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Detection of the Emerging Senecavirus A in Pigs, Mice and Houseflies

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Abstract

Senecavirus A (SVA) is an emerging picornavirus that has been recently associated with increased outbreaks of vesicular disease and neonatal mortality in swine. Many aspects of SVA infection biology and epidemiology remain unknown. Here, we present a diagnostic investigation conducted in swine herds affected by vesicular disease and increased neonatal mortality. Clinical and environmental samples were collected from affected and unaffected herds and screened for the presence of SVA by real-time PCR and virus isolation. Notably, SVA was detected and isolated from vesicular lesions and tissues from affected pigs, environmental samples, mouse feces, and mouse small intestine. SVA nucleic acid was also detected in houseflies collected in affected farms and in a farm with no history of vesicular disease. Detection of SVA in mice and housefly samples and recovery of viable virus from mouse feces and small intestine here, suggests that these pests may play a role on the epidemiology of SVA. These results provide important information that may allow the development of improved prevention and control strategies for SVA.

Key words: Senecavirus A, SVA, Seneca Valley virus, epidemiology.

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Introduction

Senecavirus A (SVA) is a single-stranded positive sense RNA virus of the genus *Senecavirus*, family *Picornaviridae* (1). The SVA genome is ~7.2 kb in length and contains a unique open reading frame encoding a large polyprotein (2,181 aa) which is cleaved to produce twelve mature proteins (5'-L-1A-1B-1C-1D-2A-2B-2C-3A-3B-3C-3D-3') (1). Among the picornaviruses, SVA is more closely related to members of the genus *Cardiovirus* (1), including the encephalomyocarditis virus (ECMV) and Theiloviruses which are known to infect a wide range of vertebrate animals including pigs, mice, and humans (2).

Senecavirus A was originally identified as a cell culture contaminant in the US in 2002 (1, 3); however, subsequent sequencing of picorna-like viruses isolated from pigs revealed the presence of the virus in the US swine population since 1988 (3–5). In the past ten years, sporadic reports have described the association of SVA with cases of swine idiopathic vesicular disease (SIVD) in Canada and in the US (3–6). Notably, since November 2014 several reports of SVA associated with vesicular disease in swine have been described in Brazil, and since March 2015 an increased number of cases of SVA associated with vesicular lesions and neonatal mortality in swine have been reported in the US (7–11).

Characteristic lesions associated with SVA infection in pigs include vesicles on the snout, oral mucosa, hoofs, and coronary bands, and typical clinical signs include lameness, lethargy, anorexia and fever (4–7, 9). Recently, SVA has also been associated with increased mortality (30-70%) in piglets less than seven days of age (7). In these cases, SVA has been detected in serum and multiple tissues of affected piglets, including brain, spleen, liver, heart, kidney, small

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intestine, and colon; however, no pathological changes have been observed in SVA positive tissues (10).

Many aspects of SVA infection biology, pathogenesis and epidemiology remain unknown, including the origin of the virus, its natural reservoirs and transmission pathways (11). Notably, a serologic survey conducted in the US, demonstrated neutralizing antibodies against SVA in swine, cattle and mice (3), suggesting a potential role for these species in the epidemiology of the virus. To date, however, SVA (live virus and/or nucleic acid) has been detected only in pigs and the actual contribution of other species for the virus ecology is still unknown.

Here, we present the results of a diagnostic investigation conducted in swine herds affected by vesicular disease and neonatal mortality in the US and in Brazil. By using real-time PCR (rRT-PCR) screening followed by virus isolation in cell culture, we detected SVA in clinical and environmental samples, including environmental swabs, mouse feces, and mouse small intestine. SVA nucleic acid was also detected in whole fly homogenates. Detection of SVA in mice and houseflies and isolation of the virus from mice suggest that these pests may play a role on SVA epidemiology.

Materials and Methods

Characteristics of target swine herds. A diagnostic investigation was conducted in swine herds located in the Midwest and Southern regions of the US and Brazil, respectively. The US case herd (herd A) is a 2800 sow farrow-to-wean commercial farm located in a high swine density area of south-central Minnesota. Mouse and fly samples were also collected from an unaffected farm (absence of vesicular disease; herd B) located at approximately 0.3 kilometers from herd A.

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Herd B consists of a 5400 sow farm divided into a 4000 sow farrow-to-wean herd and a 1400 sow gilt developer unit (GDU). These farms contain air filtration systems to minimize the risk of introduction of air borne pathogens.

Case herds C and D are small farrow-to-finish research farms (518 and 843 animals, respectively) that belong to the Brazilian Agricultural Research Corporation (Embrapa Swine and Poultry) and are located in Western Santa Catarina, Brazil. Case herd D is located at approximately 0.2 km from case herd C. Samples were also collected from an unaffected farrow-to-finish farm (absence of vesicular disease; herd E; 179 animals) located at approximately 0.2 km from case herds C and D. It is important to note that the farms sampled in our study have closed herds and no external animals had been introduced to their herds for at least six months preceding the vesicular disease outbreaks.

