Pathogenesis of Senecavirus A infection in finishing pigs

Lok R. Joshi,[1] Maureen H. V. Fernandes,[1] Travis Clement,[1] Steven Lawson,[1] Angela Pillatzki,[1] Talita P. Resende,[2] Fabio A. Vannucci,[2] Gerald F. Kutish,[3] Eric A. Nelson[1] and Diego G. Diel[1]

Correspondence
Diego G. Diel
diego.diel@sdstate.edu

[1]Animal Disease Research and Diagnostic Laboratory, Department of Veterinary and Biomedical Sciences, South Dakota State University, Brookings, SD, USA

[2]Veterinary Diagnostic Laboratory, University of Minnesota, St. Paul, MN, USA

[3]Department of Pathobiology, University of Connecticut, Storrs, CT, USA

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Senecavirus A (SVA) is an emerging picornavirus that has been associated with vesicular disease and neonatal mortality in swine. Many aspects of SVA infection biology and pathogenesis, however, remain unknown. Here the pathogenesis of SVA was investigated in finishing pigs. Animals were inoculated via the oronasal route with SVA strain SD15-26 and monitored for clinical signs and lesions associated with SVA infection. Viraemia was assessed in serum and virus shedding monitored in oral and nasal secretions and faeces by real-time reverse transcriptase quantitative PCR (RT-qPCR) and/or virus isolation. Additionally, viral load and tissue distribution were assessed during acute infection and following convalescence from disease. Clinical signs characterized by lethargy and lameness were first observed on day 4 post-inoculation (pi) and persisted for approximately 2–10 days. Vesicular lesions were first observed on day 4 pi on the snout and/or feet, affecting the coronary bands, dewclaws, interdigital space and heel/sole of SVA-infected animals. A short-term viraemia was observed between days 3 and 10 pi, whereas virus shedding was detected between days 1 and 28 pi in oral and nasal secretions and faeces. Notably, RT-qPCR and *in situ* hybridization (ISH) performed on tissues collected on day 38 pi revealed the presence of SVA RNA in the tonsils of all SVA-infected animals. Serological responses to SVA were characterized by early neutralizing antibody responses (day 5 pi), which coincided with decreased levels of viraemia, virus shedding and viral load in tissues. This study provides significant insights into the pathogenesis and infectious dynamics of SVA in swine.

INTRODUCTION

Senecavirus A (SVA), previously known as Seneca Valley virus, is a non-enveloped, single-stranded, positive-sense RNA virus of the genus *Senecavirus*, family *Picornaviridae* (Hales *et al.*, 2008; Venkataraman *et al.*, 2008). The *Picornaviridae* comprises over 50 viruses that cause disease in humans and several animal species (Racaniello, 2013). Some of the most important picornaviruses include enterovirus A through J, rhinovirus-A, -B and -C, and foot-and-mouth disease virus (FMDV) (ICTV, 2015; Racaniello, 2013). SVA is closely related to members of the genus *Cardiovirus*, including cardiovirus A (encephalomyocarditis virus, EMCV) and -B (Theiler's murine encephalomyelitis virus, TMEV) (ICTV, 2015), which infect a wide range of vertebrate species, including humans, pigs and mice (Carocci & Bakkali-Kassimi, 2012; Hales *et al.*, 2008).

The SVA genome is approximately 7.2 kb in length and contains a unique open reading frame (ORF) that is flanked by 5′ and 3′ untranslated regions (UTRs), with the 3′-UTR being followed by a poly(A) tail (Hales *et al.*, 2008). The single ORF present in the SVA genome, encodes a large polyprotein that is cleaved by virus-encoded proteases into 12 mature viral proteins (5′–L–VP4–VP2–VP3–VP1–2A–2B–2C–3A–3B–3C–3D-3′) (Hales *et al.*, 2008). Notably, amino acid sequence comparisons between SVA and other picornaviruses revealed high similarity between SVA and cardioviruses within the VP4–VP1 region and in the 2C, 3C and 3D proteins, whereas the remaining proteins of SVA are considerably different from their homologues in other picornaviruses (Hales *et al.*, 2008).

Genbank accession no. for SVA SD15-26: KX778101.

Three supplementary tables are available with the online Supplementary Material.

SVA was originally identified as a cell culture contaminant in the USA in 2002 (Knowles *et al.*, 2006). Sequencing of picorna-like viruses isolated from pigs with various clinical presentations, however, revealed the presence of SVA in the USA swine population since the late 1980s (Knowles *et al.*, 2006). Since its identification in 2002, SVA has been associated with sporadic cases of vesicular disease in pigs in the USA (Bracht *et al.*, 2016; Singh *et al.*, 2012) and Canada (Pasma *et al.*, 2008). Interestingly, since November 2014 several outbreaks of vesicular disease associated with SVA have been reported in Brazil (Joshi *et al.*, 2016; Leme *et al.*, 2015; Vannucci *et al.*, 2015). Additionally, in March 2015 SVA was detected during an outbreak of vesicular disease in China (Wu *et al.*, 2016), and since July 2015 an increasing number of cases have been reported in the USA (Bracht *et al.*, 2016; Canning *et al.*, 2016; Joshi *et al.*, 2016). The factors that led to the surge in the number of SVA outbreaks remain unknown. Interestingly, sequence analysis of current SVA isolates circulating in the USA, Brazil and China, and comparison of these isolates to historical SVA strains, revealed a marked genetic diversity (91–93 %) between contemporary and historical SVA strains (Joshi *et al.*, 2016). Whether these genetic changes have contributed to the emergence of SVA remains to be determined.

Characteristic lesions observed in recent SVA outbreaks include vesicles on the snout, oral mucosa and coronary bands, while common clinical signs include lameness and lethargy. Additionally, diarrhoea and increased neonatal mortality in piglets less than seven days of age have also been reported (Canning *et al.*, 2016; Joshi *et al.*, 2016). While studies conducted with historical SVA isolates failed to reproduce the disease in pigs (Knowles *et al.*, 2006; Yang *et al.*, 2012). A recent study with a contemporary SVA isolate from the USA reports the development of vesicular disease in experimentally infected animals, confirming the role of the virus in the aetiology of vesicular disease in pigs (Montiel *et al.*, 2016). Clinically, lesions induced by SVA are indistinguishable from those observed in other vesicular diseases of swine including foot-and-mouth disease, vesicular stomatitis, swine vesicular disease and vesicular exanthema of swine (Alexandersen *et al.*, 2002; Pasma *et al.*, 2008), which highlights the importance of rapid and accurate diagnosis and differentiation of vesicular disease-causing agents of swine.

Although SVA has recently been linked to vesicular disease in pigs (Montiel *et al.*, 2016), many aspects of its infection biology and pathogenesis remain unknown. In the present study, the pathogenesis of a contemporary SVA strain SD15-26 was evaluated following experimental inoculation of finishing pigs. The dynamics of infection and viraemia, patterns of virus shedding and viral load and tissue distribution were assessed by real-time reverse transcriptase quantitative PCR (RT-qPCR) and virus isolation. Serological responses to SVA were evaluated by virus neutralization, and immunofluorescence assays. Results here provide important insights into the pathogenesis of SVA-induced vesicular disease in pigs.

RESULTS

Characterization of Senecavirus A isolate SD15-26

The SVA isolate SD15-26 was obtained from a vesicular swab collected from finishing pigs presenting vesicles on the snout and coronary bands. The virus was isolated in H1299 human non-small-cell lung carcinoma cells, and low-passage viral stocks (passage 4) were used in all experiments described here. All samples subjected to virus isolation were positive for SVA by RT-qPCR and tested negative for other vesicular disease agents of swine [FMDV, vesicular stomatitis virus (VSV), swine vesicular disease virus (SVDV) and vesicular exanthema of swine (VES)] at the Foreign Animal Disease Diagnostic Laboratory at the Plum Island Animal Disease Center (PIADC). The complete genome sequence of SVA SD15-26 was determined by next-generation sequencing using the Illumina MiSeq sequencing platform, and complete genome sequence comparisons between the SVA SD15-26 isolate and other SVA strains available in GenBank revealed 98–99 % nucleotide identity with contemporary SVA strains and only 93 % nucleotide identity with the historical SVA prototype strain SVV-001 (Hales *et al.*, 2008). The genome of SVA SD15-26 is 7266 nt in length (RNA G + C content of 52 %) and encodes a single open reading frame of 2181 amino acids (GeneBank accession number KX778101). A summary of the genomic features of SVA SD15-26, including the predicted amino acid cleavage sites of individual proteins, is presented in Table S1 (available in the online Supplementary Material).

