Serological and Molecular Detection of Senecavirus A Associated with an Outbreak of Swine Idiopathic Vesicular Disease and Neonatal Mortality

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Abstract
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Disciplines
Large or Food Animal and Equine Medicine

Comments

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We performed a longitudinal field study in a swine breeding herd that presented with an outbreak of vesicular disease (VD) that was associated with an increase in neonatal mortality. Initially, a USDA Foreign Animal Disease (FAD) investigation confirmed the presence of Senecavirus A (SVA) and ruled out the presence of exotic agents that produce vesicular lesions, e.g., foot-and-mouth disease virus and others. Subsequently, serum samples, tonsil swabs, and feces were collected from sows (n = 22) and their piglets (n = 33) beginning 1 week after the onset of the clinical outbreak and weekly for 6 weeks. The presence of SVA RNA was evaluated in all specimens collected by reverse transcriptase quantitative PCR (RT-qPCR) targeting a conserved region of the 5′ untranslated region (5′-UTR). The serological response (IgG) to SVA was evaluated by the weekly testing of sow and piglet serum samples on a SVA VP1 recombinant protein (rVP1) indirect enzyme-linked immunosorbent assay (ELISA). The rVP1 ELISA detected seroconversion against SVA in clinically affected and non-clinically affected sows at early stages of the outbreak as well as maternal SVA antibodies in offspring. Overall, the absence of vesicles (gross lesions) in SVA-infected animals and the variability of RT-qPCR results among specimen type demonstrate that a diagnostic algorithm based on the combination of clinical observations, RT-qPCR in multiple diagnostic specimens, and serology is essential to ensure an accurate diagnosis of SVA.

Senecavirus A (SVA), formerly Seneca Valley virus (SVV), is a nonenveloped, single-stranded, positive-sense RNA virus that belongs to the genus Senecavirus in the family Picornaviridae (1). The virus was first described as a contaminant of a PER C6 cell line (2). The virus encodes one polyprotein that is posttranslationally processed by virus-encoded proteases into 4 structural (VP1 to VP4) and 7 nonstructural (2A to 2C and 3A to 3D) proteins (2, 3). The pathogenic role of these SVA proteins is unknown, but VP1 is considered to be the most immunogenic protein in viruses of the Picornaviridae family (4, 5).

Idiopathic vesicular disease (IVD), a sporadic and transient condition affecting swine, has been reported in pigs in Australia, New Zealand, and the United States (6–8). It was not until 2007 that the presence of SVA was linked with IVD outbreaks in Canada (9). Most recently, SVA was detected in sporadic and transient outbreaks of IVD in the United States, Brazil, and China (10–13). Lesions observed in cases of IVD associated with SVA infection are characterized by vesicle formations and epidermal erosions that progress to ulcers of the coronary band, oral cavity, and nasal planum. Affected animals present transient fever and lameness. Vesicular lesions in pigs resemble those in other foreign vesicular diseases, such as foot-and-mouth disease (FMD), vesicular stomatitis (VS), swine vesicular disease (SVD), and vesicular exanthema of swine (VES). In addition, numerous reports associate the presence of SVA with increased neonatal mortality in piglets that are ≤7 days of age (10, 11).

Although SVA has been described in the U.S. swine population since the late 1980s (14), due to the rapid increase in incidence and geographic range (15), SVA can be considered an emerging infectious disease. Beginning in July 2015, there has been a rise in the number of reported cases of vesicular disease (VD) and epidemic transient neonatal losses (ETNL) associated with the presence of SVA in the United States (16). Affected breeding herds reported an increase in neonatal morbidity and mortality ranging from 30% to 70%, mainly affecting piglets that are ≤7 days of age. SVA-associated mortality in neonatal pigs is not often characterized by specific clinical signs, and no suggestive lesions are observed; however, SVA was consistently detected in multiple tissues (i.e., brain, lung, spleen, kidneys, intestine, feces, and blood) (16). Phylogenetic analyses, based on either whole-genome sequences or VP1 nucleotide sequences, have shown that contemporary SVA strains detected in the United States form a distinct cluster and are more closely related to contemporary Brazilian strains than to historical U.S. SVV strains (17).