Diagnostic investigation. A diagnostic investigation was conducted in case herds A, C and D two-to-three days after the onset of the vesicular disease outbreaks. Clinical samples collected from case herd A included oral swabs, vesicular fluids, serum, and whole piglets. These samples were submitted to the University of Minnesota Veterinary Diagnostic Laboratory (UMN VDL), and referred to the Foreign Animal Disease Diagnostic Laboratory at the Plum Island Animal Disease Center for foreign animal disease investigation. Oral swabs and tissue samples were subjected to rRT-PCR for Senecavirus A and other vesicular diseases of swine, including Foot-and-mouth disease virus (FMDV), Vesicular stomatitis virus (VSV), Swine vesicular disease (SVD) and Vesicular Exanthema of Swine (VES). Tissue samples collected from piglets including small and large intestine pools were subjected to rRT-PCR assays.

Clinical samples from case herds C and D including vesicular fluid, nasal swabs, skin from ruptured vesicles, intestine and tonsil were subjected to conventional RT-PCR for SVA at

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Embrapa. At necropsy, a complete pathological examination was conducted in affected piglets. The diagnostic investigation at Embrapa was conducted under the supervision of Cidasc (Company of integrated agricultural development of Santa Catarina), the State's official agricultural inspection agency.

Environmental specimen collection and processing. Multiple environmental samples were collected from affected herd A following the diagnostic confirmation of SVA (Table 2). Samples were collected from the interior and exterior premises of herd A (Table 2). Interior samples included lesion swabs, 2 mL aliquots of injectable veterinary products (antibiotics, anti-inflammatory, etc), swabs from surfaces of walkways, personnel entry swabs, aliquots of semen, flies, whole mice, mouse feces and interior surface swabs of rodent bait boxes. Exterior samples included dust from exhaust fans, concrete pads and walkways, swabs of interior surfaces of feed bins, whole mice, interior surface swabs of bait boxes and mice feces, equipment swabs and flies. Since several mouse and fly samples collected from herd A tested positive for SVA nucleic acid, these samples were collected from an unaffected herd B. Additionally, a few mouse and fly samples were also collected from SVA affected (C and D) and unaffected herds (E) in Brazil.

Paint rollers were used to sample all interior and exterior surfaces and equipment, collecting dust from the exterior shutters of exhaust fans and sample feed bins (1). Insects were collected using sticky traps and jug traps (Wellmark International, Schaumberg, IL, USA) and whole fly homogenates processed as previously described (2, 11). Mouse fecal samples were removed from bait boxes using phosphate buffered saline (PBS) moistened Dacron swabs and stored in sterile conical tubes containing 3 mL of PBS (Fisher Scientific, Hanover Park, IL, USA). Similar methods were used to collect interior surface samples from bait boxes and the hands and soles of footwear of incoming personnel (3). Mice were dissected under a biosafety

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cabinet and tissues (small intestine, heart, lung, brain, liver spleen and kidneys) collected using standard aseptic technique. All external samples were collected in the immediate perimeter (within 5 m) of affected or unaffected farm barns.

RNA extraction and real-time PCR. Viral nucleic acid was extracted using the MagMAX viral RNA/DNA isolation kit (Life Technologies, Carlsbad, CA) following the manufacturer's instructions. Detection of SVA RNA was performed using a commercial real-time PCR kit targeting the SVA 3D polymerase gene (EZ-SVA, Tetracore Inc., Rockville, MD) or a conventional RT-PCR with primers targeting a region of the SVA VP1-VP3 genes (3). All real-time PCR screenings on environmental samples from herds A and B were performed at the SDSU Animal Disease and Research Diagnostic Laboratory (ADRDL).

Conventional RT-PCR was performed using SVA specific primers (SVV-1C556F and SVV-1D441R (3)) and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Carlsbad, CA) following the manufacturer's instructions. PCR amplicons were subjected to electrophoresis in 1% agarose gels and analyzed under a UV transilluminator.

Virus isolation. Virus isolation was performed in NCI-H1299 non-small cell lung carcinoma cell lines (ATCC CRL-5803). All samples were processed in PBS (10% v/w). After homogenization samples were cleared by centrifugation at 1,200 rpm at 4^{o}C. The supernatant was diluted (1:1) in RPMI 1640 medium supplemented with penicillin (300 U/mL), streptomycin (300 µg/mL) and amphotericin B (7.5 µg/mL), filtered (0.45 µm) and inoculated into semi-confluent (60-80%) monolayers of NCI-H1299 cells cultured in 24 well plates. After 1 h adsorption, 1 mL complete growth medium (RPMI 1640, 10% fetal bovine serum, 4 mM L-glutamine, penicillin [100 U/mL], spreptomycin [100 µg/mL] and amphotericin B [2.5 µg/mL]) was added to each well, and cells were incubated at 37^{o}C with 5% CO_2 for five days. Each

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sample was inoculated in duplicate wells and cell monolayers were monitored daily for cytopathic effect (CPE). Samples were subjected to five blind passages and those samples that did not induce CPE in inoculated monolayers after the fifth passage were considered negative. Mock-inoculated control cells were included as negative controls on each passage. Isolation of SVA was confirmed by rRT-PCR and immunofluorescence assays.

