RESEARCH REPORT
SWINE HEALTH

Title: Development and evaluation of antibody detection methods in oral fluids for cost-effective and efficient surveillance and control of Senecavirus in swine population. **NPB#18-099**

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Industry Summary:
Senecavirus A, also known as Seneca Valley Virus, has been identified in the U.S. swine population since the late 1980s and has most commonly been associated with "Swine Vesicular Disease." In 2015, a reemergence of clinical cases associated with SVA was first reported in the US and Brazil. Since its reemergence, outbreaks of SVA have been reported in multiple swine-producing countries, with cases appearing in new locations each year. Thus, SVA appears to be on the path to becoming a virus with global prevalence. One of the most concerning problems for practitioners besides the presence of transient vesicular lesions that can impair final production goals is arriving at an accurate clinical diagnosis. Lesions induced by SVA cannot be clinically differentiated from other vesicular diseases such as foot-and-mouth disease (FMD), swine vesicular disease (SVD), swine vesicular exanthema (SVE) and vesicular stomatitis virus (VSV). Today, SVA diagnosis is mostly based on direct pathogen detection. However, lesions and viremia are transient, which might preclude etiological diagnosis. Thus, detection of antibodies against SVA should be considered an important tool for making the diagnosis of virus contact/circulation in the farm.

The objective of this study was to evaluate potential epitopes that can be used to improve the current serological assay and develop a new antibody assay that allows determination of specific antibodies on oral fluids.

Multiple overlapping epitopes from two structural proteins were synthetically constructed. The immune reactivity of each epitope was evaluated against monoclonal antibodies and clinical samples collected during an outbreak of Senecavirus swine vesicular disease. In addition, in order to evaluate the presence of specific antibodies in oral fluids, a total of 30 pigs were randomly housed in three different rooms and allocated in pens containing two pigs per pen. One group

(n=10) was inoculated with SVV historical strain, one group with a contemporary SVA strain (n=10) and one control (n=10) with cell culture media. Oral fluids and serum were collected at 0, 3, 7, 10, 14, 21, 28, 35, 42, 49, 54, 60 and 67 dpi. The presence of different antibodies isotypes was evaluated in serum and oral fluids by a recombinant protein ELISA.

Results of this study showed that the humoral response could be detected using a small set of epitopes targeting small regions of two main structural proteins. This small set of epitopes used as a target antigen in the indirect ELISA format were capable of discriminating not only positive samples generated experimentally, but also clinical samples collected during an outbreak of vesicular disease. Although further studies are necessary to identify if these specific regions of the structural proteins play a major role in the induction of immune response, these linear epitopes will provide the basis to improve and develop more specific immunological tests.
In this study, specific antibodies against two different strains of Seneca virus were detected in serum one week post-infection by a recombinant protein ELISA. The same immunological test was adapted for oral fluids and demonstrated that specific SVA antibodies can also be detected after seven days post-infection. Interestingly, this test was also capable of identifying antibodies generated by two different SVA strains. Thus, oral fluid has the potential to replace individual pig serum samples for SVA routine and emergency disease surveillance. This is a faster and less expensive method to evaluate pathogen circulation in a farm, which can accurately reflect the prevalence of SVA in the population.

Keywords: Senecavirus, Oral fluids, ELISA, Seroconversion, Structural proteins

Scientific Abstract:

Senecavirus A (SVA) is an RNA virus in the family Picornaviridae. The virus has recently been detected in swine production systems, causing vesicular disease and neonate mortality. The viral capsid is composed of four structural proteins: VP1-VP4. Although the VP1 protein has been reported as the most immunogenic protein *in vivo*, no information on immunodominant regions of the SVA polyprotein is available. Besides the large amount of information generated regarding the humoral immune response and different tests reported to detect antibodies in serum, there is no information available regarding the antibody response in oral fluids.
The objectives of this study include **1)** identifying immunodominant regions of SVA polyprotein using epitope mapping, **2)** developing and validating SVA oral fluid ELISA in known status samples, **3)** determining the dynamic of immunoglobulins isotype in oral fluid during SVA active infection, and **4)** evaluating performance of assays.
The binding effect of a SVA polyclonal antibody (SVA-pAb), a set of SVA-VP1 monoclonal antibodies (mAb), and SVA positive sera from clinically affected animals was characterized using a set of SVA-VP1- and SVA-VP2-derived peptides by indirect and blocking ELISAs. Structural proteins' epitope array was based on 18 SVA-VP1 and 31 SVA-VP2 peptides, overlapping 20-mer and shifted by five amino acids. All VP1-derived peptides yielded significant signal against SVA-pAb and VP1-mAb by indirect ELISA. One peptide (aa 1-20) showed significantly higher OD on rVP1 and SVA whole virus indirect ELISAs. Peptides spanning aa 1-20, 60-80, 105-125 and 120-140 showed a significant half-maximal inhibitory concentration (IC50) on VP1-mAb against rVP1 by blocking ELISA. The VP2 epitope array was capable of detecting significantly higher signal from five overlapping amino acid residues (peptides 8, 9, 10, 11, and 12) spanning amino acids 105-185 and two non-overlapping amino acid residues (peptides 27 and 29) spanning

amino acids 390-410 and 420-440. Although several peptides showed variable binding inhibitory effects of SVA-pAb and SVA-positive clinical samples, none of the peptides reached a 50% inhibitory effect. These results suggest that the humoral immune response against SVA-VP1 and SVA-VP2 can be defined by a set of linear epitopes.
The presence of specific antibodies in oral fluids was evaluated in experimentally infected animals. Thirty pigs were randomly allocated to three different treatment groups and housed in pens, with two pigs per pen. Each pig received 5 mL 1 x 10^9 TCDI50/ml of historical SVV-1 strain (SVV group; n=10), SVA contemporary strain (SVA group; n=10) or RMPI media (Negative control group; n=10). Oral fluids and serum were collected at 0, 3, 7, 10, 14, 21, 28, 35, 42, 49, 54, 60 and 67 dpi. The presence of different antibody isotypes was evaluated in serum and oral fluids by a VP1 recombinant protein ELISA. Although animals inoculated with SVA showed a moderate IgG response in serum that lasted four weeks, no significant increment on IgG levels on oral fluids was observed. The presence of IgA was observed by the SVA-VP1 IgA ELISA in both inoculated groups after 7 dpi. The presence of specific Senecavirus antibodies was detected and sustained throughout the duration of the study. Although there were significant differences in S/P detected between both strains used for this study on specific time points (42 dpi), the test was able to discriminate positive groups. ROC analysis was performed for both Ig isotypes among animals infected with both Senecavirus strains. For IgG, an S/P value of 0.17 showed a sensitivity of 39% and specificity of 93%, while for IgA, an S/P value of 0.25 showed a sensitivity of 77% and specificity of 93%.
Based on these findings, it can be concluded that SVA immunological response could be driven by a small set of linear epitopes in the VP1 and P2 structural proteins. These immunodominant epitopes could be used as a target for immunological diagnostic assays. The immune response against Senecavirus infection can be detected in oral fluids and is largely IgA isotype based. This result supports the idea that oral fluid has the potential to replace individual pig serum samples for both SVA routine and emergency disease surveillance.