The replication properties of SVA SD15-26 were assessed in H1299 cell cultures and compared to its replication ability in cell lines derived from swine (porcine kidney-15, PK-15). Single-step [multiplicity of infection (MOI)=10] or multistep (MOI=0.1) growth curves were performed in both cell lines, and viral yields determined at different intervals post-inoculation (pi). SVA SD15-26 replicated efficiently in both cell lines with peak viral titres being detected at 72 h pi. Replication kinetics of SVA in H1299 and PK-15 cells were similar at low and high MOIs (data not shown).

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

The pathogenicity of SVA SD15-26 and its ability to cause vesicular disease were assessed in finishing pigs. Eight 15-week-old finishing pigs (approximately 55 kg weight), were inoculated with SVA SD15-26 via the oronasal route (half the inoculum was given orally and the other half intra-nasally) and were monitored for characteristic SVA clinical signs and lesions during a 38-day experimental period. All SVA-inoculated pigs (n=8) presented lameness and lethargy by day 4 pi and these signs persisted for approximately 2–10 days. Vesicular lesions were first observed on the feet and/or snout of SVA-inoculated animals on day 4 pi (six out of eight animals) (Fig. 1).Initial observations consisted of erythema in the skin, which progressed and developed into vesicles ranging from

0.5 to 3 cm in diameter. The vesicles ruptured around day 5–6 pi, leaving an erosion on the skin that subsequently evolved, forming a crust in the affected area (day 8–9 pi). Lesions were completely resolved by days 12–16 pi (Fig. 1a). Two animals presented (two out of eight; numbers 45 and 46) only erythematous areas on the snout which persisted for 2–4 days (Fig. 1b). A summary of the clinical signs and lesions observed in SVA-inoculated animals is presented in Table S2. It is important to note that animal number 46 died of unrelated cause on day 7 pi.

The presence of SVA in the lesions was confirmed by RT-qPCR (data not shown) and/or by *in situ* hybridization (ISH) (Fig. 1c). The lesions that developed on the feet of SVA-infected animals presented similar progression to those observed on the snout (Fig. 1a, b), with vesicles being observed on the heel/sole of the feet, interdigital spaces, dewclaws and/or coronary bands (Fig. 1b). Control group pigs (n=4) did not exhibit any clinical signs or pathological changes. No significant differences were observed in the rectal temperature of control and SVA-infected pigs (data not shown).

SVA-inoculated pigs present a short-term viraemia

Viraemia was assessed in serum of SVA-inoculated pigs. Serum samples collected on days 1, 3, 5, 7, 10, 14, 21, 28, 35 and 38 pi were evaluated for the presence of SVA using RT-qPCR. SVA was detected between days 3 and 10 pi (Table 1; Fig. 2a). Peak SVA genome copies were detected on day 3 pi, progressively decreasing thereafter, with no viral RNA being detected after day 10 pi (Table 1; Fig. 2a). One SVA-inoculated animal did not present viraemia (animal 44, Table 1). Notably, virus isolation attempts in RT-qPCR-positive serum samples led to isolation of SVA from only two samples (out of six) on day 3 pi (data not shown), with no virus being isolated from serum after day 3 pi. These results indicate that SVA induces a short-term viraemia in pigs, with viral RNA being detected in serum during a period of approximately 7 days (3–10 days pi), which coincided with the acute clinical phase of the disease.

SVA-inoculated pigs shed virus in oral and nasal secretions and in faeces

The dynamics and patterns of virus shedding were evaluated in oral and nasal secretions and in faeces. Oral, nasal and rectal swabs were collected on days 0, 1, 3, 5, 7, 10, 14, 21, 28 and 35 pi and screened for the presence of SVA. Virus shedding was detected up to day 28 pi by RT-qPCR, with all infected animals shedding SVA in oral and nasal secretions and in faeces between days 3 and 14 pi (Table 1). After day 14 pi a transient shedding pattern was observed, with a few animals (four out of seven) shedding virus up to day 28 pi (Table 1). No virus shedding was detected on day 35 pi (Table 1). The amount of SVA present in oral and nasal secretions and in faeces was quantitated and expressed as genome copy number per ml (Fig. 2b). Peak viral shedding was observed between days 1 and 5 pi, with higher amounts of SVA RNA being detected in oral secretions until day 5 pi. The levels and frequency of SVA shed by infected animals gradually decreased between days 5 and 28 pi, with no virus being detected on samples collected on day 35 pi (Table 1; Fig. 2b).

Shedding of infectious SVA in oral and nasal secretions and faeces was assessed by virus isolation (Joshi *et al.*, 2016) in RT-qPCR-positive samples. SVA was isolated from oral secretions up to day 21 pi, while nasal and faecal samples contained infectious virus up to days 7 and 10 pi, respectively (Table 2). The amount of infectious SVA was quantitated by limiting dilution and viral titres determined as per Spearman and Karber's method (Hierholzer & Killington, 1996), and expressed as tissue culture infectious dose 50 ($TCID_{50}$) per ml on samples collected on days 3, 7 and 21 pi (Table S3). Higher viral titres were detected in oral secretions on day 7 pi (Table S3), with individual titres ranging from 2 to 3.5 $\log_{10}$ $TCID_{50}$ ml^{-1} (Table S3). Notably, five out of seven animals were still shedding SVA in oral secretions on day 21 pi (1.75–2.25 $\log_{10}$ $TCID_{50}$ ml^{-1}; Table S2). In general, lower amounts of SVA were detected in nasal secretions and faeces of SVA-infected animals (Fig. 2b; Table S3).

Viral load and tissue distribution during the acute stage of SVA infection

Viral load and tissue distribution were investigated during the acute stage of SVA infection. For this, four animals used as mock-infected controls during the clinical phase of the experiment were inoculated with SVA (experimental day 35; same dose and inoculation route as above) and euthanized on days 3, 4, 5 and 6 pi (one animal per day). A fifth animal (number 46), which died of unrelated cause on day 7 pi, was included in this study. Tissues including lung, mediastinal lymph node, heart, liver, spleen, mesenteric lymph node, small intestine, large intestine, kidney and tonsil were collected and processed for RT-qPCR, virus isolation, histological examination and/or ISH. Two of these animals (out of four) developed vesicular lesions on the coronary bands and the presence of SVA on the skin lesions was confirmed by ISH (data not shown). SVA nucleic acid was consistently detected between days 3 and 7 pi in the lung, mediastinal and mesenteric lymph nodes, liver, spleen, small and large intestines and tonsils (Table 3; Fig. 3a), and viable SVA was isolated from these tissues between days 3 and 5 pi (Table 3). Notably, quantitation of SVA RNA in these tissues revealed higher levels of the virus in the tonsils of SVA-infected animals (Fig. 3a), with infectious virus being isolated from the tonsils up to day 7 pi. Replication of SVA in the tonsils was confirmed by ISH and virus isolation (Fig. 3b; Table 3). These results indicate that the tonsil might be one of the primary sites of SVA replication during the acute stage of infection.

