeted genetic changes on virus replication and pathogenesis [24]. Currently, few infectious clones of SVA have been developed [25–29], however they demonstrated to be attenuated in pigs [25, 26].

In the present study, we generated an infectious clone of SVA and evaluated its virulence, pathogenicity and infection dynamics (including virological parameters and host immune responses), in comparison to the parental SVA strain SD15-26 strain, a well-characterized and virulent field strain of the virus obtained from the index SVA outbreak in the US in 2015 [11–13].

METHODS

Cells and virus

NCI-H1299 non-small human lung carcinoma cell lines (ATCC CRL-5803) and BHK-21 (CCL-10) were cultured at 37°C with 5% CO₂ in RPMI (Corning) or MEM (Corning) supplemented with 10% foetal bovine serum (FBS; VWR), penicillin/streptomycin (100 IU ml⁻¹; Corning), gentamicin (50 µg ml⁻¹; Corning) and 2 mM L-glutamine (Corning). Senecavirus A stocks were prepared at low passage (passages 5 and 4, respectively) using the wild-type SVA strain SD15-26 (Joshi *et al.*, 2016 [11]) or the infectious clone rSVA SD15-26 obtained as described below.

Generation and rescue of the rSVA SD15-26 virus

A cDNA clone of SVA strain SD15-26 (pBrick-FLSVA-SD15-26) containing the full-length SVA genome under control of a T7 RNA polymerase promoter and flanked

by the restriction enzymes NheI and NotI was constructed following a three-step strategy. A pUC57 plasmid containing an RNA polymerase promoter T7 and the 5' end of SVA SD15-26 between the restriction enzymes NheI and SfiI and a pBrick plasmid containing the NheI restriction site followed by a DNA fragment encoding the P1, P2 and P3 regions of SVA SD15-26 were synthesized by Genscript. The plasmids were digested using the restriction enzymes NheI and SfiI (NEB) and the T7-5'-end fragment was ligated into the pBrick-P1,2,3 plasmid using T4 ligase (NEB) according to the manufacturer's instructions. The 3' end of the virus was amplified by RACE-PCR (Takara) from RNA extracted from the SVA strain SD15-26 according to the manufacturer's instructions and a NotI restriction site was added after the poly A tail. This fragment was digested and cloned into the pBrick-T7-5'P1,2,3 plasmid between the restriction enzymes SbfI and NotI originating the full-length cDNA clone plasmid pBrick-FLSVA-SD15-26. The assembly strategy of pBrick-FLSVA-SD15-26 is shown in the diagram depicted in Fig. 1. The integrity and identity of the SVA sequences cloned in the plasmid were confirmed by DNA sequencing.

To rescue the infectious recombinant SVA from the pBrick-FLSVA-SD15-26 cDNA clone, the plasmid was linearized with NotI (NEB) restriction enzyme, purified using standard phenol:chloroform purification and ethanol precipitation method and used as template in *in vitro* transcription reactions using the MEGAscript T7 Transcription Kit (Invitrogen) following the manufacturer's protocol. Full-length viral genomic RNA was purified by phenol:chloroform purification and isopropanol precipitation and approximately 2 µg of viral RNA were transfected in BHK-21 cells using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions. The cells were incubated for 72 h post-transfection and subjected to three freeze-and-thaw cycles. The rescued virus was then inoculated in H1299 cells for amplification. Two passages in H1299 cells were performed. After 72 h of incubation of the second passage, cells were fixed with 3.7% formaldehyde and the presence of the infectious clone rSVA SD15-26 was confirmed by indirect immunofluorescence (IFA) using a rabbit polyclonal antibody against SVA (Fig. 1). The *in vitro* transcription and virus rescue protocol were repeated three times independently. A negative mock-transfected well was included in all experiments. Complete genome sequencing of rSVA SD15-26 was used to confirm the identity and integrity of the virus genome using the MinIon sequencing platform (Nanopore). A low passage stock of rSVA SD15-26 (passage 4) was obtained using H1299 cells, and 1 ml vials were stored at –80°C.

Growth curves

Replication kinetics of wt SVA SD15-26 and rSVA SD15-26 were assessed using multi- and single-step growth curves *in vitro*. H1299 cells were cultured in six-well plates, inoculated at a multiplicity of infection (m.o.i.) of 0.1 or 10, and harvested at 4, 8, 12 and 24 h post-infection (p.i.). Virus titres were determined by end-point dilutions and the titres were calculated using Spearman and Karber's method [30] and

Fig. 1. Schematic representation of generation and rescue of rSVA SD15-26. The plasmid construction was performed using a three-step strategy based on the sequence of the wild-type (wt) SVA strain SD15-26 (11). A pUC57 plasmid containing an RNA polymerase promoter T7 and the 5' end of SVA SD15-26 and a pBrick plasmid containing the P1, P2 and P3 coding region of SVA SD15-26 were synthesized by Genscript. The 3' end of SVA SD15-26 was amplified by RACE PCR and a Not I enzyme added after the poly A tail. These three segments were assembled by restriction digestion/ligation generating a pBrick-FLSVA-SD15-26 plasmid. Following restriction enzyme linearization of pBrick-FLSVA-SD15-26 using the NotI enzyme, full length genomic viral RNA was *in vitro* transcribed and was transfected in BHK-21 cells. After 72 h, cells and supernatant were harvested and inoculated in H1299 cells. Rescue of SVA was confirmed by indirect immunofluorescence (IFA) assay using an SVA specific antibody. (Figure created with BioRender.com).

expressed as TCID₅₀ ml⁻¹. At 48 h.p.i. of the viral dilutions, cells were fixed (3.7% formaldehyde) and subjected to IFA using a rabbit polyclonal antibody against SVA. Fluorescence positive wells were used to determine the viral titers on each time point.

