RESEARCH REPORT
SWINE HEALTH

Title: Evaluation of carrier state and protection against a contemporary Senecavirus A isolate in pigs previously infected with an historical strain – **NPB #18-034**

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Industry Summary

In the recent years, cases of vesicular disease in pig due to Senecavirus A (SVA) infections have been responsible for an emerging concern in the swine industry for being clinically indistinguishable from high-consequence foreign animal diseases, including foot-and-mouth (FMD) disease. Data from previous studies showed that the virus can be found in the tonsils of naturally-infected pigs up to approximately 90 days after showing clinical disease, suggesting a potential for establishing persistent infections and an asymptomatic carrier state, and that specific mutations on the viral capsid may be driving the ability of contemporary SVA isolates to escape from the host immune responses previously built against historical isolates. This research project's goal is to investigate the ability of SVA to induce asymptomatic carrier state in pigs, and evaluate the susceptibility of pigs experimentally infected with a contemporary isolate after previous exposure with an historical strain.

Forty nine days after inoculation with the contemporary or historical strains, 2/3 and 4/4 necropsied animals respectively showed positive PCR results in the tonsils of the soft palate. Tonsil scraping showed to be a promising method to detect SVA RNA in the tonsils of clinically recovered animals, by detecting SVA in 39.1% of the animals from both groups 48 days after exposure. Shedding of SVA in saliva and feces was greatly reduced after the re-challenge with a contemporary strain in both groups previously infected with either the historical or contemporary isolate. These results show that animals can be asymptomatically infected with SVA after being thought to be clear of the virus, and could potentially be a source of infection to naïve animals. Also, animals that were previously exposed to a historical or contemporary strains were not susceptible to the contemporary strain in the second inoculation.

Key findings

- Pigs can be asymptomatically carrying SVA and be a potential source of infection to naïve animals.
- Animals previously infected with either historical or contemporary SVA were protected against the contemporary strain after re-challenge.
- Tonsil scrapping is a promising method to detect SVA in clinically recovered animals.

Keywords: swine, Senecavirus A, asymptomatic carrier

Scientific Abstract

An increasing concern in the swine industry due to Senecavirus A (SVA) infections has been arising since the previous recent years, due to it causing a vesicular disease that is indistinguishable from high-consequence foreign animal diseases, including foot-and-mouth disease. In the year of 2018, the total number of foreign animal investigations carried out in the United States was 2,072, of which 1,592 were caused by SVA infections in pigs. Such number highlights the amount of resources that are allocated into these investigations in order to rule out other vesicular diseases, and also brings attention to the matter of how to better deal with having an endemic vesicular disease in the swine population. Preliminary data demonstrated the presence of viable virus in the tonsils of naturally-infected pigs approximately 90 days after developing clinical disease, suggesting a potential for establishing persistent infections and an asymptomatic carrier state. Also, amino acid changes on the viral capsid may be driving the ability of contemporary isolates to escape from host immune responses previously built against historical isolates. This research project's goal is to investigate the ability of SVA to induce asymptomatic carrier state in pigs, and evaluate the susceptibility of pigs experimentally infected with a contemporary isolate after previous exposure with an historical strain. In addition, a comparison of different diagnostic methods was done on different time points of infection. A total of 28 three-week-old piglets were divided into three groups: Group HC, inoculated with the historical isolate from 1999 (n=12); Group CC, inoculated with contemporary isolate from 2017 (n=12); and a negative control group (n=4). At 49 days post-inoculation (dpi), 4 animals from each inoculated group and 2 from the control group had been necropsied, and then both groups were inoculated again at 50 dpi, with the contemporary strain. Sampling methods included oral, fecal and tonsil swabs, as well as tonsil scrapings, sera collection, and oral fluids. Samples were tested by SVA RT-qPCR, and serological testing was done by indirect immunofluorescence (IFA). The time points for collections were on 1, 3, 7, 10, 14, 21, 28, 35, 42, and 48 dpi, and 2, 5, 7, 14 and 21 after re-challenge. Serological response was first detected at 10 dpi on the CC group and 14dpi on the HC group, with 100% of animals from each group showing seropositivity at 42 and 28 dpi respectively. The tonsils of the soft palate from pigs in both groups showed to be able to harbor SVA up to 49 dpi, and it was also