Keywords: Senecavirus, Oral fluids, ELISA, Seroconversion, Structural proteins

Introduction:

Senecavirus A (SVA) is a non-enveloped, single-stranded RNA virus of the genus Senecavirus in the family Picornaviridae (Adams et al., 2015). The virus possesses four structural proteins (VP1-4) that compose an icosahedral capsid and seven non-structural proteins (Hales et al., 2008). SVA was originally associated with swine idiopathic vesicular disease (IVD) in Canada and the United States (Hales et al., 2008; Pasma et al., 2008). Although the presence of SVA has been described in the U.S. swine population for over 30 years (Knowles et al., 2006), since 2015, several outbreaks of IVD and epidemic transient neonatal losses (ETNL) associated with the presence of SVA have been reported in the United States (Piñeyro et al., 2015; Canning et al., 2016). In addition, numerous countries including Brazil (Leme et al., 2015; Vannucci et al., 2015a), China (Wang et al., 2017; Wu et al., 2016a), Colombia (Sun et al., 2017) and Thailand (Saeng-Chuto et al., 2017) have reported an increment in the incidence of IVD cases associated with the presence of SVA. A phylogenetic analysis of the contemporary SVA isolated in the United States, Brazil, China, Thailand and Colombia shows small genomic differences compared with historical SVV strains (Zhang et al., 2015; Vannucci et al., 2015b; Wu et al., 2016b).

The diagnosis of the diseases is based on the presence of clinical signs, detection of the virus and presence of antibodies against SVA. Outbreaks of SVA IVD are characterized by abrupt onset of

vesicles that progress to ulcerative dermatitis on the nostril and coronary bands that affects a high percentage of sows or finisher pigs (Guo et al., 2016; Canning et al., 2016). Lesions associated with SVA are clinically indistinguishable from those observed in foot-and-mouth disease (FMD), vesicular stomatitis (VS), swine vesicular disease (SVD) and vesicular exanthema of swine (VES). Etiological diagnosis can be achieved by qRT-PCR, virus isolation from vesicular material (Fowler et al., 2017; Joshi et al., 2016b), *in situ* hybridization (Joshi et al., 2016a; Resende et al., 2017) and immunohistochemistry (Leme et al., 2016). Available antibody detection methods include indirect immunofluorescence assay (IFA), virus neutralization assays (VN), and competitive enzyme-linked immunosorbent assay (cELISAs) and indirect ELISAs targeting different structural proteins (VP1, VP2, VP3) (Goolia et al., 2017; Dvorak et al., 2017; Gimenez-Lirola et al., 2016; Joshi et al., 2016b; Montiel et al., 2016). However, it has been demonstrated that animals can shed the virus and present SVA-specific IgG without evidence of clinical disease, suggesting that the virus may be circulating subclinically (Gimenez-Lirola et al., 2016).

It has been demonstrated clinically and experimentally that SVA infection induces antibodies that can last for at least 6 weeks post-infection, and the dynamic of antibodies detected during the infection might vary based on the target of the serological test (Gimenez-Lirola et al., 2016; Joshi et al., 2016a; Dvorak et al., 2017). Thus, the presence of IgG against VP1 protein can be observed as early as 7-10 days post-infection (dpi) (Dvorak et al., 2017; Gimenez-Lirola et al., 2016), while response against VP2 seems to appear earlier (approximately 5 dpi) and can be detected for approximately 60 dpi (Dvorak et al., 2017). The IgG response against VP3 has been demonstrated to be weak, and lasts approximately only two weeks post-infection (Dvorak et al., 2017). The detection of SVA IgG by IFA has been reported from 10 to over 40 dpi (Joshi et al., 2016b; Montiel et al., 2016; Goolia et al., 2017). The neutralizing antibodies also seem to appear early during infection and last for approximately 7 weeks post-infection (Joshi et al., 2016b; Goolia et al., 2017).

Oral fluids are routinely collected in commercial production settings and accurately reflect the prevalence of different pathogens in the population (Ramirez et al., 2012). The veterinary diagnostic laboratories at the University of Minnesota, Iowa State University and South Dakota State University ran a cumulative total of 334,083 diagnostic tests on swine oral fluids in 2016. The data have shown that oral fluids are both more diagnostically sensitive and economical than serum for the detection of pathogens at the herd level (Kittawornrat et al., 2014; Olsen et al., 2013; Panyasing et al., 2014). Thus, oral fluid has the potential to replace individual pig serum samples for both routine and emergency disease surveillance.

Besides the large amount of information generated regarding the serological immune response and different tests reported to detect antibodies in serum, there is no information available regarding the antibody response in oral fluids, and no validated test to detect SVA antibodies in oral fluids has been described. Development of SVA antibody detection methods in oral fluid will not only help to further understand the immune response against SVA, but also will provide an inexpensive, highly accessible, and high-throughput-compatible testing tool much needed for SVA surveillance. In addition, an oral fluid antibody detection method will make surveillance more affordable and will allow for evaluation of large populations without the burden of individual sampling (i.e., serum collection), reducing time, labor and supplies necessary for serological evaluation.