Western blots

Western blots were performed to assess expression of structural (VP1 and VP2) and non-structural (3C) SVA proteins in rSVA- and wt SVA SD15-26-infected cells. H1299 cells were cultured in six-well plates, inoculated at a m.o.i. of 10, and harvested at 4, 8, 12 and 24 h p.i. SDS-PAGE and Western blot were performed as previously described [25]. Nitrocellulose membranes were incubated with anti-VP1, anti-VP2 (kindly provided by Drs. Eric Nelson and Steve Lawson, SDSU), and anti-β-actin (Santa Cruz) mouse monoclonal antibodies, and anti-3C polyclonal antibody [31]. IRDye 800CW Goat anti-Mouse IgG (H+L) and IRDye 680CW Goat anti-Rabbit IgG (H+L) were used as secondary antibodies (LI-COR Biosciences, Lincoln, NE). Blots were developed using ChemiDoc MP Imaging System (BioRad).

Confocal microscopy

Staining patterns of SVA proteins and the presence and localization of double-stranded viral RNA (dsRNA) were investigated using IFA followed by confocal microscopy. For this, H1299 cells cultured in eight-well chamber glass slides (ibidi) were infected with either rSVA SD15-26 or wt SVA SD15-26 (m.o.i. = 1) for 24 h. Indirect immunofluorescence staining was performed as previously described [31]. An anti-SVA polyclonal antibody and anti-dsRNA monoclonal antibody (clone J2; Scicons) were utilized, followed by appropriate secondary antibodies conjugated with Alexa Fluor 594 or 488, respectively (Invitrogen). Nuclear staining was performed with DAPI (Thermo Scientific). Cells were visualized using a confocal microscope (LSM710 Confocal Zeiss, 63× magnification).

Animal pathogenesis study

Eighteen SVA negative pigs were randomly allocated in three experimental groups as follows: Group 1: mock-infected control (n = 6); Group 2: rSVA SD15-26-infected (n = 6); and Group 3: wt SVA SD15-26-infected (n = 6). Animals were

Fig. 2. Characterization of the recombinant rSVA SD15-26 virus *in vitro*. Multi-step or (a) single-step (b) growth curves were performed. For this, H1299 cells were infected with (a) 0.1 and (b) 10 m.o.i. of rSVA SD15-26 or wt SVA SD15-26 and virus titres were determined at 4, 8, 12 and 24 h p.i. Error bars represent the standard error of the mean (SEM) calculated based on results of three independent experiments (*P*-values were determined by multiple *t*-test; ***P* < 0.01). (c) Western blots were performed to assess SVA-VP1, -VP2 and -3C protein expression of wt SVA SD15-26 and rSVA SD15-26 in infected cells. H1299 cells were infected with a m.o.i. of 10 of each virus harvested on 4, 8, 12 and 24 h.p.i. and subjected to western blots using a VP1-, VP2- and 3C-specific mAb. β-actin control antibody was used to show protein load in each sample and a mock-infected sample was added as a negative control. (d) Staining patterns of SVA proteins and the presence and localization of double-stranded viral RNA (dsRNA) were investigated. H1299 cells were infected with either rSVA SD15-26 or wt SVA SD15-26 (m.o.i.=1) for 24 h, then fixed. Indirect immunofluorescence staining was performed with an anti-whole SVA polyclonal antibody and anti-dsRNA monoclonal antibody. Secondary antibodies conjugated with Alexa Fluor 594 target the anti-whole SVA polyclonal antibody (red) and Alexa Fluor 488 target dsRNA (green). Nuclear stain was performed with DAPI (blue). Cells were visualized using a confocal fluorescence microscope (LSM710 Confocal Zeiss, 63x magnification).

Fig. 3. Experimental design. Eighteen finishing pigs were randomly allocated into three groups: Group 1: mock-infected control; Group 2: rSVA SD15-26-inoculated; and Group 3: wt SVA SD15-26-inoculated. Animals were inoculated upon arrival oronasally [10^8 TCID₅₀ in 5 ml, 2 ml orally and 3 ml intranasally (1.5 ml into each nostril)] with either rSVA SD15-26 or wt SVA SD15-26 suspension. Blood and oral, nasal and rectal swabs were collected as indicated in the figure. All animals were euthanized on day 14 p.i. for collection of lymphoid tissues. (Figure created with BioRender.com).

inoculated oronasally upon arrival at our animal facilities [10^8 TCID₅₀ in 5 ml, 2 ml orally and 3 ml intranasally (1.5 ml into each nostril)] with either rSVA SD15-26 or wt SVA SD15-26 suspension. Mock-infected G1 animals were inoculated with the same volume of RPMI medium. Each group was maintained in separate rooms in ABSL-2 conditions and strict biosecurity protocols were followed. Animals received food and water *ad libitum* during the experimental period. The animals were monitored daily during the experiment for the presence of clinical signs and lesions.