found in tonsils of animals that were necropsied at 21 days after the second inoculation. Tonsil scraping in live animals was able to detect SVA positive animals up until 48dpi in both groups, 13 days after last detection in fecal swabs, with 7/12 in the HC and 2/12 in the CC groups yielding mean Ct values of 34 in both groups. Shedding of viruses in saliva and feces was only detected on day 2 after the second inoculation, while it was found up to 35 days after the first inoculation. These results show that animals can be asymptomatically infected with SVA after being thought to be clear of the virus, and could potentially be a source of infection to naïve animals. Also, animals that were experimentally infected with historical and contemporary strains were not susceptible to the contemporary strain after the first exposure. Tonsil scraping might be an important tool for the detection of asymptomatically infected animals.

Introduction

Senecavirus A (SVA) infections have been an emerging concern in the swine industry by causing vesicular disease clinically indistinguishable from high-consequence foreign animal diseases, including foot-and-mouth disease. While only sporadic cases had been reported between 1988 and 2014, the number of SVA cases have increased significantly and was responsible for the majority of the foreign animal disease (FAD) investigations in the United States in the year of 2018. Out of the 2,072 FAD investigations in this year, 1,729 were due to vesicular diseases with 1,592 in pigs only. The amount of resources allocated into these investigations is only one of the responsible factors contributing to the economic impact of the disease that is also bringing attention to the matter of how to better deal with having an endemic vesicular disease in the swine population.

Preliminary data demonstrated the presence of viable virus in the tonsils of naturally-infected pigs approximately 90 days after showing clinical disease, suggesting a potential for establishing persistent infections and an asymptomatic carrier state. Despite of a clear genetic divergence observed between historical and contemporary SVA isolates, there is no evidence whether animals previously exposed to a SVA strain are protected or susceptible against a heterologous isolate. Specific amino acid changes on structural proteins (VP1, VP2 and VP3) on the surface of the viral capsid allow picornaviruses (i.e. FMDV) to evade neutralizing antibodies, and then succeed as a pathogen throughout the years. Preliminary data comparing whole genomes of SVA isolates also allowed the identification of amino acid changes on the viral capsid that may be driving the ability of contemporary isolates to escape from host immune responses previously built against historical isolates.

This research project's goal is to investigate the ability of SVA to induce asymptomatic carrier state in pigs, and evaluate the susceptibility of pigs experimentally infected with a contemporary isolate after previous exposure with an historical strain.

Research objectives

Objective 1: To investigate persistence and tissue distribution of SVA in pigs experimentally-infected with an historical and a contemporary strain.

Research question: How long can pigs become persistently-infected with SVA? What are the tissues where SVA is harbored in persistently-infected pigs?

Objective 2*:* To evaluate the susceptibility of pigs infected with a contemporary isolate after previous exposure with an historical SVA strain.

Research question: Are animals protected against a heterologous SVA strain after previous exposure?

Materials & Methods

Animals and facilities: A total of 28 crossbred three-week-old piglets sourced from a sow system with no prior history of SVA infections were enrolled in the study. Sera and pooled oral swabs were collected for confirmation of naïve status in all piglets by indirect immunofluorescence (IFA) and real time qPCR respectively. Animals were housed in the University of Minnesota's Veterinary Isolation Unit (BSL-2) and were fed *ad libitum* commercial feed and water.

Virus isolates: An historical (99-14900\MN) and contemporary (19-19343\MN) isolates showing 87.5% nucleotide identity obtained from clinically-affected pigs in 1999 and 2017, respectively, were used.