Objectives

Objective 1: Development and validation of SVA oral fluid ELISA in known status samples
We will validate our current SVA rVP1 ELISA for determination of the antibody response in oral fluid. In addition, we will evaluate other SVA immunological targets. We have explored experimentally the use of a set of specific peptides within VP1 and VP2 proteins. The use of these peptides will allow for a better discrimination of the antibodies generated within acute SVA infection.

Objective 2: Determine the dynamic of immunoglobulins isotype in oral fluid during SVA active infection
Besides the large amount of information generated regarding the serological immune response and different tests reported to detect antibodies in serum, there is no information available regarding the antibody response in oral fluids. However, we have reported differences in oral fluid antibody isotype responses for different pathogens. Therefore, oral fluid immunoglobulin isotype-specific kinetics (IgG, IgA and IgM) will be evaluated during the optimization process to identify the isotype that gives the best assay performance.

Objective 3: Assays performance and evaluation
The assay sensibility and specificity of the SVA oral fluid ELISA will be calculated based on samples of known status and a set of negative samples obtained from the ISU-VDL. The diagnostic performance will be calculated using receiver operating characteristic (ROC) curve analysis.

Material and methods

Generation of 1) Potential antigenic targets for ELISA development, 2) specific polyclonal and monoclonal antibodies against Senecavirus A (SVA), and 3) oral fluid indirect ELISAs based on a recombinant VP1

1.1 *Development of targets for SVA ELISA antibody detection*: The first step of this project is to generate targets for the detection of SVA antibodies in oral fluids. We will evaluate the performance of a recombinant VP1 protein for the detection of SVA antibodies in oral fluids. This protein was generated before this study and tested for the detection of SVA IgG in serum in indirect ELISA format by our group (Gimenez-Lirola, L. G. et al.). In short, production of SVA recombinant VP1 protein was achieved by the Escherichia coli codon-optimized version of the VP1 (1,359 nucleotides [nt]) gene (GenBank number DQ641257) by *in vitro* synthesis (Shanghai Genery Biotech Co. Ltd., Shanghai, China). The gene was amplified using the forward primer 5=-CATCATCATCATCATCATATGTCTACAGATAATGCA GAAACG-3= and reverse primer 5=-AGACTGCAGGTCGACAAGCTT TTA ACC TGA CTG CAT CAG CAT 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 g/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-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 X 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 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-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 (50mMTris, 240mMNaCl, 10mMKCl, 2mMMgCl2, 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).

Eighteen overlapping 20-mer peptides from VP1 and 31 overlapping 20-mer peptides from VP2 shifted by five amino acids, spanning the complete SVA-VP1 and SVA-VP2 amino acid sequence, were synthesized (Novoprotein Scientific INC., Summit, NJ) and conjugated to keyhole limpet hemocyanin (KLH) at the N-terminus and hydroxyl at the C-terminus (Table 1 and Table 2). Reactivity of synthetic peptides was measured by two SVA-pAbs and three VP1-mAbs.

Name of peptide	Sequence of peptide	Position of amino acid	Length (aa)
SVA-VP1-PEP1	STDNAETGVIEAGNTDTDFS	1-20	20
SVA-VP1-PEP 2	DTDFSGELAAPGSNHTNVKF	15-35	20
SVA-VP1-PEP 3	TNVKFLFDRSRLLNVIKVLE	30-50	20
SVA-VP1-PEP 4	IKVLEKDAVFPRPFPTQEGA	45-65	20
SVA-VP1-PEP 5	TQEGAQQDDGYFCLLTPRPT	60-80	20
SVA-VP1-PEP 6	TPRPTVASRPATRFGLYANP	75-95	20
SVA-VP1-PEP 7	LYANPSGSGVLANTSLDFNF	90-110	20
SVA-VP1-PEP 8	LDFNFYSLACFTYFRSDLEV	105-125	20
SVA-VP1-PEP 9	SDLEVTVVSLEPDLEFAVGW	120-140	20
SVA-VP1-PEP 10	FAVGWFPSGSEYQASSFVYD	135-155	20
SVA-VP1-PEP 11	SFVYDQLHVPFHFTGRTPRA	150-170	20
SVA-VP1-PEP 12	RTPRAFASKGGKVSFVLPWN	165-185	20
SVA-VP1-PEP 13	VLPWNSVSSVLPVRWGGASK	180-200	20
SVA-VP1-PEP 14	GGASKLSSATRGLPAHADWG	195-215	20
SVA-VP1-PEP 15	HADWGTIYAFVPRPNEKKST	210-230	20
SVA-VP1-PEP 16	EKKSTAVKHVAVYIRYKNAR	225-245	20
SVA-VP1-PEP 17	YKNARAWCPSMLPFRSYKQK	240-260	20
SVA-VP1-PEP 18	SYKQKMLMQ	255-264	9

Table 1: VP1 epitope regions. Eighteen peptides spanning the length of SVA-rVP1 were generated. Peptides were 20 amino acids in length and overlapping by five peptides. Peptide purity was at 85%, and all peptides except SVA-VP1-PEP5, 8, and 17 were optimized through addition of cysteine.

Name of peptide	Sequence of peptide	Position of amino acid	Length (aa)
SVA-VP2-PEP1	SGDVETNPGPASDNPILEFL	1-20	20
SVA-VP2-PEP 2	ILEFLEAENDLVTLASLWKM	15-35	20
SVA-VP2-PEP 3	SLWKMVHSVQQTWRKYVKND	30-50	20

