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Standardization of ELISA with Senecavirus A recombinant VP2 protein and its use in swine herds in Brazil

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ABSTRACT. Senecavirus A (SVA) is a nonenveloped, single-stranded RNA virusThe icosahedral viral particle is composed of four structural proteins: VP1, VP2, VP3 and VP4, among which VP2 is strongly involved in the antibody immune response. The virus causes vesicles on the snout and feet in pigs, which are clinically indistinguishable from other vesicular diseases such as foot-and-mouth disease. Outbreaks of SVA have been reported worldwide since 2014; however, its prevalence in Brazil remains unknown. In this study, the VP2 structural protein was produced and purified from *E. coli*, and recombinant VP2 (rVP2), based on the most recent Brazilian strain, was used to develop an indirect ELISA to identify antibodies against SVA in Brazilian swine herds. Sensitivity and specificity values of the rVP2 ELISA were determined using receiver operating characteristic analysis performed on 43 SVA positive and 219 negative serum samples. In addition, serum samples from pigs immunized with eight distinct Brazilian SVA inactivated strains were tested with the rVP2 ELISA. For the specificity of the assay,

17 serum samples from vesicular stomatitis virus (VSV) from naturally infected pigs were tested. The rVP2 ELISA was found to have 100% specificity and 74.4% sensitivity. The performance of the assay using samples collected during the SVA outbreak, had a sensitivity of 100%, and with those collected nine months after the outbreak it had a sensitivity of 73.4%. The rVP2 ELISA developed here was able to detect specific SVA antibodies in acute disease and recovered pigs, and no cross-reactivity with VSV was observed. This assay has potential as a useful tool for monitoring SVA infection and could help to improve disease diagnosis.

Key words: Enzyme-linked immunosorbent assay; Serology; Diagnostics; Seneca valley virus; SVV; SVA

INTRODUCTION

Senecavirus A (SVA), also known as Seneca Valley virus, is composed of an icosahedral capsid with positive-sense single-stranded RNA; it belongs to the *Senecavirus* genus, *Picornaviridae* family (Hales et al., 2008; Adams et al., 2015). The virus was first described in 2002, as a cell contaminant in human retinoblast cells (PER.C6®), presumedly introduced via porcine trypsin or fetal bovine serum (Hales et al., 2008). In retrospective studies, SVA was detected in 1988 from samples of healthy pigs in the U.S., but its first association with vesicular disease (VD) was reported in 2007 in the U.S. (Pasma et al., 2008). Outbreaks of VD associated with SVA have been reported since 2014 and the incidence of SVA infections has increased in several countries, such as China (Qian et al., 2016; Wu et al., 2017), the U.S. (Canning et al., 2016, Preis et al., 2022), and Brazil (Leme et al., 2015; Vannucci et al., 2015; Vieira et al., 2022). A retrospective serological survey conducted in Brazil suggested that the virus was not circulating prior to 2014 (Saporiti et al., 2017).

SVA causes an acute VD in pigs, characterized by vesicles and erosions on the snout, oral cavity and coronary band. Acute loss between 30-70% of neonatal piglets associated mainly with claudication, lethargy, neurological signs and/or diarrhea has been reported (Vannucci et al., 2015; Zhang et al., 2018, Houston et al., 2020). A major concern about SVA-VD is that it is clinically indistinguishable from other VDs, such as foot-and-mouth disease (FMDV), swine vesicular disease (SVD), vesicular stomatitis (VS) and vesicular exanthema of swine (VES) (Leme et al., 2015; Segales et al., 2017; Liu et al., 2020). Identification of SVA infection helps to rule out mainly FMDV, an economically devastating disease (Zhang et al., 2018).