Experimental design and sampling: Animals were divided into three groups: group CC, inoculated intranasally with 9.6 x 10^6 $TCID_{50}$ of the contemporary isolate (n=12); group HC, inoculated intranasally with 1.7 x 10^7 $TCID_{50}$ of the historical isolate (n=12); and a negative control group inoculated with sterile culture media (n=4). One pig from group CC was necropsied 39 days post-inoculation (dpi) due to SVA-unrelated death; and 3 additional animals from group CC, 4 animals from group HC and 2 animals from the control group were euthanized and necropsied 49 dpi. At day 50 post-inoculation (pi), the remaining 8 pigs from groups CC and HC were re-challenged intranasally with 2 x 10^7 $TCID_{50}$ of the contemporary strain, and the two control pigs were re-challenged with the sterile cell culture media. Sampling methods included oral, fecal and tonsil swabs, as well as tonsil scrapings, sera collection and oral fluids. Samples were tested by SVA real time qPCR, and serological testing was done by indirect immunofluorescence (IFA). The time points for collection of samples were on 1, 3, 7, 10, 14, 21, 28, 35, 41 and 48 dpi in the first challenge, and 2, 5, 7, 14 and 21 days post re-challenge (dpr).

	Acclimation	Inoculation of HC and CC groups[1]	Monitoring: clinical disease; viral shedding and neutralizing antibodies.										Necropsy 3 CC and 4 HC animals[2]
Days post-inoculation	-1	0	1	3	7	10	14	21	28	35	41	48	49

	Re-challenge[1]	Monitoring: clinical disease; viral shedding and neutralizing antibodies.				
Days post inoculation	50	52	55	57	64	71
Days post re-callenge	0	2	5	7	14	21

[1]CC group inoculated with contemporary and HC group with historical strain on day 0. Re-challenge of both groups with contemporary strain.
[2]One animal from group CC died and was necropsied on day 39.

Figure 1 – Representation of the experimental design and timeline (days).

Results and Discussion

Objective 1 - How long can pigs become persistently-infected with SVA? What are the tissues where SVA is harbored in persistently-infected pigs?

All the pigs were *Senecavirus A* IgG negative through IFA at the beginning of the study; and were also PCR negative in fecal swabs.

Detection of SVA RNA from oral swabs was possible until day 21 in the HC, with 3/12 animals yielding positive CT values (with an average CT of 35), and the last detection in the CC group was in one out of 12 animals (with a CT of 35). In fecal swabs, one out of the 12 animals from group HC had a positive PCR (CT 35) at 28dpi, and 5/12 animals from group CC were PCR positive (mean Ct value of 35). With this, we can conclude that shedding was occurring at low levels through saliva up until 21 and 28 dpi, and through feces until 28 and 35 dpi in HC and CC groups, respectively. However, tonsil scraping in live animals was able to detect SVA positive animals up until 48dpi in both groups, 13 days after last detection in fecal swabs, with 7/12 in the HC and 2/12 in the CC groups yielding mean Ct values of 34 in both groups.

One animal from the CC group died of SVA unrelated cause at day 39, and tissue testing showed PCR positive submandibular and retropharyngeal lymph nodes, with Cts of 29 and 28, respectively. At 49dpi, 2/3 animals from the CC group were PCR positive in the tonsils, submandibular and retropharyngeal lymph nodes, with mean CT values of 31, 30 and 30 respectively, and all were negative in the tissue homogenate. In the HC group, 4/4 animals necropsied at 49dpi had positive PCR results from the tonsils and submandibular lymph nodes, with mean CTs of 29 and 28 respectively, while 3/4 had a mean CT value of 30 in the retropharyngeal lymph node and 1/4 had a CT of 35 in the tissue homogenate. PCR results can be seen in table 1.