SVA-VP2-PEP 4	YVKNDNFWPNLLSELVGEGS	45-65	20
SVA-VP2-PEP 5	VGEGSIALAATLSNQASVKA	60-80	20
SVA-VP2-PEP 6	ASVKALLGLHFLSRGLNYTD	75-95	20
SVA-VP2-PEP 7	LNYTDFYSLLIEKCSSFFTV	90-110	20
SVA-VP2-PEP 8	SFFTVEPPPPPAENLMTKPS	105-125	20
SVA-VP2-PEP 9	MTKPSVKSKFRKLFKMQGPM	120-140	20
SVA-VP2-PEP 10	MQGPMDTVKDWNQIAAGLKN	135-155	20
SVA-VP2-PEP 11	AGLKNFQFVRDLVKEVVDWL	150-170	20
SVA-VP2-PEP 12	VVDWLQAWINKEKASPVLQY	165-185	20
SVA-VP2-PEP 13	PVLQYQLEMKKLGPVALAHD	180-200	20
SVA-VP2-PEP 14	ALAHDAFMAGSGPPLGDDQI	195-215	20
SVA-VP2-PEP 15	GDDQIEYLQNLKSLALTLGK	210-230	20
SVA-VP2-PEP 16	LTLGKTNLAQSLTTMINAKQ	225-245	20
SVA-VP2-PEP 17	INAKQSSAQRVEPVVVVLRG	240-260	20
SVA-VP2-PEP 18	VVLRGKPGCGKSLASTLIAQ	255-275	20
SVA-VP2-PEP 19	TLIAQAVSKRLYGSQSVYSL	270-290	20
SVA-VP2-PEP 20	SVYSLSVYSLPPDPDFFDGY	285-305	20
SVA-VP2-PEP 21	FFDGYKGQFVTLMDDLGQNP	300-320	20
SVA-VP2-PEP 22	LGQNPDGQDFSTFCQMVSTA	315-335	20
SVA-VP2-PEP 23	MVSTAQFLPNMADLAEKGRP	330-350	20
SVA-VP2-PEP 24	EKGRPFTSNLIIATTNLPHF	345-365	20
SVA-VP2-PEP 25	NLPHFSPVTIADPSAVSRRI	360-380	20
SVA-VP2-PEP 26	VSRRINYDLTLEVSEAYKKH	375-395	20
SVA-VP2-PEP 27	AYKKHTRLNFDLAFRRTDAP	390-410	20
SVA-VP2-PEP 28	RTDAPPIYPFAAHVPFVDVA	405-425	20
SVA-VP2-PEP 29	FVDVAVRFKNGHQSFNLLEL	420-440	20
SVA-VP2-PEP 30	NLLELVDSICADIRAKQQGAR	435-455	20
SVA-VP2-PEP 31	NMQTLVLQ	450-458	8

Table 2: VP2 epitope regions. Thirty-one peptides spanning the length of SVA-rVP2 were generated. Peptides were 20 amino acids in length and overlapping by five peptides. Peptide purity was at 85%, and all peptides were optimized through addition of cysteine.

1.2 Production and characterization of polyclonal (pAb) and monoclonal (mAb) antibodies against SVA: Polyclonal antibodies (pAbs) were generated by inoculation of mice with inactivated SVA. A total of 1 x 10mL of $1x10^6$ TCID50/mL of passage one SVA was treated with 5% formalin and ultracentrifugated for two hours at 35,000 rpm. The viral pellet was resuspended in 400 µL of phosphate-buffered saline (PBS), and an aliquot of 50 µL was mixed with incomplete Freund's adjuvant at a 1:1 volume ratio. This was injected intraperitoneally into BALB/c mice, followed by a booster injection two weeks after primary inoculation. Blood was harvested and the presence of SVA antibodies evaluated weekly by SVA-rVP1 ELISA. When the SVA-rVP1 antibody titer reached a maximum concentration, mice were treated intraperitoneally with Pristane (Sigma-Aldrich, St. Louis, MO) and three days later given an intraperitoneal injection of SP2/O cells

(approximately 5 to 8 x 10^6 cells) to produce polyclonal fluid. After 10 days, the two mice were euthanized. Ascites fluid was collected and stored at -20ºC.

Monoclonal antibodies (mAbs) against the recombinant SVA-VP1 (SVA-rVP1) protein were generated by GenScript (Piscataway, NJ, USA). Five six-week-old BALB/c mice were injected subcutaneously with 0.1 mg of SVA-rVP1 protein using the MonoExpress immunization technique, twice at two-week intervals. Spleen cells were gathered and fused with SP2/0 myeloma cells. Hybridoma supernatants of parental clones were suspended in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS, 1x HT supplement, 1x penicillin streptomycin, and 0.02% sodium azide. A total of 20 hybridomas were screened using indirect SVA-rVP1 ELISA, and three hybridomas were selected for mAb production and purification: 4D10, 8F12 and 10C2.

1.3 *Development of an oral fluid indirect ELISAs based on a recombinant VP1 protein:*

Ninety-six wells of microtiter plates (MaxiSorp; Nunc, Thermo Fisher Scientific, Agawam, MA, USA) were coated with 100µl of SVA rVP1 protein (0.18 µg/ml) per well in carbonate buffer (pH 8.2) and 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. Oral fluid samples were tested undiluted, and VP1-coated plates were loaded with a 200 µl (IgG testing) or 100 µl (IgM testing) 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 2 h (IgG) or 1h (IgA) and then washed 5 times with PBST, followed by 100 µl of a 1:1,500 dilution of peroxidase-conjugated goat anti-pig IgG (Fc) or 1:3,000 goat anti-pig IgA (Bethyl Laboratories, Inc., Montgomery, TX, USA) incubated at 37°C for 1 h (IgG testing) or 30 min (IgA testing). The peroxidase reaction was visualized by adding 100 µl 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 µl 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 were expressed as sample-to-positive (S/P) ratios: S/P ratio = (sample OD-negative-control mean OD)/(positive control mean OD- negative-control mean OD).

Oral fluid collection and confirmation of humoral status in Senecavirus experimentally infected pigs.

A total of 30 pigs (>5 weeks old) were acquired from an SVA-free farm. SVA-negative status was confirmed by the absence of antibodies by FFN, IFA and ELISA and of viral circulation by PCR (rRT-PCR) before their arrival to the ISU-animal facilities 3 days before inoculation. At DPI 0, 10 pigs per group were challenged intranasal, either with 5 mL 1 x 10^9 TCDI50/ml of historical SVV-1 strain (SVV group), SVA contemporary strain (SVA group) or RMPI media (Negative control group). All animals were allocated to pens holding two animals. Oral fluid and serum were collected at 0, 3, 7, 10, 14, 21, 28, 35, 42, 49, 54, 60 and 67 dpi.

Table 3:

Inoculation groups	Number of pigs	Total serum samples	Total oral fluid samples
SVA group	10	130	402
SVV group	10	130	402
Negative control	10	130	335

Evaluation of the assay performance.