The SVA genome is 7280 nucleotides long, with an open reading frame encoding a single polyprotein. It is structured by the standard L-4-3-4 picornaviruses layout: a leader protein, four polypeptides of P1 (structural), three polypeptides of P2 and four polypeptides of P3 (nonstructural) (Hales et al., 2008). The 3C protease is responsible for cleaving the P1 region into VP0, VP3 and VP1. The VP0, when mature, is cleaved in VP2 and VP4 forms that are found in the inner surface (Racaniello, 2007; Hales et al., 2008; Wen et al., 2022). The antigenic sites are located in VP's external viral capsid proteins (VP1, VP2 and VP3), which are involved in the neutralization of picornaviruses (Maggioli et al., 2018). Among these VP proteins, VP2 showed to be an ideal target for the specific detection of SVA

antibodies as it is more conserved and immunogenic than VP1 and VP3 (Dvorak et al., 2017). This study aimed to develop and standardize an indirect ELISA based on a recombinant VP2 (rVP2) of SVA to detect antibodies against the virus in Brazilian pig herds.

MATERIAL AND METHODS

SVA protein selection

A total of 51 Brazilian Senecavirus sequences were retrieved from Genbank (https://www.ncbi.nlm.nih.gov/genbank/); however, only 14 sequences (GenBank accession numbers: KR063107, KR0631008, KR063109, MF615501, MF615502, MF615503, MF615504, MF615505, MF615506, MF615507, MF615508, MF615509, MF615510, and MZ456812), which presented the full length genome, were used in the study. As VP2 has been shown to be the viral protein with a higher antibody response (Dvorak et al., 2017) and has more epitopes for antibody recognition (Yang et al., 2007), it was selected to be the target in this study. From the viral polyprotein, the full-length VP2 gene, composed of 852 nucleotides, was identified based on its respective cleavage sites (Hales et al., 2008). To determine the consensus sequence of the VP2 gene, the 14 nucleotide sequences were aligned using the PRRN online program (https://www.genome.jp/tools-bin/prrn). As the consensus sequence generated too many degenerate bases, the sequence MZ456812 (BRMSA 2598 - SISGEN A91DB6D) was selected. This sequence was chosen because it represents the most recent Brazilian SVA circulating in Brazil and is available on GenBank. This virus was isolated from a SVA outbreak that occurred at Embrapa Suínos e Aves swine facility, in Santa Catarina State in 2020, under the official veterinary service investigation.

VP2 3D modeling and epitope identification

The 14 Brazilian VP2 SVA amino acid sequences were aligned for the generation of the 3D model and the identification of epitopes. The three-dimensional model of VP2 was constructed using the PyMol software. The conserved epitopes were identified based on a previous study of Fan et al. (2020). The epitopes sites were colored on the 3D model and marked on the sequence alignment.

Expression and production of the rVP2

The VP2 full length gene sequence of SVA was optimized, synthesized, and ligated into pET-28a(+) plasmid (Novagen, USA) for expression in *E. coli*. The plasmid pET28a-VP2 was introduced into competent *E. coli* BL21(DE3) (Thermo Fisher Scientific, USA) for culture. Gene expression was induced by incubation of liquid 2yt supplemented with 1mM de isopropyl β-D-1-thiogalactopyranoside (IPTG, Thermo Fisher Scientific, USA) for 3 h at 37°C.

The recombinant protein was solubilized with 8M urea and purified using nickel affinity chromatography by modifying the centrifugation method to a gravitational method (Probond Resin, Thermo Fisher Scientific, USA) following and adapting the manufacturer's instructions. The recombinant protein was dialyzed with 1x phosphate-buffered saline at pH

7.2 (PBS) for 72 h at 4°C. Purification and sample quality was performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis 12% (SDS-PAGE) and Western blotting, by using an anti-6x-histidine monoclonal antibody (Sigma, USA). The concentration of protein was measured by the Bradford method (Bradford, 1976).

Swine serum samples

A total of 43 serum samples from SVA-VD affected pigs were tested. From these, 23 positive serum samples were obtained from sows naturally infected with SVA from the outbreak in which the isolated MZ456812 was used for VP2 protein design. Serum samples were collected during the acute phase of the infection (seven days after the beginning of vesicular lesions on the snout and coronary band - 7 DPI) (n= 8 - Group A) and after the convalescent phase (nine months after the outbreak - 9 MPI) (n= 15 - Group B). In addition, positive pig serum samples collected during the acute phase (2 to 15 DPI) (n= 20 - Group C), which were confirmed by serum virus neutralization (SVN), were obtained from the Ministry of Agriculture, Livestock and Food Supply (MAPA).

