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# Pathogenicity and cross-reactive immune responses of a historical and a contemporary Senecavirus A strains in pigs

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ABSTRACT

The goals of this study were to compare the pathogenicity and infection dynamics of a historical and a contemporary SVA strains (SVV 001 and SD15-26) and to assess cross-neutralizing and cross-reactive T cell responses following experimental infection in pigs. Both SVA strains successfully infected all inoculated animals, resulting in viremia and robust antibody and cellular immune responses. SVA SD15-26 infection resulted in characteristic clinical signs and vesicular lesions, however, SVA SVV 001 did not cause overt clinical disease with inoculated animals remaining clinically normal during the experiment. Notably, neutralization- and -recall IFN-γ expression-assays revealed marked cross-neutralizing antibody and cross-reactive T cell responses between the two viral strains. Together these results demonstrate that the historical SVA SVV 001 strain presents low virulence in pigs when compared to the contemporary SVA SD15-26 strain. Additionally, immunological assays indicate that SVA SVV 001 and SD15-26 are antigenically related and share conserved antigenic determinants.

## 1. Introduction

Senecavirus A (SVA) is a non-enveloped, single-stranded positive sense RNA virus, which belongs to the genus *Senecavirus* family *Picornaviridae* (ICVT, 2018). The SVA genome is ~ 7.2 kb in length and contains a single open reading frame (ORF) flanked by untranslated regions (UTRs) in the 5′ and 3′ ends, with a poly(A) tail at the 3′ end of the genome (Hales et al., 2008). The ORF is translated in a large polyprotein (2181 aa) that is cleaved by 3Cpro, a viral encoded protease, into 12 viral proteins (5′–L–VP4–VP2–VP3–VP1–2A–2B–2-C–3A–3B–3C–3D-3′) (Hales et al., 2008). The VPs form the capsid that consists of an icosahedral arrangement of 60 protomers, each composed by VP1, VP2, VP3 and VP4 (Semler and Wimmer, 2002; Venkataraman et al., 2008). The remaining polypeptides are nonstructural proteins that function on virus replication (Lin et al., 2009) and may play important roles on virus virulence and pathogenesis (Hales et al., 2008; Lin et al., 2009).

Senecavirus A was isolated for the first time in the United States (US) in 2002 as a contaminant of PER.C6 cell cultures (Hales et al., 2008). Electron microscopy performed on infected cells revealed icosahedral viral particles of about 27 nm in diameter. Based on the size and morphology of the virion, replication kinetics, and genomic sequences, the newly discovered virus was identified as a novel picornavirus and designated Seneca Valley virus (SVV), with the isolate being named SVV 001 (Hales et al., 2008). Recently, SVV was renamed and is currently known as Senecavirus A (SVA), with SVV 001 being the prototypic SVA strain (ICVT, 2014). Following SVA SVV 001 discovery in 2002, a seroepidemiologic study detected antibodies against the virus in pigs and other animal species in the US (Knowles et al., 2006). Additionally, screening of archived swine samples using molecular assays and sequencing confirmed the circulation of SVA in the US swine population since 1988 (Knowles et al., 2006). From 2002 until 2014, SVA was only associated with few cases of vesicular disease in pigs in Canada and in the US (Amass et al., 2004; Knowles et al., 2006; Singh et al., 2012). Late in 2014, several outbreaks of vesicular disease (VD) in pigs, resembling foot-and-mouth disease (FMD), and characterized by the development of vesicles on the snout and coronary bands of affected animals were reported in Brazil (Leme et al., 2015; Vannucci et al., 2015). The virus detected during these outbreaks shared 94.2% nucleotide identity with SVA SVV 001 (Vannucci et al., 2015). Since the

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detection of SVA in Brazil in 2014, the virus has been associated with various outbreaks of VD in other countries, including China (Wu et al., 2016), the US (Bracht et al., 2016; Canning et al., 2016; Joshi et al., 2016a), Thailand (Saeng-chuto et al., 2018) and Colombia (Sun et al., 2017). Notably, since its re-emergence in the US, in July 2015, more than 300 cases of SVA have been confirmed (SHMP, 2018). The factors underlying the emergence of SVA and the increase in disease incidence remain largely unknown. Interestingly, genetic comparisons between historical- (obtained prior to 2006) and contemporary SVA isolates (obtained between 2007 and 2015) revealed a marked genetic divergence and evolution of contemporary SVA isolates (Joshi et al., 2016a). Whether these genetic changes contributed to an increase in virulence and pathogenicity of contemporary SVA strains, however, remains unknown.

The role of SVA on the etiology of VD in pigs was recently demonstrated (Joshi et al., 2016b; Montiel et al., 2016) and its pathogenesis and the host immune responses to infection were studied in detail in finishing pigs (Joshi et al., 2016b; Maggioli et al., 2017). After a short incubation period (3–5 days post-infection [pi]), animals present lethargy and lameness (Joshi et al., 2016b; Maggioli et al., 2017). The clinical phase is also characterized by the development of vesicles on the snout and/or feet (dewclaw, coronary band, and/or sole) of affected animals. With the progression of the disease, the vesicles rupture, leaving ulcered lesions on the skin, that eventually resolve by day 14-16 pi (Joshi et al., 2016b; Maggioli et al., 2017). A short-term viremia (3–10 days pi) is detected and infected animals develop early neutralizing antibody (NA) responses (Joshi et al., 2016b; Maggioli et al., 2017). Early cellular immune responses to SVA are characterized by increased frequencies of IFN-γ-expressing $CD4^+$ T cells, while later cell mediated responses involve increased frequencies of SVA-specific IFN-γ-expressing $CD8^+$ T cells (Maggioli et al., 2017). Virus shedding in oral and nasal secretions and feces are detected up to 21–28 days pi (Joshi et al., 2016b).

Here we compared the pathogenicity of the historical SVA strain SVV 001 with a virulent contemporary SVA strain SD15-26. The goals of the study were to assess the infection dynamics and pathogenicity of these two strains in susceptible pigs as well as to assess cross-neutralizing and cross-reactive T cell responses following infection.

## 2. Results

### 2.1. Genetic comparison between SVA strains SVV 001 and SD15-26

Complete genome sequence comparisons between SVA SVV 001 and SD15-26 showed that these viral strains share 93.8% nucleotide identity (id) (Table 1), whereas comparison of SVA SD15-26 with other contemporary strains revealed 96–98% nucleotide id (data not shown). Pairwise comparisons of the ORF1 region showed 93.9% nucleotide identity between SVA SVV 001 and SD15-26. Additionally, 94.6% and 95.6% nucleotide id was observed when nucleotide sequences of the 5′- or 3′ UTRs were compared (Table 1). Pairwise comparisons of the polyprotein sequences revealed 97.8% amino acid similarity between SVV 001 and SD15-26. A total of 49 aa substitutions are present in the ORF of SVA SD15-26 when compared to SVA SVV 001 (Table 1). Among the capsid proteins, VP3 presents the greatest variability (7.4%; 95.8% aa similarity), while VP4 is the most conserved VP (100% identity) (Table 1). Several amino acid substitutions were also observed in the non-structural proteins (Table 1), with 2A and 3A being the most variable proteins, while 3B and 3D are the most conserved non-structural proteins (Table 1). These results suggest a marked genetic divergence and evolution of the contemporary SVA strain (SD15-26) when compared to the historical prototypic SVA strain SVV 001.