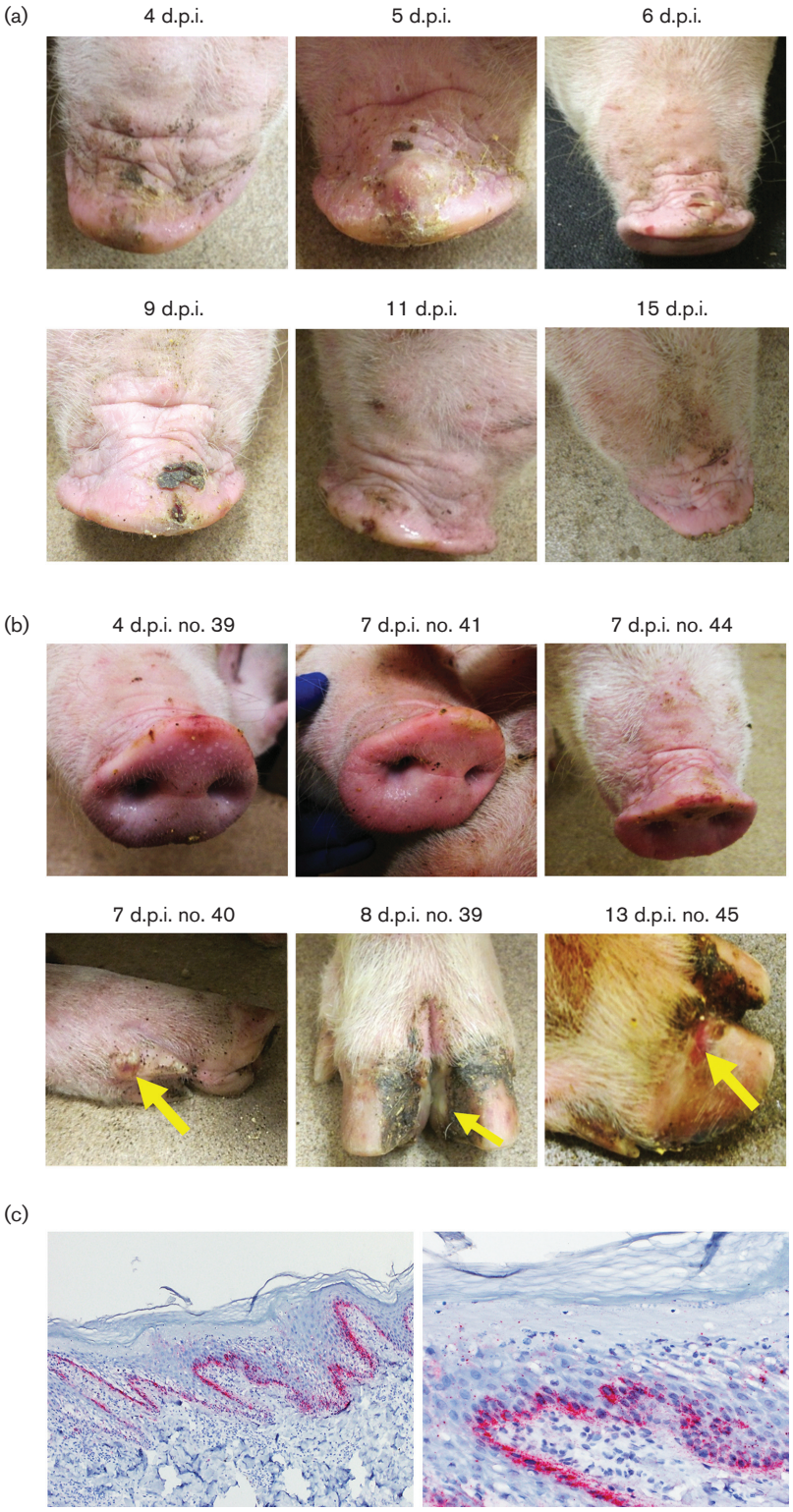

Fig. 1. Senecavirus A-induced vesicular disease in swine. (a) Progression of SVA-induced vesicular disease in swine. Animals were inoculated oronasally with SVA strain SD15-26. Vesicular lesions on the snout were first observed on day 4 pi, and by day 6 pi vesicles ruptured, leaving an eroded/ulcered lesion on the skin which progressed, leaving a crust covering the affected area of the skin (days 8–9 pi). By day 11 pi only a small scar remained on the snout and by day 15 pi the lesion was completely resolved. (b) Distinct lesions observed in SVA-inoculated pigs. While some animals presented erythema or small vesicles on the snout (top panels), other animals presented large vesicles on the snout or feet, located in the interdigital spaces, dewclaws

and/or coronary band (bottom panels; yellow arrows). (c) *In situ* hybridization demonstrating the presence of SVA RNA (red coloration) in the skin of the coronary band of SVA-inoculated swine. Staining was observed in keratinocytes in intraepidermal vesicles in the stratum spinosum and stratum basale (day 4 pi).

SVA RNA is present in tissues from convalescent pigs

The presence of SVA in tissues was assessed following convalescence from disease. Tissues including lung, mediastinal lymph node, heart, liver, spleen, mesenteric lymph node, small intestine, large intestine, kidney and tonsil were collected on day 38 pi (approximately 3.5 weeks after complete resolution of vesicular lesions) and processed for RT-qPCR and virus isolation. SVA RNA was detected in the mediastinal lymph node (six out of seven animals), spleen (four out of seven), mesenteric lymph node (two out of seven), small intestine (two out of seven), large intestine (one out of seven), kidney (one out of seven) and tonsils (seven out of seven) of SVA-infected animals. Notably, quantitation of viral nuclei acid by RT-qPCR demonstrated high viral load in the tonsils of all SVA-inoculated animals (average of approximately 10^5 genome copies) (Fig. 3c). The presence of SVA RNA in the tonsils was confirmed by ISH, with most viral RNA being detected in the subepithelial area and occasionally in lymphoid follicles (Fig. 3d). No viral RNA was detected in the lungs, heart and liver of SVA-inoculated animals. No infectious SVA was isolated from any of the RT-qPCR-positive tissues on day 38 pi (data not shown). These results demonstrate the presence of SVA RNA in lymphoid (lymph nodes and tonsils) and non-lymphoid tissues (small and large intestine and kidney) following convalescence from vesicular disease in pigs.

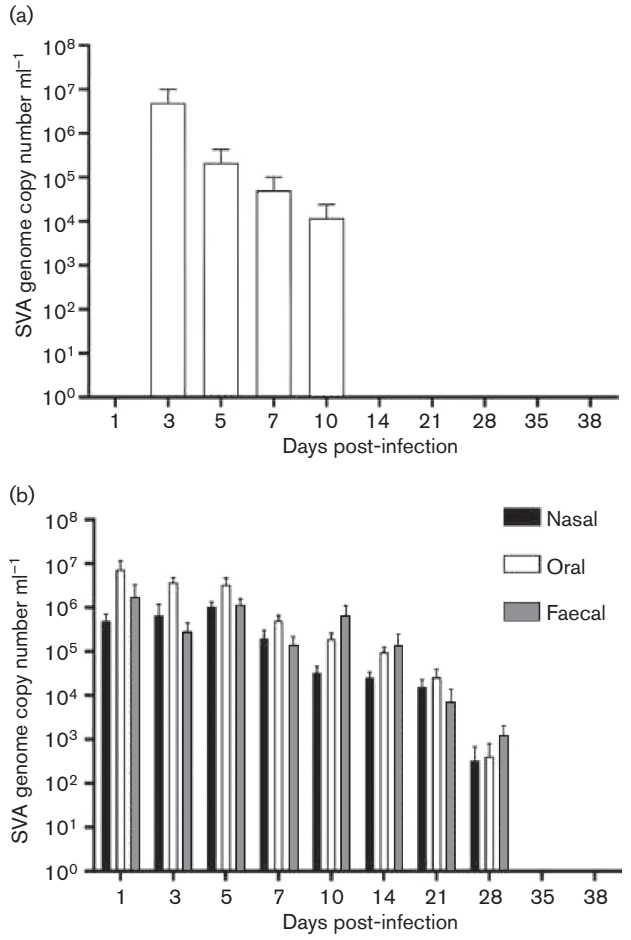

Fig. 2. Infection dynamics of SVA in swine. (a) Viraemia detected in serum by RT-qPCR and expressed as $\log_{10}$ genome copy number ml^{-1}. Error bars represent the standard error of the mean (SEM). (b) Virus shedding in nasal and oral secretions and in faeces of SVA-infected animals. Levels of virus shedding were determined by RT-qPCR and expressed as $\log_{10}$ genome copy numbers ml^{-1}. Error bars represent the SEM. No viraemia or virus shedding was detected in control non-infected animals.