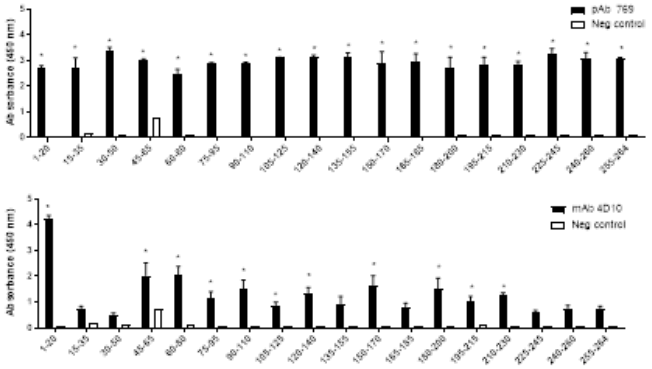
The SVA rVP1 IgG and IgM ELISA cutoffs were evaluated by receiver operator characteristic (ROC) analysis and diagnostic sensitivity and specificity estimated for various S/P thresholds (GraphPad Prism 8).

Results

1. Development and validation of SVA oral fluid ELISA in known status samples

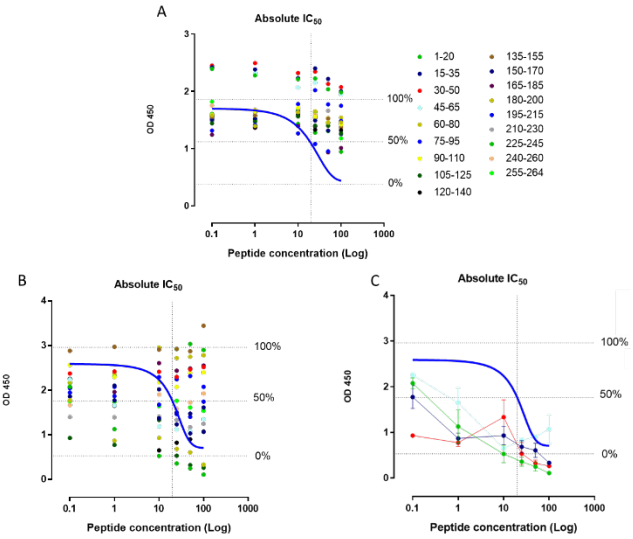
1.1. SVA VP1-mAb but not SVA pAb show differential binding activity against SVA-VP1 epitopes by indirect ELISA epitope mapping

To localize linear B-cell epitopes of SVA VP1 involved in SVA immunogenicity, the binding activity of SVA pAb and mAb was evaluated against sequential, overlapping peptides of the SVA-rVP1 protein by indirect peptide ELISA. SVA pAb showed significant reactivity against all peptides spanning the entire SVA VP1 protein compared to a negative control. In addition, no significant differences in intensity were observed among peptides by SVA pAb detection (Figure 1A). However, SVA mAb was capable of detecting significantly higher signals on only four non-overlapping amino acid residues spanning amino acids 1-20, 45-140, 150-170 and 180-230. The peptide spanning amino acid 1-20 showed a particularly high and significant reactivity compared to the peptides spanning amino acids 45-140, 150-170 and 180-230 (Figure 1B).



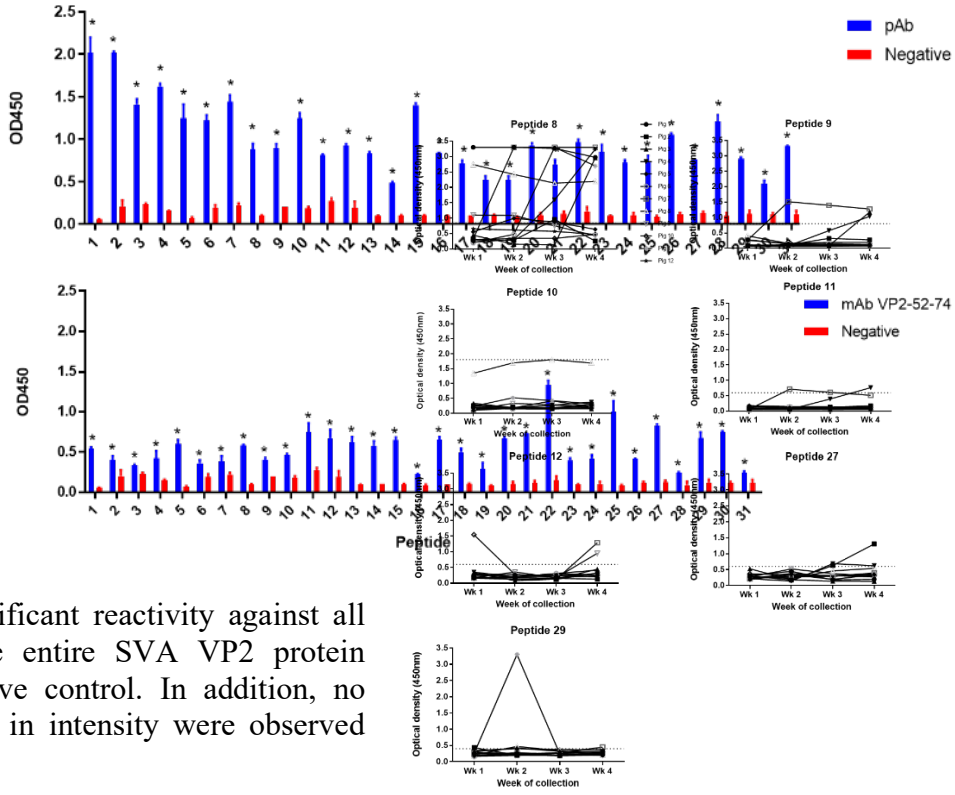
1.2. A group of SVA VP1 linear epitopes shows a differential inhibitory effect on SVA mAb but not in SVA pAbs against rVP1 protein

Specific peptide inhibitory activity on SVA pAb and SVA mAb against SVA rVP1 protein was evaluated by blocking ELISA. Ten log dilution concentrations of each peptide were incubated for 1 hour, either with SVA pAb or mAb. Then, inhibitory binding concentration 50 (IC_{50}) against SVA rVP1 protein was evaluated by SVA rVP1 ELISA. As a positive control, inhibitory activity of the whole rVP1 protein was also evaluated. Pre-incubation of rVP1 protein with SVA mAbs resulted in significant binding inhibition in a dose-dependent manner, while SVA pAbs showed a minimal reduction in binding activity against rVP1 protein. When SVA pAb binding activity against rVP1 was evaluated after individual peptide blockage, all epitopes showed a partial binding inhibitory effect. However, none of the peptides demonstrated a 50% or greater inhibitory effect on SVA pAb binding activity to SVA rVP1 protein (Figure 2A). All eighteen peptides also possessed some ability to block binding activity of SVA mAb to SVA-rVP1. However, four peptides spanning amino acids 1-20, 60-80, 105-125 and 120-140 of SVA-rVP1 showed potent inhibition, reducing SVA mAb binding activity to rVP1 protein by more than 50% (Figure 2B, C).