**Table 1**
Comparative genome analysis between SVA SVV 001 and SD15-26.

| | SVA SD15-26 | | |
|---|---|---|---|
| SVV 001 | nt id % | aa id % | Substitution(s) |
| 5′UTR[a] | 94.6 | – | |
| L | 95.8 | 98.7 | R56K |
| VP4 | 98.6 | 100 | – |
| VP2 | 94.2 | 98.6 | T165I, N368S, Y426F, A428T |
| VP3 | 92.6 | 95.8 | L452I, E491G, V493A, P494S, V497E, T502A, T511A, R516K, T575A, V603I |
| VP1 | 91.8 | 97 | Q735A, E736T, A766V, G770D, F834Y, A845T, V894I, I912V |
| 2A | 81.5 | 87.5 | I941V |
| 2B | 93.5 | 98.4 | D988N, V1003I |
| 2 C | 93.4 | 98.8 | K1079T, S1148G, N1366S, T1378A |
| 3 A | 91.5 | 90 | S1397N, S1415T, T1427A, E1429D, R1462K, A1469T, G1478E, S1479P, V1480A |
| 3B | 94.2 | 100 | – |
| 3 C | 93.3 | 97.6 | T1547A, I1589L, S1628A, E1649D, L1685M, |
| 3D | 95.5 | 98.9 | I1729V, N1839S, V1850S, A1856P, V1860M |
| 3′UTR | 95.6 | – | |
| ORF1 | 93.9 | 97.8 | |
| Genome | 93.8 | – | |

id: identity.
[a] 2 nt insertion (TC) at position 553–554 in SVA SD15–26 5′UTR.

### 2.2. Infection of pigs with SVA SD15-26, but not with SVA SVV 001, results in vesicular disease

The pathogenicity and infection dynamics of the historical SVV 001 and contemporary SD15-26 SVA strains were compared in pigs. For this, twelve SVA-negative finishing pigs (~60 kg), were inoculated oronasally with SVV 001 (n = 6) or SVA SD15–26 (n = 6) strains (5 mL orally and 5 mL intranasally [half into each nostril]; titer $10^{7.5}$ $TCID50\,mL^{-1}$) (Joshi et al., 2016b), and monitored for clinical signs and vesicular lesions for 14 days. One animal from each group was euthanized on days 3 and 7 post-inoculation (pi). All pigs inoculated with SVA SD15-26 (5), except the animal euthanized on day 3 pi, presented characteristic clinical signs of SVA, including lameness, lethargy and/or snout irritation. In contrast to the clinical presentation observed in SVA SD15-26-inoculated animals, none of the animals inoculated with the historical SVA strain SVV 001 presented clinical signs. Vesicular lesions were observed on the feet and/or snout of SVA SD15-26-inoculated animals starting on day 4 pi (two out of six animals). All SVA SD15-26-inoculated animals developed characteristic vesicular lesions on the snout and/or on the feet, with lesions being observed on the coronary band, sole, dewclaw, and/or interdigital space (Fig. 1A). Vesicular lesions evolved through the stages of erythema, fluid-filled vesicles, ruptured vesicles, skin ulcers/erosions, to scabby lesions and finally normal skin. Notably, no lesions were observed in SVV 001-inoculated pigs (Fig. 1B). Clinical scores were calculated by attributing one point (1) to the snout and each foot (right front, left front, right hind and left hind) presenting lesions on any day of the 14-day experiment (maximum score is 5 per animal per day). A score of zero (0) was attributed to areas (snout or foot) presenting no lesions. Peak clinical scores for SVA SD15-26-inoculated animals were observed on day 7 pi, when one animal with a score of 5 was euthanized. After day 7 pi, the lesions on the snout started to subside, while scabby lesions were still observed on the feet of several animals at the end of the experiment on day 14 pi (Fig. 1C). As no gross lesions were observed in SVV 001-inoculated animals, clinical scores for this group remained 0 throughout the experiment (Fig. 1C). The identity of the SVA strain detected in inoculated animals was confirmed by sequencing of a fragment of the P1 region, amplified from the tonsil of infected animals (data not shown).

Skin samples collected from SVA SD15-26 and SVV 001-inoculated animals on days 3, and 7 pi were subjected to histological examination.

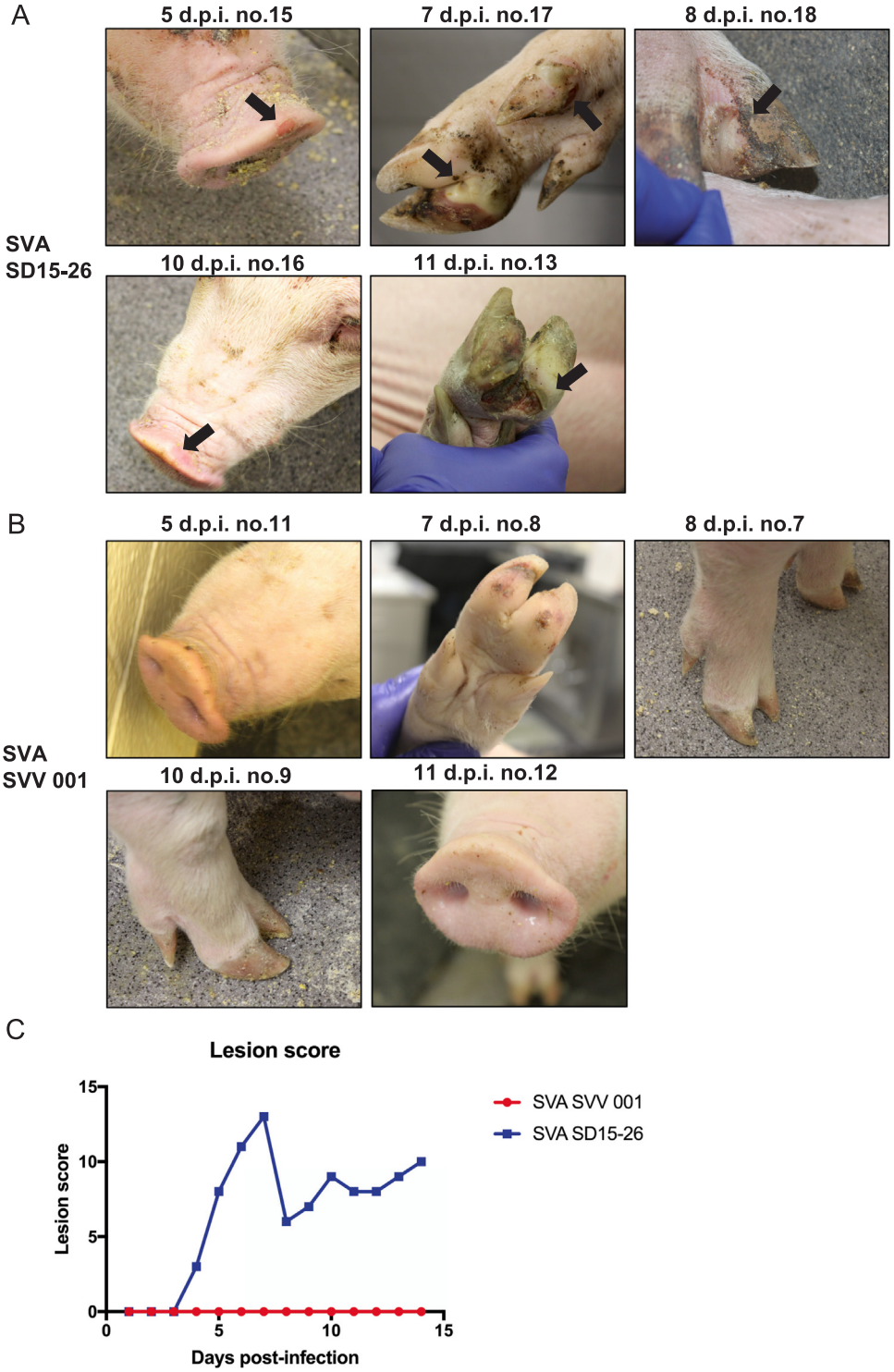


**Fig. 1.** Comparative pathogenicity of SVA SVV 001 and SD15-26 in finishing pigs. (A) Clinical course and progression of vesicular disease observed in SVA SD15-26-inoculated animals. All the animals inoculated with SD15–26 presented lesions starting on day 4 pi. Lesions were observed on the snout, interdigital space, dewclaws, heel and/or coronary band. The black arrows indicate the lesions. (B) Clinical course observed in SVA SVV 001-inoculated animals. Animals inoculated with the SVA strain SVV 001 did not present any vesicular lesion and remained clinically normal throughout the experiment. (C) Lesion score. Daily average group lesion scores were calculated. One point was attributed to each foot or/and snout presenting a vesicular lesion for a total of five points per animal per day. SVA SD15-26 (blue) and SVV 001 (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