Currently, the diagnosis of SVA is based on the detection of nucleic acid by PCR targeting VP1 or the 5′-UTR and is confirmed by virus isolation (18). Previous reports have demonstrated serological evidence of SVA by compet-

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itive enzyme-linked immunosorbent assay (ELISA) and virus neutralization (19). However, to our knowledge, there are no ELISAs available for the detection of IgG against SVA-VP1. Here, we demonstrate serological evidence of SVA infection in a breeding herd after an outbreak ofVD and ETNL that were associated with the presence of SVA.

MATERIALS AND METHODS

The study. In July 2015, a 4,000-head breed-to-wean herd located in northeast Iowa reported an acute outbreak of vesicular disease. Clinically affected sows presented vesicular lesions in the coronary bands and nostrils that last for a period of 7 to 10 days and progressed to dermal ulcers. More chronically affected animals presented areas of fibrosis and granulation tissue affecting the coronary bands and nostrils. In addition, unspecific systemic signs, such as fever and transient anorexia lasting for 48 h, were observed. In piglets, a significant increase in neonatal mortality (30% to 40%) with no specific clinical signs other than lethargy and anorexia was observed for a period of 2 weeks. Due to the resemblance of the vesicular lesions observed during this outbreak to those of other exotic vesicular diseases, all samples were submitted to the National Veterinary Service Laboratories (NVSL) for Foreign Animal Disease (FAD) investigation. Samples were negative for foot-and-mouth disease virus (FMDV), SVD, VES, vesicular stomatitis virus (VSV) serotype Indiana 1 (VSV-IND1), and VSV serotype New Jersey (VSV-NJ). The presence of SVA was confirmed by the NVSL and the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL).

One week after the onset of clinical signs, a longitudinal study was conducted in clinically affected sows (CAS) and non-clinically affected sows (NCAS) in the herd as well as their offspring beginning at 1 week of age. The objective of this longitudinal study was to assess the presence of SVA and the dynamics of the SVA-VP1 antibody response. CAS were defined as sows that presented active vesicles and/or cutaneous ulcers in the interdigital region, coronary band, and/or nostril; lameness; fever; and anorexia during clinical evaluation. NCAS were housed in the same farrowing barn, and only sows from pens contiguous to clinically affected animals were selected. Serum samples, tonsil swabs, and feces collected by rectal swabs were obtained from CAS (n = 11), NCAS (n = 11), 2 piglets (n = 22) from each CAS, and 1 piglet from each NCAS (n = 11) 1 week after the onset of the clinical outbreak and weekly thereafter for 6 weeks.

The presence of SVA RNA was evaluated in all specimens collected by the ISU-VDL. An animal was considered SVA positive if SVA RNA was detected by reverse transcriptase quantitative PCR (RT-qPCR) in at least 1 sample type at ≥1 time point. Serological confirmation of SVA exposure and dynamic SVA antibodies were evaluated using a recombinant VP1 protein (rVP1) indirect ELISA.

SVA real-time RT-PCR. Fecal and tonsillar swabs were placed into 1 or 0.5 ml phosphate-buffered saline (PBS), respectively, and agitated manually for 3 s prior to extraction. Samples (PBS containing the fecal or tonsillar material or serum) were extracted using the MagMAX viral RNA kit or Pathogen RNA/DNA kit (Life Technologies, Carlsbad, CA) and a KingFisher 96 instrument (Thermo Scientific, Waltham, MA) following manufacturer recommendations. In order to control the integrity of the PCR, 20,000 copies of Xeno RNA control (Life Technologies) were included in each reaction. The viral RNA was eluted in 90 ml of elution buffer. The primers and probe were designed to target the conserved region of the 5′ untranslated region (5′-UTR) of Senecavirus A. The forward primer sequence was 5′-AACCGCGCTGTGTTTGTAGAG-3′, the reverse primer sequence was 5′-GAACCTCGACACACACACC-3′, and the probe sequence was 5′-CCAAAGGTGTTAGCGCACCCAAACG-3′. The probe was labeled using 6-carboxyfluorescein (6-FAM) at the 5′ end and ZEN internal quencher and Iowa Black quencher at the 3′ end (Integrated DNA Technologies, Coralville, IA, USA). Real-time reverse transcriptase PCR (RT-PCR) was conducted on nucleic acid extracts using the TaqMan Fast Virus 1-Step master mix (Life Technologies, Carlsbad, CA) following manufacturer recommendations, with the addition of 1 μl per reaction of Xeno internal control reagent (Life Technologies, Carlsbad, CA) labeled with either VIC or LIZ. Real-time RT-PCR was performed on an ABI 7500 Fast instrument (Life Technologies) with the following conditions in fast mode: 1 cycle of 50°C for 5 min, 1 cycle of 95°C for 20 s, and 40 cycles of 95°C for 3 s and 60°C for 30 s. For analysis of PCR data, the baseline was set to automatic. The threshold setting was set to 0.1 for SVA and to 10% of the maximum of the curve for Xeno. In order for a negative result to be considered valid, the Xeno for the sample required a threshold cycle (Ct) value of less than 40.

