Persistent Infection and Transmission of Senecavirus A from Carrier Sows to Contact Piglets

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ABSTRACT Senecavirus A (SVA) is a picornavirus that causes acute vesicular disease (VD), that is clinically indistinguishable from foot-and-mouth disease (FMD), in pigs. Notably, SVA RNA has been detected in lymphoid tissues of infected animals several weeks following resolution of the clinical disease, suggesting that the virus may persist in select host tissues. Here, we investigated the occurrence of persistent SVA infection and the contribution of stressors (transportation, immunosuppression, or parturition) to acute disease and recrudescence from persistent SVA infection. Our results show that transportation stress leads to a slight increase in disease severity following infection. During persistence, transportation, immunosuppression, and parturition stressors did not lead to overt/recrudescent clinical disease, but intermittent viremia and virus shedding were detected up to day 60 postinfection (p.i.) in all treatment groups following stress stimulation. Notably, real-time PCR and *in situ* hybridization (ISH) assays confirmed that the tonsil harbors SVA RNA during the persistent phase of infection. Immunofluorescence assays (IFA) specific for double-stranded RNA (dsRNA) demonstrated the presence of double-stranded viral RNA in tonsillar cells. Most importantly, infectious SVA was isolated from the tonsil of two animals on day 60 p.i., confirming the occurrence of carrier animals following SVA infection. These findings were supported by the fact that contact piglets (11/44) born to persistently infected sows were infected by SVA, demonstrating successful transmission of the virus from carrier sows to contact piglets. Results here confirm the establishment of persistent infection by SVA and demonstrate successful transmission of the virus from persistently infected animals.

IMPORTANCE Persistent viral infections have significant implications for disease control strategies. Previous studies demonstrated the persistence of SVA RNA in the tonsil of experimentally or naturally infected animals long after resolution of the clinical disease. Here, we showed that SVA establishes persistent infection in SVA-infected animals, with the tonsil serving as one of the sites of virus persistence. Importantly, persistently infected carrier animals shedding SVA in oral and nasal secretions or feces can serve as sources of infection to other susceptible animals, as evidenced by successful transmission of SVA from persistently infected sows to contact piglets. These findings unveil an important aspect of SVA infection biology, suggesting that persistently infected pigs may function as reservoirs for SVA.

KEYWORDS SVA, Seneca Valley virus, SVV, persistence, carrier

Senecavirus A (SVA), formerly known as Seneca Valley virus, is a positive-sense single-stranded RNA virus in the genus *Senecavirus*, family *Picornaviridae* (1). SVA was first identified as a cell culture contaminant in 2002 (1, 2). Retrospective sequencing of archived/unclassified swine picornavirus isolates revealed that SVA has been circulating in the U.S. swine population since at least 1988 (2), being associated with multiple clinical presentations, including idiopathic vesicular disease (VD) in pigs (1–4).

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An increased number of outbreaks of SVA-induced VD have been reported worldwide since 2014, affecting swine in the United States (5–8), Brazil (7–9), China (10), Thailand (11), Colombia (12), and Vietnam (13). Clinically, SVA-induced VD is indistinguishable from foot-and-mouth disease (FMD) (14, 15), a highly contagious VD caused by the related picornavirus, foot-and-mouth disease virus (FMDV) (16). Interestingly, several cases of natural SVA infection have been reported following common swine production stressors, such as transportation (3, 4) and parturition (7, 17, 18), suggesting that stressors may contribute to SVA-induced VD (19).

The etiologic role of SVA on VD in swine has been recently confirmed experimentally (14, 15). After a short incubation period (3 to 5 days), infected animals present clinical signs characterized by lethargy and lameness, followed by the development of classic vesicles on the snout and/or feet (14). A short-term viremia (1 to 10 days) is observed in infected animals (14), with decreasing levels of viremia paralleling the appearance of neutralizing antibodies in serum (20). SVA lesions usually subside within 14 to 16 days of infection (14, 20, 21); however, virus shedding has been observed up to 28 days postinfection (dpi) (14). Notably, SVA RNA has been detected in tissues (mainly the tonsil) of experimentally infected pigs several weeks after resolution of the clinical disease (up to 38 dpi) (14), in spite of robust CD8⁺ T cell responses elicited upon infection (20). These observations were confirmed in animals naturally infected with SVA, in which viral RNA was detected in the tonsil of affected sows approximately 60 days after the VD outbreak (22). The presence of SVA nucleic acid in tissues from infected animals long after disease resolution (14, 22) suggests that, like other picornaviruses (23–25), SVA may establish persistent infection in susceptible animals.

Persistence is a common feature of many picornaviruses of significance to animal and human health (23–26). The carrier state of FMDV, for example, has been defined by the presence of infectious virus in oropharyngeal fluids of infected animals for 28 or more days after infection (23). Other picornaviruses that are known to establish long-term persistence include the human pathogens poliovirus (PV), rhinovirus (RV), and coxsackievirus (CV) and the animal pathogens encephalomyocarditis virus (EMCV) and Theiler's murine encephalomyelitis virus (TMEV) (24, 25, 27–29). Despite the significance of persistent infections and their implications for the control of picornaviruses, little is known about the mechanisms underlying establishment, maintenance, and potential recrudescence of persistent picornaviral infections. Notably, rare cases of disease recrudescence and, more frequently, recurrent virus shedding from persistently infected hosts have been linked with stress or immunosuppression for picornaviruses affecting humans and animals (24, 25, 29).

The present study focused on three important aspects of SVA infection biology: (i) the effect of stressors (transportation) on acute SVA-induced VD; (ii) the occurrence of chronic/persistent SVA infection; and (iii) the effect of stressors (transportation, immunosuppression, or parturition) on potential recrudescence from persistent SVA infection.