Immunofluorescence. Indirect immunofluorescence was used to confirm isolation of SVA. Infected cell cultures showing CPE were fixed with 3.7% formaldehyde for 30 min at room temperature, washed three times with PBS and permeabilized with 0.2% Triton-X100-PBS for 10 min at RT. After permeabilization, cells were washed three times with PBS and incubated with SVA specific monoclonal antibody (F61 SVV-9-2-1) (13) or swine convalescent serum. After primary antibody incubation, cells were washed as above and incubated with anti-mouse- (Alexa Fluor® 488; Life Technologies, Carlsbad, CA) or anti-swine-IgG (DyLight® 488; Bethyl Laboratories, Montgomery, TX) secondary antibodies, respectively.

SVA genome sequencing. The complete genome sequence of select Senecavirus A isolates obtained in this study were determined by using a primer walking RT-PCR sequencing approach. For this, nine sets of overlapping primers (F1 to F9) covering the entire SVA genome were designed (primer sequences available upon request) using the PRIMO primer design software (14). Viral cDNA was synthesized using the ProtoScript II reverse transcriptase (New England Biolabs, Ipswich, MA) and a mixture of oligo $d(T)_{18}$ and random primer 6 (NEB, Ipswich, MA). Two microliters of cDNA were used in PCR amplification reactions with the Q5® Hot Start High-Fidelity 2X Master Mix (NEB, Ipswich, MA) following to the manufacturer's protocols. The PCR amplicons (F1 to F9) were subjected to electrophoresis in 1% agarose gels to confirm correct target amplification and purified by using the GenJet PCR purification kit (Thermo

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Fisher Scientific, Waltham, MA). Purified PCR products were sequenced with fluorescent dideoxynucleotide terminators in an ABI 3700 automated sequencer (Applied Biosystems Inc., Foster City, CA). Sequence assembly and editing were performed with the LaserGene analysis software package (LaserGene, version 5.07; DNAStar Inc., Madison, WI).

SVA genome comparison and phylogenetic analysis. Alignment and comparison of the complete genome and VP1 nucleotide sequences between SVA isolates obtained here and those of SVA strains available on GenBank (Accession numbers, DQ641257, KC667560, KR063108, KR063107, KR063109, KT757280, KT757281, KT757282, KT321458, KR075677, KR075678, EU271758, EU271763, EU271757, EU271762, EU271761, EU271760, EU271759) were performed by using ClustalW (15) in the MEGA 6 software (16). The complete SVA genome and a 539 nt region of the VP1 gene were used to construct phylogenetic trees. Phylogenetic analysis was performed using the neighbor-joining method in MEGA 6 (MEGA, version 6) (16). The codon positions included in the analysis were the 1st, 2nd, 3rd, and non-coding.

Nucleotide sequence accession numbers. The complete genome sequences of SVA isolates MN15-84-4 (swab from mortality tractor bucket), MN15-84-8 (mouse fecal sample), MN15-84-M3 (mouse small intestine), MN15-84-21 (swab from internal hallway) and MN15-84-22 (lesion swab from sow) are available on GenBank under accession numbers KU359210, KU359211, KU359212, KU359213, KU359214.

Results

Clinical history and SVA diagnosis (Case herd A). On September 26, 2015 an outbreak of acute watery diarrhea followed by profound lethargy was observed in 3-7-day old piglets in two farrowing rooms of herd A. Morbidity of approximately 75% and mortality rates of 50% were

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observed in piglets housed in the affected farrowing rooms. Clinical signs including lethargy and diarrhea were subsequently observed in piglets from two other farrowing rooms of this farm (over 5-7 days), with morbidity and mortality rates of 25% and 15%, respectively. On September 29, 2015, an acute outbreak of vesicular disease was observed in approximately 75% of the sows in the gestation barns of herd A. Vesicles ranging from 1-3 cm in diameter were observed on the snout and coronary bands of affected sows and after 5-10 days these vesicles ruptured leaving a dry scab in the affected skin. Affected sows also presented vesicles in the dewclaws and demonstrated lameness.

The diagnostic investigation conducted at the UMN VDL revealed the presence of SVA nucleic acid in oral swabs collected from affected sows (seven out of seven tested [7/7]) and in intestinal homogenates from affected piglets (3/3). A summary of the diagnostic results is presented in Table 1. All tests performed for other vesicular diseases (FMDV, VSV, VES, and SED) resulted negative.

Clinical history and SVA diagnosis (Case herds C and D). Clinical signs were first observed in sows in the gestation barn of herd C on October 30, 2015. Initially, five animals presented vesicular lesions, including one sow with vesicles on the snout and coronary band of the hoof (Fig. 1) and four sows with ruptured vesicles on the snout. A total of 28 sows (25 in the gestation barn and three in the maternity barn) and two boars were affected (morbidity of 15%). Piglets were not affected in this herd. Six sows presented lameness and three days later ulcerative lesions were observed on the wall of the hoofs.

On November 3, clinical signs were observed in two sows in the maternity room of herd D (located at 0.2 kilometers from herd C). One sow presented an intact vesicle on the snout and another sow had a ruptured vesicle on the snout (morbidity of 1.5%). Interestingly, a sudden

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increase in neonatal mortality was observed in 3-5 day-old piglets. Mortality rates in piglets reached up to 34% one week after the first vesicular lesions were observed in the sows. In addition, the suckling piglets presented lethargy and watery diarrhea, over a period of approximately 5-7 days.