Production of SVA recombinant VP1 protein. An Escherichia coli codon-optimized version of the VP1 (1,359 nucleotides [nt]) gene (GenBank number DQ641257) was synthesized in vitro (Shanghai Genery Biotech Co. Ltd., Shanghai, China). The gene was amplified using the forward primer 5′-CAT CAT CAT CAT CAT ATG TCT ACA GAT AAT GCA GAA ACG-3′ and reverse primer 5′-AGA CTG CAG GTC GAC AAG CTT TTA ACC TGA CTG CAT CAG C-3′. The PCR product was cloned into the pCold II expression plasmid using a NovoRec PCR One-Step Directed cloning kit (Novoprotein Scientific, Inc., Shanghai, China). The construction of pCold II-VP1-SVA was confirmed by sequencing (Genewiz, Inc., Suzhou, China) and then transformed into E. coli BL21(DE3) pLysS (Rosetta cells; Invitrogen, Carlsbad, CA, USA). The bacterial clone was grown in Luria-Bertani (LB) medium (Invitrogen), containing 100 μg/ml ampicillin for plasmid selection at 16°C by shaking at 250 rpm. When an A600 of 0.9 was reached, 0.1 M isopropyl-thio-β-D-galactopyranoside (IPTG) was added to induce the overexpression of VP1, and cultures were grown for an additional 16 h at 16°C. Cells were chilled at 4°C.
and harvested by centrifugation at 3,500 \( \times \) g for 15 min, resuspended in 20 mM phosphate-buffered saline (PBS) and 500 mM NaCl, pH 7.4, and lysed by ultrasonication. The crude extracts were centrifuged at 50,000 \( \times \) g for 30 min at 4°C, and fractions were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). VP1 protein (30.9 kDa) was mainly expressed in the precipitate of cell lysate as an inclusion body. The rVP1 protein (30.9 kDa) was solubilized from inclusion bodies using a denaturing buffer (20 mM Tris, 6 M guanidine-HCl, and 10 mM \( \beta \)-mercaptoethanol, pH 8.0). Then, the rVP1 protein present in the supernatant of the solubilized inclusion body was refolded in vitro into the native conformation and concentrated by dialysis against a refolding buffer (50 mM Tris, 240 mM NaCl, 10 mM KCl, 2 mM MgCl\(_2\), 0.4 M sucrose, 0.5 M arginine, 0.05% Triton X-100, and dithiothreitol, pH 8.2). The amino acid sequence (278 amino acids [aa]) identity of the purified rVP1 protein (GenBank number DQ641257) was compared to the contemporary and historical SVA strains (Fig. S1 in the supplemental material).

**VP1 indirect ELISA procedure.** Ninety-six wells of microtiter plates (MaxiSorp; Nunc, Thermo Fisher Scientific, Agawam, MA, USA) were coated with 100 \( \mu l \) of SVA rVP1 protein (0.18 \( \mu g/\mu l \)) per well in carbonate buffer (pH 8.2) and were incubated at 4°C for 16 h. After incubation, plates were washed 5 times with phosphate-buffered saline, pH 7.4, containing 0.1% Tween 20 (PBST), blocked with a 1% (wt/vol) bovine serum albumin solution (Jackson ImmunoResearch, Inc.), incubated at 25°C for 2 h, dried at 37°C for 3 h, and stored at 4°C in a sealed bag with desiccant packs.