Histological changes observed on a snout lesion of a SVA SD15-26-inoculated animal, revealed vesiculopustular dermatitis characterized by vesicles containing eosinophilic homogenous fluid (edema), accumulation of fibrin and necrotic debris (Fig. 2A). Additionally, large pustules presenting aggregates of neutrophils admixed with variable amounts of eosinophilic material, cellular and karyorrhectic debris and bacteria (lytic necrosis) were also observed (Fig. 2A). The lesions on the coronary band of SVA SD15-26-inoculated animals consisted vesiculopustular dermatitis, characterized by mild, multifocal infiltration of inflammatory cells composed of lymphocytes and plasma cells surrounding blood vessels in the dermis (Fig. 2B). Consistent with the absence of gross vesicular lesions in SVV 001-inoculated animals, no histological changes were observed on the skin of the snout and coronary bands of animals from this group (Fig. 2C and D).

### 2.3. Comparative viremia levels and virus shedding

The levels of viremia in SVA SVV 001- and SVA SD15-26-inoculated animals were assessed in serum. Serum samples collected on days 0, 1, 3, 7, 10 and 14 pi were tested for the presence of SVA RNA using RT-qPCR. SVA was first detected in serum on day 1 pi, with peak SVA SD15-26 viremia levels being detected on day 3 pi (Fig. 3A). On day 10

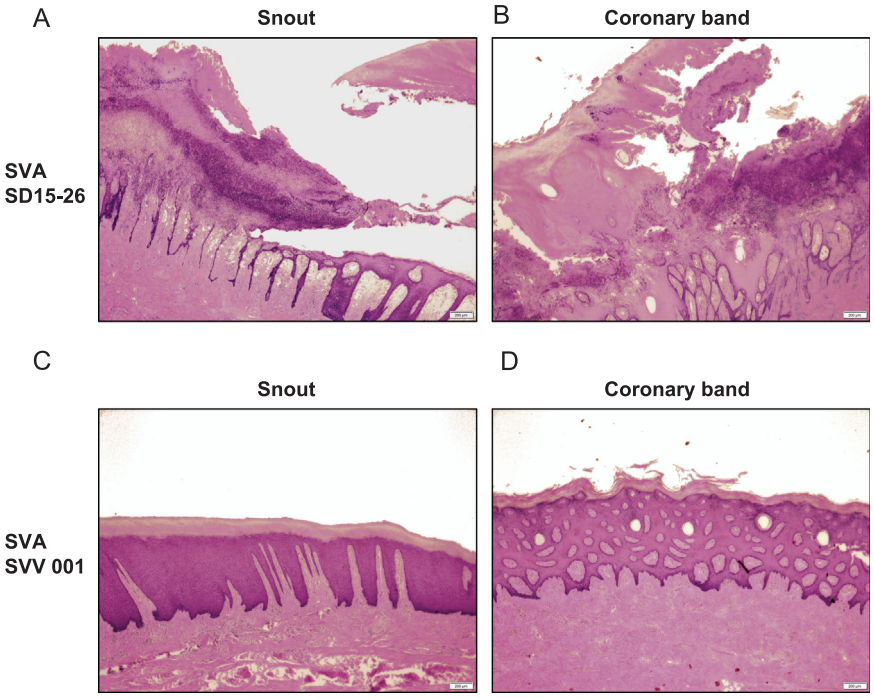


**Fig. 2.** Histological sections of the skin of SVA SD15-26 and SVV 001-inoculated animals. (A-B) Vesiculopustular dermatitis observed in histological sections of SVA SD15-26-inoculated animals on day 3 pi. Histological examination of the snout skin revealed extensive areas of the epidermis presenting erosions to ulcerations with multifocal to coalescing vesicles, which contained homogenous eosinophilic fluid (edema), fibrin and necrotic debris, neutrophils and eosinophilic material, cellular and karyorrhectic debris and numerous mixed bacteria (A). The lesion on the coronary band was characterized by vesiculopustular dermatitis with mild, multifocal infiltration of inflammatory cells composed of lymphocytes and plasma cells around the blood vessels in the dermis (B). (C-D) Normal histological morphology observed in SVA SVV 001-inoculated animals. The histology of the snout (C) and coronary band (D) of the animals inoculated with SVA strain SVV 001 were unremarkable. Haematoxylin and eosin stain.