RESULTS

Effect of stressors on acute SVA-induced vesicular disease. The effect of transportation stress on SVA infection and disease dynamics was investigated. Fourteen SVA-negative cull sows were allocated to three experimental groups, as follows: group 1 (G1), mock-infected controls ($n = 4$); group 2 (G2), animals infected with SVA upon arrival (transportation stress; $n = 5$); and group 3 (G3), animals infected with SVA after acclimation (7-day acclimation; $n = 5$) (Fig. 1). Animals were inoculated oronasally with SVA strain SD15-26 (G2 and G3) (14) or mock-inoculated with RPMI 1640 medium (G1). After inoculation, animals were monitored at 6- to 12-h intervals for characteristic clinical signs and lesions of SVA. All animals from G2 (transportation stress) and G3 (acclimated) presented characteristic clinical signs of SVA, including lethargy and lameness. Clinical signs were first observed around 18 h postinoculation (p.i.) in animals from G2 and around 36 h p.i. in animals from G3.

FIG 1 Experimental design. (A) Acute phase (phase 1): effects of transportation stress on acute SVA-induced VD. Group 1 (G1), mock-infected control group ($n = 4$); group 2 (G2), transportation stress with SVA infection of sows upon arrival (2-h trip from farm to animal facility; $n = 5$); and group 3 (G3), SVA-infected sows after acclimation (7-day acclimation/no-stress group; $n = 5$). (B) Chronic/persistent phase (phase 2): occurrence of persistent infection and effect of stressors on chronic/persistent SVA infection. SVA- or mock-infected sows from the first phase of the study were subjected to transportation stress (2-h trip; G1 and G2) or received immunosuppressive dexamethasone treatment (1.5 mg/kg, i.v.) for five consecutive days (G3). The effects of these stressors on persistent SVA infection were evaluated for 14 days. To assess the effect of the stress associated with parturition, a fourth group (G4) consisting of SVA-infected pregnant gilts was included in the study (parturition stress, $n = 5$). Gilts in G4 were inoculated with SVA upon arrival at the animal facility on gestational day 68. Parturition was induced on day 44 p.i. (gestational day 112), and its effects on SVA persistent infection were evaluated for 14 days. During the chronic/persistent phase, all four groups (G1 to G4) were monitored in parallel.

Vesicular lesions were initially observed at 36 h p.i. in two animals from G2, while lesions in animals from G3 were observed at ~42 h p.i. The mean times to lesion development were 58 h for animals from G2 and 66 h for animals from G3. The first lesions were observed on the feet of animals from both G2 and G3, with snout lesions developing 1 to 2 days later (Fig. 2A). Vesicular lesions on the snout were first observed in animals from G2 (Fig. 2B) on days 3 to 4 p.i. and 1 to 2 days later in animals from G3 (Fig. 2C). Most lesions were resolved by day 18 to 20 p.i. Lesions were observed in four of five (4/5) animals from both G2 and G3. No lesions or clinical signs were observed in animals from the mock-infected control G1. It is important to note that lesions observed in animals from G2 were, in general, larger than the lesions in animals from G3 (Fig. 2C).

Clinical scores were recorded for each animal based on the presence or absence of vesicular lesions (21). A score of 1 was assigned to the snout or each foot affected by vesicular lesions, for a total score of 5 per animal per day. Average daily scores were calculated for each group. As shown in Fig. 2D, slightly higher clinical scores were observed in animals subjected to transportation stress (G2) during days 1 to 4 p.i. than in animals acclimated prior to SVA inoculation (G3).

Dynamics of viremia and virus shedding during acute SVA infection in sows.

The dynamics of viremia and virus shedding were assessed in serum, in oral and nasal secretions, and in feces. Oral, nasal, or rectal swabs collected on days 0, 1, 3, 5, 7, 14, 21, 28, and 35 p.i. were tested for SVA RNA using real-time reverse transcriptase quantitative PCR (RT-qPCR). Viremia was detected between days 1 and 21 p.i. (Fig. 3A). High SVA genome copy numbers were consistently detected in serum between days 1

FIG 2 Clinical presentation of acute SVA-induced vesicular disease following transportation stress or acclimation. (A) Time to lesion development in animals in G2 (transport stress) and G3 (acclimated). Vesicular lesions were first observed on the feet of SVA-infected animals at day 2 p.i. (top panels) and subsequently on the snout on day 3 p.i. (bottom panels). (B) Severity of vesicular lesions in animals in G2 (transport stress) and G3 (acclimated). Vesicular lesions were detected on the snout of SVA-infected sows starting at day 3 p.i. (transport stress group [G2] and acclimated group [G3]). Overall, lesions observed in G2 transport-stressed animals were more severe than those observed in G3 acclimated animals. (C) Clinical scores. Animals were evaluated daily for the presence of vesicles and lameness. One point was assigned for each foot or snout displaying vesicular lesions, and another point was assigned for lameness (maximum of 6 points/animal/day), and daily group averages were calculated. Arrows indicate vesicular lesions.

FIG 3 Kinetics of viremia and virus shedding during acute SVA infection in sows (0 to 35 dpi). Viremia (A) and virus shedding in oral (B) and nasal (C) secretions and in feces (D) were determined by RT-qPCR quantitated based on a relative quantitation method.

and 7 p.i., with only one animal from G2 and one animal from G3 remaining viremic on days 14 and 21 p.i., respectively (Fig. 3A).