Diagnostic investigation conducted at Embrapa revealed the presence of SVA nucleic acid, in various clinical samples including vesicular fluid collected from the lesions, skin of ruptured vesicles, and in the small intestine, tonsil and coronary band of affected piglets. No SVA was detected in nasal swabs (Table 1). At necropsy, the piglets presented enlargement and edema of inguinal lymph nodes, empty stomach, ascites, severe edema of the mesocolon, and severe necrosis of the coronary bands (data not shown).

Detection of Senecavirus A in environmental samples. Environmental samples collected from case herd A and from unaffected herd B were screened by rRT-PCR. All samples were collected following standard sampling protocols (17–19), processed for viral RNA isolation and screened by SVA rRT- PCR at the SD ADRDL. A summary of the rRT-PCR screening results is presented in Table 2. Senecavirus A was detected in clinical samples (lesion swabs) and multiple environmental samples collected in the interior and exterior areas of the affected farm. Notably, the virus was detected in mouse fecal samples collected in bait boxes in the interior and exterior areas of case herd A. Additionally, the small intestine collected from a mouse on farm A was positive for SVA (Table 2; Fig. 2). All other tissues collected from this mouse, including heart, brain and lungs, were negative for SVA (Table 2). Fly samples collected in the affected farm (herd A) and in the unaffected farm (herd B) were also positive for SVA nucleic acid. In addition, a pool of flies collected in herd C (Brazilian farm) tested positive for SVA (Table 2).

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Isolation of Senecavirus A from environmental samples. Virus isolation (VI) was performed in select SVA PCR positive samples to assess the presence of viable virus. All VIs were performed in a highly permissive human non-small lung carcinoma cell line (H1299) (20). SVA isolation, as evidenced by development of CPE in inoculated cell cultures (Fig. 2A), was confirmed by rRT-PCR (Table 2) and by immunofluorescence assays (Fig. 2B). A summary of the VI results is presented in Table 2. Senecavirus A was isolated from multiple environmental samples. Most interestingly, SVA was consistently isolated from mouse fecal samples, and from a mouse small intestine collected in case herd A. No virus was isolated from PCR positive fly samples.

Genetic comparisons and phylogenetic analysis. Complete genome sequences of five SVA isolates (SVA-MN15-84-4, -MN15-84-8, -MN15-84-21, -MN15-84-22, and -MN15-308-M3) obtained from case herd A were compared to other SVA sequences available on GenBank. Complete genome sequence comparisons revealed that the isolates characterized here share 93-94% nucleotide identity with the prototype US SVA strain SVV001, 96% nt identity with an isolate obtained in Canada in 2011 (SVA-11-55910-3), 98-99% nt identity with other contemporary isolates recently obtained in the US (IA40380/2015, IA46008/2015 and SD41901/2015, 97% nt identity with Brazilian isolates (BRA-MG1/2015, BRA-MG2/2015 and BRA-GO3/2015) and 96% nt id with a recent Chinese isolate (CH-1-2015) (Table 3). Comparison of the amino acid sequences of SVA polyprotein (2181 aa) revealed that the MN isolates here share 97-99% aa identity with other SVA strains (Table 3). Comparisons based on a 541 nt region of the VP1 gene, revealed a similar genetic heterogeneity between these isolates (Table 4). A greater genetic divergence (86-88% nt id), however, was observed when the MN isolates were compared to historical isolates obtained prior to 2002 (Table 4).

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Phylogenetic reconstructions based on complete genome sequences or the VP1 gene show a clear phylogenetic separation between historical- (1988-2002) and contemporary- (2007-2015) SVA strains (Fig. 3; Fig. 4). Phylogenetic analysis based on the VP1 gene revealed that all the isolates obtained here cluster with other SVA strains currently circulating in swine in the US (Fig. 3). Similarly, contemporary SVA isolates from distinct geographic locations (i.e US, Brazil and China) form separate phylogenetic clusters (Fig. 3; Fig. 4). The virus that appears most closely related to the isolates characterized here is the strain SVA-IA40380/2015, obtained from an exhibition pig in Iowa (8) (Fig. 3 and Fig. 4).

Discussion

Senecavirus A has been associated with sporadic outbreaks of vesicular disease in pigs in the US since the late 1980's (3–6). Recently, however, an increased number of reports have described the association of SVA with vesicular disease and neonatal mortality in swine (7, 8). Notably, the number of cases of SVA has jumped from two in 2014 to over 100 in 2015 (Swine Health Monitoring Project, December 2015 report), which represents a significant increase in the incidence of infection. Since November 2014, SVA has also been frequently reported in swine in Brazil (7, 9). However, the factors that contributed for the re-emergence of SVA in the US and its emergence in Brazil remain unknown.