Sample collection and processing

Blood samples and swabs (oral, nasal and rectal/faecal) were collected on days 0, 1, 3, 7, 10 and 14 p.i., and processed as previously described [11, 32]. Animals were euthanized on day 14 p.i. and lymphoid tissues (tonsil, mesenteric lymph node and mediastinal lymph node) were collected and stored at -80°C until further processed.

Virus isolation and titration

Virus isolation (VI) was performed in NCI-H1299 non-small cell lung carcinoma cell lines. All samples were processed in PBS (10%, weight/volume). Swab samples were vortexed for 10 s, cleared by centrifugation (6000 *g* for 3 min) and filtered (0.45 μm). One hundred μl of each sample were inoculated into monolayers of cells cultured in 24-well plates (cells prepared 24 h in advance) containing 100 μl of RPMI 1640 medium supplemented with penicillin (300 U ml^{-1}), streptomycin

(300 $\mu\text{g ml}^{-1}$) and gentamicin (150 $\mu\text{g ml}^{-1}$). After 1 h of adsorption, the inoculum was removed and 1 ml of fresh culture media was added to each well, and cells were incubated at 37°C with 5% CO_2 for 72 h. Samples were subjected to three blind passages then fixed (3.7% formaldehyde) and subjected to IFA using a rabbit polyclonal antibody against SVA to confirm virus isolation. Mock-inoculated control cells were included as negative controls on each passage. Virus titrations of VI positive samples were performed by endpoint limiting dilution. Each original sample was subjected to ten-fold serial dilutions in RPMI media and each dilution inoculated into monolayers of H1299 cell culture in 96-well plates. At 48 h post-inoculation, cells were fixed and stained with an SVA-specific rabbit polyclonal antibody followed by incubation with an anti-rabbit IgG Alexa-594 conjugated secondary antibody (ImmunoReagents). Virus infectivity was assessed under a fluorescence microscope and viral titres were calculated by the Spearman and Karber's method [30] and expressed as TCID₅₀ ml^{-1} [11].

RNA extraction and rRT-PCR

Viral RNA was extracted from serum, swab samples and lymphoid tissues using the IndiMag Pathogen Kit (Indical) and the IndiMag 48 (Indical) automated extractor following the manufacturer's instructions. The samples were processed as previously described [25], then 200 μl of each sample was used for nucleic acid extraction. VetMAX Xeno Internal Positive Control – VIC Assay (Applied Biosystems) was used in

Fig. 4. Comparative pathogenicity of rSVA SD15-26 and wt SVA SD15-26 in 15-week-old finishing pigs. Vesicular lesions were observed in the rSVA- and in the wt SVA SD15-26-inoculated animals. Lesions were observed on the snout, dewclaws and/or coronary band. The white arrows indicate the lesions. The severity, progression and resolution of the VD-disease were similar in both rSVA SD15-26 and wt SVA SD15-26 groups. Mock-infected animals did not present any vesicular lesion throughout the experiment.

all extracted samples to ensure proper nucleic acid extraction and verify the absence of PCR inhibitors. The presence of SVA RNA was assessed using RNA-to-Ct 1 Step-kit (Applied Biosystems) and custom designed primers and probe (Prime-Time qPCR probe assays, Integrated DNA Technologies, USA) targeting the SVA 3D gene as previously described [25]. Amplification and detection were performed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). A standard curve was established by using ten-fold serial dilutions from 10^{-1} to 10^{-8} of SVA SD15-26 virus suspension containing 10^8 TCID₅₀ ml⁻¹. Relative viral genome copy numbers were calculated based on the standard curve and determined using the CFX Maestro software (Bio-Rad). The amount of viral RNA detected in samples were expressed as log₁₀ (genome copy number) ml⁻¹.

Virus neutralization assay

Neutralization assays were performed using a recombinant SVA SD15-26 clone expressing an enhanced green fluorescent protein (eGFP) (Cloning strategy and rescue of rSVA SD15-26 eGFP are described below). For this, serial two-fold dilutions of serum (1:40 to 1:81920) were incubated with 200 TCID₅₀ of rSVA-eGFP for 1 h at 37°C. H1299 cells were added to each well and plates were incubated at 37°C for 72 h. Plates were visualized using a fluorescence microscope (Olympus CKX53) to determine neutralizing antibody (NA) titres,

which were expressed as the reciprocal of the highest serum dilution capable of completely inhibiting SVA infection/replication based in expression of eGFP by the rSVA-eGFP virus. This assay was previous optimized in our lab and the NA titres from the assay using wt SVA SD15-26 was comparable to the NA titres using rSVA-eGFP.