Negative swine serum samples (n= 219 - Group D) were randomly selected from a serum database at the Embrapa Swine and Poultry institute, from pigs of different ages and Brazilian states from 1990-2012, before the emergence of SVA around the world.

In addition, to verify the specificity of the assay, 17 serum samples from VSV naturally infected sows (Group E), which were positive by SVN to VSV under official investigation by MAPA, were tested. These samples were obtained from Bahia state in 2020, from sows showing vesicular clinical signs.

Experimental SVA immunization

Eight distinct Brazilian SVA strains (GenBank accession numbers: MF615501, MF615502, MF615503, MF615505, MF615506, MF615507, MF615509, and MZ456812) containing 10^6 50% Tissue Culture Infectious Dose per mL ($TCID_{50}$/mL) in H1299 non-small lung carcinoma cell line (ATCC CRL-5803) was used for serum production. The SVA isolates were inactivated with binary ethylenimine as previously described (Bahnemann, 1990) and 20% Emulsigen as an adjuvant (MVP Laboratories Inc., USA) was added to the preparation.

To obtain specific antibodies against SVA, eight specific pathogen-free pigs40-days-old were intramuscularly immunized with 2 mL of an inactivated SVA preparation. Three boosters were administered two weeks apart and blood was collected from each pig 15 days after the final booster. The sera were obtained (n= 8 - Group F) and stored at -20°C until analysis.

RT-qPCR and serum virus neutralization assays for SVA

The official veterinary service was notified of an occurrence of VD and the SVA infection was confirmed by RT-qPCR in vesicular fluid and associated epithelium. Viral RNA was extracted from 50 µL of vesicular fluid and serum (from the outbreak) and of infected H1299 cells (SVA isolates) using the magnetic particle processor procedure (MagMAXTM-96 Viral RNA Isolation kit, Applied Biosystems, USA). RT-qPCR was

performed using a commercial SVA RT-qPCR reagent targeting the conserved SVA 3D polymerase region (EZ-SVA, Tetracore Inc., USA).

For the serum virus neutralization assay (SVN), serial two-fold dilutions of heat-inactivated positive and hyperimmune sera (1:40 to 1:40,960) were incubated for 1 h at 37°C with 200 $TCID_{50}$/mL of each SVA strain. H1299 cells were added to each well in a 96-well plate and after 48 h of incubation at 37°C with 5% CO_2, were fixed with 3.7% formaldehyde. Cells were permeabilized with 0.2% Triton-X and incubated with primary antibody (anti-SVA rabbit polyclonal antibody - gently provided by Dr Diego G. Diel) diluted 1:3,000 for 1 h at 37°C. Then, goat anti-rabbit IgG (H+L) highly cross-adsorbed secondary antibodies - Alexa 594 conjugated (Thermo Fisher Scientific, USA) diluted 1:1,200 were incubated for 1 h at 37°C. Plates were read under a fluorescence microscope and antibody titers were expressed as the reciprocal of the highest dilution of serum capable of completely inhibiting SVA replication.

VP2 antibody detection by rVP2 SVA ELISA

Polystyrene 96 well plates (Costar, ref 3590, USA) were absorbed with purified rVP2 previously solubilized in 2% sodium dodecyl sulfate (Lechtzier et al., 2002). The rVP2 was serially diluted from 20 to 0.0195 µg/mL in 0.05 M carbonate/bicarbonate buffer at a pH of 9.6 and incubated in a humid chamber overnight at 4°C. The plates were blocked with 200 µL/well of 5% skim milk in phosphate buffer saline with 0.05% Tween 20 (PBST) for 1 h at 37°C. The plates were washed five times with PBST. After this, a pool of serum samples from SVA positive pigs (Group A, B and F) and a pool of serum samples from negative pigs (Group D) were diluted 1:100 and incubated for 1 h at 37°C. To evaluate the antigen-antibody interaction, the anti-IgG pig antibody conjugated with peroxidase (Sigma, USA) was tested in three dilutions (1:50,000; 100,000; and 150,000) in PBST. The enzymatic reactions were revealed with 100µL/well of 3,3',5,5'-tetramethylbenzidine (TMB - Sigma, USA) for 15 minutes and interrupted by adding 50µL/well of H2SO4 2M. The optical densities were determined by spectrometry in an ELISA reader with a 450 nm filter.