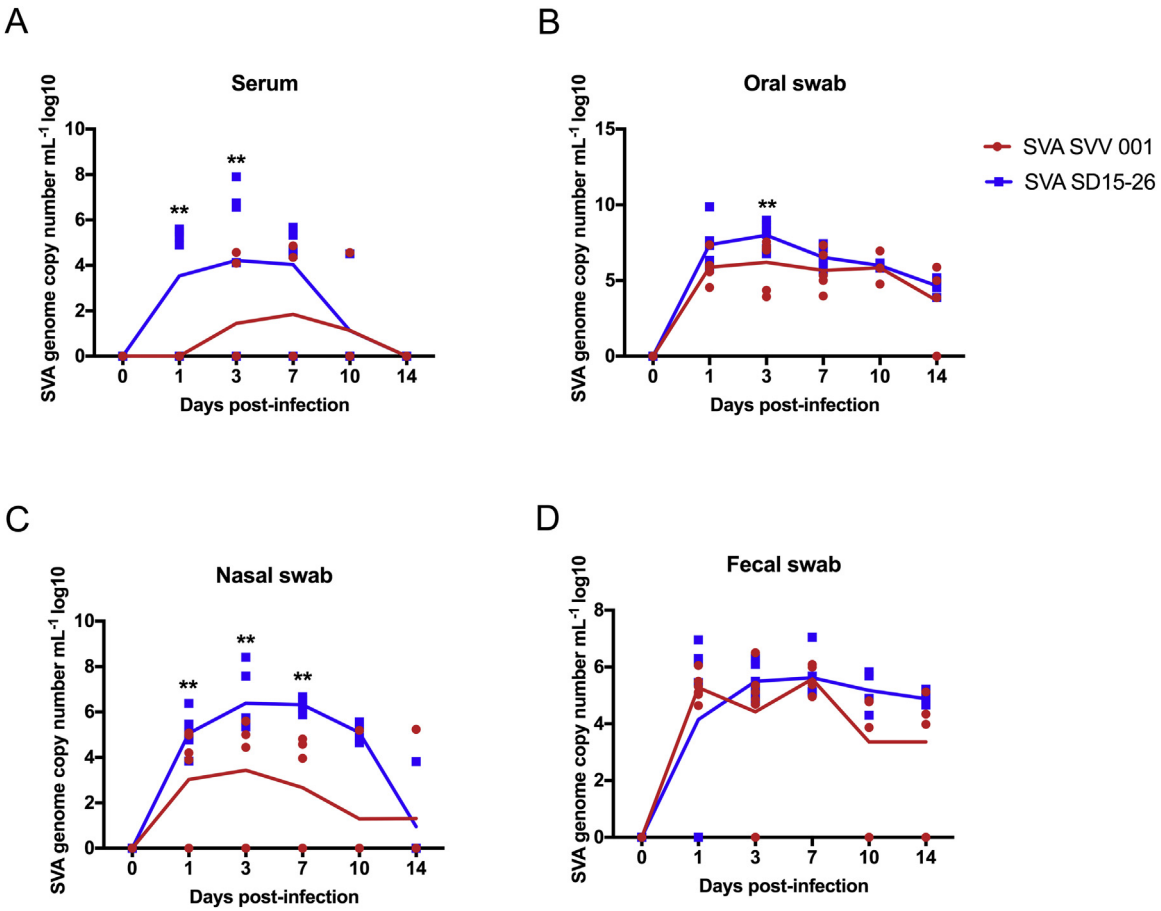


**Fig. 3.** Viremia and virus shedding in animals inoculated with Senecavirus A strains SVV 001 and SD15-26. (A) Viremia levels in serum as determined by RT-qPCR. Viral loads are expressed as $\log_{10}$ genome copy number per $mL^{-1}$. Virus shedding in oral (B) and nasal (C) secretions and in feces (D) were determined by RT-qPCR and expressed as $\log_{10}$ genome copy number per $mL^{-1}$. The animals inoculated with SVA strain SD15-26 are represented in blue and the animals inoculated with SVV 001 strain are in red. The lines represent the average of each group. *P* values were determined by Student's *t*-test (**$P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

pi, only one SVA SD15-26-inoculated animal was still viremic, and on day 14 pi viral RNA was not detected in any inoculated animal (Fig. 3A). SVA SVV 001 genome was first detected in serum of two animals on day 3 pi and similar to the results in SVA SD15-26-inoculated animals, SVA SVV 001 viremia was detected until day 10 pi (Fig. 3A). It is important to note that, not all inoculated animals presented viremia (Fig. 3A), with a higher frequency of viremic animals being detected in SVA SD15-26-inoculated group. Notably, the levels of viremia detected in SVA SD15-26 inoculated animals were significantly higher than those detected in SVA SVV 001-inoculated animals (Fig. 3A).

Virus shedding was assessed in oral and nasal secretions and feces of SVA SD15-26 and SVV 001-inoculated animals. Oral, nasal and rectal swabs collected on days 0, 1, 3, 7, 10 and 14 pi were subjected to RT-qPCR. Virus shedding was detected until the end of the experiment on day 14 pi in animals from both groups (Fig. 3B, C and D). Peak viral shedding on oral and nasal secretions and feces was detected on day 3 pi in animals from both SVA SD15-26 and SVV 001-inoculated groups (Fig. 3B). Interestingly, while levels of virus shedding in oral secretions and feces were similar between both groups, significantly higher amounts of SVA RNA were detected on nasal swabs of SVA SD15-26-inoculated animals when compared to SVA SVV 001-inoculated animals (Fig. 3C). No differences in virus shedding in feces were observed between SVA SD15-26 and SVV 001 inoculated animals throughout the 14-day experimental period (Fig. 3D).

### 2.4. Detection of SVA RNA in non-lymphoid tissues

Viral load and tissue distribution of SVA SVV 001 and SD15-26 were assessed in non-lymphoid tissues. Tissues including heart, lungs, kidney, liver, small intestine and large intestine were collected on days 3, 7, and 14 pi and processed for RT-qPCR. Viral load detected in each tissue is presented in Fig. 4. Lungs and large intestine were the tissues in which a greater number of animals from both groups were positive for SVA RNA (Fig. 4B and F). In general, SVA SD15-26-inoculated animals present higher amounts of viral RNA in these tissues when compared to SVV 001-inoculated animals, which corroborates the levels of viremia detected in animals inoculated with this viral strain (Fig. 4).

### 2.5. Detection of SVA RNA in lymphoid tissues

Viral load was also investigated in lymphoid tissues using RT-qPCR. Viral load detected in the thymus, spleen, mediastinal and mesenteric lymph nodes and tonsil are presented in Fig. 5. Notably, while SVA RNA was detected in at least one animal in all examined tissues (Fig. 5), the tonsil was the tissue that presented the highest viral load and the highest number of positive animals with all SVA SD15-26 and SVV 001-inoculated animals being positive for SVA RNA throughout the experiment (days 3, 7, and 14 pi) (Fig. 5E). In contrast, thymus was the tissue with the lowest number of positive animals, with only one animal from the SVA SD15-26-inoculated group being positive on day 7 pi (Fig. 5A). There was an increase in the number of animals that were positive for SVA in lymphoid tissues with the progression of the infection, as evidenced by a higher number of positive animals in both SVA SD15-26 and SVV 001-inoculated groups (Fig. 5). Together these results suggest that lymphoid tissues may represent active sites of SVA replication during the acute phase of infection. Additionally, early detection of SVA in the tonsil in both SVA SD15-26 and SVV 001 strengthen the hypothesis that the tonsil may be the primary site of SVA replication.

### 2.6. Cross-neutralization between SVA SVV 001 and SD15-26

The serological responses to both SVA strains, SVV 001 and SD15-26, were evaluated by virus neutralization assay (VN). All inoculated animals seroconverted and presented high levels of neutralizing antibodies (NA) on day 5 pi (Fig. 6A and B). Peak NA titers for both groups were observed on day 10 pi (Fig. 6A and B). Notably, marked cross-neutralization between SVA SVV 001 and SD15-26 was observed when serum from one group of animals was used in VN assays against the heterologous strain of virus (Fig. 6A and B). Interestingly, even when SVA SVV 001 was the virus used on VN assay, the NA titers in SVA SD15-26-inoculated animals were higher (Fig. 6A; $^{**}p < 0.05$, $^{***}p < 0.01$). These results confirmed successful infection of both SVA SD15-26 and SVV 001-inoculated animals and demonstrate significant cross-neutralization between the historical and contemporary SVA strains.

### 2.7. Cross-reactive T cell responses between SVA SVV 001 and SD15-26

Cellular responses to SVA SVV 001 and SVA SD15-26 were evaluated by flow cytometry (Fig. 7). Peripheral blood mononuclear cells from SVA SVV 001 or SVA SD15-26 were stimulated with either SVA strain and IFN-γ expression by T cells (total $CD3^+$), and individual T cells subsets, including $CD4^+$, $CD4^+CD8^+$, and $CD8^+$ T cells were investigated on days 0 and 14 pi. Animals inoculated with either SVA strain (SVA SVV 001 or SD15-26) presented antigen-specific IFN-γ expression by T cells on day 14 pi (Fig. 7A-G). Although recall IFN-γ expression by SVA SD15-26-inoculated animals (Fig. 7I) were slightly higher than by SVA SVV 001-inoculated animals (Fig. 7H), no statistical differences were detected between the two groups ($p > 0.05$). Substantial T cell cross-reactivity between SVA SD15-26 and SVV 001 was observed. The frequency of IFN-γ-expression T cells in SVA SVV 001 inoculated animals were similar upon recall stimulation with SVA SVV 001 (Fig. 7H, left panel) or SVA SD15-26 (Fig. 7H, right panel). A similar phenotype was observed in SVA SD15-26-inoculated animals (Fig. 7I). These results indicate that SVA SVV 001 and SD15-26 may share relevant T cells epitopes.