Cloning strategy and rescue of rSVA-eGFP

A full-length cDNA clone expressing eGFP was constructed. For this, we inserted the eGFP gene followed by a teschovirus 2A peptide (T2A) fusion protein between SVA 2A/2B [26]. A pUC57 plasmid containing the eGFP plus T2A sequence flanked upstream by the P1 region of the SVA SD15-26 genome and downstream by the SVA SD15-26 2A sequence was synthesized. This construct was flanked by two unique restriction enzymes SfiI (NEB) and BglII (NEB) present in the SVA genome. This plasmid and the backbone pBrick-FLSVA-SD15-26 were digested with SfiI and BglII enzymes and ligated using T4 ligase (Invitrogen) according to the manufacturer's instructions. After plasmid amplification using standard protocols, *in vitro* transcription and virus rescue were performed as described above (see Generation and rescue of rSVA SD15-26 virus). A stock of rSVA SD15-26 passage 3 was obtained using H1299 cells, and 1 ml vials were stored at -80°C for use in the virus neutralization assays described here.

Table 1. Summary lesions observed in rSVA SD15-26 and SVA SD15-26 inoculated pigs

Group	Animal ID	Lesions	Duration
rSVA SD15-26	7	Vesicle on dewclaw of back right foot	6–14 days p.i.
		Vesicle on dewclaw of back left foot	6–14 days p.i.
rSVA SD15-26	8	Vesicles on snout	7–9 days p.i.
		Vesicle on dewclaw of front left foot	7–14 days p.i.
rSVA SD15-26	9	Vesicle on dewclaw of back left foot	7–14 days p.i.
rSVA SD15-26	11	Vesicle on the coronary band and	
		dewclaw of back left foot	7–14 days p.i.
		Vesicle on dewclaw of front left foot	7–14 days p.i.
wt SVA SD15-26	14	Vesicle on dewclaw of back left foot	7–14 days p.i.
wt SVA SD15-26	17	Vesicle on snout	6–10 days p.i.
		Vesicle on dewclaw of front right foot	7–14 days p.i.
wt SVA SD15-26	18	Vesicles on snout	6–10 days p.i.
		Vesicle on the sole and dewclaw of front right foot	7–14 days p.i.
		Vesicle on dewclaw of back right foot	7–14 days p.i.

Statistical analysis

Statistical analysis and data plotting were performed using GraphPAD Prism 8.4.3(471) software (GraphPAD Software).

RESULTS

Recovery of the infectious rSVA SD15-26 clone

A full-length cDNA clone based on the sequence of the wild-type (wt) SVA strain SD15-26 [11] was cloned under a T7 promoter into a pBrick plasmid. Following restriction enzyme linearization, full-length genomic viral RNA was *in vitro* transcribed and transfected in BHK-21 cells. After 72 h, cells and supernatant were harvested and inoculated in H1299 cells. At ~24 h p.i., characteristic SVA cytopathic effect (CPE) was observed in H1299 cells. Rescue of SVA was confirmed on passage 2 in H1299 cells with indirect immunofluorescence (IFA) assay using SVA-specific antibodies (Fig. 1). Complete genome sequencing confirmed the identity and integrity of the genome of the virus recovered from the cDNA clone (rSVA SD15-26). No nucleotide changes were observed in

the rSVA SD15-26 genome in comparison to the wt SVA SD15-26. The virus rescue procedure was repeated three times independently. Neither CPE nor fluorescent positive cells were detected in mock-transfected cells.

In vitro characterization of the rSVA SD15-26

Replication kinetics of the rSVA SD15-26 was evaluated and compared to wt SVA SD15-26 using multi- and single-step growth curves *in vitro* (Fig. 2a, b). The recombinant rSVA SD15-26 and wt SVA SD15-26 presented similar growth kinetics in H1299 cells (Fig. 2a, b). Additionally, no noticeable differences in expression levels of important structural and non-structural viral proteins (VP1, VP2 and 3C) were observed in rSVA- and wt SVA SD15-26 infected cells by western blot (Fig. 2c). Cells infected with either the rSVA SD15-26 or the wt SVA SD15-26 for 12 h were also analysed by confocal microscopy after immunofluorescence staining (Fig. 2d). Staining patterns of SVA proteins and the presence and localization of double-stranded viral RNA (dsRNA), a product of virus replication, were equivalent in cells infected with both viruses (Fig. 2d). These results demonstrate that the rSVA SD15-26 presents similar replication properties to the wt SVA SD15-26 *in vitro*.