Virus shedding was detected in oral and nasal secretions and feces up to day 35 p.i., with all inoculated animals shedding SVA between days 1 and 14 p.i. (Fig. 3B to D). After day 14 p.i., intermittent virus shedding was observed, with a few animals from G2 and G3 (2 to 3 animals) shedding SVA up to day 35 p.i. (Fig. 3B to D). Higher levels of SVA

FIG 4 SVA neutralizing antibody levels during acute SVA infection of sows and detection of SVA in tonsil. (A) Virus-neutralizing (VN) antibody responses elicited by SVA infection were evaluated weekly from day 0 to day 35 p.i. NA titers represent the reciprocal of the highest serum dilution capable of completely inhibiting SVA infectivity. The gray line represents the cutoff value of the VN assay. (B) Viral load in the tonsil of SVA-infected animals. To confirm viral presence in the tonsil, one animal from each group was necropsied on day 44 p.i. SVA presence in the tonsil was determined by RT-qPCR, and viral load was determined by a relative quantitation method.

shedding were detected between days 3 and 14 p.i., with excretion levels gradually decreasing thereafter. No differences in virus shedding levels were detected between animals from G2 and G3.

Neutralizing antibody (NA) titers elicited by SVA infection were determined by virus neutralization assays as previously described (14, 20). High levels of NA were detected in all SVA-inoculated sows starting on day 5 p.i. NA titers remained elevated throughout the acute phase of SVA infection (Fig. 4A).

Clinical outcome following stress stimulation during chronic/persistent SVA infection. SVA RNA has been detected in the tonsil of SVA-infected pigs long after clinical disease resolution (up to day 38 p.i.) (14), suggesting that the virus may establish persistent infections in affected animals. Notably, stress and immunosuppression have been associated with recrudescence from persistent picornaviral infections (24–26). Here, we investigated the establishment of persistent SVA infection and the effect of stressors (i.e., transportation, immunosuppression, and parturition) on disease recrudescence and recurrent virus shedding. The chronic/persistent experimental phase comprised four groups as follows: group 1 (G1), mock-infected control animals ($n = 4$); group 2 (G2), SVA-infected sows subjected to transportation stress ($n = 5$); group 3 (G3), SVA-infected sows subjected to dexamethasone (Dx)-induced immunosuppression ($n = 5$); and group 4 (G4), SVA-infected sows subjected to parturition stress ($n = 5$) (Fig. 1). Sows from G1, G2, and G3 comprised the animals from the acute phase of the experiment (Fig. 1), while G4 consisted of SVA-negative pregnant gilts inoculated with SVA on gestational day 70 (~44 days prior to the anticipated farrowing date) (Fig. 1). To confirm SVA infection and the presence of virus RNA in the tonsil of inoculated animals, one sow each from each G2, G3, and G4 was euthanized on day 42 p.i., and the tonsil was collected and tested by SVA RT-qPCR. All three sows presented high levels of SVA RNA in the tonsil on day 42 p.i. (Fig. 4B), confirming the presence/persistence of SVA RNA in this tissue. On day 46 p.i. animals from G2 were subjected to transportation

stress (2-h trailer ride), while animals from G3 were subjected to Dx-induced immunosuppression (Dx administration for 5 consecutive days at 46 to 50 days p.i.). Animals in G4 farrowed between days 44 and 46 p.i., and parturition and nursing served as natural stressors for the animals in this group. Mock-infected animals from G1 were used as negative controls.

After stress stimulation, all animals from G1 ($n = 4$), G2 ($n = 4$), G3 ($n = 4$), and G4 ($n = 4$) were monitored daily during 14 days for characteristic clinical signs and lesions of SVA (Fig. 1). No clinical signs or lesions were observed after stress stimulation (transportation [G2], immunosuppression [G3], or parturition [G4]) (data not shown). Additionally, piglets born to sows in G4 remained clinically normal throughout the 14-day experimental period.

The effect of stressors on peripheral blood leukocyte counts was assessed by flow cytometry (Fig. 5A and B). White blood cell (WBC) counts were lower in animals subjected to Dx administration (G3) (Fig. 5A) than in control G1 animals (days 53 and 60 p.i.) ($P < 0.05$). Additional analysis revealed reduced levels of circulating T cells ($CD3^+$) in animals from G3 (days 46 to 60 p.i.) ($P < 0.05$) (Fig. 5D). No differences in the levels of granulocytes, monocytes, and B cells ($CD21^+$) were observed between treatment groups (Fig. 5C, E, and F). Additionally, animals in G1, G2, and G4 presented WBC counts within the normal range throughout the experiment (Fig. 5A to F).

Intermittent long-term viremia and virus shedding following SVA infection.

The dynamics of viremia and virus shedding in oral and nasal secretions and in feces were assessed after stress stimulation of SVA-inoculated sows. Samples collected daily between days 46 and 60 p.i. (or 0 and 14 days poststress [p.s.]) were tested for SVA by RT-qPCR. Viremia was detected intermittently between day 46 and 60 p.i. in one to four animals in G2, G3, and G4 (Fig. 6A). Notably, on day 50 p.i., the last day of Dx administration, all animals in G3 were viremic. The levels of viremia (determined as genome copy numbers) observed during the chronic/persistent phase of infection were lower than the levels detected during acute SVA infection (Fig. 6A). No differences in the levels of viremia were observed between the treatment groups (G2, G3, and G4).

Virus shedding was detected in oral and nasal secretions and in feces up to day 60 p.i. (day 14 p.s.) by RT-qPCR. Notably, SVA shedding was detected at low frequency (1 to 3 animals) in all three groups (G2, G3, and G4) subjected to stress stimulation (Fig. 6B to D). Virus shedding during the chronic/persistent phase of infection was characterized by an intermittent/transient pattern of virus excretion (Fig. 6A to C). Interestingly, one animal in G4 (sow 600) presented the most consistent SVA shedding pattern throughout the experiment. Similar to the levels of viremia, the amounts of SVA excreted during the chronic/persistent phase of infection were markedly lower than the levels detected during the acute phase of infection (Fig. 2). No differences in virus shedding were observed between the treatment groups (G2, G3, and G4). Levels of SVA-specific NAs remained elevated in all animals until day 60 p.i. (Fig. 7).