## 3. Discussion

In the present study, we compared the pathogenicity and infection dynamics of a historical (SVV 001) and a contemporary (SD15-26) SVA strains in finishing pigs. Senecavirus A strain SVV 001 was isolated in PER.C6 cell cultures (Hales et al., 2008), presumably originating from porcine trypsin (Knowles et al., 2006). Whereas SVA SD15-26, a virulent SVA strain, was isolated from a vesicular lesion during an outbreak of VD in the US, in 2015 (Joshi et al., 2016b). Results here show that oronasal inoculation of finishing pigs with both SVA strains resulted in infection of all inoculated animals, however, only SVA SD15-26-infection led to overt clinical disease, while SVA SVV 001-inoculated animals remained clinically normal for the duration of the experiment. These observations indicate that SVA SVV 001 presents low virulence in pigs.

Genetic determinants of picornavirus virulence and pathogenicity have been identified in both coding and non-coding regions of the genome (Kanno et al., 1999; Li et al., 2011; Rohll et al., 1994), and even silent nucleotide changes may lead to altered virulence phenotypes (Kanno et al., 1999). Nucleotide changes in coding and non-coding regions may alter secondary RNA structures that contribute to virus replication (Burrill et al., 2013; Simmonds and Smith, 1999), and amino acid substitutions in individual viral proteins may result in altered protein functions, thus potentially leading to a distinct fitness of the virus in the host. In comparison to SVA SVV 001, the contemporary SVA strain SD15-26 presents multiple nucleotide changes involving the 5′- and 3′ UTRs and the long polyprotein-encoding ORF. The role and contribution of the genetic changes observed in contemporary SVA strains for the virus virulence and pathogenesis remain unknown. However, given the extent and distribution of these changes (involving non-coding and coding regions), it is likely that they contributed, at least in part, for the emergence of virulent contemporary SVA strains.

Both SVA SVV 001 and SD15-26 strains successfully established

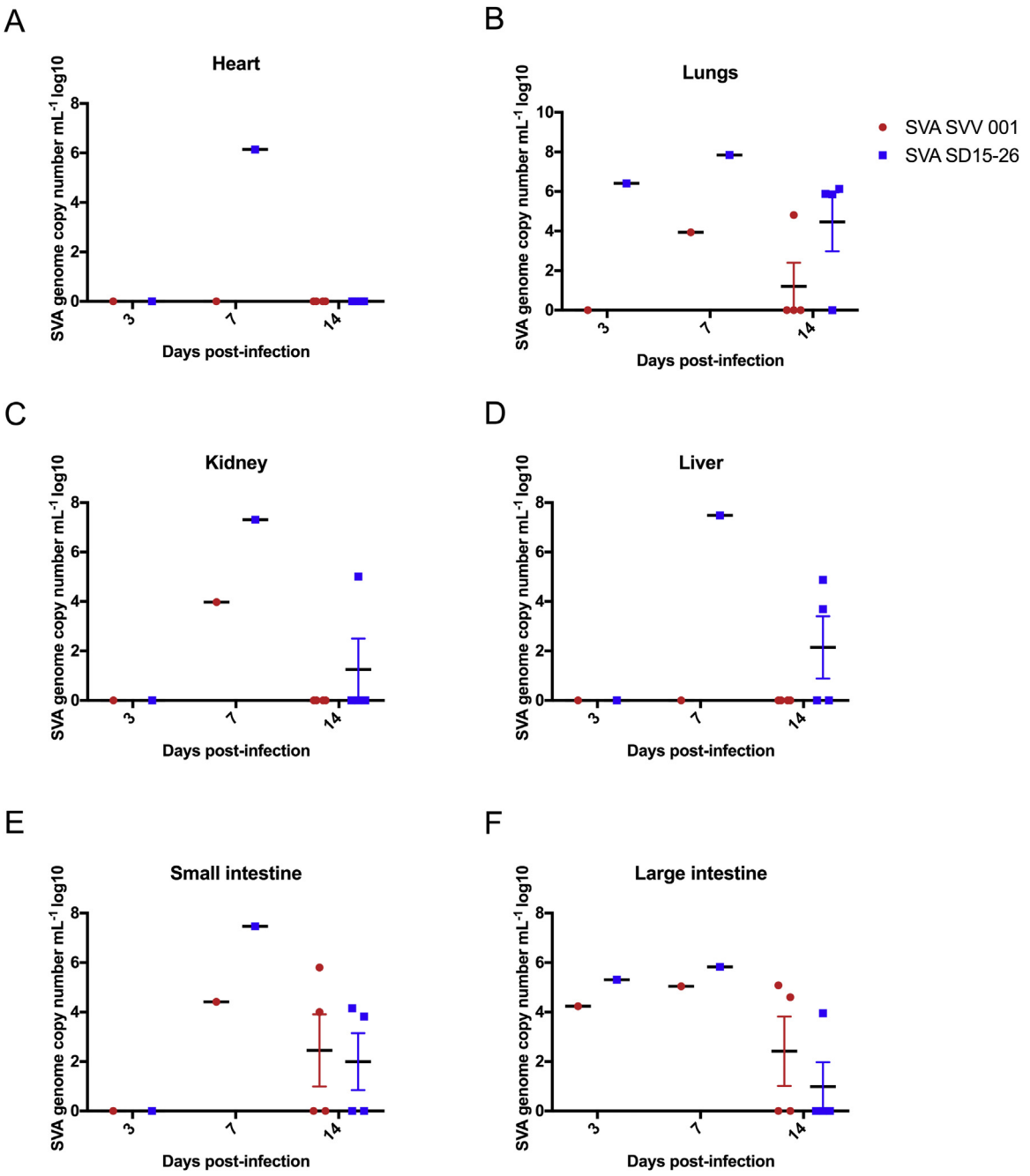


**Fig. 4.** Viral load and tissue distribution of Senecavirus A (SVA) strains SVV 001 and SD15-26 were assessed in non-lymphoid tissues during acute infection in pigs. Viral load in different tissues collected on days 3, 7 and 14 post-infection. Levels of SVA RNA in heart (A), lungs (B), kidney (C), liver (D), small intestine (E) and large intestine (F) were determined by RT- qPCR and expressed as $\log_{10}$ genome copy number $mL^{-1}$. The animals inoculated with SVA strain SD15-26 are represented in blue and the animals inoculated with SVV 001 strain are represented in red. Error bars represent the standard error of the mean (SEM). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

infection in all inoculated animals, however, marked differences in the pathogenicity and infection dynamics were observed. Notably, while all SVA SD15-26 inoculated pigs developed characteristic VD, none of the animals inoculated with SVA SVV 001 presented clinical signs nor lesions. These observations indicate that SVA SVV 001 is less pathogenic than SVA SD15-26 to pigs, corroborating the findings of previous pathogenesis studies conducted with other historical SVA strains (Knowles et al., 2006; Yang et al., 2012), in which inoculated animals did not develop clinical VD. It is important to note, however, that in the study by Yang and collaborators (2012), inoculation was performed through injection of SVA directly in the coronary bands or tongue of experimental animals (Yang et al., 2012). This represents an important difference from the inoculation route used here and it may explain the failure of the isolate used in Yang's study to induce VD in inoculated pigs. Here, SVA SVV 001-inoculated animals also presented delayed viremia (day 3 *vs* day 1) with lower levels of viral RNA being detected in serum throughout the 14-day experiment. Differences in virus shedding were observed mostly in nasal secretions, with SVV 001-inoculated animals shedding lower amounts of virus. Whereas similar levels of virus shedding were observed in oral secretions and feces. Consistent with this, the number of positive animals and the viral load in non-lymphoid tissues were slightly lower in SVV 001 inoculated animals. Together these results indicate that SVA SVV 001 presents a lower virulence when compared to SVA SD15-26, reinforcing the idea