To titrate the SVA positive serum samples, the rVP2 SVA ELISA was carried out following the standardized steps and diluting the pig serum from 1:100 up to 1:6,400 in PBST. ELISA absorbances were normalized to minimize the effect of intra-ass ay and inter-assay variation according to Ramanakumar et al. (2010).

VP2 antibody detection by Western blotting

Positive samples were confirmed by Western blotting. The recombinant VP2 protein was transferred from SDS-PAGE gels to nitrocellulose membranes by electrophoretic elution. SVA positive serum diluted 1:100 was added and incubated for 1 h at room temperature, followed by adding protein A conjugate for 1 h at room temperature (Sigma, USA). The reveal was done using a 3'3'-Diaminobenzidine (DAB) reagent (Sigma, USA).

Statistical analysis

The diagnostic accuracy of the assay was performed by using the software Epitools Epidemiological Calculators (https://epitools.ausvet.com.au/roccurves) which applies the curve ROC analysis to measure the variation in sensitivity and specificity of the antigen tested by ELISA. The area under the ROC curve (AUC) was determined, which is an effective way to summarize the overall diagnostic accuracy that gives values between 0-1, where a value more than 0.9 is considered an outstanding accurate test and 1 is perfect (Mandrekar, 2010).

The Youden Index (J) was used to evaluate the maximum potential effectiveness of the VP2. It is considered the main summary statistic of the ROC curve, with the ability to differentiate when the sensitivity and the specificity have equal weight.

The graphs were designed using GraphPad Prism, version 6 for Windows (GraphPad®).

RESULTS

VP2 3D modeling and epitope identification

The VP2 layout presented one conformation in α-helix on the epitope ^{12}DRVITQT^{18} (red), two β-sheet conformations on the epitopes ^{98}GGAFTA^{103} (green) and ^{150}KSLQELN^{156} (magenta), and two loops on the epitopes ^{71}WTKAVK^{76} (yellow) and ^{248}YKEGAT^{253} (blue). As loops are disposed on the surface of the VP2, they are more likely exposed to the action of solvents or to the interaction with biological molecules, which could explain the epitope location matching with these regions (Figure 1A).

The VP2 amino acids alignment of the 14 Brazilian SVA sequences showed five epitope regions (^{12}DRVITQT^{18}, ^{98}GGAFTA^{103}, ^{150}KSLQELN^{156}, ^{71}WTKAVK^{76} and ^{248}YKEGAT^{253}), which were marked with colored boxes (Figure 1B). In total, five mutations were identified, which were highlighted in orange. The SVA sequence selected for VP2 synthesis (MZ456812), showed 4/284 mutations (K42R, S70P, T180A and T280A), all of them located outside of an epitope region; while a SVA sequence from 2016 (MF615505) showed one mutation (I15M) located on the epitope DRVITQT. However, this unique mutation did not interfere with the performance of the rVP2 SVA ELISA using the corresponding serum.