Histopathological changes induced by SVA infection

A complete histological examination was performed in tissues collected during the acute (days 3–7 pi) and convalescent stages of SVA infection (day 38 pi). Histological changes observed in the skin of animals presenting vesicular lesions in the coronary bands (days 4–6 pi) consisted of coronary band dermatitis, which was characterized by multifocal separation of the dermis from the epidermis with cleft formation and exocytosis of inflammatory cells (Fig. 4a). Clefts contained large numbers of necrotic keratinocytes admixed with oedema, mild haemorrhage, fibrin and inflammatory infiltrates consisting of macrophages, lymphocytes, plasma cells and neutrophils (Fig. 4a). Additionally, the epidermis adjacent to the vesicular lesions presented hyperplasia with rete peg formation and anastomoses, multifocal intracellular oedema and mild spongiosis (Fig. 4a).

All other tissues including lung, mediastinal lymph node, heart, liver, spleen, mesenteric lymph node, small intestine, large intestine, kidney and tonsils collected during the acute stage of SVA infection (days 3–7 pi) or after convalescence (day 38 pi) were also subjected to histological examination. Notably, during the first 3–7 days pi, histological changes were restricted to lymphoid tissues (tonsils, spleen and lymph nodes) and lung. Typical histological changes observed in the tonsils, spleen and lymph nodes consisted of mild to moderate lymphoid hyperplasia (Fig. 4b). The lungs presented multifocal mild atelectasis and occasionally diffuse congestion with multifocal mild perivascular accumulation of lymphocytes, plasma cells and macrophages (data not shown). No histological changes were observed in

Table 1. Virus shedding by SVA SD15-26-inoculated animals as detected by RT-qPCR

		Virus shedding at days post-infection (pi)																										
		1			3			5			7			10			14			21			28			35		
	Animal number	O*	N†	F‡	O	N	F	O	N	F	O	N	F	O	N	F	O	N	F	O	N	F	O	N	F	O	N	F
Control	35	–§	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	36	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	37	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	38	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SVA	39	+‖	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–
	40	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–
	41	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–
	42	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	+	–	–	–
	43	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	NA	NA	NA
	44	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	–	–	–	–	–
	45	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	–	–
	46	+	+	+	+	+	+	+	+	+	+	+	+	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA, not available.
*Oral secretions.
†Nasal secretions.
‡Faeces.
§No SVA RNA detected by RT-qPCR.
‖SVA RNA detected by RT-qPCR.

Table 2. Virus shedding by SVA-inoculated animals as detected by virus isolation

Group	Animal no.	Virus shedding at days post-infection (pi)																									
		1			3			5			7			10			14			21			28			35		
		O*	N†	F‡	O	N	F	O	N	F	O	N	F	O	N	F	O	N	F	O	N	F	O	N	F	O	N	F
SVA	39	+	+‖	+	+	+	+	+	+	+	+	–	+	+	–	–	–	–	–	+	–	NT	NT	NT	NT	NT	NT	NT	NT
	40	+	+	–	+	+	+	–	+	+	+	–	+	+	–	–	–	–	–	–	–	NT	–	NT	NT	NT	NT	NT	NT
	41	+	+	–	+	+	+	–	–	+	+	+	+	+	–	+	–	–	–	+	–	NT	NT	NT	NT	NT	NT	NT	NT
	42	+	+	–	+	+	+	–	+	+	+	+	–	+	–	–	–	–	–	NT	NT	–	NT	NT	NT	NT	NA	NA	NA
	43	+	+	+	+	+	+	–	+	+	+	–	–	–	–	–	–	NT	–	+	NT	NT	NT	NT	–	NT	NT	NT	NT
	44	+	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–	–	NT	+	–	NT	NT	–	–	NT	NT	NT	NT
	45	+	+	+	+	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	NT	NT	–	NT	NT	NT	NT	NT	NT
	46	–§	+	–	+	+	+	+	+	+	+	–	+	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NT, not tested. SVA was not detected by RT-qPCR.
NA, not available.
*Oral secretions.
†Nasal secretions.
‡Faeces.
§Negative sample after inoculation in cell cultures.
‖Positive sample after inoculation in cell cultures.

the other tissues collected during acute infection (days 3 and 7 pi). The only histological change observed in tissues collected on day 38 pi was moderate lymphoid hyperplasia, which was restricted to the tonsils (data not shown).

SVA infection induced early neutralizing antibody (NA) response in pigs.

The serological response to SVA was evaluated by indirect immunofluorescence (IFA) and virus neutralization assay (VN). All SVA-infected animals seroconverted to SVA and developed an early antibody response against the virus (days 5–10 pi; Fig. 5a, b). SVA-specific IgG antibodies were first detected by IFA on day 10 pi, with most animals presenting high levels of SVA-specific IgG antibodies until the end of the experiment on day 38 pi. Additionally, all SVA-infected animals developed high levels of neutralizing antibodies (NA) against the virus. Notably, NA were first detected on day 5 pi, with peak NA titres observed on day 10 pi. High levels of NA were still detected at the end of the experiment on day 38 pi. Pigs in the control groups did not present antibodies against SVA throughout the experiment. These results demonstrate an early and robust NA response following SVA infection in pigs.

DISCUSSION

The goal of this study was to assess the pathogenesis and infection dynamics of SVA-induced vesicular disease in swine. Despite being detected in pigs presenting vesicular disease (Pasma *et al.*, 2008; Singh *et al.*, 2012), early studies conducted with historical SVA strains failed to reproduce disease in pigs (Knowles *et al.*, 2006; Yang *et al.*, 2012). Recently, however, experimental infection of pigs with contemporary SVA isolates demonstrated the ability of SVA to cause vesicular disease in swine (Chen *et al.*, 2016; Montiel *et al.*, 2016). Results here corroborate findings from the latter studies, confirming the role of SVA as the aetiological agent of vesicular disease in swine. Animals that were inoculated with SVA strain SD15-26 presented characteristic clinical signs and vesicular lesions similar to those that have been reported in cases of natural infection with the virus (Canning *et al.*, 2016; Guo *et al.*, 2016; Joshi *et al.*, 2016; Leme *et al.*, 2015). Clinical disease was observed on days 4–5 pi and lasted for approximately 10–12 days. These observations are consistent with the findings of Chen and Montiel and collaborators (Chen *et al.*, 2016; Montiel *et al.*, 2016), indicating that SVA has a short incubation period and induces an acute self-limiting vesicular disease in pigs. Although all SVA-inoculated pigs seroconverted, only 8 out of 12 animals (approximately 66 %) developed vesicular disease. This seems to be a common feature of SVA, with similar morbidity rates being observed in natural cases (Canning *et al.*, 2016; Guo *et al.*, 2016; Joshi *et al.*, 2016; Leme *et al.*, 2015; Vannucci *et al.*, 2015) and in experimental infections in pigs (Montiel *et al.*, 2016).

Table 3. Tissue distribution of SVA during the acute stage of infection

Animal no. (dpi)	Tissues																			
	Lung		Med. LN*		Heart		Liver		Spleen		Mes. LN†		Small Intestine		Large Intestine		Kidney		Tonsil	
	RT-qPCR	VI	RT-qPCR	VI	RT-qPCR	VI	RT-qPCR	VI	RT-qPCR	VI	RT-qPCR	VI	RT-qPCR	VI	RT-qPCR	VI	RT-qPCR	VI	RT-qPCR	VI
35 (3 dpi)	+‡	+	+	+	–	NT	+	+	+	+	+	+	+	+	+	+	–	NT	+	+
36 (4 dpi)	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+
38 (5 dpi)	+	-§	+	+	–	NT	+	–	+	+	+	+	+	–	+	+	–	NT	+	+
37 (6 dpi)	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–
46 (7 dpi)	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	–	NT	+	+

VI: virus isolation; NT: Not tested. No SVA detected by RT-qPCR.
*Mediastinal lymph node.
†Mesenteric lymph node.
‡Positive sample.
§Negative sample.