Here, we present the findings of a diagnostic investigation conducted in two swine breeding farms located in high swine density areas in the US and Brazil. In September and October 2015, outbreaks of vesicular disease and neonatal mortality were confirmed in two swine breeding farms located in Minnesota and in the Brazilian state of Santa Catarina. Senecavirus A nucleic acid was detected in vesicular fluid, oral and lesion swabs (coronary bands) from affected sows and in intestinal homogenates, tonsil and coronary swabs from

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affected piglets (Table 1). These findings corroborate the results of recent reports that describe the association of SVA with vesicular disease and neonatal mortality in swine (7, 9, 10). Additionally, results here are consistent with studies that demonstrated the detection of SVA in vesicular fluid, lesion swabs, lesion scrapings, and oral swabs, indicating that these specimens are appropriate for SVA diagnosis (9, 21). SVA nucleic acid has also been detected in serum and various tissues from affected animals (lymph nodes, tonsil, spleen and lung) (7, 21). Notably, piglets that died during SVA outbreaks presented high viral load in multiple tissues including brain, liver, lung, heart, kidney, small intestine and colon (10). Interestingly, SVA nucleic acid was not detected in nasal swabs collected from affected sows from herd C here (Table 1), whereas the possibility that they contained low amounts of viral nucleic acid bellow the detection limit of the RT-PCR assay used for testing cannot be formally excluded.

Clinically, the outbreaks investigated here were characterized by lethargy, watery diarrhea and increased neonatal mortality (15-50% in piglets less than seven days of age), which occurred concomitantly with vesicular lesions (snout and coronary bands) and lameness in sows (morbidity ranging from 1.5% to 75%). Clinical signs and neonatal mortality lasted for approximately five to ten days, when lesions started to subside and neonatal mortality decreased to pre-outbreak rates (5-8%). The morbidity and mortality rates observed here are similar to those described in recent reports of SVA outbreaks in Brazil and the US (7–10). Historically, SVA has been associated with vesicular lesions in adult animals (4–6, 9), whereas common findings in recent outbreaks include diarrhea and sudden death in suckling piglets (7). Similarly, in the outbreaks investigated here, piglets presented diarrhea, lethargy and mortality, which were followed by the development of vesicular lesions in sows. This was observed in two of the three affected herds (A and D), while in the third herd (C) only sows presented clinical signs (vesicular

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lesions and lameness). Although several aspects of SVA infection biology remain unknown (i.e. the pathogenesis of the disease in swine), our study presents additional evidence demonstrating the association of SVA with vesicular disease and neonatal mortality in pigs. Detection and isolation of the virus from lesions and tissues from affected animals coupled with the absence of other pathogens known to cause vesicular and/or enteric disease in swine strengthen the notion that SVA could be the cause of these distinct clinical presentations in swine. Indeed, vesicular disease has been recently reproduced in pigs inoculated with SVA (L. R. Joshi and D. G. Diel, unpublished data; K.J. Yoon, presented at the 2015 NA PRRSV Symposium and Conference for Research Workers on Animal Health Meeting, Chicago, IL, 5 to 8 December 2015), confirming the role of the virus as the etiologic agent of SIVD.

After the confirmation of SVA infection in pigs (Table 1), an investigation was conducted to assess possible sources of SVA within affected herds. For this, multiple environmental samples (Table 2) were collected from affected herd A and tested for the presence of SVA. SVA nucleic acid was detected and viable virus recovered from various environmental samples from herd A, including swabs from internal and external building surfaces, and farm tools and equipment. Interestingly, SVA was also detected and isolated from mouse fecal samples and from a mouse (*Mus musculus*) small intestine collected from farm A (Table 2; Fig. 2). Additionally, SVA nucleic acid was detected in houseflies (*Musca domestica*) collected within the premises of the affected farm A. Following detection of SVA in mouse and houseflies in herd A, we collected these samples from an unaffected farm in the US (herd B) and from affected (herd D) and unaffected farms (herd E) in Brazil. Notably, SVA nucleic acid was detected in houseflies collected on the exterior of the unaffected farm in the US (herd B; located at 0.3 kilometers of affected farm A), and on a pool of flies collected in the affected farm in

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Brazil (herd D) (Table 2). Results here demonstrate viable SVA in various environmental samples including farm tools, building surfaces and equipment and in multiple mouse samples (feces and intestine) collected in the SVA affected farm A. Given that these samples were collected after the SVA outbreak in swine, it is not possible to draw definitive conclusions about the source of the virus responsible for the outbreak. However, these results suggest that farm tools and equipment can potentially function as fomites and transfer SVA to susceptible animals within affected herds. Most interestingly, our data demonstrating that mice can carry and, perhaps, shed SVA in feces (Table 2), suggest that these pests may contribute to the spread of SVA within affected herds. Additional sampling and testing are needed, however, to determine the actual role of this species on the ecology of SVA. It would be interesting, for example, to collect mouse samples in areas where SVA has not been detected in swine to assess whether this species functions as a natural reservoir for SVA. Although no viable SVA was recovered from rRT-PCR positive housefly samples tested here, detection of the virus on this pest deserves consideration.