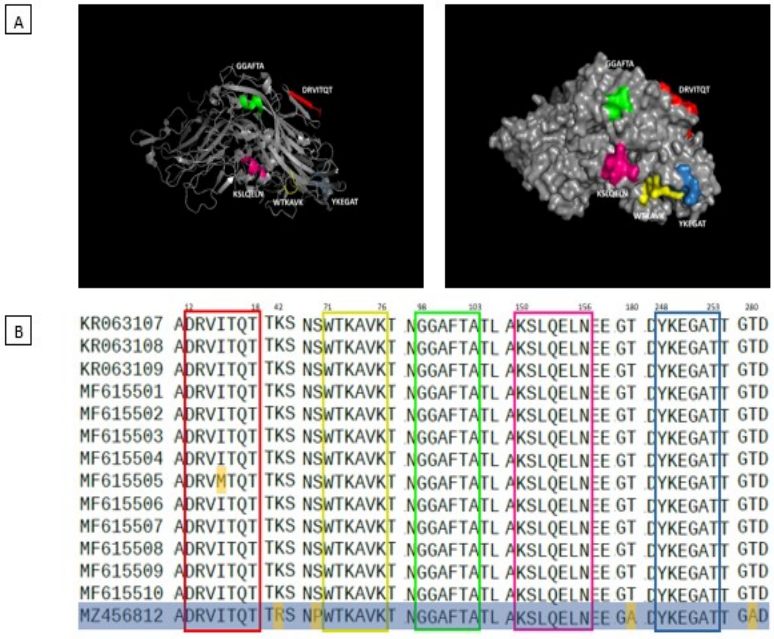

Figure 1. Analysis of VP2 epitopes and mutations of SVA amino acid sequences. (A) Simulated 3D model of VP2. The epitopes are colored as follows: ^{12}DRVITQT^{18} (red), ^{98}GGAFTA^{103} (green), ^{150}KSLQELN^{156} (magenta), ^{71}WTKAVK^{76} (yellow), and ^{248}YKEGAT^{253} (blue). (B) VP2 amino acid sequences of the 14 Brazilian SVA, in which colored boxes indicate the corresponding epitope regions. The strain MZ456812, the focus of this study, is highlighted in blue. All mutations are marked in orange.

rVP2 protein expression

The rVP2 protein was expressed and determined through electrophoresis in SDS-PAGE, which revealed a mass of approximately 35 kilodaltons (kDa) for the recombinant protein after purification (Figure 2A). In Western blotting, the rVP2 protein was recognized by the anti-6x-histidine antibody, which confirmed the gene expression (Figure 2B). Then, swine positive (Group A) and negative SVA (Group D) sera were tested by Western blotting. The positive serum showed a strong signal at the expected 35 kDa size, corresponding to VP2 (Hales et al. 2008; Houston et al. 2020) (Figure 2C).

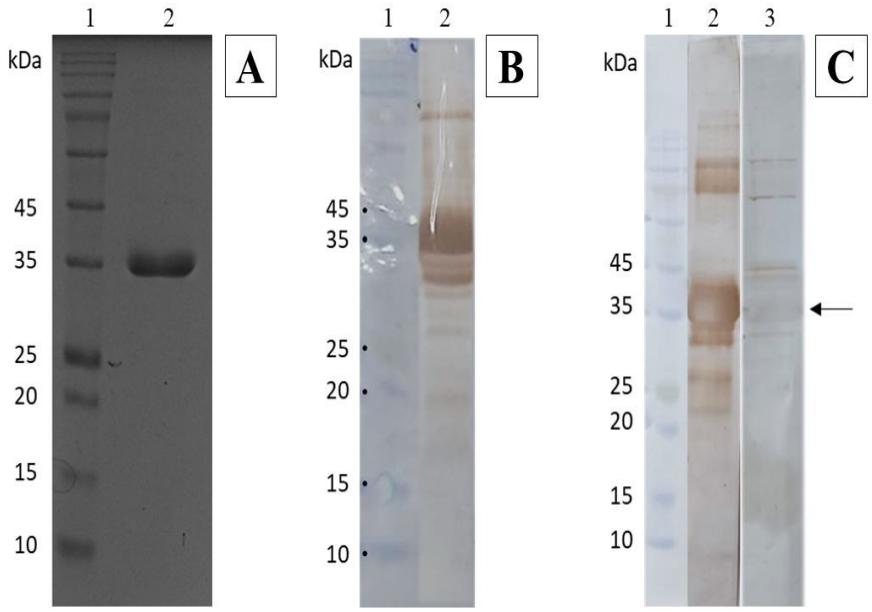

Figure 2. Purification and confirmation of SVA rVP2 protein in SDS-PAGE and Western blotting. (A) rVP2 purified: Lane 1 - protein marker - Sinapse (S2600). Lane 2 - rVP2 after purification (ProBond). (B) Western blotting with anti-histidine: Line 1 - protein marker - Sinapse (S2600). Line 2 - rVP2 recognized by the anti-6x-histidine antibody. (C) - Western blotting with SVA positive and negative pig serum: Line 1 - protein marker - Sinapse (S2600). Line 2 - VP2 signal in a positive serum. Line 3 - absence of VP2 signal in a negative serum.