Other aspects of SVA pathogenesis were assessed in our study, included viraemia, virus shedding and viral load and tissue distribution. Viraemia coincided with the clinical phase of the disease (days 3–10 pi), with peak viral RNA (approximately $1\times10^{6.5}$ SVA genome copies ml^{-1}) being detected in serum on day 3 pi, just prior to the appearance of vesicular lesions on day 4 pi. Despite the high amounts of viral RNA being detected in serum during seven days, SVA was isolated only on day 3 pi, with no infectious virus being recovered from serum thereafter. Notably, levels of viraemia decreased after the appearance of circulating neutralizing antibodies (Figs 2a and 5b), with the detection of NAs on day 5 pi correlating with the absence of infectious virus in serum. Similar patterns of viraemia have been observed in nursing pigs that were inoculated with another contemporary SVA strain, with the virus RNA being detected in serum on days 3 and 7 pi (Chen *et al.*, 2016). These observations indicate that SVA induces a short-term viraemia in infected animals and further suggest that, as with other picornaviruses (Dotzauer & Kraemer, 2012), neutralizing antibodies may play a role in clearance of SVA from the blood.

The dynamics of virus shedding following oronasal infection with SVA were monitored in oral and nasal secretions and faeces. Viral nucleic acid was consistently detected in all SVA-infected animals, with at least one specimen of each animal testing positive for SVA for approximately 3 weeks pi (Table 1). Notably, oral secretions contained larger amounts of SVA, with infectious SVA being recovered from oral secretions for 21 days when compared to only 7 days from nasal secretions and faeces (Tables 3, S2). These findings are consistent with results from a previous study, in which higher amounts of SVA were detected in oral fluid from SVA-infected animals (Chen *et al.*, 2016). The prolonged shedding of SVA in oral secretions (Tables 2, S2; Fig. 4b) indicates that the virus may replicate in the oral mucosa or, perhaps, in another associated tissue. Infectious SVA was, indeed, detected in the tonsils between days 3 and 7 pi, indicating that this tissue supports active replication of the virus and may serve as a source of virus that is shed in oral secretions. Collectively, these data suggest that oral and nasal secretions and faeces may serve as a source of SVA infection to susceptible animals. In addition to vesicular fluid, which contains large amounts of SVA (Canning *et al.*, 2016; Joshi *et al.*, 2016; Leme *et al.*, 2015; Vannucci *et al.*, 2015), these secretions/excretions may play a role in transmission of SVA in the field.

Tissue distribution and viral load were investigated during the acute phase of SVA infection. SVA was consistently detected in the lung, lymph nodes (mediastinal and mesenteric), liver, spleen, small and large intestines and tonsils of SVA-infected animals between days 3 and 7 pi, with infectious virus being recovered from most of these tissues between days 3 and 5 pi (Table 3). Consistent with the virological findings, histological examination of the tonsils, spleen and lymph nodes revealed mild to moderate lymphoid hyperplasia, indicating that these tissues may

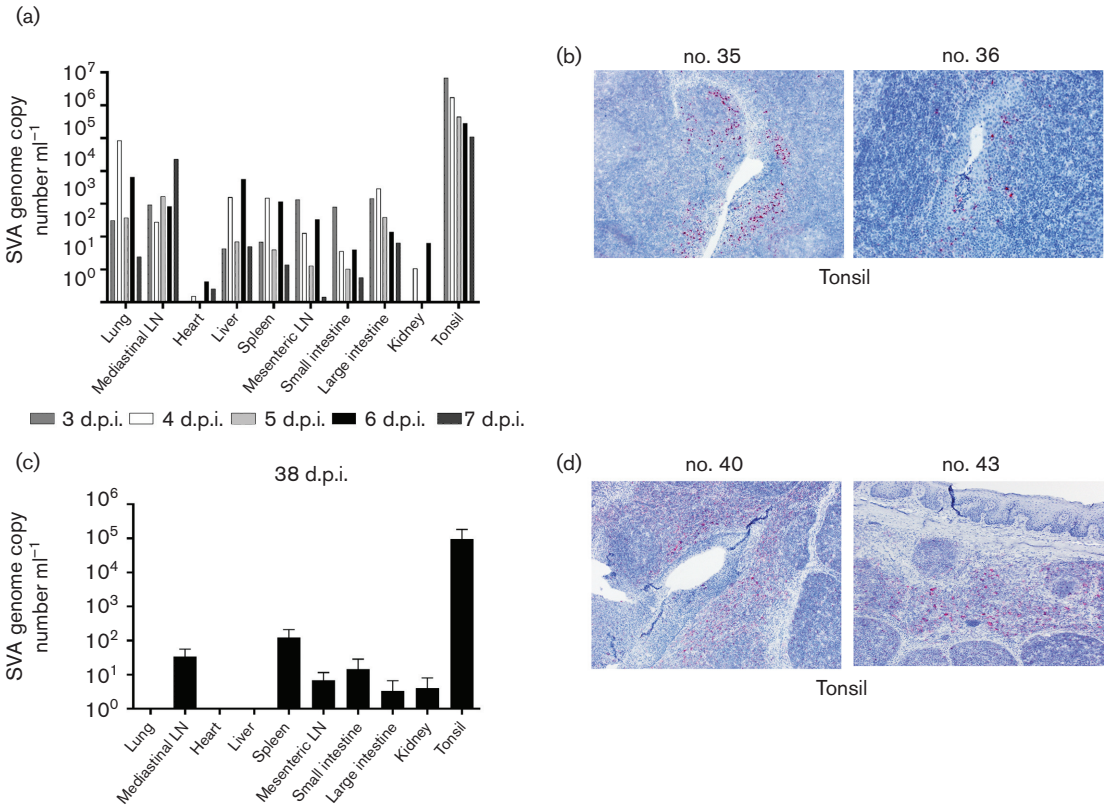

Fig. 3. Viral load and tissue distribution of SVA during infection in finishing pigs. (a) Viral load in different tissues collected between days 3 and 7 post-infection. Levels of SVA were determined by RT- qPCR and expressed as $\log_{10}$ genome copy number ml^{-1}. (b) *In situ* hybridization demonstrating the presence of SVA nucleic acid (red coloration) in the tonsils of SVA-infected pigs on days 3 (number 35) and 4 pi (number 36). Multifocal staining was observed in crypt epithelium and subepithelial lymphocytes. (c) Viral load in different tissues collected on day 38 post-infection. Levels of SVA were determined by RT-qPCR and expressed as $\log_{10}$ genome copy number ml^{-1}. Error bars represent the standard error of the mean (SEM). (d) *In situ* hybridization demonstrating the presence of SVA nucleic acid (red coloration) in the tonsils of SVA-infected pigs on days 28 (number 43) and 38 pi (number 40). Multifocal staining was observed in crypt epithelium, subepithelial lymphocytes and lymphoid follicles.

represent potential sites of SVA replication. However, given that the virus was still viraemic at the time of tissue collection, the possibility that detection of SVA in some of these tissues was due to the virus circulating in the blood cannot be formally excluded. Notably, levels of SVA nucleic acid detected in the tonsils were markedly higher than those in other tissues, with infectious virus being detected in this tissue for at least seven days pi (Fig. 3a; Table 3). Previous studies have indicated that the tonsil is one of the primary sites of replication for multiple picornaviruses (Gelmetti *et al.*, 2006; He *et al.*, 2014; Murphy *et al.*, 2010; Papaioannou *et al.*, 2003). Likewise results here, demonstrating infectious SVA and virus-specific ISH staining in crypt epithelium and subepithelial mononuclear cells in the tonsils, indicate that this tissue is one of the sites of SVA replication during the acute phase of infection.