Infection of pigs with rSVA SD15-26 results in vesicular disease

The pathogenicity and infection dynamics of rSVA SD15-26 were evaluated in finishing pigs and compared to those of the wt SVA SD15-26. For this, eighteen 15-week-old finishing pigs were randomly allocated into three groups, as follows: Group 1: mock-infected (RPMI); Group 2: rSVA SD15-26-inoculated; and Group 3: wt SVA SD15-26-inoculated (Fig. 3). Animals were inoculated oronasally [2 ml orally and 3 ml nasally (1.5 ml into each nostril)] as previously described [11]. Following inoculation, animals were monitored daily for 14 days and clinical signs and lesions were recorded. Pigs in the rSVA and wt SVA SD15-26 groups presented characteristic clinical signs of SVA, including lameness and lethargy starting on days 3–5 p.i. Vesicular lesions were observed in four animals in the rSVA SD15-26-inoculated group and in three animals in the wt SVA SD15-26 group, starting at 6 days p.i. (Table 1). Vesicles developed on the snout and/or feet [dewclaw, sole and coronary band (Fig. 4; Table 1)] of the affected animals in each group, and were resolved by day 14 p.i. Overall, the clinical outcome of SVA infection, including disease onset, severity and duration was similar in rSVA- and the wt SVA SD15-26-inoculated animals. Together, these results demonstrate that the rSVA SD15-26 virus is virulent and retained its pathogenicity in swine.

The rSVA SD15-26 clone induces viremia and virus shedding comparable to wt SVA SD15-26

The levels of viremia were assessed in serum by determining the presence of SVA RNA using a real-time reverse transcriptase PCR (rRT-PCR) assay. While the mock-infected group remained negative throughout the experiment, SVA RNA was detected in serum samples of rSVA and wt SVA

Fig. 5. Kinetics of viremia and virus shedding in animals inoculated with rSVA SD15-26 and wt SVA SD15-26. Viremia (a) and virus shedding in oral (b) and nasal (c) secretions and in faeces (d) were determined by rRT-PCR and expressed as $\log_{10}$ genome copy number per ml^{-1} . Shedding of infectious virus in oral (e) and nasal (f) secretions and in faeces (g) were assessed by virus isolation (VI) and the amount of infectious virus in VI positive samples was further quantitated by end point dilutions and viral titres determined as per the Spearman and Karber's method [30]. The dotted line represents the limit of detection of the titration (e, f, and g). Animals from mock-inoculated group are represented in green, rSVA SD15-26-inoculated animals are pink and the animals inoculated with wt SVA SD15-26 are in black. Error bars represent the SEM.

SD15-26 groups starting on day 1 p.i. (Fig. 5a). The peak of viremia in animals in both groups was observed on day 3 p.i., with some animals being viremic on day 7 p.i. in both groups (Fig. 5a). On day 10 p.i., just one animal in the wt SVA SD15-26 group was still viremic, and all animals were negative on day 14 p.i. (Fig. 5a). No statistical differences were observed in the levels of viremia between the rSVA- and wt SVA SD15-26-inoculated animals.

Virus shedding was detected using rRT-PCR in both rSVA- and wt SVA SD15-26-inoculated groups starting on day 1 p.i. in oral and nasal secretions and in faeces (Fig. 5b–d). Animals in both groups shed viral RNA until day 14 p.i. Importantly, no statistical differences in SVA RNA levels shed

by the rSVA- and wt SVA SD15-26-inoculated animals were observed throughout the experiment (Fig. 5b–d). All animals from the mock-control group remained negative throughout the experiment (Fig. 5b–d).

Shedding of infectious SVA in oral and nasal secretions and faeces was also assessed by virus isolation (VI) and the amount of infectious virus in VI positive samples was determined by end point dilutions and mean viral titers determined by the Spearman and Karber's method and expressed as $\log_{10}$ TCID₅₀ per ml^{-1} [30]. High infectious SVA titres were detected in oral and nasal swabs at days 1 and 3 p.i. (Fig. 5e, f) in all infected animals. At days 7 and 10 p.i., two-to-three animals in both rSVA- and wt SVA SD15-26-inoculated groups were still

Fig. 6. Viral load and tissue distribution of Senecavirus A (SVA) in lymphoid tissues. Mediastinal lymph node (a), mesenteric lymph node (b) and tonsil (c) were collected on day 14 p.i. and levels of SVA RNA were determined by rRT-PCR and expressed as $\log_{10}$ genome copy number ml^{-1} . Animals from mock-inoculated group are represented in green, rSVA SD15-26-inoculated animals are pink and the animals inoculated with wt SVA SD15-26 are in black. Error bars represent the SEM.

shedding infectious SVA in oral and nasal swabs, but the titres were lower than on day 3 p.i. (Fig. 5e, f). Two animals in the wt SVA SD15-26 were still positive on day 14 p.i. (Fig. 5f) in nasal swabs. Low titres of infectious SVA was also detected in faeces in all time points throughout the 14-day experimental period in the rSVA- and wt SVA SD15-26-inoculated animals (Fig. 5g). These results confirmed successful infection of all inoculated animals and further demonstrated that the rSVA SD15-26 clone generated here presented comparable infection dynamics, viremia and patterns of virus shedding to those observed in wt SVA SD15-inoculated animals.