Mice and houseflies are among the most common and widely distributed pests, and have been frequently associated with infectious disease transmission to humans and animals (22, 23). They can serve as natural reservoirs or mechanical vectors for several bacterial and viral pathogens, including various picornaviruses such as enteroviruses and ECMV which are known to infect humans, pigs and other animal species (22, 23). Results here showing that mice can carry viable SVA (small intestine and feces) and flies can carry the virus' nucleic acid, suggest that these species may play a role in SVA ecology. Detection of SVA in mice, corroborate early serologic surveys that have shown the presence of neutralizing antibodies against SVA in this species (14% of samples tested were positive) (3). Although the results presented here

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359 demonstrate the association of SVA with mice and houseflies, additional studies are needed to
360 define the actual role and contribution of these species for SVA infection biology and
361 epidemiology. Studies designed to assess the susceptibility of mice to SVA infection and to
362 define sites of virus replication and routes and duration of virus shedding, and to assess the
363 survival of SVA in houseflies would shed light on the potential role of these pests on SVA
364 ecology and the actual risk they may pose to swine.

365 Many factors may have contributed for the recent emergence of SVA in swine (24),
366 including the genetic evolution of contemporary virus strains (Fig. 3 and Fig. 4). Genetic
367 comparisons between historical- (obtained between 1988 and 2002) and contemporary SVA
368 isolates (obtained between 2007 and 2015) here, revealed a high genetic diversity between these
369 viruses (86-88% at the VP1 gene; and 93-94% nt identity when the complete genome sequence
370 was compared). All contemporary isolates sequenced to date (including those obtained in the US,
371 Brazil and most recently in China), however, share 96-99% nt identity. Additionally,
372 phylogenetic analysis and inference of the evolutionary distances (data not shown) between SVA
373 isolates, revealed a marked evolution of contemporary SVA isolates, when compared to
374 historical viral strains (Fig. 3, and Fig.4). These evolutionary changes may have resulted in: 1. an
375 increased adaptation of SVA to pigs; or yet, 2. an improved ability of the virus to spread between
376 hosts and/or natural reservoirs. These possibilities, however, await experimental confirmation.

377 In summary, here we describe the detection of Senecavirus A in swine presenting
378 vesicular disease and neonatal mortality and in two common and widely distributed pests, mice
379 and houseflies. These results provide the first evidence of live SVA in mice, corroborating the
380 findings of Knowles at al. who detected neutralizing antibodies against SVA in this species (3).
381 These observations suggest that mice and houseflies may play a role on SVA epidemiology.

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TABLE 1 Detection of SVA in clinical samples following outbreak of vesicular disease in sow herds in the US and in Brazil.

Herd	Sample ID	Sample Description	PCR Result
A[a]	Sow 1	Oral swab	Positive
	Sow 2	Oral swab	Positive
	Sow 3	Oral swab	Positive
	Sow 4	Oral swab	Positive
	Sow 5	Oral swab	Positive
	Sow 6	Oral swab	Positive
	Sow 7	Oral swab	Positive
	Piglet 1	Intestine[c]	Positive
	Piglet 2	Intestine	Positive
	Piglet 3	Intestine	Positive
C[b]	Sow 150	Nasal swab	ND
	Sow 154*	Vesicular fluid	Positive
		Nasal swab	ND
	Sow 190	Nasal swab	ND
	Sow 271*	Vesicular fluid	Positive
		Nasal Swab	ND
		Coronary band of the hoof	Positive
	Sow 278	Nasal swab	ND
	Sow 346	Nasal swab	ND
	Sow 798	Vesicular fluid	Positive
	Sow 767	Vesicular fluid	Positive
	Sow 820	Nasal swab	ND
	Sow 966	Nasal swab	ND
	Sow 975	Nasal swab	ND
	Sow 988*	Vesicular fluid	Positive
		Nasal swab	ND
	Sow 1170	Nasal swab	ND
	Sow 1551	Nasal swab	ND
	Piglet 262	Intestine	ND
	Piglet	Intestine	Positive
D[b]	Piglet 1	Tonsil	Positive
	Piglet 2	Tonsil	Positive
	Piglet 2	Coronary band of the hoof	Positive

[a]Herd A located in Minnesota; [b]Herds C and D located in Santa Catarina, Brazil. Samples from herd A were tested by rRT-PCR while samples from herd C and D were tested by conventional RT-PCR. [c]Pooled small and large intestine homogenate. ND: not detected.

*These animals presented vesicular lesions. Vesicular fluids tested positive for SVA while nasal swabs were negative.

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503 TABLE 2 Detection and isolation of SVA from environmental samples collected in swine herds
504 in the US and in Brazil.