One of the most intriguing findings of our study was the detection of SVA RNA in tissues from convalescent animals. While the virus RNA was detected in lymph nodes, spleen, intestine and kidney of a few animals, all animals (seven out of seven) presented high levels of SVA nucleic acid in the tonsils (1–3 logs higher than in other tissues; Fig. 3c). ISH performed on sections from the tonsil demonstrated a similar staining pattern to that observed during the acute phase of infection, with SVA nucleic acid being detected mainly in epithelial cells in the crypt and in subepithelial mononuclear cells. Despite the detection of high levels of SVA RNA in these tissues, no infectious virus was recovered after three passages in highly susceptible H1299 cells (data not shown) (Joshi *et al.*, 2016; Poirier *et al.*, 2013). The fact that SVA RNA was not cleared from tissues approximately 3.5 weeks after resolution of the clinical disease raises important questions about the strategies that the virus may have evolved for its maintenance in nature. Several picornaviruses, including EMCV and FMDV, are known to establish persistent infections in natural hosts, with carrier animals playing an important role in the epidemiology of these viruses (Alexandersen *et al.*, 2002; Billinis *et al.*, 1999; Zhang &

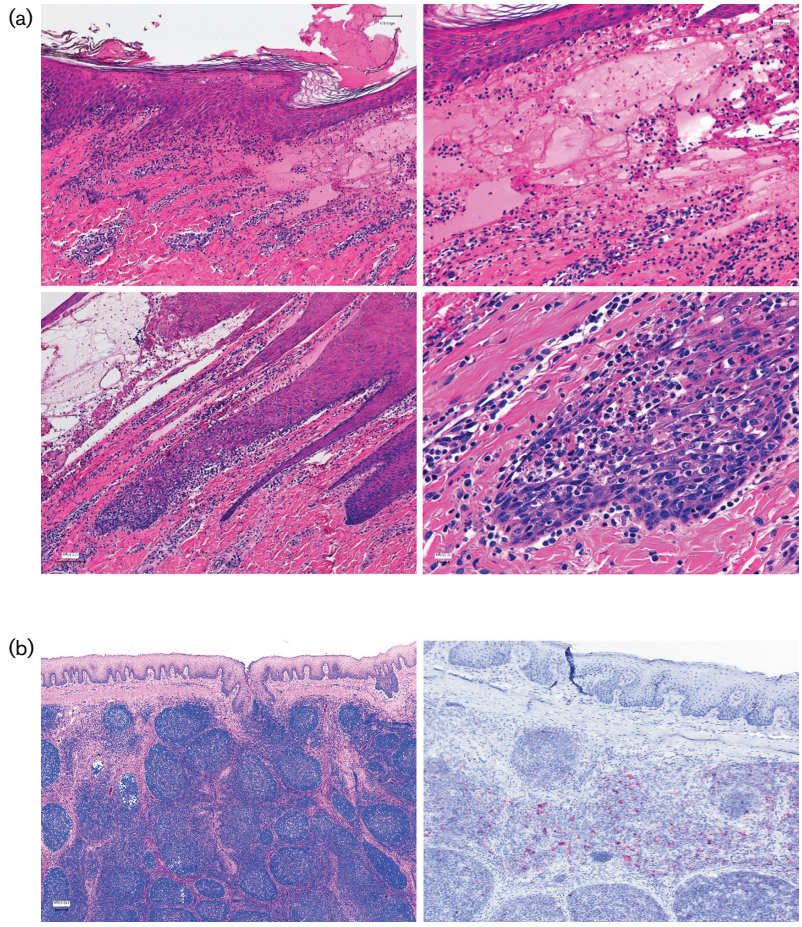

Fig. 4. Histological changes observed in SVA-inoculated swine. (a) Histological sections of skin recovering the coronary band of an animal that presented a vesicular lesion after SVA-infection (day 4 pi). Lesions were characterized by perivascular inflammation with separation of the epidermis from the dermis and accumulation of fibrin, oedema, haemorrhage and inflammatory cells within the cleft [top panels; 100× (left); 200× (right)]. Bottom panels show intense inflammation and separation at the dermal–epidermal junction, with accumulation of inflammatory exudate and necrotic keratinocytes [100× (left); 400× (right)]. (b) Histological section of the tonsil of a SVA-infected animal (day 4 pi). Histological changes in the tonsil were characterized by lymphoid hyperplasia (left panel). *In situ* hybridization demonstrating the presence of SVA nucleic acid (red coloration) in lymphoid follicles (right panel).

Alexandersen, 2004). Therefore, in the future it would be interesting to assess whether SVA is capable of establishing chronic/subclinical and/or persistent infection in pigs. Most important would be to assess whether infectious virus can be recovered from tissues and/or body secretions/excretions of convalescent animals under physiological conditions or, perhaps, after stressful stimuli.

SVA-specific antibodies were detected at early stages of SVA infection, with neutralizing antibodies first detected around day 5 pi. Early antibody responses are a hallmark of several picornaviruses, with initial detection of antibodies usually coinciding with progressive clearance of the viruses from the circulation and most organs, excretions and secretions (Dotzauer & Kraemer, 2012). Similarly, here, rising levels of SVA-specific NA in serum paralleled decreased disease severity (Figs 1 and 5), lower levels of viraemia (Fig. 2a), and virus shedding (Fig. 2b), and decreased viral load in tissues (Table 3, Fig. 3a).

In summary, this study provides important insights into the infection dynamics and pathogenesis of SVA in swine. Results presented here demonstrate the acute nature of the vesicular disease caused by SVA in swine and shed light on the patterns of viraemia and virus shedding by infected animals. Virus isolation and RT-qPCR performed on tissues indicate that SVA has a tropism for lymphoid tissues, with higher viral loads being detected in the tonsils of infected animals. Results demonstrating the presence of SVA nucleic acid in tissues from convalescent animals highlight similarities between SVA and other picornaviruses. Additional studies are needed, however, to assess

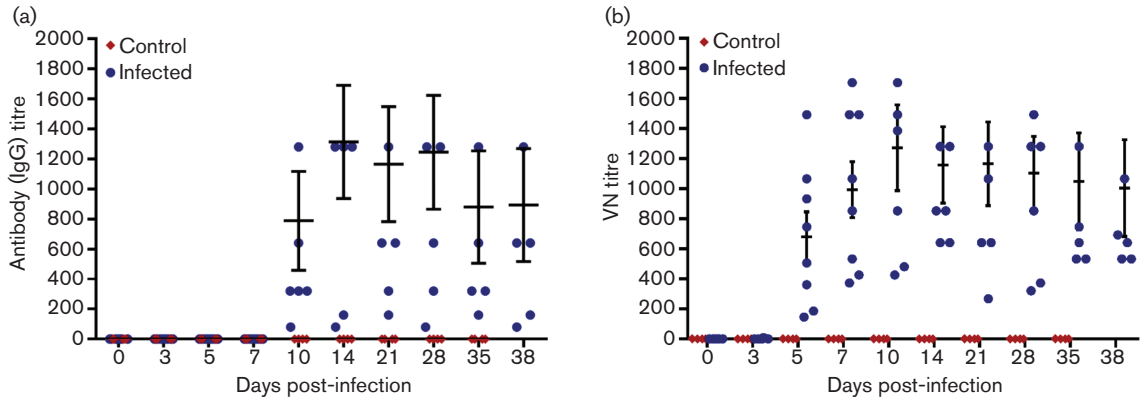

Fig. 5. Serological responses to Senecavirus A. (a) Indirect immunofluorescence assay demonstrating serum IgG antibody responses against SVA. (b) Virus neutralization assay demonstrating neutralizing antibody responses against SVA. Red diamonds represent individual control uninfected animals. Blue dots represent individual SVA-inoculated animals. Error bars represent the standard error of the mean (SEM).

the importance of these findings for SVA infection biology and epidemiology.

METHODS

Cells and virus. H1299 were obtained from the American Type Culture Collection (ATCC-CRL 5803) and cultured at 37 °C with 5 % CO_2 in RPMI 1640 medium supplemented with 10 % fetal bovine serum and 2 mM L-glutamine and containing penicillin (100 U ml^{-1}), streptomycin (100 µg ml^{-1}) and gentamycin (50 µg ml^{-1}). PK-15 cells were cultured at 37 °C with 5 % CO_2 in MEM containing the supplements as above.

Senecavirus A isolate SD15-26 was obtained from a lesion swab collected from a finishing pig presenting vesicular disease in the state of South Dakota in 2015. Swab and serum samples were submitted to the SD Animal Disease Research and Diagnostic Laboratory and referred to the Foreign Animal Disease Diagnostic Laboratory at the Plum Island Animal Disease Center (PIADC) for foreign animal disease (FAD) investigation. SVA-positive swab samples were processed for virus isolation as previously described (Joshi *et al.*, 2016) and inoculated into semi-confluent (60–70 %) monolayers of H1299 cells. Cells were monitored daily for five days for the development of cytopathic effect (CPE), and SVA isolation was confirmed by RT-qPCR and immunofluorescence assays. Low-passage (passage 4) SVA stocks were prepared and titrated in H1299 cells and used in all experiments described here.