Serum samples were diluted 1:50, and VP1-coated plates were loaded with a 100-\( \mu l \) sample per well for ELISA testing. Antibody-positive and -negative controls were run in duplicate on each ELISA plate. Samples were incubated at 37°C for 1 h and then washed 5 times with PBST, followed by 100 \( \mu l \) of a 1:20,000 dilution of peroxidase-conjugated goat anti-pig IgG (Fc) antibody (Bethyl Laboratories, Inc., Montgomery, TX, USA) incubated at 37°C for 1 h. The peroxidase reaction was visualized by adding 100 \( \mu l \) of tetramethylbenzidine-hydrogen peroxide (TMB) substrate solution (SurModics IVD, Inc., Eden Prairie, MN, USA) per well and incubated at room temperature for 5 min. Reactions were stopped with 100 \( \mu l \) of stop solution per well (SurModics IVD, Inc.) and measured for optical density (OD) at 450 nm using an ELISA plate reader (BioTek Instruments, Inc., Winooski, VT) operated with commercial software (Gen5; Biotek Instruments, Inc.).

Serum antibody responses was expressed as sample-to-positive (S/P) ratio: S/P ratio = (sample OD – negative-control mean OD)/(positive-control mean OD – negative-control mean OD).

The optimal cutoff value of the SVA rVP1 IgG ELISA was estimated using 480 presumed antibody-negative serum samples collected during the summer of 2014 from a commercial farm with no history of IVD.
Serological and Molecular Detection of SVA

Serum samples were collected from 20 pigs in 3 barns starting at ~6 weeks of age and weekly thereafter for a period of 8 weeks. Test results on serum samples (n = 72) from SVA CAS and NCAS collected from weeks 3 to 6 after the onset of the clinical outbreak were used as antibody-positive samples.

Data analysis. The proportions of RT-qPCR-positive animals and 95% confidence intervals (CIs) were calculated. Pearson’s chi-square test was used to detect differences in the proportions of RT-qPCR-positive results among CAS, NCAS, and their offspring by specimen, i.e., tonsil, serum, and feces. The Fisher exact test was used when 25% of the cells in a contingency table contained <5 counts (SAS 9.4). One-way analysis of variance (ANOVA) with Tukey’s correction was used for multiple comparisons with an α of 0.05 (GraphPad Prism 6).

The SVA rVP1 IgG ELISA cutoffs were evaluated by receiver operator characteristic (ROC) analysis and diagnostic sensitivity and specificity estimated for various S/P thresholds (SAS version 9.4; SAS Institute, Inc., Cary, NC). The selected cutoff was used to calculate the percentage of positive animals and 95% CIs for groups (CAS and NCAS) over time.

RESULTS

Detection of SVA RNA by RT-qPCR. The proportion of positive animals according to serum samples and the proportion of animals positive for SVA in tonsil swabs and feces varied over the 6 weeks after the appearance of clinical signs (Fig. 1). The overall proportion of positive sows (50.75% [95% CI, 42.2% to 59.3%]) detected by RT-qPCR was greater than that of their offspring (28.12% [95% CI, 21.8% to 34.5%]). However, there were no significant differences (P > 0.05) in the proportion of RT-qPCR-positive animals among specimens evaluated (Fig. 1) or clinical status (CAS versus NCAS) (Table 1).

SVA RNA was detected in all specimen types from CAS, NCAS, and their offspring by RT-qPCR (Fig. 2). A total of 290 samples from sow and 406 samples from piglets were evaluated. The overall detection rates among samples were 27.9% (95% CI, 22.7% to 33.09%) in sows and 22.3% (95% CI, 18.53% to 26.09%) in piglets. The overall proportions of positive samples by RT-qPCR were significantly variable (P > 0.05) among the three specimens evaluated in sows (Fig. 2A). However, no significant differences (P < 0.05) in the proportion of positive samples among the specimens evaluated were observed in piglets (Fig. 2B).