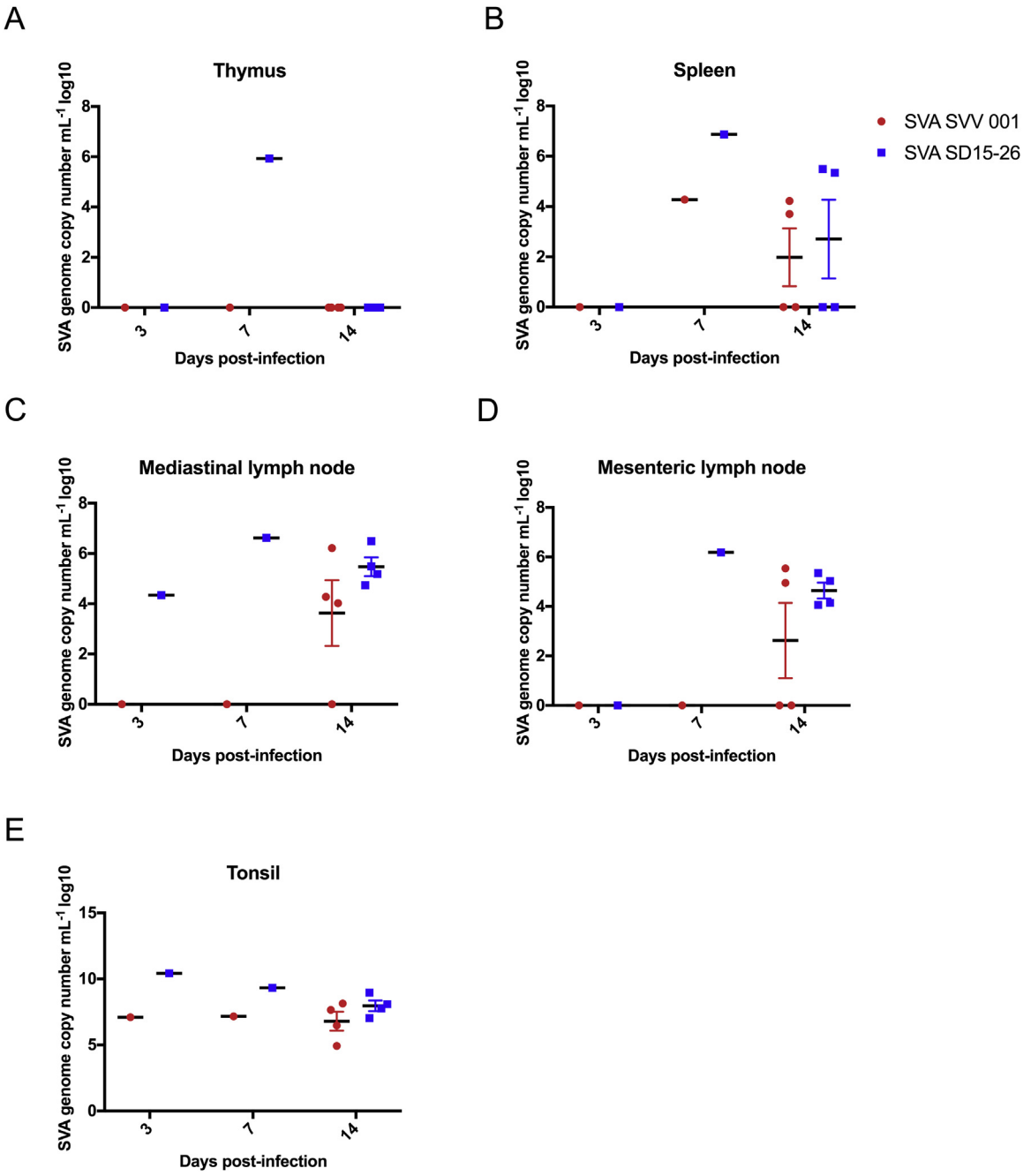


**Fig. 5.** Viral load and tissue distribution of Senecavirus A (SVA) strains SVV 001 and SD15-26 were determined in lymphoid tissues during acute infection in pigs. Viral load in different tissues collected on days 3, 7 and 14 post-infection. Thymus (A), spleen (B), mediastinal lymph node (C), mesenteric lymph node (D) and tonsil (E) were collected on days 3, 7 and 14 post-infection. Levels of SVA RNA were determined by RT- qPCR and expressed as $\log_{10}$ genome copy number $mL^{-1}$. The animals inoculated with SVA strain SD15-26 are represented in blue and the animals inoculated with SVV 001 strain are in red. Error bars represent the standard error of the mean (SEM). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

that historical SVA strains may be less pathogenic to pigs. The possibility that other historical SVA strains are virulent and capable of inducing VD in pigs, however, cannot be formally excluded.

Previously we have shown that SVA SD15-26 appears to have tropism for lymphoid tissues, with the tonsil being one of the target tissues during the first seven days of infection (Joshi et al., 2016b). Results here corroborate with these observations, demonstrating the presence of high amounts of viral RNA in the tonsil of both SVA SVV 001- and SD15-26-inoculated animals. Interestingly, when compared to the other lymphoid tissues tested (thymus, spleen, mediastinal and mesenteric lymph nodes) the tonsil was the only tissue in which both viral strains were detected in all animals throughout the experiment. These results provide evidence of the importance of the tonsil as a replication site for SVA and further suggest that this organ may be the primary site of SVA replication.

Given the great genetic and antigenic diversity of picornaviruses (Simmonds, 2006), and the nucleotide and inferred amino acid divergence between the historical (SVV 001) and the contemporary (SD15-26) SVA strains used here, we sought to assess cross-reactive immune responses elicited in infected animals. Since NA are one of the most important correlates of protection against picornaviruses (Morimoto, 2001; Reed and Cardosa, 2016), and provide the basis for the classification of picornaviruses into serotypes (ICTV, 2011), we initially assessed cross-NA responses in SVA SVV 001 and SD15-26-inoculated

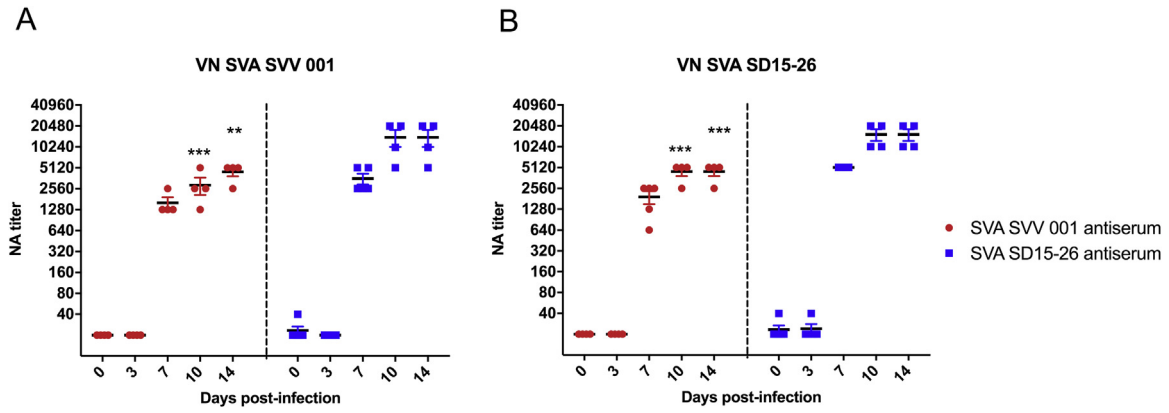


**Fig. 6.** Virus neutralization (VN) assays demonstrating cross-neutralizing antibody responses against Senecavirus A (SVA) strains SVV 001 (A) and SD15-26 (B). The serum of animals inoculated with SVA strain SD15-26 are represented in blue and the animals inoculated with SVV 001 strain are in red. Error bars represent the standard error of the mean (SEM). (**$P < 0.05$; ***$P < 0.01$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