Sequencing of Senecavirus A. The complete genome sequence of SVA SD15-26 was determined using the Illumina MiSeq sequencing platform. Prior to viral RNA extraction, 0.5 ml of the virus suspension was subjected to DNAse (10 U; Turbo DNAse, Ambion) and 5′ – phosphate-dependent exonuclease (Terminator 5′- Phosphate-Dependent Exonuclease; Epicentre) treatment for 30 min at 37 °C. Viral RNA was purified using the QiaAmp viral RNA mini kit (Qiagen). First-strand SVA cDNA was synthesized using the Protoscript II reverse transcriptase kit (New England Biolabs) and a pool of 10 SVA-specific primers targeting the entire viral genome (sequences available upon request). Second-strand SVA cDNA was synthesized using the NEBNext mRNA second-strand synthesis module (New England Biolabs) and purified using Agencourt AMPure XP beads (Beckman Coulter) according to the manufacturer's protocols. One nanogram of double-stranded cDNA was used for DNA library preparation using the Nextera XT DNA library kit (Illumina) according to the manufacturer's instructions. Following DNA library preparation, the sample was subjected to DNA sequencing using the Illumina MiSeq platform at the South Dakota Animal Disease Research and Diagnostic Laboratory (SD ADRDL). The SVA genome was assembled using Ray v2.3.1 (Boisvert *et al.*, 2012). Variants were assembled with Vicuna v1.3 (Yang *et al.*, 2012) and Trinity r2011-11–26 (Grabherr *et al.*, 2011). All variants were mapped with Consed v29 (Gordon & Green, 2013), samtools pileup (Li, 2011) and VarScan.v2.4.1 (Koboldt *et al.*, 2012) and aligned with the CLUSTALW 2.1 multiple sequence alignment program (Thompson *et al.*, 1994).

Replication kinetics. Replication properties of SVA SD15-26 was assessed *in vitro*. H1299 and PK-15 cells were cultured in six-well plates, inoculated with SVA SD15-26 at a multiplicity of infection (MOI) of 0.1 (multi-step growth curve) or 10 (single-step growth curve), and harvested at various time points post-infection (2, 4, 6, 8, 12, 24, 36, 48 and 72 h post-infection). Virus titres were determined for each time point using Spearman and Karber's method and expressed as $TCID_{50}$ ml^{-1}.

Pathogenesis studies in pigs. The pathogenesis of SVA was evaluated in 15-week-old finishing pigs. Two experiments were conducted to assess SVA pathogenesis, clinical course of disease, patterns of virus shedding and virus load and tissue distribution. In the first experiment 12 SVA-negative finishing pigs (approximately 55 kg in body weight) were randomly allocated into two experimental groups as follows: Group 1, control group (RPMI 1640; n=4) and Group 2, SVA-inoculated group (SVA SD15-26; n=8). Animals from Group 2 were inoculated with 5 ml of a virus suspension containing $10^{8.07}$ $TCID_{50}$ ml^{-1} via the oral (2 ml) and intranasal (1.5 ml to each nostril) (oronasal) routes. Animals from control Group 1 were inoculated with RPMI 1640 medium in the same fashion. All animals were challenged on the day of arrival, and Group 1 and Group 2 pigs were kept in individual rooms and received food and water *ad libitum*.

Animals were monitored daily for clinical signs and lesions. Rectal temperatures were measured daily for 21 days. Oral, nasal and rectal swabs and blood samples were collected on days 1, 3, 5, 7, 10, 14, 21, 28, 35 and 38 pi. Dacron-tipped swabs were used for sample collection and placed in tubes containing 1 ml PBS (Mediatech Inc.). Blood samples were collected by jugular venipuncture and serum samples were aliquoted and stored at −20 °C until further processed. All animals

from Group 2 were humanely killed on day 38 pi. Tissues including tonsil, heart, lung, mediastinal lymph node, liver, spleen, mesenteric lymph node, small intestine and large intestine were collected and half were frozen and processed for RT-qPCR and virus isolation while the other half were fixed in 10 % formalin and processed for histological examinations and ISH. ISH was performed at the Veterinary Diagnostic Laboratory of the University of Minnesota .

To assess SVA tissue distribution and viral load during the acute phase of the infection, the four control animals from Group 1 were inoculated with SVA SD15-26 as described above, on day 35. Animals were monitored daily for clinical signs and lesions. One animal was euthanized on days 3, 4, 5 and 6 pi, and tissues including tonsil, heart, lung, mediastinal lymph node, liver, spleen, mesenteric lymph node, small intestine and large intestine were collected and processed as described above. A fifth animal from Group 1 (number 46) that died of an unrelated cause on day 7 pi was included in this study. All experiments were conducted at the SDSU Animal Resource Wing (ARW) and followed the protocols and guidelines approved by the SDSU Institutional Animal Care and Use Committee (approval number 16002A).

Viral RNA extraction and real-time PCR (RT-qPCR). Viral nucleic acid was extracted from swab samples and tissues using the MagMAX viral RNA/DNA isolation kit (Life Technologies) following the manufacturer's instructions. Detection of SVA RNA was performed using a commercial RT-qPCR kit targeting the SVA 3D polymerase gene (EZ-SVA; Tetracore). All RT-qPCR screenings were performed at the SDSU Animal Disease Research and Diagnostic Laboratory (ADRDL).

Virus isolation (VI) and titrations. Virus isolation was performed in RT-qPCR positive samples as previously described (Joshi *et al.*, 2016). The amount of SVA present in select VI positive samples was determined by the Spearman and Karber's method and viral titers expressed as $TCID_{50}$ per ml^{-1}. Samples (swabs or tissue homogenates) were subjected to tenfold serial dilutions (10^{-1} to 10^{-6}) and 100 µl of each dilution was inoculated into H1299 cells cultured in 96-well plates (four replicate wells per dilution). Cells were incubated at 37 °C with 5 % CO_2 for 48 h, fixed with 3.7 % formaldehyde for 20 min and stained with a SVA whole-virus rabbit polyclonal antibody followed by incubation with an anti-rabbit IgG-Alexa 594 conjugated antibody (Thermo Fisher Scientific).

Virus neutralization assay. Neutralizing antibody responses elicited by SVA infection were assessed using a virus neutralization assay. Serial two-fold dilutions of serum (1 : 40 to 1 : 2560) were incubated with 200 $TCID_{50}$ of SVA SD15-26 for 1 h at 37 °C. H1299 cells were added to each well (approximately 0.2×10^5 cells per well) and plates were incubated at 37 °C for 48 h. After incubation, cells were fixed with 3.7 % formaldehyde for 20 min and stained with an anti-SVA rabbit polyclonal antibody followed by incubation with an anti-rabbit IgG-Alexa 594 conjugated antibody (Thermo Fisher Scientific). VNs were read under a fluorescence microscope and NA titres were expressed as the reciprocal of the highest dilution of serum capable of completely inhibiting SVA infection/replication. Negative and positive control sera were included in all assays. All samples were tested in triplicate and the average titres are presented.

Immunofluorescence assay. Antibody responses elicited by SVA infection were evaluated by indirect immunofluorescence assay. To assess the levels of anti-SVA antibodies, H1299 cells were cultured in 96-well plates and infected with 200 $TCID_{50}$ of SVA strain SD15-26. Cells were fixed at 48 h pi with 3.7 % formaldehyde, permeabilized with 0.2 % Triton X-100 and incubated with twofold serial dilutions of serum (1 : 40 to 1 : 2560) for 1 h at 37 °C. Cells were washed three times with PBS (Mediatech Inc.), incubated with an anti-swine IgG-FITC conjugated antibody (Bethyl Laboratories) and evaluated under the fluorescence microscope. Antibody titres were determined as the reciprocal of the highest dilution of serum where SVA-infected cells were observed. Negative and positive control samples were included in all assay plates. All samples were tested against infected and uninfected control cells simultaneously.