Diagnostic performance of the rVP1 indirect ELISA. Based on an ROC analysis of the SVA rVP1 indirect ELISA, the optimum

| Sow ID
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</tbody>
</table>

Detection rate (no. positive/total no. [%])

95% CI (%) 0–41.0 25.1–83.9 52.2–100 100–100 100–100 100–100

Overall detection rate (no. positive/total no. [%])

95% CI (%) 0–27.9 36.2–78.5 46.4–99.0 46.4–99.0 59.0–100 59.0–100

95% CI (%) 0–26.1 26.4–90.7 46.4–99.0 46.4–99.0 59.0–100 59.0–100

95% CI (%) 0–27.9 36.2–78.5 58.0–94.4 70.7–100 77.8–100 77.8–100

α, ID, identification number.
β, −, negative result; +, positive for SVA-VP1 IgG antibody; N/A, not collected or not available.
† Week 1 was defined as the first week of collection.
S/P cutoff was determined to be S/P = 0.3, which provided a diagnostic sensitivity and specificity of 93% and 99%, respectively. At the selected cutoff, the rVP1 indirect ELISA detected SVA-VP1 IgG antibodies the first week after the appearance of clinical signs (13.6% [95% CI, 0.0% to 27.9%]), regardless of SVA clinical status (Table 2). Once the rVP1 indirect ELISA detected a positive animal, the test showed consistency in SVA-VP1 IgG antibody detection throughout the rest of the study (Table 2). The rVP1 IgG ELISA detected antibodies in 100% (10/10) of CAS at weeks 4 to 6 after the onset of the clinical outbreak, whereas in NCAS the detection rates were 72.7% (8/11) at weeks 3 and 4 and 81.8% (9/11) at weeks 5 and 6. However, no significant difference in detection rate between CAS versus NCAS was observed (P > 0.05). Interestingly, SVA-VP1 IgG antibodies were not detected in 18.2% of NCAS (2/11) by the rVP1 indirect ELISA throughout the study (Table 2).

The declines in SVA-VP1 IgG antibodies in neonatal pigs from CAS and NCAS are illustrated in Fig. 3B and Table 3, respectively. In 42.8% (9/21) and 63.6% (7/11) of piglets from CAS and NCAS, respectively, SVA antibody levels were greater than the cutoff (S/P ≥ 0.3) during the first week after the onset of the clinical outbreak. SVA-VP1 IgG antibodies were undetectable by rVP1 indirect ELISA by week 5 in piglets from CAS. The same serological profile was found in piglets from NCAS, with the exception of 1 piglet that remained positive throughout the study. The distribution of cumulative SVA antibody ELISA S/P ratios in serum samples from SVA-negative pigs versus serum samples from SVA-positive sows and their offspring is presented in Fig. 4.

**SVA antibody response of rVP1 indirect ELISA.** During the second and third week after the onset of clinical signs, there was a significant increase in SVA-VP1 IgG levels (P ≤ 0.05), reaching a plateau that lasted until the end of the observational period (Fig. 3A). No differences in SVA-VP1 IgG S/P ratio antibody response or proportion of seropositive animals were observed over time regardless of the clinical status of the sows (CAS versus NCAS).

Piglets born to CAS and NCAS showed a reduction in the pattern of SVA-VP1 IgG levels during the evaluated period. The SVA-VP1 IgG levels in serum were significantly higher during the first and second week after the onset of the clinical outbreak compared with the levels observed between weeks 4 and 6 (P ≤ 0.05) after clinical onset. Further analysis showed that there were no significant differences over time in SVA-VP1 IgG S/P ratios between piglets from CAS or NCAS.

**DISCUSSION**

In July 2015, we initiated a longitudinal field study in a swine breeding herd farm that presented with an outbreak of IVD associated with an increase in neonatal mortality. The presence of SVA was initially confirmed and systematically identified over time in different specimens from CAS and NCAS and their offspring. In addition, the presence of exotic vesicular disease etiologies was ruled out.