Viral load in tissues

The presence of SVA RNA was investigated in lymphoid tissues by rRT-PCR on day 14 p.i. All virus-inoculated animals were positive for SVA RNA in the mediastinal lymph node and tonsil (Fig. 6a, c), while four animals of rSVA- and wt SVA SD15-26-inoculated group were positive for SVA RNA in the mesenteric lymph node (Fig. 6b). All animals from the mock-infected control group were negative for SVA RNA (Fig. 6a–c). These findings suggest that the rSVA SD15-26 virus generated here presents a similar tissue tropism as the wt SVA SD15-26, with the tonsil likely serving as one of the main sites of virus replication during acute infection.

Comparative neutralizing antibody responses

Neutralizing antibody (NA) titres elicited by rSVA and wt SVA SD15-26 were determined by virus neutralization assays using a recombinant SVA expressing eGFP generated here (detailed in Methods the construction and rescue). All animals inoculated with the rSVA or wt SVA SD15-26 seroconverted, presenting high levels of NA titres (Fig. 7). Neutralizing antibody titres remained elevated until the last day of the experiment (day 14 p.i.). No differences were observed in humoral immune responses elicited by rSVA- and wt SVA SD15-26 infection.

DISCUSSION

Senecavirus A (SVA) is a picornavirus that has been circulating in pigs in the USA with sporadic outbreaks being reported since the late 1980s [33]; however, the virus was just recently confirmed as the causative agent of VD in swine [9, 15]. In spite of its clinical relevance, several aspects of SVA biology, virulence and pathogenesis remain unknown. To begin to study the molecular interactions of SVA with the swine host and define molecular determinants of the virus virulence, pathogenesis and persistence, we generated an infectious cDNA clone based on the contemporary virulent SVA strain SD15-26 [11]. Our results demonstrate that the recombinant virus (rSVA SD15-26) rescued from the cDNA clone retained its pathogenicity and caused VD similar to the wild-type virus in inoculated pigs.

The rSVA SD15-26 clone was designed to be identical to the wt SVA SD15-26 strain genome, as previous studies showed that a few mutations in the cDNA infectious clone could lead to attenuation of the virus in pigs [25, 26]. Interestingly, the mutations that led to attenuation of SVA that were described by Chen and collaborators [26] and by our group [25] accumulated or were introduced in the 5' and 3' ends and in the P1 region of the virus genome [25, 26]. These regions contain important secondary RNA structures including the internal ribosomal entry site (IRES) and stem-loops that are critical for the picornavirus life cycle [17, 18]. Small changes in the conformation of these RNA structures caused in some cases by SNPs are known to cause impaired protein expression and/or picornavirus replication, leading to attenuated disease phenotypes *in vivo* [34–36]. The identity of the rSVA SD15-26 was confirmed by whole-genome sequencing, and no SNPs were identified in the virus genome. It is important to note that the rescue procedure was repeated three times independently and negative controls were added in all steps of the process, to ensure that no cross-contamination occurred during virus rescue.

Fig. 7. SVA neutralizing antibody (NA) levels in serum of animals. Animals from mock-inoculated group are represented in green, rSVA SD15-26-inoculated animals are pink and the animals inoculated with wt SVA SD15-26 are in black. NA titres represent the reciprocal of the highest serum dilution capable of completely inhibiting SVA infectivity. The dotted line represents the cutoff value of the VN assay. Error bars represent the standard error of the mean (SEM).

Given that the rSVA SD15-26 replicated efficiently and similarly to wild-type virus *in vitro* (Fig. 2), an animal study using the rSVA SD15-26 infectious clone and wt SVA SD15-26 was performed. Importantly, the rSVA SD15-26-inoculated animals presented clinical signs and lesions indistinguishable from those observed in animals inoculated with the wt SVA SD15-26 virus (Fig. 4). The clinical presentation was characterized by lethargy and lameness, which were followed by the development of vesicles on the snout and/or feet (dewclaw, coronary band, sole) of inoculated animals. These are characteristic clinical signs and lesions observed in VD outbreaks caused by SVA [1–7]. Additionally, disease onset, duration and resolution were similar in rSVA- and wt SVA SD15-26-inoculated animals and corresponded to those observed in animals naturally or experimentally infected with SVA [2, 37, 38]. These results demonstrate that the rSVA SD15-26 clone is virulent and as pathogenic as the wild-type virus in pigs.

All animals inoculated with rSVA or wt SVA SD15-26 presented a short-term viremia, with viral RNA being detected in serum between days 1 and 10 p.i. (Fig. 5a). Similar patterns of viremia have been observed in nursing [26] and finishing pigs [11–13], and sows [14] infected with wild-type strains of SVA. No differences in virus shedding in oral/nasal secretions and faeces between rSVA- and wt SVA SD15-26-inoculated groups were noted (Fig. 5b–g). These observations demonstrate that the replication and infection dynamics of rSVA SD15-26 in pigs mirrors the properties of the wild-type virus.