Group	Necropsy Day	Pig Id	Tonsil Soft Palate	Submandibular lymph node	Retropharyngeal lymph node	Tissue Homogenate
Contemporary	39	908	Neg	29.42	28.27	Neg
Control	49	911	Neg	Neg	Neg	Neg
Control	49	920	Neg	Neg	Neg	Neg
Historical	49	992	31.64	28.12	26.34	35.18
Historical	49	907	30.1	27.65	33.86	Neg
Historical	49	925	27.76	29.19	Neg	Neg
Historical	49	915	28.1	27.84	30	Neg
Contemporary	49	909	Neg	Neg	28.45	Neg
Contemporary	49	924	35.88	33.31	Neg	Neg
Contemporary	49	914	27.08	27.56	30.56	Neg

Table 1: Real time PCR results from days 39 and 49, before the re-challenge.

Interestingly, PCR results from the necropsies from 21 days after the re-challenge (71 days after the first inoculation) showed similar positivity rates in the different tissues, but with higher CT values. In the HC group, 7/8, 6/8 and 5/8 animals had positive PCR results from the tonsils, submandibular and retropharyngeal lymph nodes with average CTs of 33, 32 and 31 respectively, between the positive samples. The CC group had 5/8 animals with positive tonsils, submandibular and retropharyngeal lymph nodes with average CT values of 33, 33 and 32 respectively, among the positive samples. Tissue homogenates from both groups were all negative at this time point. PCR results from the necropsies after the re-challenge can be seen in table 2.

Group	Necropsy Day	Pig Id	Tonsil Soft Palate	Submandibular lymph node	Retropharyngeal lymph node	Tissue Homogenate
Control	71	913	Neg	Neg	Neg	Neg
Control	71	921	Neg	Neg	Neg	Neg
Historical	71	916	Neg	Neg	Neg	Neg
Historical	71	905	33.02	29.27	30.6	Neg
Historical	71	903	34.32	35.31	Neg	Neg
Historical	71	923	35.04	35.08	28.66	Neg
Historical	71	917	34.7	30.33	30.97	Neg
Historical	71	919	31.01	Neg	27.54	Neg
Historical	71	927	32.59	35.44	Neg	Neg
Historical	71	902	29.08	27.32	35.18	Neg
Contemporary	71	904	Neg	Neg	32.62	Neg
Contemporary	71	926	26.88	28.46	29.71	Neg
Contemporary	71	918	33.4	31.8	35.17	Neg
Contemporary	71	929	Neg	34.46	Neg	Neg
Contemporary	71	906	35.64	Neg	32.27	Neg
Contemporary	71	928	34.34	34.22	Neg	Neg
Contemporary	71	910	Neg	34.37	Neg	Neg
Contemporary	71	922	33.96	Neg	30.61	Neg

Table 2: Real time PCR results from 21 days after the re-challenge (71 days after first inoculation).

Results show a high detection rate of SVA RNA in the tonsils of the soft palate, submandibular and retropharyngeal lymph nodes up to 49dpi. These results corroborate with previous findings where the tonsils of naturally infected animals were PCR positive up until 90 days post infection. The tonsil of the soft palate is known to be colonized by other pathogens, such as porcine reproductive and respiratory syndrome virus, *Salmonella spp.* and classical swine fever virus, being a possible reservoir for infection of other animals. Due to the known tropism of SVA to lymphoid tissues, the lymph nodes might also be acting as harboring sites along with the tonsils. Even though it is not known if these sites can harbor SVA for the same amount of time as the tonsils, a previous study funded by the National Pork Board (#17-215) has shown that replication

of SVA happens as early as 6 and 12 hours post-inoculation at the submandibular and retropharyngeal lymph nodes, respectively. Despite PCR ability to detect SVA RNA in the tonsils and lymph nodes 21 days after the re-challenge, which would be expected, this same time point represents a 71 day period from the first contact with the virus and yielded higher CT values than the necropsies from day 49. While this finding show the protective effect of the humoral and cellular responses, it could also mean most animals would show negative results if followed for a longer period.