During this investigation, we observed that clinical signs and vesicular lesions were highly transient, i.e., their diagnostic value decreased rapidly over time. In contrast, SVA RNA and/or antibody were detected in 100% of affected animals, regardless of their clinical status, throughout the study. Notably, the SVA-VP1 IgG antibody levels in sows showed a significant increase during the first 3 weeks after the appearance of vesicular lesions, which suggested a causal role for SVA infection. The presence of SVA RNA was corroborated in different specimens (i.e., serum, tonsil, and feces) and at different time points during the study. However, SVA RNA detection was inconsistent in sows, suggesting variation in the pattern of shedding and/or the amount of virus available in each specimen type. There is no available information regarding the duration of SVA viremia or viral shedding in natural or experimental conditions. However, we observed that the proportion of positive animals in serum reached undetectable levels 3 weeks after the onset of the clinical outbreak (Fig. 1). This finding is in line with the time point when the highest antibody levels were detected (Fig. 3). Although there is no information available about SVA-VP1-neutralizing properties, this might suggest that viremia was reduced by the presence of VP1 neutralizing antibodies.

The neonatal mortality rate reported in this study was similar to that previously reported in 4- to 7-day-old piglets (10). The pattern of declining SVA-VP1 IgG detected in neonatal piglets by rVP1 ELISA was consistent with a passive antibody profile. However, SVA in serum was detected in 36.4% (12/33) of piglets by RT-qPCR between weeks 1 and 3 in postclinical outbreaks (Fig. 1), while rVP1 IgG ELISA did not demonstrate active immune response (Fig. 3B). Among positive piglets in serum (n = 12), only 1 piglet had detectable levels of SVA-VP1 IgG maternal antibodies.
During the first 4 weeks, viremia in the absence of detectable levels of SVA-VP1 IgG antibodies might reflect their role in reducing infection or neutralizing activity. Nevertheless, the potential neutralizing activity of VP1 antibodies against SVA has not been described. FMDV VP1 elicited a neutralizing antibody that induced serogroup-specific immunity (20). In addition, the roles of nonstructural and structural proteins other than SVA-VP1 as well as the differences in the reactivity between linear and conformational epitopes of VP1 need to be investigated further.

VP1 has been described as the most immunogenic capsid protein among the members of the *Picornaviridae* family, e.g., FMDV (4, 21). The amino acid sequence of this protein is highly conserved among the SVA strains reported, varying from 95.8% to 100% percentage identity with the contemporary SVA strains. Conversely, the SVA-VP1 amino acid sequence presents /H11002/ identity with other members of the *Picornaviridae* family (e.g., Cardiovirus). It has been reported that VP1 elicits an antibody response that allows us to differentiate it from other picornaviruses (20); however, this information needs to be evaluated further for SVA. The rVP1 ELISA developed in this study detected seroconversion in sows at early stages of SVA outbreak, regardless

### TABLE 3 Detection of Senecavirus A VP1 IgG antibodies by rVP1-ELISA collected weekly after onset of clinical outbreak from piglets born from clinically affected sows and nonclinically affected sows

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<tr>
<td>50952</td>
<td>156</td>
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Detection rate (no. positive/total no. [%])

<table>
<thead>
<tr>
<th>Detection rate (no. positive/total no. [%])</th>
<th>8/21 (38.1)</th>
<th>6/21 (28.6)</th>
<th>4/21 (19.0)</th>
<th>4/20 (20.0)</th>
<th>0/21 (0)</th>
<th>0/21 (0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>95% CI (%)</td>
<td>17.3–58.9</td>
<td>9.3–47.9</td>
<td>2.2–35.8</td>
<td>2.5–37.5</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

**a** Negative result; **+**, positive for SVA-VP1 IgG antibody; N/A, not collected or not available.

**b** Week 1 was defined as the first week of collection.
of their clinical status. Overall, based on the clinical evidence in the present study, it can be concluded that SVA induces an early immune response in SVA-positive sows under field conditions. Here, we evaluated the time of detection after the onset of the clinical outbreak; however, questions regarding the duration of the antibody response remain unanswered. It can also be concluded that seropositive sows can transfer maternal antibodies to their offspring, but their protective role in neonatal pigs is still unknown.

There is a growing awareness that SVA vesicular lesions resemble those of other vesicular foreign animal diseases, i.e., FMDV, SVD, VES, and VSV. The transience and variability of clinical signs and the inconsistency of PCR results among specimens observed in sows demonstrated that serological confirmation is essential to ensure an accurate diagnosis of SVA. A diagnostic algorithm based on a combination of clinical observations, PCR in multiple specimens, and antibody detection methods would be more effective than a single diagnostic method.

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