rVP2 ELISA optimization and validation

The rVP2 ELISA was standardized with 500 ng/well of rVP2 to coat the plates, serum samples dilution of 1:100 and secondary antibody conjugated with peroxidase dilution of 1:1000,000. The cut-off value was determined using ROC analysis, performed with 262 samples, among which 43 were positive (Group A, B and C) and 219 were negative (Group D) for SVA. The results of the ROC analysis are shown in Table 1. There were no large changes in the sensitivity values according to different specificity levels. Assuming a specificity of 100%, we used a cut-off value of OD = 0.549, showing an area under the curve (AUC) of 0.965, and a sensitivity of 74.4% (Table 2; Figure 3A).

Table 1. Analysis of the sensitivity and specificity values (in %) of the rVP2 SVA ELISA at different cut-off points.

Parameters	Cut-off points						
	0.549	0.54	0.54	0.53	0.511	0.476	0.434
Specificity	100	99.5	99.1	98.2	95.4	90.4	80.4
Sensitivity	74.4	76.7	76.7	76.7	79.1	90.7	97.7

When the ROC analysis was applied separately, the positive samples from 7 DPI (Group B) and 9 MPI (Group C) along with negative samples (Group D), the cut-off values for that data were 0.728 and 0.551, resulting in AUC= 1 and AUC= 0.94 respectively (Table 2). Assuming a specificity of 100%, the sensitivity was 100% for Group B and 73.3% for Group C (Table 2; Figure 3B and 3C).

Table 2. ROC curve parameters of rVP2 SVA ELISA, according to the different serum samples grouped by SVA status of infection.

Samples	Cut-off points	Specificity	Sensitivity	AUC (IC 95%)	Youden's statistic
Groups A-D (n= 262)	0.549	100%	74.4%	0.965(0.936-0.995)	0.721
Groups A and D - 7 DPI (n= 227)	0.728	100%	100%	1 (1-1)	1
Groups B and D - 9 MPI (n= 234)	0.551	100%	73.3%	0.94(0.836-1)	0.733

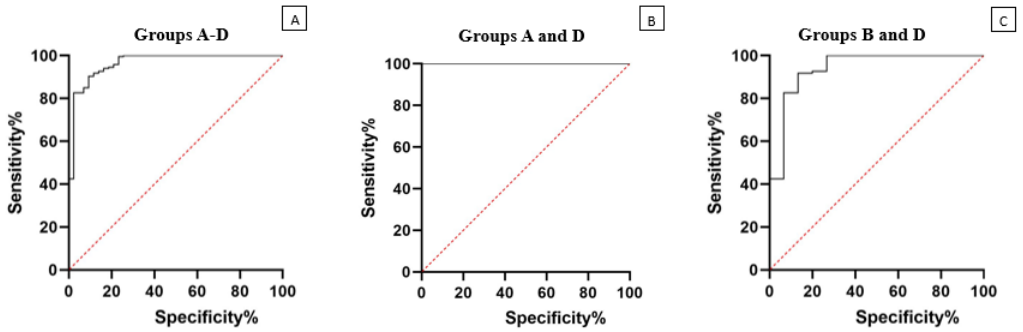

Figure 3. Receiver operating characteristic (ROC) curve of rVP2 SVA ELISA with a confidence interval of 95%. (A) ROC curve with 43 positive samples (Groups A, B and C) and 219 negative samples (Group D) (AUC=0.965). (B) ROC curve with eight positive samples of 7 DPI (Group A) and 219 negative samples (Group D) (AUC=1). (C) ROC curve with 15 positive samples of 9 MPI (Group B) and 219 negative samples (Group D) (AUC=0.94).

Positive serum samples showed OD absorbance above the cut-off (Figure 4A), which was confirmed by IgG titer analyses. The IgG titers varied from 100 to 1,600 (Figure 4B). Positive samples for VSV (Group E) showed no cross-reactivity in the SVA VP2 ELISA (Figure 4C).