1.3. SVA mAb and pAbs show differential binding activity against SVA-VP2 epitopes by indirect ELISA epitope mapping

In order to determine linear B-cell epitopes of SVA VP2 structural protein potentially involved in SVA immunogenicity, we evaluated the binding activity of SVA pAb and VP2-mAb against sequential, overlapping peptides of the SVA-rVP2 protein by indirect peptide ELISA. SVA mAb and pAb showed significant reactivity against all peptides spanning the entire SVA VP2 protein compared to a negative control. In addition, no significant differences in intensity were observed

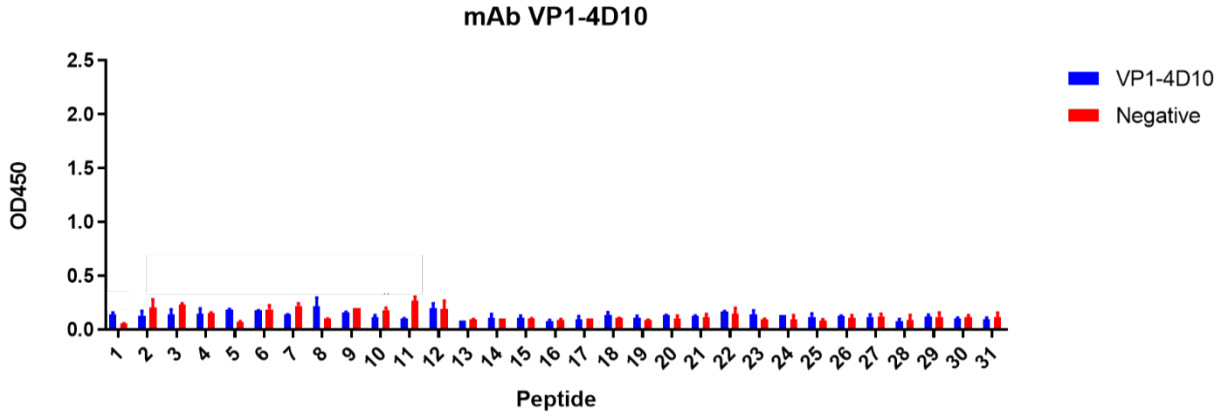


among peptides by SVA VP2 mAb or SVA pAb detection (Figure 3A, B).

However, in clinical samples obtained from a clinical break of vesicular disease positive to SVA by rVP1 ELISA, the SVA VP2 epitope array was capable of detecting significantly higher signal from five overlapping amino acid residues (peptides 8, 9, 10, 11 and 12) spanning amino acids 105-185 and two non-overlapping amino acid residues (peptides 27 and 29) spanning amino acids 390-410 and 420-440. In addition, a single peptide spanning amino acid 105-125 showed a particularly high and significant reactivity compared to other peptides within the VP2 structural protein (Figure 4).

1.4. SVA VP1 mAb do not show cross-reaction against VP2 linear epitopes

Final characterization of target peptides was performed in order to evaluate potential cross-reaction between different VP2 peptides against VP1 mAb. No significant difference in reactivity with any of the VP2 peptides against VP1 mAb was observed (Figure 5).

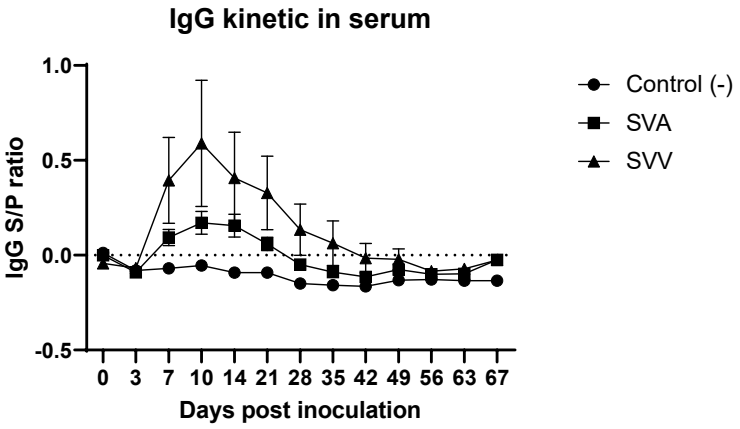


2. Determine the dynamic of immunoglobulins isotype in oral fluid during SVA active infection

2.1. Senecavirus infection induces specific detectable IgA antibodies on oral fluids during the acute infection

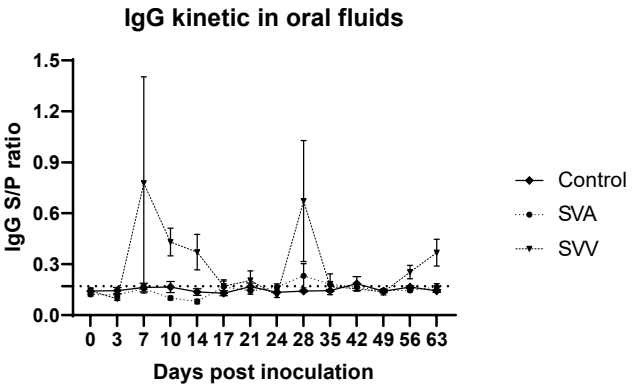
Oral fluid and serum were collected at 0, 3, 7, 10, 14, 21, 28, 35, 42, 49, 54, 60 and 67 dpi from three different inoculated groups as follows. Negative control group (NCG): 5 pens containing 2 pigs each, inoculated by intramuscular and intranasal administration of 2 ml of RMPI media (Control -). SVA group (SVA): 6 pens containing 2 pigs each, inoculated intramuscularly and intranasal with 2 mL of $1x10^9$/ml of a contemporary SVA-isolate. SVV group (SVV): 6 pens containing 2 pigs each, inoculated with 2 ml intramuscularly and 2 ml intranasal of $1x10^9$ of an SVV-001 acquired from ATCC.