SVA establishes persistence in the tonsil. The tonsil is one of the primary sites of SVA replication during acute infection (14), and it was shown to harbor SVA RNA long after resolution of the clinical disease (14, 22). Therefore, here we investigated the occurrence of persistent SVA infection in the tonsil of SVA-infected animals. Viral load and distribution in the tonsil were assessed by RT-qPCR and *in situ* hybridization (ISH), respectively. All animals from G1 to G4 were euthanized on day 60 p.i., and the tonsil was collected and processed for SVA detection. High levels of SVA nucleic acid were detected in the tonsil of all SVA-inoculated animals by RT-qPCR (Fig. 8A and Table 1). Notably, infectious SVA was isolated from the tonsil of two animals on day 60 p.i. (one animal from G2 and one animal from G4) (Table 1) after extraction of viral particles from the tissue homogenates with Vertrel, a solvent known to extract lipids and lipid bilayers from cells (30).

In situ hybridization (ISH) staining revealed the presence of SVA RNA in tonsillar epithelial cells, crypt epithelial cells, subepithelial lymphoid tissue and lymphoid follicles (Fig. 8B). In addition to cytoplasmic staining in epithelial cells, a closer look at the

FIG 5 Dexamethasone-induced changes in peripheral white blood cells count in SVA-infected sows. Peripheral white blood cell count and leukocyte discrimination were performed by flow cytometry during SVA infection of control sows (G1) and sows subjected to transport stress (G2), dexamethasone treatment (G3), or parturition stress (G4). (A) A representative flow cytometry gate strategy used to determine white blood cell subpopulations is shown. The plot shows red blood cells, platelets, and cell debris exclusion and leukocyte selection (based on forward scatter area [FSC-A] and side scatter height [SSC-H]; top left plot). A dot plot depicts lymphocyte, monocyte, and granulocyte populations (top right plot). This plot served as confirmatory gates used for selection based on the expression of cell markers (bottom plots). B and T lymphocytes were

(Continued on next page)

lymphoid region revealed the presence of SVA RNA in monocular cells (data not shown).

Next, we investigated the potential mechanism for SVA persistence in the tonsil. Notably, a dsRNA-specific immunofluorescence assay (IFA) revealed the presence of dsRNA in the tonsil of SVA-infected sows on day 60 p.i., whereas no dsRNA was detected in the tonsil of mock-infected G1 animals (Fig. 9). Together, these results demonstrate the persistence of SVA in the tonsil of infected sows.

Persistently infected sows transmit SVA to contact piglets. Persistently infected carrier animals may play an important role in the epidemiology of picornaviruses, serving as a source of infection to susceptible animals (31, 32). Thus, the potential for transmission of SVA from persistently infected sows to their offspring was investigated. After farrowing of G4 sows, piglets were maintained with their mothers and monitored daily for clinical signs and lesions of SVA. Additionally, oro-rectal swabs were collected daily from five randomly selected piglets from each sow ($n = 20$) and tested for SVA by RT-qPCR. All piglets born to SVA-infected sows remained clinically normal throughout the experiment. While SVA RNA was detected in oro-rectal secretions from piglets born to all four sows (animals 600, 1737, 1802, and 1807), a higher frequency of positive piglets born to sow 600 (the sow with most consistent pattern of virus shedding) was detected throughout the experiment (Fig. 10).

To assess whether piglets born to sows persistently infected with SVA were in fact infected with the virus, all piglets ($n = 44$) were euthanized on experimental day 60 (day 14 of life), and the tonsil was collected to assess the presence of SVA RNA. As shown in Table 2, SVA RNA was detected in 8 of 44 (8/44) piglets. Notably, piglets born to three SVA-inoculated sows (600, 1737, and 1802) were positive for SVA, with 6 of 11 piglets born to sow 600 being positive for SVA. Virus neutralization assays in serum of piglets collected on day 60 p.i. (day 14 of life) revealed high levels of NAs, likely acquired through passive transfer from the sows (Table 2). Together, these results demonstrate efficient SVA transmission from persistently infected sows to their offspring.

DISCUSSION

Senecavirus A (SVA) recently emerged worldwide, causing several outbreaks of VD, which is clinically indistinguishable from FMD in pigs (4, 6, 7, 9, 11, 13, 17, 33–35). Interestingly, many natural cases of SVA have been reported following stressful conditions, such as transportation (3, 4) and parturition (7, 17, 18). Thus, it has been hypothesized that stress and/or immunosuppression may contribute to SVA-induced VD (4, 19). Previously we developed an SVA infection model in which animals were inoculated upon arrival at the animal facility after a 2-h trip from the farm (14). Using this model, we successfully and consistently reproduced VD in SVA-inoculated animals, with lesions being observed on the snout and/or feet of ~50 to 100% of inoculated animals within 3 to 5 days of infection (14, 20, 21). Here, using a sow infection model, we investigated the effect of transportation stress on SVA infection dynamics and disease pathogenesis. Results from these studies showed that sows subjected to transportation stress prior to infection with SVA developed vesicular lesions slightly earlier than the acclimated animals. The size and severity of the lesions also seemed to be accentuated in animals inoculated after transportation stress. Interestingly, the sows inoculated with SVA in the present study displayed clinical signs and developed vesicular lesions earlier than nursery (36) or finishing pigs used in previous SVA pathogenesis studies (14, 20, 21), suggesting that age, sex, and/or weight may potentially contribute to disease onset and severity in pigs. A recent study conducted to

FIG 5 Legend (Continued)

selected based on the expression of CD21 and CD3, respectively (panel 1 plots). Granulocytes and monocytes were determined by the expression of CD172a, as well as by their differential size and complexity properties (panel 2 plot). (B) Absolute white blood cell counts. (C) Relative granulocyte count (CD172a⁺; high complexity). (D) Relative T cell count (CD3⁺). (E) Relative monocyte count (CD172a⁺; low complexity). (F) Relative B cell count (CD21⁺). Percentages in panels C to F are based on the total WBC count.