Objective 2 - Are animals protected against a heterologous SVA strain after previous exposure?

All animals were absent of clinical signs throughout the whole study after both the first inoculation with only the historical or contemporary strain and also after the re-challenge of both groups with the contemporary strain. However, viral shedding differed much between after both time points.

First challenge:
Shedding through saliva and feces was evaluated by oral and fecal swabs, and all animals from both groups had positive PCR results in both methods on days 3 and 7. Positivity rate in the oral swabs declined earlier, with 9/12 animals from the HC group and 6/12 from the CC group yielding positive PCR results at 14dpi, with the last detections in 3/12 HC and 1/12 CC on days 21 and 28 respectively. At day 21, all animals from both groups showed positive fecal swab PCR results, with the last detections on 1/12 HC and 5/12 CC animals at days 28 and 35 dpi, respectively. The last time points where tonsil swabs were able to yield positive results were the same as in the fecal swabs, with 2/12 and 1/12 animals from HC and CC groups showing positive results at days 28 and 35 respectively. Tonsil scrapings were the most efficacious in detecting SVA RNA in the experimentally infected animals, being able to yield positive results in all time points, with 7/12 and 2/11 positive PCRs from HC and CC groups at 48 dpi. Serum PCR results from HC and CC groups were positive in 12/12 and 11/12 animals respectively at 3dpi, and was detected up until days 28 and 35 in one animal from each group. Serological response was first detected at 10 dpi in the CC group and 14 dpi in the HC group, in 1/12 and 5/12 animals respectively. At 28 dpi, HC group had 12/12 animals with positive IFA results, while the CC group had 11/12. First time point where 12/12 animals from CC group had IFA positive results as at 42 dpi.

Second challenge:
Shedding of viruses in saliva and feces was greatly reduced after re-challenge of HC and CC groups with the contemporary strain. The only time point with positive PCR results was two days after the re-challenge. Three out of eight HC and CC animals had positive oral swabs and 8/8 HC and CC animals had positive fecal swabs. In the tonsil swabs, 0/8 HC animals were PCR positive, while 5/8 CC animals yielded positive results. Tonsil scrapings from the HC and CC animals were positive in 3/8 and 4/8 animals, respectively. All swab collections from the subsequent time points of collection yielded negative PCR results. Serum PCR results had one positive result from groups

HC and CC on days 5 and 7 after re-challenge respectively.

Results from after the first and second challenges show a great reduction in viral shedding after re-challenge with a homologous and heterologous strain.

Additional deliverable information:

Detection of SVA positive animals can be achieved easily by oral or fecal swabs in recently infected animals due to the high shedding of virus in the first 21 days of infection, and serological tests are also good tools to determine if an animal has had contact with the virus in the past. However, since SVA was known to be harbored in the tonsils of recovered animals, we decided to compare different diagnostic methods for detection of SVA positive animals across time. The methods for diagnostics were both in the individual (oral, fecal and tonsil swabs, and tonsil scrapings) and group level (oral fluids).

Oral, fecal and tonsil swabs were able to detect SVA RNA constantly in more than 90% of the animals from both groups up until 10 dpi, after which oral swabs had a more rapid decline in the following time points, followed by tonsil swabs and then fecal swabs. Last positive results in the three methods before the re-challenge were on 28 dpi in 4.2% of the oral swabs and 35 dpi in 20.8% fecal and 4.2% tonsil swabs. Tonsil scrapings started with a slightly lower detection rate in the first days, 79.2% positive results at 1 dpi, remaining positive in more than 90% of samples from day 3 to 10 post inoculation. The decline in the detection of SVA in the tonsil scrapings was less pronounced than in the other methods, with 41.7% of all animals being positive by tonsil scrapings at 35 dpi, while oral swabs was not detecting any positive animal, and fecal and tonsil swabs had 20.8% and 4.2% positive samples, respectively. The tonsil scrapings continued to detect positive animals in the two following samplings at 42 and 48 dpi, with 17.4% and 39.1% of positive samples in each time point respectively. Oral fluids were successful in detecting SVA constantly in both groups up until 35 dpi, but did not detect SVA on days 42 and 48.