Productive infection was confirmed by the presence of specific SVA-VP1 IgG antibodies in serum. The detected humoral response was stronger and more sustained in animals inoculated with SVV historical strain. Specific SVA-VP1 IgG antibodies were detected 7 dpi with a peak response at 10 dpi, followed by a rapid decline. SVA-VP1 IgG antibodies were undetectable by rVP1-indirect ELISA after 35 dpi.

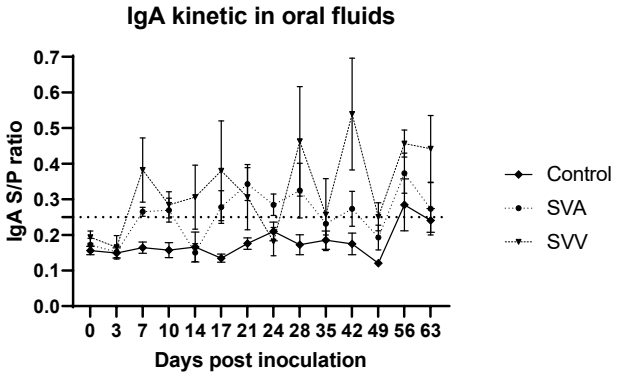


The response in the SVA infected group was more attenuated with detectable levels at 7 dpi, but only lasted until 21 dpi (Figure 6).

The IgG response in oral fluids was evaluated in animals experimentally infected with SVA and SVV. The same time point's periods were evaluated in order to correlate the IgG dynamic on serum and oral fluids. Although animals inoculated with SVA showed a moderate IgG response in serum that lasted for four weeks, no significant increment on IgG levels on oral fluids was observed.



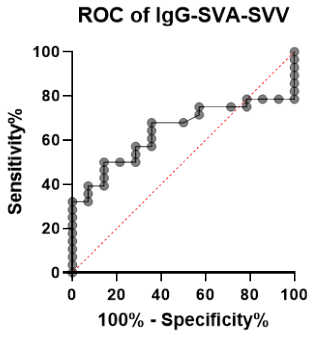
The IgG response on SVV-inoculated animals was erratic, with a large variation response detected by SVA-VP1 IgG ELISA. Thus, we detected 1 positive animal at 7 dpi and 3 animals at 10, 14 and 28 dpi. No antibodies were detectable between 35 and 56 dpi, and then 5 animals were detected at 56 and 63 dpi (Figure 7A).

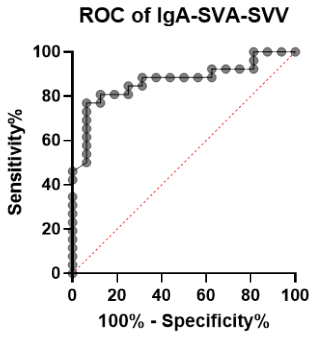


The presence of IgA was observed by the SVA-VP1 IgA ELISA in both inoculated groups after 7 dpi. The presence of specific Senecavirus antibodies was detected and sustained throughout the duration of the study. Although there were significant differences in S/P detected between both strains used for this study on specific time points (42 dpi), the test was able to discriminate positive groups (Figure 7B).

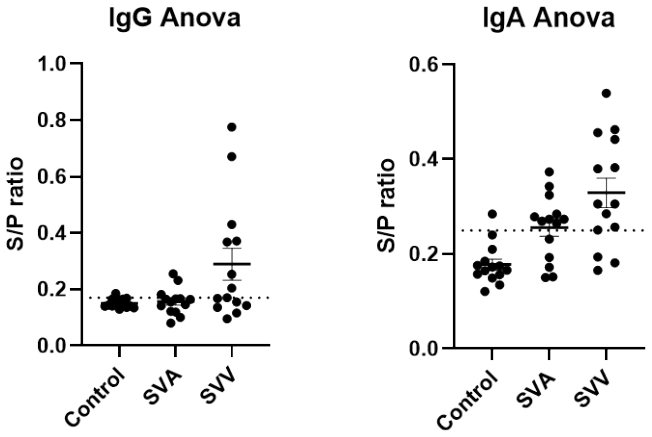
3. Assays performance and evaluation

In order to determine the S/P cutoff for positive samples determination, a ROC analysis was performed for both Ig isotypes among animals infected with both Senecavirus strains. For IgG, a S/P value of 0.17 showed a sensitivity of 39% and specificity of 93%, while for IgA, a S/P value of 0.25 showed a sensitivity of 77% and specificity of 93% (Figure 8A-B).





The assay performance showed no significant difference in the overall detection rate on oral fluids IgG levels between SVV-infected and control animals. The IgG detection rate was shown to be more efficient and significantly different compared with control groups (p = 0.01) on SVA-infected animals only. Meanwhile, the overall detection rate of IgA was significantly higher in both infected groups compared with the negative control (SVA p= 0.03; SVV p > 0.0001) (Figure (9A-B).