FIG 6 Kinetics of viremia and virus shedding during chronic/persistent SVA infection (42 to 60 dpi). Viremia (A) and virus shedding in oral (B) and nasal (C) secretions and in feces (D) were determined by RT-qPCR and quantitated based on a relative quantitation method.

evaluate the effect of immunosuppression on acute SVA-induced VD concluded that immunosuppressive doses of Dx had no significant effect on disease onset and severity or on the levels of viremia and virus shedding (19). Results here, showing early lesion development and enlarged lesions following transportation stress, suggest that while stressors are not required for SVA-induced VD in pigs, exposure of animals to stressful conditions may contribute to or accelerate the development of vesicular lesions following infection with SVA.

By using a natural swine host/SVA infection model, we unveiled important aspects of SVA infection biology, pathogenesis, and the host immune responses to infection

FIG 7 SVA neutralizing antibody levels in serum of SVA-infected animals during chronic/persistent SVA infection. Virus-neutralizing (VN) antibody responses during chronic/persistent SVA infection from day 42 to 60 p.i. NA titers represent the reciprocal of the highest serum dilution capable of completely inhibiting SVA infectivity.

(14, 20, 21). Most importantly was the discovery that SVA RNA persists in the tonsil of infected animals long after the clinical/acute phase of the disease (day 38 p.i.) (14), suggesting that the virus could potentially establish persistent infections in susceptible animals. These findings were later confirmed in naturally infected sows, which were shown to carry SVA RNA in the tonsil for up to 60 days post-SVA outbreak (22). In the present study, we investigated the occurrence of persistent SVA infection in sows. Consistent with our previous findings (14), high levels of SVA nucleic acid were detected in the tonsil of all (15/15) SVA-inoculated animals on day 42 or 60 p.i. These results confirmed that SVA RNA persists in host tissues for extended periods of time following resolution of the clinical disease. Most importantly, detection of infectious SVA in the tonsil of two animals on day 60 p.i. after treatment of the tissue homogenate with Vertrel confirmed that SVA establishes persistent infection in the tonsil of infected animals. These findings demonstrate that, in addition to being one of the primary sites of SVA replication during acute infection (14), the tonsil also harbors the virus during persistence.

The exact mechanism(s) by which picornaviruses establish, maintain, and recrudescence from persistent infections is not completely understood. One of the requirements for establishment of persistent viral infections is the maintenance of the viral genome in persistently infected cells (37, 38). To preserve genome stability during persistence, positive-stranded RNA viruses may maintain their genome as double-stranded RNA (dsRNA) in the absence of active replication (39) while other viruses establish persistence through continuous low-level replication in persistently infected cells (37, 40). There is evidence suggesting that both mechanisms may be at play during picornavirus persistence (41–43). Tam and Messner have shown that persistence of coxsackievirus RNA in muscle cells occurs through stable dsRNA complexes that are produced following resolution of the acute viral infection (41). These observations suggest that the virus genome could be maintained in “sanctuary” cells that survive acute infection as a dsRNA molecule during nonproductive persistent infection (41). It is important to note, however, that dsRNA is produced by positive-sense RNA viruses, including picornaviruses (i.e., encephalomyocarditis virus), as an intermediate of viral genome replication (44). Therefore, the presence of dsRNA in tissues persistently infected with picornaviruses, as demonstrated in the tonsil of SVA-inoculated sows here, could represent either stable dsRNA complexes in a nonreplicating form or, more likely, dsRNA intermediates produced during low-level virus replication in persistently infected cells.

Interestingly, a series of recent studies has shown that persistent coxsackievirus genomes mutate, undergoing 5′-terminal deletions (~7 to 49 nucleotides [nt]) that are associated with long-term viral persistence (43, 45–48). Notably, the 5′-terminally

FIG 8 Detection of SVA RNA in the tonsil of SVA-infected animals on day 60 p.i. (A) Viral load in the tonsil of SVA-infected animals, as determined by RT-qPCR and quantitated based on a relative quantitation method. (B) Representative *in situ* hybridization demonstrating the presence of SVA nucleic acid (in red) in the tonsils of SVA-infected sows on day 60 p.i. G1 control, SVA RNA detected; G2, transport stress; G3, immunosuppression by dexamethasone; and G4, parturition. Animal numbers are indicated. Multifocal staining was observed in tonsillar epithelial (TE) cells, crypt epithelial (CE) cells, subepithelial lymphoid tissue (SLT), and lymphoid follicles (LF).

deleted picornavirus genomes have been shown to be capable of replicating, resulting in low-yield infectious virus production (48, 49). Collectively, these observations suggest at least two potential mechanisms for picornavirus genome persistence: (i) continuous low-level replication in persistently infected cells and/or (ii) cycles of productive and nonproductive infection. Results here demonstrating intermittent low-level virus shedding by persistently infected animals and detection of dsRNA support both hypotheses. It is important to note, however, that in addition to maintenance of the viral genome

TABLE 1 Detection of SVA in the tonsil of SVA-inoculated sows

Group no. (stressor)	Animal no.	Viral load by RT-qPCR (C_T) ^a	Virus isolation after Vertel treatment ^b
1 (control)	225	ND	—
	418	ND	—
	979	ND	—
	1615	ND	—
2 (transport)	1176	27.29	—
	1549	25.20	—
	1623	24.79	+
	1661	23.93	—
3 (Dx immunosuppression)	333	26.20	—
	845	26.34	—
	1096	27.44	—
	1658	26.00	—
4 (parturition)	600	26.45	—
	1737	23.98	—
	1802	23.34	—
	1807	24.59	+

^aViral load in tonsil was detected by RT-qPCR. C_T , cycle threshold; ND, not detected.