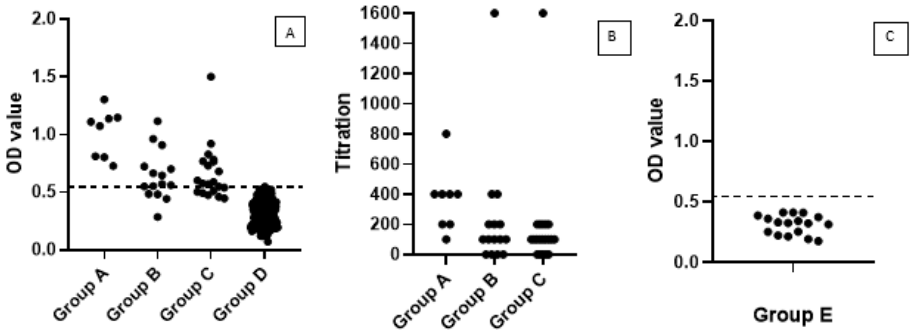

Figure 4. Analysis of rVP2 SVA ELISA with SVA positive and negative serum samples. The cut-off value for the assay is shown by the dashed line (0.549) (A) OD values for 43 SVA positive samples (Groups A, B and C) and 219 negative samples (Group D) (B) IgG SVA titers for 43 SVA positive samples (Groups A, B and C). (C) Seventeen pig serum samples were positive for VSV.

Experimental immunization analysis

Eight distinct inactivated SVA strains were administered in pigs to obtain specific antibodies against SVA. Serum samples collected from pigs before and after three boosters were tested by rVP2 SVA ELISA. From the eight Brazilian SVA strains, four were able to stimulate specific immune responses against the recombinant VP2 protein: MF615506, MF615507, MF615502, and MF615505 (Figure 5).

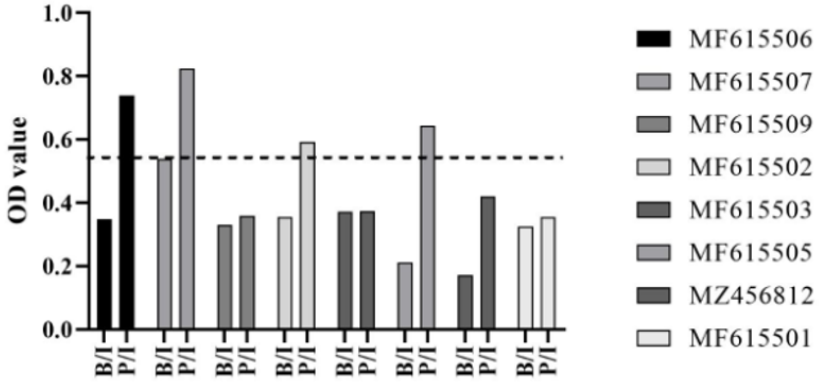

Figure 5. Analysis of rVP2 SVA ELISA of serum from eight pigs immunized with inactivated Brazilian SVA strains. The cut-off value for the assay is shown by the dashed line (0.549). B/I = Before Immunization, P/I = Post Immunization.

Comparison of rVP2 SVA ELISA and SVN/RT-PCR

Twenty-eight serum samples from Group C (n= 20) and Group F (n= 8), previously positive for SVA antibodies by SVN were tested by the rVP2 ELISA. Twenty out of 28 samples (71.43%) were positive in both assays (Figure 6).

Twenty-three SVA RT-PCR positive serum samples collected during the SVA outbreak, from Group A (n= 8) and B (n= 15), were also tested by the rVP2 ELISA. Nineteen of the 23 samples were positive in both assays, despite that the RT-PCR corresponds to virus detection and the ELISA indicates antibody response to virus infection (Figure 6).