Discussion and conclusion

Due to their highly conserved sequence, immunogenicity and virus specificity, viral structural proteins including VP1 and VP2 have been used as targets for SVA diagnostic testing, which so far includes serological (ELISA, IFA, VN) and molecular testing (PCR, ISH). While the other capsid proteins are highly conserved among picornaviruses, VP1 is unique to the *Senecavirus* genera and is involved in cell tropism. In addition, we evaluated the humoral response of VP2, which seems to play a major role in the induction of neutralizing antibodies. However, there is a gap in information associated with immunodominant regions associated with these two structural proteins. **Thus, the first part of this study aims to examine the role of linear epitopes of the VP1 and VP2 proteins in the SVA humoral immune response.**

Experimental studies have shown that after 5 dpi, SVA-specific antibodies can be detected in serum. Different structural proteins, including VP1, VP2 and VP3, have been evaluated as potential antigen targets in swine serum for IgG detection through indirect ELISA. While the VP3 protein showed minimal immunoreactivity, both VP1 and VP2 reacted with serum from naturally infected animals. A VP1 indirect ELISA yielded a sensitivity of 93% and a specificity of 99%. The VP2 protein ELISA was optimized and validated, with 94.2% sensitivity and 89.7% specificity. IFA testing of swine serum showed similar results, with increased sensitivity of the ELISA test during early infection. Cross-reactivity has been demonstrated between historical SVV strains and contemporary SVA strains. Comparative serological studies have shown that a competitive ELISA using mAbs decreased cross-reactivity and allowed a more rapid test conclusion than IFA.

Here, SVA pAb and SVA rVP1 mAb characterization showed that SVA rVP1-mAb present a differential binding activity against different regions of the VP1 protein when evaluated on indirect ELISA. It is possible that conformational epitopes are responsible for this interaction. Therefore, differences in folding and interaction among conformational epitopes in whole virus

particles may mask specific binding sites needed for SVA VP1 mAb antigen interaction. SVA VP1 is part of a polyprotein, and interaction with other areas of the polyprotein may be vital for viral infection and replication. In foot-and-mouth disease virus, the GH loop demonstrates flexibility and an ability fold in different configurations. The hydrophobic pocket of VP1 has been shown in other picornaviruses to be related to capsid stability and genome release. This could be a contributing aspect to the immunogenicity observed for SVA VP1. The pocket is located under a canyon in the protein structure, and its entrance is almost entirely sealed by other residues of VP1. In other viruses, mAb for VP1 has been shown to bind deep in the canyon and have more than one region of receptor binding. The SVA-rVP1 could possess differences in this structural pocket due to folding changes that prevent natural epitopes from being exposed. Thus, a combination of these VP1 protein structural features could contribute to the difference in SVA mAbs binding activity to whole virus particles. The SVA VP1 epitope, spanning amino acids 1-20 of the SVA-rVP1 protein, showed a high reactivity and was capable of inhibiting interaction between SVA mAb and SVA rVP1. These results suggest that this linear region of SVA-VP1 plays an important role in the humoral immune response. While other peptides showed variable binding activity and inhibitory effects on SVA mAb interaction with rVP1 protein, peptides spanning amino acid 60-80, 105-125 and 120-140 were capable of significantly inhibiting mAb binding activity to SVA-rVP1. These regions may act as additional linear epitopes capable of inducing a humoral immune response.

All VP2-derived peptides showed significant reactivity against SVA-pAb and VP2-mAb by indirect ELISA compared to negative controls. None of the VP2-derived peptides showed cross-reactivity against VP1-mAb. Twenty-five peptides yielded significant OD signals against clinical positive serum samples, with a significantly higher OD signal on aa 105-125, 120-140, 150-170 and 255-275. The 20 amino acid segments generated in this study were all recognized by SVA pAb, mAb and clinical samples**.** In addition, relative to VP2, no difference in reactivity was observed against VP2 Map or SVA polyclonal antibodies. However, the binding activity of each VP2 peptide varies with clinical samples. It should be noted that the swine sera were collected from sows during a clinical outbreak, while mouse sera were collected from experimentally inoculated animals boostered with a specific, known dose of virus at specific time intervals. Thus, differences observed in clinical samples could be associated with time and dose of exposure. All monoclonal antibodies evaluated in this study were IgG isotype. Previous experiments in swine demonstrated that neutralizing activity is likely related to the presence of IgM. In addition, the same study also showed an earlier and higher neutralizing activity against VP2 compared with VP1 antibodies. In this study, sera collected from clinically affected sows also showed neutralizing activity, which might suggest that VP1 is not sufficient to generate antibody protection, and interaction with multiple structural proteins expressed during SVA replication might be necessary.

Antibody detection in oral fluids is a faster and less expensive method to evaluate pathogen circulation in a farm. Oral fluids are routinely collected in commercial production settings and accurately reflect the prevalence of different pathogens in the population. However, targets used for antibody detection in serum might not be appropriate for antibody detection in oral fluids. In addition, dynamics of antibodies or isotypes detected in serum may not correlate with those detected in the humoral response. **Thus, in the second part of this study, we evaluated an**

appropriate target for the development of an ELISA that allows detection of antibodies against Senecavirus in oral fluids. First, we determined that animals infected with two different Senecavirus strains can mount an appropriate humoral response. We observed, as previously described, a strong IgG response in serum that lasts for approximately five to six weeks post-infection. However, IgG isotype was not consistently detected when evaluated in oral fluids. IgG antibody response in oral fluids was only intermittently detected in a small subset of animals inoculated with SVV strain, but no IgG response was observed on those animals infected with SVA. It can be speculated that IgG isotype antibodies are not secreted in oral fluids. Although the inconsistency of IgG detection might be related to the target used for the IgG ELISA, the VP1 protein structure shares a 99% similarity between the two virus strains used in this study. When the same target protein was used to evaluate the dynamic of IgA in oral fluids, we observed a quick and consistent response in oral fluids. Although the protective role of IgA has not been reported, the presence of specific Senecavirus IgA antibodies can be a useful marker for early detection of Senecavirus infection in swine populations after a break of vesicular disease. Veterinary diagnostic laboratories, including the University of Minnesota, Iowa State University and South Dakota State University, ran a cumulative total of 334,083 diagnostic tests on swine oral fluids in 2016. The data have shown that oral fluids are both more diagnostically sensitive and economical than serum for the detection of pathogens at the herd level. Thus, oral fluid has the potential to replace individual pig serum samples for both routine and emergency disease surveillance.

Based on these findings, it can be concluded that SVA immunological response could be driven by a small set of linear epitopes in the VP1 and P2 structural proteins. These immunodominant epitopes could be used as a target for an immunological diagnostic assay. The immune response against Senecavirus infection can be detected in oral fluids and is largely IgA isotype based. These results support the idea that oral fluid has the potential to replace individual pig serum samples for both SVA routine and emergency disease surveillance.

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