^bNo SVA was isolated in H1299 cells after three 5-day passages in untreated tissues. Virus isolation from tissue homogenate of treated tissues is indicated as follows: —, infectious SVA not detected; +, infectious SVA reisolated.

in persistently infected tissues, picornaviruses likely evolved to evade host responses and maintain a long-term population of persistently infected cells. SVA persistence seems to occur independently of the immune responses that infected animals elicit against the virus (20). In the present study, persistent SVA RNA was detected in animals presenting robust NA titers while in a previous study we detected persistent SVA RNA in animals presenting SVA-specific humoral and T cell responses (20). This suggests that the ability of the virus to evade host immune responses may play a significant role in establishment and maintenance of persistent infections.

The effect of stressors on disease recrudescence and/or recurrent viremia and virus shedding was evaluated during chronic/persistent infection in our study. SVA-infected animals were subjected to stressors that have been associated with natural SVA outbreaks in the field, including transportation, immunosuppression, and parturition. Although no clinical signs or overt disease were observed after stress stimulation, intermittent virus shedding was detected in all SVA-infected groups. Notably, all animals subjected to Dx immunosuppression presented viremia on the last day of Dx administration, suggesting that Dx-induced immunosuppression (evidenced by significantly lower levels of circulating T cells in G3 animals) may favor recurrent SVA viremia. It is important to note, however, that the virus was rapidly cleared from the circulation of Dx-treated animals, likely as a result of the high levels of circulating neutralizing antibodies, leading to a transient/intermittent pattern of viremia in persistently infected animals. Similarly, low-frequency (1 to 3 animals)/intermittent virus shedding was detected in oral and nasal secretions and/or feces of animals subjected to transportation, immunosuppression, or parturition stressors.

Given that many of the natural cases of SVA have been reported in sows after parturition, we investigated the effect of parturition stress on recurrent SVA-induced VD. To simulate natural stress conditions and evaluate potential transmission of SVA from persistently infected sows to their offspring, piglets were maintained with their mothers and monitored daily for clinical signs. While no clinical signs were observed in infected sows after parturition or in contact piglets after birth, SVA RNA was detected in oro-fecal secretions of piglets born to all four inoculated sows. Interestingly, piglets born to sow 600, which presented the most consistent shedding pattern after parturition, presented the highest frequency of positive oro-fecal swabs throughout the experiment. Most importantly, testing of the tonsil of all piglets at the end of the

FIG 9 *In vivo* detection of viral dsRNA in tonsil of SVA-infected pigs during chronic/persistent infection. Frozen tonsil sections from mock-inoculated (top) or SVA-infected animals at day 60 p.i. dsRNA was stained with a mouse monoclonal antibody specific for viral dsRNA (J2 clone), and the cell nuclei were counterstained with DAPI (right panel; blue). Red fluorescence indicates the presence of dsRNA in the cytoplasm of cells. G1, control animal 979; G2, transport stress animal 1623; G4, parturition stress animal 1807.

experiment revealed that piglets born to three of four (3/4) sows were infected with SVA. The highest frequency of positive piglets was again detected within the litter of sow 600 (6/10). These findings indicate that SVA shed by persistently infected animals can be efficiently transmitted to susceptible animals, confirming that persistently infected animals function as carriers of the virus and serve as a source of infection to other susceptible animals. The low frequency of piglets carrying SVA in the tonsil, however, is likely a result of the protective function of neutralizing antibodies, which have been linked to control of SVA infection (20) and which were detected in all piglets in the present study (likely due to passive transfer from the sow) (Table 2).

In conclusion, results here confirm that SVA establishes persistent infection in the natural swine host and further demonstrate chronic virus shedding from persistently infected animals. These observations corroborate observations described for other important picornaviruses, including PV (25, 26, 31) and rhinovirus (29) in humans, FMDV in cattle (23, 50), and EMCV in pigs (24), suggesting that these viruses may share

FIG 10 Detection of SVA RNA in oro-rectal secretions from piglets born to sows in the parturition stress group (G4). (A) Oro-rectal secretions from piglets born to sows in G4 (5 piglets from each sow were randomly sampled) were collected daily. Levels of SVA RNA were determined by RT-qPCR and quantitated by a relative quantitation method.

mechanisms to establish, maintain, and perhaps recrudesce from, persistent infections. Isolation of SVA from tissues and results demonstrating efficient transmission of SVA from persistently infected sows to their offspring confirm that infected animals may function as carriers of the virus and effectively serve as a source of infection to other susceptible animals. The occurrence of persistent SVA infection, demonstrated here, has significant implications for the epidemiology of the disease and will impact the design and implementation of SVA control strategies in the future.