After the re-challenge, oral, fecal and tonsil swabs were only able to detect SVA RNA on the second day after this second inoculation with the contemporary strain. Tonsil scraping was able to detect SVA in 38.9% of samples on day 2 post re-challenge, 0% on day 5, and 5.6% on day 7. No positive samples were detected through any method on days 14 and 21 after the re-challenge. Oral fluids only detected SVA on day 2 post re-challenge. Proportions of positive samples per sampling method can be seen on table 3.

Days after inoculation	Oral swabs	Fecal swabs	Tonsil swabs	Tonsil scrapings	Oral Fluids
0	0.0%	0.0%	0.0%	0.0%	0.0%
1	91.7%	95.8%	100.0%	79.2%	100.0%
3	100.0%	100.0%	100.0%	91.7%	100.0%
7	100.0%	100.0%	100.0%	95.8%	100.0%
10	95.8%	91.7%	95.8%	100.0%	100.0%
14	62.5%	83.3%	95.8%	79.2%	100.0%
21	16.7%	100.0%	29.2%	75.0%	100.0%
28	4.2%	16.7%	8.3%	45.8%	100.0%
35	0.0%	20.8%	4.2%	41.7%	100.0%
42	0.0%	0.0%	0.0%	17.4%	0.0%
48	0.0%	0.0%	0.0%	39.1%	0.0%
52 (2)	33.3%	88.9%	27.8%	38.9%	100.0%
55 (5)	0.0%	0.0%	0.0%	0.0%	0.0%
57 (7)	0.0%	0.0%	0.0%	5.6%	0.0%
64 (14)	0.0%	0.0%	0.0%	0.0%	0.0%
71 (21)	0.0%	0.0%	0.0%	0.0%	0.0%

Table 3: Proportions of positive samples in across time in the different diagnostic methods. Numbers in parenthesis represent the number of days after re-challenge.

Tonsil scraping has shown to be a promising method to detect SVA RNA in the tonsils of clinically recovered animals during the late-phase infection, and was able to detect SVA in 39.1% of the animals on day 48 post inoculation. It is not clear whether the second inoculation with the contemporary strain served as a booster dose for the immunological response in the animals which caused them to reduce the viral load to undetectable levels, or if the decrease in the detection would be seen regardless of the second mass exposure. Nevertheless, tonsil scraping should be considered as a tool for detection of asymptomatic carrier animals, being able to detect SVA positive animals up to 48 days after exposure.

Conclusions

Due to the concern that previously infected animals could be serving as a potential source of infection to naïve animals as reservoirs, we evaluated the ability of SVA to induce asymptomatic carrier state in pigs. Forty nine days after inoculation with the contemporary or historical strains, 2/3 and 4/4 necropsied animals from both groups showed positive PCR results in the tonsils of the soft palate, respectively. Other sites such as the submandibular and retropharyngeal lymph nodes, showed to be potentially important sites for the persistence of the virus. While oral, fecal and tonsil swabs were able to detect SVA positive animals up to 28, 35 and 35 days after inoculation respectively, tonsil scraping has shown to be a promising method to detect SVA RNA in the tonsils

of clinically recovered animals, by detecting SVA in 39.1% of the animals from both groups at 48 dpi.

Shedding of SVA in saliva and feces was greatly reduced after the re-challenge with a contemporary strain in both groups previously infected with either the historical or contemporary isolate.

These results show that animals can be asymptomatically infected with SVA after being thought to be clear of the virus, and could potentially be a source of infection to naïve animals. Also, animals that were experimentally infected with historical and contemporary strains were not susceptible to the contemporary strain after previous exposure with the heterologous or homologous strain. Tonsil scraping might be an important tool for the detection of asymptomatically infected animals.