Viral load in lymphoid tissues was also evaluated in this study since SVA seems to have tropism and usually is found on lymph nodes and tonsil during acute infection [11, 13]. Senecavirus A RNA was detected in similar levels in four animals of both rSVA- and wt SVA SD15-26-inoculated group A in the mesenteric lymph node (Fig. 6b), and in all animals in the mediastinal lymph node and tonsil (Fig. 6a, c). The similar distribution and viral loads observed in rSVA- and wt SVA SD15-26-inoculated animals suggest a similar tissue tropism of the recombinant and wild-type viruses. Interestingly, high levels of SVA RNA were observed in the tonsil of the inoculated animals on day 14 p.i. (end of acute phase of infection), suggesting that similar to the wt SVA, the rSVA SD15-26 clone was also able to establish persistence in tonsil as shown previously for wild-type SVA SD15-26 [14]. The rSVA SD15-26 clone generated here will be great platform to dissect the molecular mechanisms underlying SVA persistence.

Senecavirus A infection elicits robust neutralizing antibody responses, with high NA titres being detected as early as 5–7 days p.i. [11–13]. Results here show that infection with the rSVA SD15-26 efficiently elicits high titres of NA starting on day 7 p.i. (Fig. 7), which were comparable to those elicited by wt SVA SD15-26. Notably, to perform this neutralization assay a second recombinant SVA expressing eGFP was generated. The addition of eGFP was already reported as a tool to facilitate neutralization assays for SVA [27], and assays performed in our lab showed that NA titres from the assay using wt SVA SD15-26 was comparable to the NA titres using rSVA-eGFP (data not shown). This second recombinant was

generated using the cDNA SVA SD15-26 backbone, which demonstrated the potential of this platform and its ability to express heterologous genes.

In summary, this study describes the development of an SVA infectious clone that is as virulent and pathogenic as the wt SVA SD15-26. Results presented here showed that rSVA SD15-26 can induce clinical signs and VD in finishing pigs indistinguishable to wt SVA SD15-26. Furthermore, the dynamics of viremia, virus shedding and viral load in the tissues were comparable between the rSVA- and SVA SD15-26-inoculated groups. The SVA infectious clone generated in this study is a useful platform to study virulence determinants of SVA, and to dissect other aspects of SVA biology, pathogenicity and persistence.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

Animal experiment was revised and approved by the Cornell University Institutional Animal Care and Use Committee (approval number 2019-049).