MATERIALS AND METHODS

Cells and virus. NCI-H1299 non-small human lung carcinoma cell lines (ATCC CRL-5803) were cultured at 37°C with 5% CO₂ in RPMI 1640 medium (Corning) supplemented with 10% fetal bovine serum (FBS; Seradigm), 2 mM L-glutamine (Corning), penicillin-streptomycin (100 IU/ml; Corning), and gentamicin (50 µg/ml; Corning). Stocks of SVA strain SD15-26 (passage 4) (14) were prepared and titrated in H1299 cells and used in the animal experiments described below.

Animal inoculation. To investigate the effect of stressors on SVA infection dynamics and the occurrence of persistent infection, SVA-negative sows were experimentally infected and monitored for 60 days. The experiment was divided in two phases: (i) the acute phase (–7 to 35 dpi), which focused on investigating the effect of transportation stress on lesion development and disease dynamics; and (ii) the chronic/persistent phase (day 46 to 60 p.i.), designed to investigate the occurrence of persistent infection and the effect of stressors (transportation, immunosuppression, and parturition) on disease recrudesce and recurrent virus shedding (Fig. 1).

For the acute phase, 14 SVA-negative cull sows were randomly allocated into three experimental groups as follows: group 1 (G1), mock-infected control group ($n = 4$); group 2 (G2), sows infected with SVA upon arrival (2-h transportation stress; $n = 5$); and group 3 (G3), sows infected with SVA after acclimation (7-day acclimation, no-stress group; $n = 5$). For the chronic/persistent phase, sows from G1 and G2 were subjected to transportation stress (2-h trailer ride), whereas animals in G3 received immunosuppressive doses of dexamethasone (1.5 mg/kg, intravenously [i.v.]) for 5 consecutive days (46 to 50 dpi) (51). To assess the effect of the stress associated with parturition, a fourth group of animals was included in the study (group 4 [G4], parturition stress; $n = 5$) (Fig. 1). This group consisted of five primiparous pregnant gilts that were inoculated with SVA upon arrival at the animal facility (day 0 p.i.) on gestational day 68. On day 44 p.i. (gestational day 112) parturition was induced by intramuscular (i.m.) administration of prostaglandin-F2 α (10 mg). To mimic field conditions of stress related to parturition and nursing, 11 piglets from each sow were kept with their mothers for 14 days and allowed to suckle colostrum and milk *ad libitum*.

Animals from G2, G3, and G4 were inoculated with 10 ml of a virus suspension containing 10^{7.18} 50% tissue culture infective doses (TCID₅₀)/ml by the oral (5 ml) and intranasal (2.5 ml to each nostril) routes. Noninfected G1 control animals were inoculated with RPMI 1640 medium by the oronasal route as described above. Animals were inoculated and monitored for clinical signs (every 6 to 12 h for the first 5 days and then once daily) and lesions for 60 days. One animal from each SVA-infected group was euthanized for tissue collection on day 44 p.i. All the remaining animals were euthanized on day 60 p.i. Animal experiments were conducted at the South Dakota State University (SDSU) Animal Resource Wing (ARW) and followed the protocols and guidelines approved by the SDSU Institutional Animal Care and Use Committee (approval number 17-112A).

Clinical scores. Animals were monitored for characteristic signs and lesions of SVA daily during the first 18 days of the acute phase. Individual daily lesion scores were assigned to each animal, and the daily average scores were calculated for each group. Lesions observed on the snout and/or each foot were assigned a score of 1, 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) (21). A score of zero (0) was assigned when no lesions were observed.

TABLE 2 Detection of SVA in tonsil of contact piglets by RT-qPCR

Group 4 sow and piglet no.	Viral load by RT-qPCR (C_T) ^a	NA titer ^b
600		
1	ND	5,120
2	ND	10,240
3	34.2	1,280
4	33.4	5,120
5	ND	2,560
6	35.8	10,240
7	34.3	1,280
8	37.0	5,120
9	34.1	5,120
10	ND	2,560
11	ND	2,560
1737		
1	ND	160
2	ND	640
3	ND	5,120
4	ND	2,560
5	ND	2,560
6	ND	2,560
7	ND	640
8	ND	640
9	ND	1,280
10	ND	1,280
11	36.5	2,560
1802		
1	ND	10,240
2	ND	2,560
3	ND	10,240
4	ND	5,120
5	37.4	1,280
6	ND	2,560
7	ND	5,120
8	ND	10,240
9	ND	5,120
10	ND	2,560
11	ND	5,120
1807		
1	ND	10,240
2	ND	2,560
3	ND	10,240
4	ND	5,120
5	ND	2,560
6	ND	5,120
7	ND	5,120
8	ND	5,120
9	ND	5,120
10	ND	5,120
11	ND	5,120

^aViral load in tonsil was detected by RT-qPCR. Tonsil was collected at necropsy on day 14 postbirth. C_T , cycle threshold; ND, not detected.

^bVirus neutralizing antibody titers detected in serum on day 14 postbirth. Titers represent the reciprocal of the highest dilution of serum capable of preventing SVA infectivity.

Sample collection and processing. Swabs (oral, nasal, and rectal/fecal) and blood samples (serum and whole heparinized blood) were collected during the acute phase (days 0, 1, 3, 5, 7, 14, 21, 28, and 35 p.i.) and chronic/persistent phase (days 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, and 60 p.i.) of the experiment. Serum and peripheral blood mononuclear cell (PBMCs) were processed and stored as previously described (14, 20). Additionally, oro-fecal swabs (oral swab followed by rectal swab) were collected from piglets born to sows in G4 on days 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, and 59 p.i. One animal of each group (G1, G2, G3, and G4) was euthanized on day 44 p.i., to confirm the presence of SVA in the tonsil. One animal from G4 was also euthanized, and the tonsil and five randomly selected fetuses were collected to investigate the presence of SVA. The remaining animals and piglets born to G4 sows were euthanized on day 60 p.i. Tonsil was collected from all animals and stored at -80°C or fixed in 10% formalin.