Herd	Sample ID	Sample Description	Real-time PCR (Ct)	Virus Isolation (VI)	VI Confirmation Real-time PCR (Ct)
A	1	Feed residue inside of bins	ND		
	2	Dust from exhaust fans	28.34	Positive	9.20
	3	Ground outside farm	29.62	Positive	8.48
	4¥	Mortality tractor bucket	31.6	Positive	10.37
	5	Vet vehicle cab	ND		
	6	Mouse feces in bait boxes (Gestation)	30.59	Positive	9.57
	7	Mouse feces in bait boxes (Gestation)	28.61	Positive	8.88
	8¥	Mouse feces in bait boxes (Gestation)	28.84	Positive	11.60
	9	Mouse feces in bait boxes (Farrowing)	35.74	NT	
	10	Mouse feces in bait boxes (Farrowing)	35.72	NT	
	11	Mouse feces in bait boxes (Farrowing)	36.8	NT	
	12	Antibiotic A injectable	ND		
	13	Oxytocin injectable	ND		
	14	Antibiotic B injectable	34.89	NT	
	15	Gentamycin injectable	ND		
	16	Mycoplasma hyopneumoniae vaccine	ND		
	17	Acepromazine injectable	ND		
	18	PCV2 vaccine	ND		
	19	Vitamin K injectable	ND		
	20	Prostaglandin injectable	ND		
	21¥	Internal hallway swab	22.92	Positive	11.42
	22¥	Snout lesion swab from affected sow	18.95	Positive	11.83
	23	Semen used prior to outbreak	ND		
	24	Semen used prior to outbreak	ND		
	25	Office mouse bait box	34.95	NT	
	26	Office floor	ND		
	27	Concrete pad entrance door	36.75	NT	
	28	Personnel hands & footwear swabs	ND		
	29	Personnel hands & footwear swabs	ND		
	30	Personnel hands & footwear swabs	ND		
	31	Personnel hands & footwear swabs	ND		
	32	External mouse feces	32.16	Positive	9.78
	33	External mouse feces	35.81	Positive	9.07
	34	External mouse feces	35.74	Negative	
	35	External mouse feces	ND		
	36	External mouse feces	ND		
	37	External bait box swab	33.85	Positive	9.31
	38	External bait box swab	35.02	Positive	7.62
	39	External bait box swab	ND		
	40	External bait box swab	ND		
	41	Feces collected on mice nest outside the farm	ND		

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	42	Small intestine - Mouse collected outside (M1)	ND		
		Spleen-M1	ND		
		Liver-M1	ND		
		Heart-M1	ND		
		Lung-M1	ND		
		Kidney-M1	ND		
		Brain-M1	ND		
	43	Small Intestine - Mouse outside (M2)	ND		
	44[¥]	Small Intestine - Mouse collected inside (M3)[¥]	30.93	Positive	13.06
		Heart-M3	ND		
		Lung-M3	ND		
		Brain-M3	ND		
	45	Small Intestine - Mouse collected inside (M4)	ND		
	46	Flies collected inside affected farm	27.27	Negative	
	47	Flies collected outside affected farm	26.39	Negative	
	48	Small Intestine - Bird collected outside	ND		
B	1	Internal bait box swab (Gestation)	ND		
	2	Internal bait box swab (Gestation)	ND		
	3	Internal bait box swab (Farrowing)			
	4	Internal bait box swab (Farrowing)	ND		
	5	Mouse feces exterior to farm	ND		
	6	Mouse feces in bait boxes (Gestation)	ND		
	7	Mouse feces in bait boxes (Gestation)	ND		
	8	Mouse feces in bait boxes (Gestation)	ND		
	9	Mouse feces in bait boxes (Gestation)	ND		
	10	Mouse feces in bait boxes (Gestation)	ND		
	11	Mouse feces in bait boxes (Gestation)	ND		
	12	Mouse feces in bait boxes (Farrowing)	ND		
	13	Mouse feces in bait boxes (Farrowing)	ND		
	14	Mouse feces in bait boxes (Farrowing)	ND		
	15	Mouse feces in bait boxes (Farrowing)	ND		
	16	Mouse feces in bait boxes (Farrowing)	ND		
	17	Mouse feces in bait boxes (Farrowing)	ND		
	18	Small Intestine - Mouse collected outside (M1)	ND		
		Spleen - M1	ND		
		Liver - M1	ND		
		Kidney - M1	ND		
		Lung - M1	ND		
		Brain - M1	ND		
	19	Flies collected outside unaffected farm B	31.67	Negative	
	20	Flies collected inside unaffected farm B	ND		
	21	Flies collected outside unaffected farm B	ND		
	22	Flies collected inside unaffected farm B	ND		
	23	Flies collected outside unaffected farm B	ND		
	24	Flies collected inside unaffected farm B	ND		
	25	Flies collected outside unaffected farm B	ND		
	26	Flies collected inside unaffected farm B	35.66	Negative	
C	1	Flies collected inside affected farm C	Positive	NT	
	2	Intestine mice collected inside 1	ND		
	3	Intestine mice collected inside 2	ND		
E	1	Flies collected inside unaffected farm	ND		

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A. Affected farm (Minnesota); B. Unaffected farm (MN); C. Affected farm (Santa Catarina, Brazil); E: Unaffected farm (SC, Brazil). ND: Not detected; NT: Not tested. Samples from herds A and B were tested by rRT-PCR while samples from herd C were tested by conventional RT-PCR. ¥ Virus isolates for which complete genome sequences were obtained.

TABLE 3 Complete genome sequence comparisons between the SVA isolates MN15-84-4, MN15-84-8, MN15-84-21, MN15-84-22 and MN15-308-M3 and other historical and contemporary SVA strains.