References

- Bracht AJ, O'Hearn ES, Fabian AW, Barrette RW, Sayed A. Real-Time reverse transcription PCR assay for detection of Senecavirus A in swine Vesicular diagnostic specimens. *PLoS One* 2016;11:e0146211.
- Joshi LR, Mohr KA, Clement T, Hain KS, Myers B, et al. Detection of the emerging picornavirus Senecavirus A in Pigs. *J Clin Microbiol* 2016;54:1536–1545.
- Hause BM, Myers O, Duff J, Hesse RA. Senecavirus A in pigs, United States, 2015. *Emerg Infect Dis* 2016;22:1323–1325.
- Wu Q, Zhao X, Bai Y, Sun B, Xie Q, et al. The first identification and complete genome of Senecavirus A affecting pig with idiopathic vesicular disease in China. *Transbound Emerg Dis* 2017;64:1633–1640.
- Saeng-Chuto K, Stott CJ, Wegner M, Kaewprommal P, Piriyaongsa J, et al. The full-length genome characterization, genetic diversity and evolutionary analyses of Senecavirus A isolated in Thailand in 2016. *Infect Genet Evol* 2018;64:32–45.
- Sun D, Vannucci F, Knutson TP, Corzo C, Marthaler DG. Emergence and whole-genome sequence of Senecavirus A in Colombia. *Transbound Emerg Dis* 2017;64:1346–1349.
- Arzt J, Bertram MR, Vu LT, Pauszek SJ, Hartwig EJ, et al. First detection and genome sequence of Senecavirus A in Vietnam. *Microbiol Resour Announc* 2019;8.
- Leme RA, Zotti E, Alcântara BK, Oliveira MV, Freitas LA, et al. Senecavirus A: An emerging vesicular infection in Brazilian pig herds. *Transbound Emerg Dis* 2015;62:603–611.
- Pasma T, Davidson S, Shaw SL. Idiopathic vesicular disease in swine in Manitoba. *Can Vet J = La Rev Vet Can* 2008;49:84–85.
- Knight-Jones TJD, Rushton J. The economic impacts of foot and mouth disease - what are they, how big are they and where do they occur? *Prev Vet Med* 2013;112:161–173.
- Joshi LR, Fernandes MHV, Clement T, Lawson S, Pillatzki A, et al. Pathogenesis of Senecavirus A infection in finishing pigs. *J Gen Virol* 2016;97:3267–3279.
- Maggioli MF, Lawson S, de Lima M, Joshi LR, Faccin TC, et al. Adaptive immune responses following Senecavirus A infection in Pigs. *J Virol* 2018;92.
- Fernandes MHV, Maggioli MF, Joshi LR, Clement T, Faccin TC, et al. Pathogenicity and cross-reactive immune responses of a historical and a contemporary Senecavirus A strains in pigs. *Virology* 2018;522:147–157.
- Maggioli MF, Fernandes MHV, Joshi LR, Sharma B, Tweet MM, et al. Persistent infection and transmission of Senecavirus A from carrier sows to contact piglets. *J Virol* 2019;93.
- Hales LM, Knowles NJ, Reddy PS, Xu L, Hay C, et al. Complete genome sequence analysis of Seneca Valley virus-001, a novel oncolytic picornavirus. *J Gen Virol* 2008;89:1265–1275.
- Venkataraman S, Reddy SP, Loo J, Idamakanti N, Hallenbeck PL, et al. Structure of Seneca Valley Virus-001: an oncolytic picornavirus representing a new genus. *Structure* 2008;16:1555–1561.
- Willcocks MM, Locker N, Gomwalk Z, Royall E, Bakhshesh M, et al. Structural features of the Seneca Valley virus internal ribosome entry site (IRES) element: a picornavirus with a pestivirus-like IRES. *J Virol* 2011;85:4452–4461.
- Zoll J, Heus HA, van Kuppeveld FJM, Melchers WJG. The structure-function relationship of the enterovirus 3'-UTR. *Virus Res* 2009;139:209–216.
- Rohll JB, Moon DH, Evans DJ, Almond JW. The 3' untranslated region of picornavirus RNA: features required for efficient genome replication. *J Virol* 1995;69:7835–7844.
- Kloc A, Rai DK, Rieder E. The roles of picornavirus untranslated regions in infection and innate immunity. *Front Microbiol* 2018;9:485.
- Lin JY, Chen TC, Weng KF, Chang SC, Chen L-L, et al. Viral and host proteins involved in picornavirus life cycle. *J Biomed Sci* 2009;16:103.
- Porter AG. Picornavirus nonstructural proteins: emerging roles in virus replication and inhibition of host cell functions. *J Virol* 1993;67:6917–6921.
- Wang Y, Ma L, Stipkovits L, Szathmary S, Li X. The strategy of picornavirus evading host antiviral responses: Nonstructural proteins suppress the production of IFNs. *Front Microbiol* 2018;9:2943.
- Stobart CC, Moore ML. RNA virus reverse genetics and vaccine design. *Viruses* 2014;6:2531–2550.
- Sharma B, Fernandes MH, De Lima M, Joshi LR, Lawson S, et al. A Novel Live Attenuated Vaccine Candidate Protects Against Heterologous Senecavirus A Challenge. *Front Immunol* 2019;10:2660.
- Chen Z, Yuan F, Li Y, Shang P, Schroeder R, et al. Construction and characterization of a full-length cDNA infectious clone of emerging porcine Senecavirus A. *Virology* 2016;497:111–124.
- Liu F, Huang Y, Wang Q, Shan H. Construction of eGFP-Tagged senecavirus a for facilitating virus neutralization test and antiviral assay. *Viruses* 2020;12.
- Wang M, Mou C, Chen Z, Chen Z. Infectious recombinant Senecavirus A expressing novel reporter proteins. *Appl Microbiol Biotechnol* 2021;105:2385–2397.
- Li C, Wang H, Shi J, Yang D, Zhou G, et al. Senecavirus-Specific recombination assays reveal the intimate link between Polymerase fidelity and RNA recombination. *J Virol* 2019;93.
- Hierholzer JC, Killington RA. Virus isolation and quantitation. Mahy B and Kangro H (eds). In: *Virology Methods manual*. San Diego: Academic Press; 1996. pp. 25–47.
- Fernandes MHV, Maggioli MF, Otta J, Joshi LR, Lawson S, et al. Senecavirus A 3C protease mediates host cell apoptosis late in infection. *Front Immunol* 2019;10:363.

32. Maggioli MF, Lawson S, de Lima M, Joshi LR, Faccin TC, *et al.* Adaptive Immune Responses following Senecavirus A Infection in Pigs. *J Virol* 2018;92:e01717.
33. Knowles NJ, Hales LM, Jones BH, Landgraf JG, House JA, *et al.* Epidemiology of Seneca valley virus: identification and characterization of isolates from pigs in the United States. In: *XIVth Meeting of the European Study Group on Molecular Biology of Picornaviruses*. Saariseika, 2006.
34. Sun C, Yang D, Gao R, Liang T, Wang H. Modification of the internal ribosome entry site element impairs the growth of foot-and-mouth disease virus in porcine-derived cells. *J Gen Virol* 2016;97:901–911.
35. García-Núñez S, Gismondi MI, König G, Berinstein A, Taboga O, *et al.* Enhanced IRES activity by the 3'UTR element determines the virulence of FMDV isolates. *Virology* 2014;448:303–313.
36. Li R, Zou Q, Chen L, Zhang H, Wang Y. Molecular analysis of virulent determinants of enterovirus 71. *PLoS One* 2011;6:26237.
37. Guo B, Piñeyro PE, Rademacher CJ, Zheng Y, Li G. Novel senecavirus a in swine with vesicular disease, United States, July 2015. *Emerg Infect Dis* 2016;22:1325–1327.
38. Montiel N, Buckley A, Guo B, Kulshreshtha V, VanGeelen A, *et al.* Vesicular disease in 9-Week-Old pigs experimentally infected with Senecavirus A. *Emerg Infect Dis* 2016;22:1246–1248.

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