RNA extraction and RT-qPCR. Viral RNA was extracted from swabs, serum, and tissues (tonsil and mediastinal and mesenteric lymph nodes) using the MagMAX viral RNA/DNA isolation kit (Life Technologies), according to the manufacturer's instructions, in an automated nucleic acid extractor (KingFisher Purification System; Thermo Fisher Scientific).

Nucleic acid was extracted from serum, swabs, and tissue samples as described above. Swab samples were vortexed and cleared by centrifugation ($10,000 \times g$ for 5 min), and 200 μ l of cleared supernatant was used for nucleic acid extraction. Two hundred microliters of serum was also used for nucleic acid extraction. For tissues, approximately 0.5 g of each tissue was minced using a sterile scalpel, resuspended in RPMI 1640 medium (10%, wt/vol), and homogenized using a stomacher (2 cycles of 60 s). Homogenized samples were then centrifuged at $14,000 \times g$ for 2 min at room temperature (RT), and 200 μ l of cleared supernatant was used for nucleic acid extraction.

The presence of SVA RNA was assessed using commercial SVA RT-qPCR reagents targeting the SVA 3D polymerase gene (EZ-SVA; Tetracore, Rockville, MD) (14, 20, 21). Amplification and detection were performed with an Applied Biosystems 7500 real-time PCR system under the following conditions: 10 min at 45°C for reverse transcription, 2 min at 95°C for polymerase activation, and 40 cycles of 5 s at 95°C for denaturation and 30 s at 60°C for annealing and extension. A standard curve was established by using SVA SD15-26 virus of titer $10^{7.88}$ TCID₅₀/ml and preparing 10-fold serial dilutions from 10^{-1} to 10^{-10} . Relative viral genome copy numbers were calculated based on the standard curve determined using a four-parameter logistic regression model function within MasterPlex Readerfit (version 2010) software (Hitachi Software Engineering America, Ltd., San Francisco, CA). The amount of viral RNA detected in samples was expressed as the log₁₀ (genome copy number)/ml.

Virus isolation. Virus isolations were performed using the tonsils collected on day 60 p.i., and nasal swabs were collected on days 46 to 60 p.i., which were positive for SVA by RT-qPCR. A pretreatment of the samples with 1,1,1,2,3,4,4,5,5,5-decafluoro-pentane (Vertrel, XF; Sigma-Aldrich), a solvent known to extract lipids and lipid bilayers from cells, was performed as described for trichlorotrifluoroethane (TTE) (52). After Vertrel treatment, the samples were subjected to virus isolation (three passages) in H1299 cells as previously described (14, 20).

Virus neutralization assay. Neutralizing antibodies were determined by virus neutralizing antibody assays as previously described (14, 20).

Flow cytometry. Peripheral blood leukocyte counts were determined using flow cytometry analysis. For this, whole blood from SVA-infected and control pigs was stained using a no-wash lysis and fixation method. Primary antibody concentrations were optimized by titration. Cell populations were determined using the following swine-specific antibodies (Southern Biotech, Birmingham, AL): mouse anti-pig CD3-Alexa Fluor 647 (ϵ chain specific; clone BB23-8E6-8C8, isotype IgG2a; T cells), anti-pig CD21-phycoerythrin (PE) (clone BB6-11C9.6, isotype IgG1; B cells), anti-pig monocyte/granulocyte-PE (anti-CD172a, clone 74-22-15, isotype IgG1). Monocytes and granulocytes were further differentiated by their forward scatter (FSC) and side scatter (SSC) properties. Briefly, whole-blood samples (50 μ l) were incubated with antibodies (30 μ l) for 20 min at room temperature in the dark. After incubation with antibodies, 1,520 μ l of High-Yield Lyse fixative-free buffer (Invitrogen) was added, and samples were incubated in the dark for 10 min at room temperature, as directed by the manufacturer. Single-stain controls were included in all assays. Specific cell populations in the samples were acquired using an Attune NxT flow cytometer (Thermo Fisher Scientific), and data were analyzed with FlowJo software (TreeStar, San Carlos, CA). Results are expressed as the percentage of cells of the total cells counted.

ISH. Formalin-fixed tissue sections (tonsil) were used to perform *in situ* hybridization (ISH) for SVA RNA (RNAScope). The ISH was performed as previously described (53). The ISH probe utilized was specific for the viral genome of Seneca Valley virus (nt 301 to 1345 of the VP1 gene; GenBank accession number EU271758.1) (Advanced Cell Diagnostics, Inc.) (53).

IFA. Frozen tissue sections (tonsil) were used to perform immunofluorescence assays (IFAs) for dsRNA. For this, ~6- μ m tissue cryosections were placed in positively charged glass slides, fixed with 3.7% formaldehyde solution in phosphate-buffered saline (PBS) for 30 min, and permeabilized with acetone-methanol (1:1) solution for 10 min. Slides were washed three times for 5 min with PBS and then blocked with 10% goat serum solution in PBS for 1 h in a humidified chamber. Slides were washed as described above and incubated with an anti-dsRNA monoclonal antibody (MAb) (1:200 MAb, clone J2; Scicons, Budapest, Hungary) for 4 h at RT in a humidified chamber. Slides were washed as described above and incubated with secondary anti-mouse IgG antibody (Alexa Fluor 594; Life Technologies) (1:250 in PBS–1% bovine serum albumin [BSA]). Slides were washed as described above, counterstained with 4',6'-diamidino-2-phenylindole (DAPI), mounted in using Vectashield antifade mounting medium, and visualized under a fluorescence microscope.

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