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REFERENCES

Alexandersen, S., Zhang, Z. & Donaldson, A. I. (2002). Aspects of the persistence of foot-and-mouth disease virus in animals-the carrier problem. *Microbes Infect* **4**, 1099–1110.

Billinis, C., Paschaleri-Papadopoulou, E., Psychas, V., Vlemmas, J., Leontides, S., Koumbati, M., Kyriakis, S. C. & Papadopoulos, O. (1999). Persistence of encephalomyocarditis virus (EMCV) infection in piglets. *Vet Microbiol* **70**, 171–177.

Boisvert, S., Raymond, F., Godzaridis, E., Laviolette, F. & Corbeil, J. (2012). Ray Meta: scalable *de novo* metagenome assembly and profiling. *Genome Biol* **13**, R122.

Bracht, A. J., O'Hearn, E. S., Fabian, A. W., Barrette, R. W. & Sayed, A. (2016). Real-time reverse transcription PCR assay for detection of Senecavirus a in swine vesicular diagnostic specimens. *PLoS One* **11**, e0146211.

Canning, P., Canon, A., Bates, J. L., Gerardy, K., Linhares, D. C. L., Piñeyro, P. E., Schwartz, K. J., Yoon, K. J., Rademacher, C. J. & other authors (2016). Neonatal mortality, vesicular lesions and lameness associated with Senecavirus A in a U.S. Sow farm. *Transbound Emerg Dis* **63**, 373–378.

Carocci, M. & Bakkali-Kassimi, L. (2012). The encephalomyocarditis virus. *Virulence* **3**, 351–367.

Chen, Z., Yuan, F., Li, Y., Shang, P., Schroeder, R., Lechtenberg, K., Henningson, J., Hause, B., Bai, J. & other authors (2016). Construction and characterization of a full-length cDNA infectious clone of emerging porcine Senecavirus A. *Virology* **497**, 111–124.

Dotzauer, A. & Kraemer, L. (2012). Innate and adaptive immune responses against picornaviruses and their counteractions: an overview. *World J Virol* **1**, 91–107.

Gelmetti, D., Meroni, A., Brocchi, E., Koenen, F. & Cammarata, G. (2006). Pathogenesis of encephalomyocarditis experimental infection in young piglets: a potential animal model to study viral myocarditis. *Vet Res* **37**, 15–23.

Gordon, D. & Green, P. (2013). Consed: a graphical editor for next-generation sequencing. *Bioinformatics* **29**, 2936–2937.

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R. & other authors (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat Biotechnol* **29**, 644–652.

Guo, B., Piñeyro, P. E., Rademacher, C. J., Zheng, Y., Li, G., Yuan, J., Hoang, H., Gauger, P. C., Madson, D. M. & other authors (2016). Novel Senecavirus A in Swine with vesicular disease, United States, July 2015. *Emerg Infect Dis* **22**, 1325–1327.

Hales, L. M., Knowles, N. J., Reddy, P. S., Xu, L., Hay, C. & Hallenbeck, P. L. (2008). Complete genome sequence analysis of Seneca Valley virus-001, a novel oncolytic picornavirus. *J Gen Virol* **89**, 1265–1275.

He, Y., Ong, K. C., Gao, Z., Zhao, X., Anderson, V. M., McNutt, M. A., Wong, K. T. & Lu, M. (2014). Tonsillar crypt epithelium is an important extra-central nervous system site for viral replication in EV71 encephalomyelitis. *Am J Pathol* **184**, 714–720.

Hierholzer, J. C. & Killington, R. A. (1996). Virus isolation and quantitation. In *Virology Methods Manual*, pp. 25–46. Edited by B. W. J. Many & H. O. Kangro. Amsterdam: Elsevier.

ICTV (2015). *Virus Taxonomy: 2015 Release* . London.

Joshi, L. R., Mohr, K. A., Clement, T., Hain, K. S., Myers, B., Yaros, J., Nelson, E. A., Christopher-Hennings, J., Gava, D. & other authors (2016). Detection of the emerging Picornavirus Senecavirus A in pigs, mice, and houseflies. *J Clin Microbiol* **54**, 1536–1545.

Knowles, N. J., Hales, B. H., Jones, J. G., Landgraf, J. A., House, K. L., Skele, K. D., Burroughs, K. D. & Hallenbeck, P. L. (2006). Epidemiology of Seneca Valley virus: identification and characterization of isolates from pigs in the United States. In *Eur 2006 XVIth Meet Eur Study Gr Mol Biol Picornaviruses*, p. G2. Saariselka.

Koboldt, D. C., Zhang, Q., Larson, D. E., Shen, D., McLellan, M. D., Lin, L., Miller, C. A., Mardis, E. R., Ding, L. & Wilson, R. K. (2012). VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Res* **22**, 568–576.

Leme, R. A., Zotti, E., Alcântara, B. K., Oliveira, M. V., Freitas, L. A., Alfieri, A. F. & Alfieri, A. A. (2015). Senecavirus A: an emerging vesicular infection in Brazilian pig herds. *Transbound Emerg Dis* **62**, 603–611.

Li, H. (2011). A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. *Bioinformatics* **27**, 2987–2993.

Montiel, N., Buckley, A., Guo, B., Kulshreshtha, V., VanGeelen, A., Hoang, H., Rademacher, C., Yoon, K. J. & Lager, K. (2016). Vesicular disease in 9-week-old pigs experimentally infected with senecavirus A. *Emerg Infect Dis* **22**, 1246–1248.

Murphy, C., Bashiruddin, J. B., Quan, M., Zhang, Z. & Alexandersen, S. (2010). Foot-and-mouth disease viral loads in pigs in the early, acute stage of disease. *Vet Rec* **166**, 10–14.

Papaioannou, N., Billinis, C., Psychas, V., Papadopoulos, O. & Vlemmas, I. (2003). Pathogenesis of encephalomyocarditis virus (EMCV) infection in piglets during the viraemia phase: a histopathological, immunohistochemical and virological study. *J Comp Pathol* **129**, 161–168.

Pasma, T., Davidson, S. & Shaw, S. L. (2008). Idiopathic vesicular disease in swine in Manitoba. *Can Vet J* **49**, 84–85.

Poirier, J. T., Dobromilskaya, I., Moriarty, W. F., Peacock, C. D., Hann, C. L. & Rudin, C. M. (2013). Selective tropism of Seneca Valley virus for variant subtype small cell lung cancer. *J Natl Cancer Inst* **105**, 1059–1065.

Racaniello, V. R. (2013). Picornaviridae: the viruses and their replication. In *Fields Virol*, 6th edn, pp. 453–489. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams & Wilkins.

Singh, K., Corner, S., Clark, S. G., Sherba, G. & Fredrickson, R. (2012). Seneca Valley virus and vesicular lesions in a pig with idiopathic vesicular disease. *J Vet Sci Technol* **3**, 1-3.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Vannucci, F. A., Linhares, D. C., Barcellos, D. E., Lam, H. C., Collins, J. & Marthaler, D. (2015). Identification and complete genome of Seneca Valley virus in vesicular fluid and sera of pigs affected with idiopathic vesicular disease, Brazil. *Transbound Emerg Dis* **62**, 589–593.

Venkataraman, S., Reddy, S. P., Loo, J., Idamakanti, N., Hallenbeck, P. L. & Reddy, V. S. (2008). Structure of Seneca Valley virus-001: an oncolytic picornavirus representing a new genus. *Structure* **16**, 1555–1561.

Wu, Q., Zhao, X., Bai, Y., Sun, B., Xie, Q. & Ma, J. (2016). The first identification and complete genome of *Senecavirus* a affecting pig with idiopathic vesicular disease in China. *Transbound Emerg Dis*.

Yang, X., Charlebois, P., Gnerre, S., Coole, M. G., Lennon, N. J., Levin, J. Z., Qu, J., Ryan, E. M., Zody, M. C. & Henn, M. R. (2012). *De novo* assembly of highly diverse viral populations. *BMC Genomics* **13**, 475.

Zhang, Z. & Alexandersen, S. (2004). Quantitative analysis of foot-and-mouth disease virus RNA loads in bovine tissues: implications for the site of viral persistence. *J Gen Virol* **85**, 2567–2575.