animals. For this, virus-specific antisera were used in VN assays against the homologous or heterologous virus strain. Results of these assays revealed marked cross-neutralization activity between the two SVA strains, with NA titers in SVA SD15-26 being slightly higher (1–2-fold) than in SVA SVV 001-inoculated animals. Notably, it has been previously shown that SVA SVV 001 is also serologically related and presents cross-neutralizing activity with several other historical SVA isolates obtained in the US between 1988 and 2001 (Knowles et al., 2006). These observations indicate that historical and contemporary SVA strains are serologically related suggesting that these strains share conserved antigenic determinants including neutralizing epitopes, despite the amino acid changes present in the capsid proteins.

Recently, we have shown that T cell responses to SVA infection are characterized by increased frequencies of $CD4^+$, $CD8^+$ and double positive $CD4^+CD8^+$ T cells (Maggioli et al., 2017). Picornavirus-specific T cells have been shown to recognize and respond to conserved as well as serotype-specific T cell epitopes (Gern et al., 1997). To define cross-reactive T cell responses between SVA SVV 001 and SD15–26, PBMCs collected from infected animals were stimulated *in vitro* with each viral strain and recall IFN-γ expression was assessed by flow cytometry analysis. Results from these experiments demonstrate significant cross-reactive T cell responses between the two viral strains, as evidenced by increased frequencies of IFN-γ-expressing $CD3^+CD4^+$- and $CD3^+CD8^+$ T cells following recall stimulation *in vitro*. These findings corroborate the notion of conserved picornavirus T cell epitopes that are cross-reactive between different strains- or even different species of picornaviruses (Bell et al., 2014; Muehling et al., 2016).

In summary, this study shows that there are pronounced differences in the pathogenicity and infection dynamics between the historical prototypic SVA strain SVV 001 and the contemporary SVA SD15-26 strain in pigs. These biological differences in the natural host species of the virus might reflect the evolution of SVA or perhaps its enhanced fitness in the host. Understanding differences in biological properties and pathogenesis of SVA is critical for the development of improved diagnostic tools and vaccines for prevention and control of this important emerging virus of swine.

## 4. Methods

### 4.1. Cells and viruses

NCI-H1299 human cells were purchased from the American Type Culture Collection (ATCC-CRL 5803). Cells were maintained at 37 °C with 5% $CO_2$ in RPMI-1640 medium (Corning) supplemented with 10% fetal bovine serum and 2 mM L-Glutamine. Penicillin (100 U $mL^{-1}$), streptomycin (100 μg $mL^{-1}$) and gentamycin (50 μg $mL^{-1}$) were also added to the cell culture media.

Senecavirus A strain SVV 001 (purchased from ATCC - Seneca Valley virus lot 3, July 22, 2003) was the first SVA strain isolated in PER.C6 cells in 2002 (Hales et al., 2008). SVA strain SD15-26 was isolated from swine presenting vesicular disease and has been fully characterized in our laboratory (Joshi et al., 2016b; Maggioli et al., 2017). Stocks of both SVA SVV 001 and SD15-26 strains were prepared and titrated in H1299 cells.

### 4.2. Sequence analysis

Pairwise nucleotide and amino acid sequence identities between SVA SVV 001 (GenBank accession number NC_011349) and SVA SD15-26 (GenBank accession number KX778101) were determined with the EMBOSS Needle sequence alignment tool (https://www.ebi.ac.uk/Tools/psa/).

### 4.3. Animal studies

The pathogenicity of SVA SVV 001 and -SD15-26 were compared in 15-week-old finishing pigs. For this, 12 SVA negative finishing pigs (weighing ~ 60 kg) were randomly allocated in two experimental groups: Group 1, SVV 001-inoculated group (n = 6), and Group 2, SD15-26-inoculated group (n = 6). Animals from both groups were inoculated with virus suspensions containing $10^{7.5}$ $TCID_{50}$ $mL^{-1}$ via the oronasal route (5 mL orally and 5 mL intranasally [half into each nostril]). Animals were challenged on arrival at SDSU Animal Resource Wing (ARW). Group 1 and 2 animals were maintained in separate rooms and strict biosecurity protocols were followed, to avoid cross contamination. Animals received food and water *ad libitum* for the duration of the 14-day experiment. As day 0 samples were used for baseline immune (antibody levels and T cell responses) assessments, a control mock-inoculated group was not included in the study.

Following virus inoculation, animals were monitored daily for characteristic SVA clinical signs and lesions. Clinical signs and lesions were recorded, and individual daily lesion scores were attributed to each animal. A score of 1, was attributed to lesions observed on the snout and/or each foot for a total score of 5 per animal per day (snout = 1; right front foot = 1; left front foot = 1, right rear foot = 1; and left rear foot = 1). A score of 0, was attributed to the snout and/or foot of each animal that did not present vesicles in any given day of the 14-day experiment. Average daily lesion scores were calculated for each group. Swabs (oral, nasal and rectal/fecal) and blood samples (serum and whole heparinized blood) were collected on days 0, 1, 3, 5, 7, 10 and 14 pi. Serum and PBMCs were collected and stored as previously described (Joshi et al., 2016b; Maggioli et al., 2017). One animal of