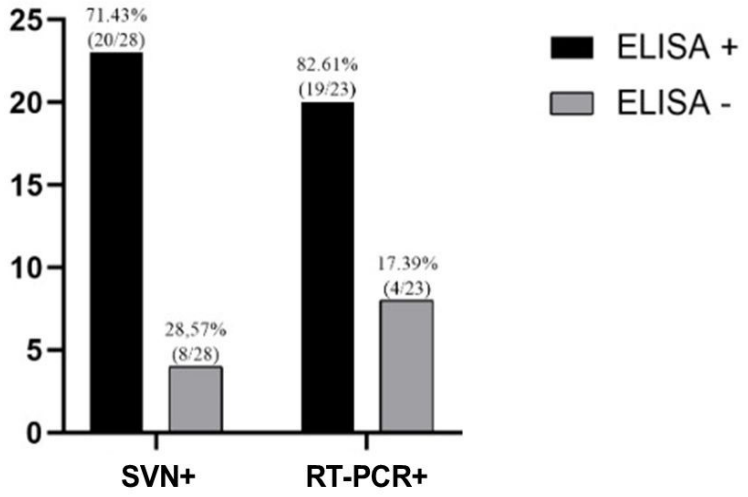

Figure 6. Comparison of rVP2 SVA ELISA with SVN and its correlation with SVA RT-PCR detection.

DISCUSSION

Senecavirus A is an emerging virus, associated with vesicular disease in swine. It poses a major concern because of the clinical similarity with other VD, particularly with FMD, a World Organisation for Animal Health listed disease (Houston et al. 2020). Outbreaks of SVA-VD have been reported in several swine-producing countries around the world since 2014 (Leme et al., 2015; Vannucci et al., 2015; Canning et al., 2016; Qian et al., 2016; Wu et al., 2017). In Brazil, the disease was characterized as the presence of vesicles and coalescing erosions on snout, oral cavity and coronary bands in different pig categories and as acute loss of neonatal piglets in the first days of age (Leme et al., 2015; Vannucci et al., 2015; Leme et al., 2017). This resemblance with FMD, losses due to piglet mortality, and other economic impact on the pig production, highlights the importance of SVA infection (Zhang et al., 2018). As some pigs can be infected and shed SVA without showing any clinical disease (Segales et al., 2017; Houston et al., 2020) a SVA specific antibody detection assay is crucial to minimize the impact of the disease (Zhang et al., 2018).

SVA has a short-term viremia, from 1-10 days, which occurs along with the production of neutralizing antibodies (nAb) (Joshi et al., 2016; Maggioli et al., 2018). Virus-specific nAb are important to control viremia, limit SVA spread to tissues and to reduce the severity of disease (Dotzauer and Kraemer, 2012). This early and robust nAb production coincides with VP2 and VP3 specific IgM antibodies at 5 DPI, followed by IgG

antibodies production around 7 DPI (Joshi et al., 2016; Maggioli et al., 2018). Notably, as SVA nAb begins to be produced, viremia starts to decrease, and the complete clinical resolution usually occurs in 2-3 weeks (Maggioli et al., 2018).

Currently, several SVA antibody detection methods are available, such as indirect immunofluorescence (IF), virus neutralization assays and enzyme-linked immunosorbent assays, targeting different SVA structural proteins, including VP1, VP2, and VP3 (Gimenez-Lirola et al., 2016; Dvorak et al., 2017; Goolia et al., 2017; Bai et al., 2021). Among the three SVA structural proteins, VP2 showed higher antibody responses than VP1 and VP3 and showed higher affinity binding on an avidity ELISA (Dvorak et al., 2017). Antigenic sites are located mainly within the external viral capsid proteins, although VP2 is the capsid protein that presents more epitope site recognition (Yang et al., 2007; Fan et al., 2020). Here we analyzed those epitopes and verified that they are conserved among Brazilian VP2 SVA sequences, as mentioned previously (Fan et al., 2020).

The rVP2 SVA ELISA developed here, showed to be specific (100%) and sensitive (74.4%) in detecting SVA antibodies in the pig population, regardless of infection status. Notably, no cross-reactivity with VSV positive serum samples was observed. A previous study based on a VP2 ELISA demonstrated a specificity and sensibility of 94.2% and 89.7%, respectively (Dvorak et al., 2017). As expected, a good correlation was identified between SVN and rVP2 SVA ELISA, and the ELISA. is more cost-effective and less labor consuming than SVN.

In conclusion, the rVP2 SVA ELISA developed in this study was able to detect specific SVA antibodies. This assay can be a useful tool for monitoring SVA infection and could help to improve the disease diagnosis. The rVP2 protein synthesized and expressed here can also be used to generate monoclonal antibodies that could be applied in future diagnosis methodologies for SVA detection.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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