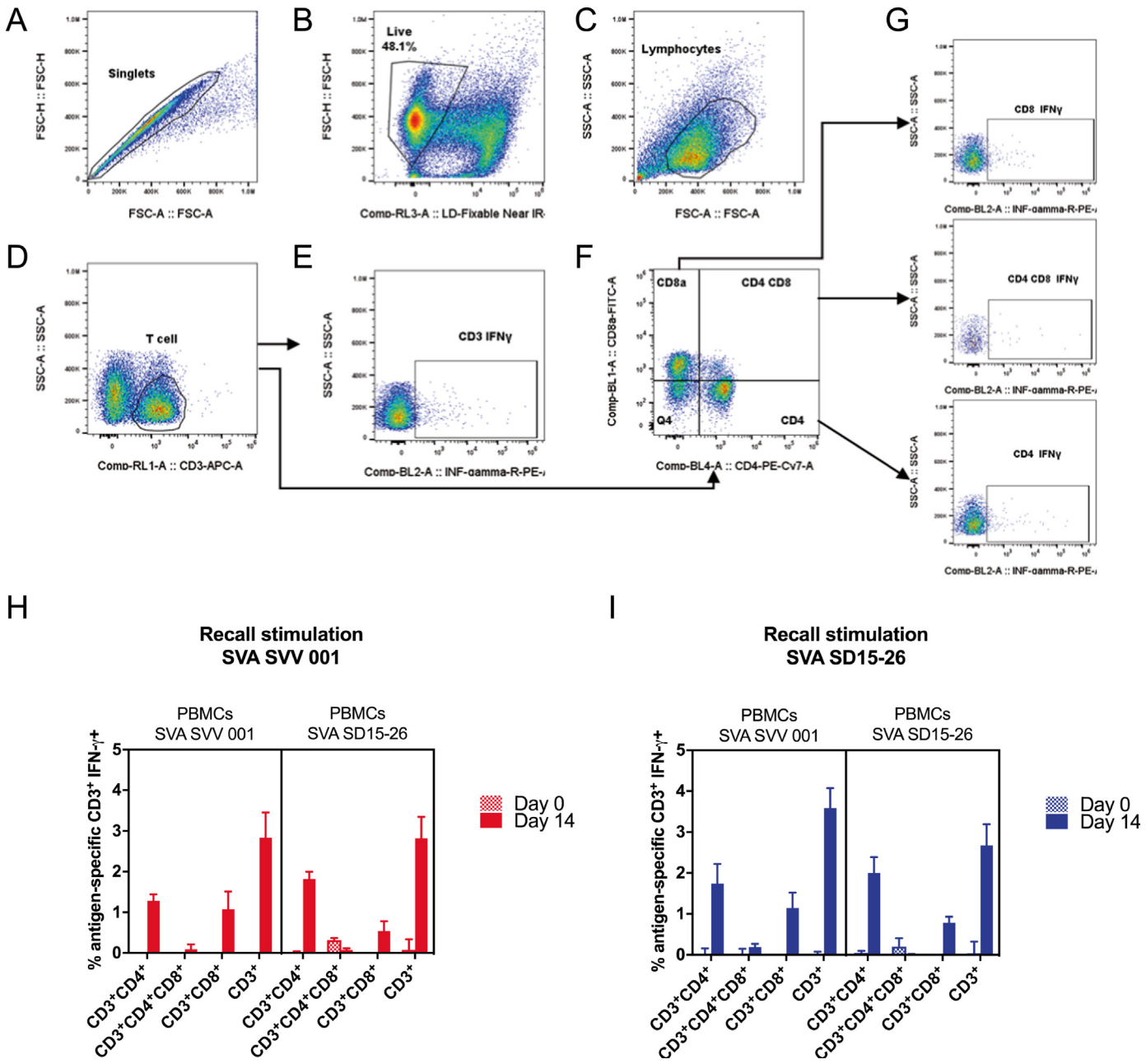


**Fig. 7.** Infection with SVA SVV 001 or SD15-26 elicits cross-reactive T-cells. Cryopreserved PBMCs isolated from SVA SVV 001- or SD15-26-inoculated pigs were thawed and allowed to rest for 6 h. Cells were then cultured in the presence of UV-inactivated SVA SVV 001 (MOI = 1), SVA SD15-26 (MOI = 1), cRPMI, or ConA (5 μg/mL) plus PHA (5 μg/mL). At 4 h post stimulation, brefeldin A was added to the culture and cells were further stimulated for 12 h (16 h culture/stimulation). After stimulation, αβ T cells were analyzed for virus-specific IFN-γ expression as measured by ICS. Gating strategy: Following doublet- (A) and dead cell- (B) exclusion, lymphocytes- (C; based on forward scatter [FSC] and side scatter [SSC] properties), and total live $CD3^+$ T cells were selected (D) and IFN-γ production by T cells (E) and the its main subsets evaluated (F and G). Each subsequent panel shows only the population of interest that was selected from the gate in the previous plot, as indicated by arrows. Collective results of flow cytometric analysis are shown for each population of cells evaluated from SVA SVV 001 (H) or SVA SD15-26 infected animals (I) in response to recall stimulation with either SVA SVV 001 (left side) or SVA SD15-26 (right side) on days 0 and 14 days post-infection. Data represent group means ± SEM. No statistical differences were detected by Sidak's multiple-comparison test.

each group was euthanized on days 3 and 7 pi, and all the remaining animals were euthanized on day 14 pi. Tissues (heart, lungs, kidney, liver, small intestine, large intestine, thymus, spleen, mediastinal lymph node, mesenteric lymph node and tonsil) were collected and stored at − 80 °C or fixed in 10% formalin. To ensure that no cross contamination occurred during the experiment, sequencing of a fragment of P1 protein of SVA present in the tonsil of all animals was performed. Animal experiments were revised and approved by the SDSU Institutional Animal Care and Use Committee (approval number 16-002A).

### 4.4. Viral RNA extraction and real-time RT-PCR (RT-qPCR)

Nucleic acid was extracted from serum, swabs and tissue samples using the MagMax viral RNA/DNA isolation kit (Thermo Fisher). One gram of each tissue was minced using a sterile scalpel, re-suspended in RPMI 1640 medium (10% w/v) and homogenized using a stomacher (two cycles of 60 s). Homogenized tissue samples were cleared by centrifugation and 50 μL of cleared tissue homogenate was used for nucleic acid extraction using the automated KingFisher Flex Purification System (Thermo Scientific). Swab samples were vortexed

cleared by centrifugation and 50 μL of cleared sample used for nucleic acid extraction as above. Fifty μL of serum were also used for nucleic acid extraction. The presence of SVA RNA in serum, oral and nasal secretions, feces and tissues were assessed using commercial SVA RT-qPCR reagents targeting a conserved region within the SVA 3D polymerase (EZ-SVA TC-9079-192 Tetracore Inc.). It is important to note that the 3D sequences targeted by the qPCR primers and probes included in the EZ-SVA TC-9079-192 assay are conserved between SVA SVV001 and SD15-26. A standard curve was generated with *in vitro* transcribed SVA RNA and viral loads were determined using the four-parameter logistic regression model function within MasterPlex 2010 software (Hitachi Software Engineering America, Ltd., San Francisco, CA).

### 4.5. Cross-neutralization assays

Neutralizing antibodies elicited by infection with SVA SVV 001 or SD15-26 were determined by virus neutralizing antibody assays as previously described (Joshi et al., 2016b). To determine the cross-neutralization levels of SVV 001 and SD15–26 serum samples from inoculated animals were tested against both viral strains in the VN assays. NA titers were expressed as the reciprocal of the highest serum dilution capable of completely inhibiting SVA infection/account inhibit replication. All assays were performed in triplicate and positive and negative control serum samples were included in all test plates.

### 4.6. Flow cytometry

Intracellular IFN-γ expression assays were performed as previously described (Maggioli et al., 2017). SVA-specific T cell phenotypes were determined using a panel of swine-specific antibodies, including mouse anti-pig CD3-Alexa Fluor 647 (ε chain specific; clone BB23-8E6-8C8, isotype IgG2a; BD Pharmingen), anti-pig CD4 (clone 74-12-4, isotype IgG2b), anti-pig CD8α (clone 76-2-11, isotype Ig2a) (all from the Washington State University MAb Center, Pullman, WA). Secondary antibodies included goat anti-mouse IgG2a-phycoerythrin (PE), goat anti-mouse IgG2a-FITC, and goat anti-mouse IgG2b-PE-Cy7 (all from Southern Biotech, Birmingham, AL). Cross-IFN-γ expression responses were assessed by stimulation of the PBMCs collected from SVV 001- or SD15-26-inoculated animals with both SVA strains used in our study (SVV 001 or SD15-26). Staining protocols and assay controls have been previously described (Maggioli et al., 2017). Flow cytometry data was acquired with the Attune NxT flow cytometer (Thermo Fisher Scientific) and data analysis was performed using FlowJo software (TreeStar, San Carlos, CA). Data was corrected for background proliferation and/or IFN-γ expression by subtracting the frequency of cells that responded under non-stimulated conditions. Results from day 0 were used as baseline comparisons on T cell responses detected on day 14 pi. Percentage of responding cells was expressed as the percentage of live T cells ($CD3^+$ cells).

### 4.7. Statistical analysis

Statistical analysis was performed by analysis of variance followed by T-Student's or Sidak's comparisons test. Statistical analysis and data plotting were performed using the GraphPAD Prism 6.0 software (GraphPAD Software Inc., La Jolla, CA).

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