	% identity for SVA isolate[a]:									
	MN15-84-4		MN15-84-8		MN15-84-21		MN15-84-22		MN15-308-M3	
SVA isolate[b]	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
SV001	93	97	94	97	93	97	93	97	94	97
11-55910-3	96	98	96	98	96	98	96	98	96	98
IA40380/15	99	99	99	99	99	99	99	99	99	99
IA46008/15	98	99	98	99	98	99	98	99	98	99
SD41901/15	98	99	98	99	98	99	98	99	98	99
BRAMG1/15	97	99	97	99	97	99	97	99	97	99
BRAMG2/15	97	99	97	99	97	99	97	99	97	99
BRAGO1/15	97	99	97	99	97	99	97	99	97	99
CH-1-15	96	98	96	98	96	98	96	98	96	98
MN15-84-4	-	-	99	99	99	100	99	99	99	99
MN15-84-8	99	99	-	-	99	100	99	100	99	100
MN15-84-21	99	99	99	100	-	-	99	100	99	100
MN15-84-22	99	99	99	100	99	100	-	-	99	100
MN15-308-M3	99	99	99	100	99	100	99	100	-	-

[a]Sequence alignment performed by using CLUSTAL W (15).
[b]GenBank accession numbers are DQ641257, KC667560, KT757280, KT757282, KT757281, KR063107, KR063108, KR063109, KT321458, KU359210, KU359211, KU359212, KU359213, KU359214, respectively.

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TABLE 4 Comparison of nucleotide sequences of VP1 gene between historical and contemporary SVA isolates

	% identity for SVA isolate[a]				
SVA isolate	MN15-84-4	MN15-84-8	MN15-84-21	MN15-84-22	MN15-308-M3
SV001	91	91	91	91	91
11-55910-3	94	94	94	94	94
131395	92	93	93	93	93
1278	90	90	90	90	90
89-47752	88	88	88	88	88
92-48963	87	88	88	88	88
90-10324	87	88	88	88	88
88-36695	86	86	86	86	86
88-23626	86	86	86	86	86
IA40380/15	97	100	100	100	100
IA46008/15	99	99	99	99	99
SD41901/15	99	99	99	99	99
BRAMG1/15	97	97	97	97	97
BRAMG2/15	97	97	97	97	97
BRAGO1/15	97	97	97	97	97
BRA/UEL-B2	97	97	97	97	97
BRA-UEL-A1	96	97	97	97	97
CH-1-15	96	96	96	96	96
MN15-84-4	-	99	99	99	99
MN15-84-4	99	-	100	100	100
MN15-84-21	99	100	-	100	100
MN15-84-22	99	100	100	-	100
MN15-308-M3	99	100	100	100	-

[a]A 541 nt region of the VP1 gene (nucleotide positions 2680-3220 of the genome of SVA prototype strain SVV001) were aligned by using CLUSTAL W (15).

[b]GenBank accession numbers are DQ641257, KC667560, EU271763, EU271757, EU271762, EU271761, EU271760, EU271759, KT757280, KT757282, KT757281, KR063107, KR063108, KR063109, KR075678, KR0756787, KT321458, KU359210, KU359211, KU359212, KU359213, KU35921,4 respectively.

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547 **Fig 1** Clinical presentation of Senecavirus A in sows. Vesicular lesions on the snout (**A**) and
548 coronary bands (**B**) a sow from herd C. (**A**) Large vesicles (1-3 cm in diameter, orange arrow)
549 were filled with fluid, ruptured and dried out after 5-10 days leaving a dry scab covering the
550 affected skin. (**B**) Dried scab (yellow arrow) on the wall of the hoof and ruptured vesicle on the
551 coronary band (orange arrow).

552 **Fig 2** Isolation of Senecavirus A in cell culture. (**A**) Cytopathic effect (rounded cells) observed in
553 H1299 non-small lung cell carcinoma cells following inoculation of mouse fecal sample into
554 semi-confluent monolayers (48 hours post inoculation; right panel). Mock-infected control cells
555 (left panel). (**B**) Immunofluorescence assay confirming isolation of SVA from environmental
556 samples. SVA specific monoclonal antibody (F61 SVV-9-2-1 (13), left panels) or convalescent
557 serum (right panels) were used. SVA isolates shown were obtained from a swab from the snout
558 of a sow (SVA-MN15-84-22), mouse small intestine (SVA-MN15-308-M3), mouse fecal sample
559 (SVA-MN15-84-8), and from an environmental swab (SVA-MN15-84-21). Magnification 200X,
560 scale bar 50 µm.

561
562 **Fig 3** Evolutionary relationships of Senecavirus A based on complete genome sequences. The
563 evolutionary history was inferred using the Neighbor-Joining method (25). The optimal tree with
564 the sum of branch length = 0.11793138 is shown. The percentage of replicate trees in which the
565 associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the
566 branches (26). The tree is drawn to scale, with branch lengths in the same units as those of the
567 evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were

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computed using the Maximum Composite Likelihood method (27) and are in the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 7098 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (16).

Fig 4 Evolutionary relationships of Senecavirus A based on the VP1 gene. The evolutionary history was inferred using the Neighbor-Joining method (25). The optimal tree with the sum of branch length = 0.21986844 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (26). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (27) and are in the units of the number of base substitutions per site. The analysis involved 22 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 539 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (16).

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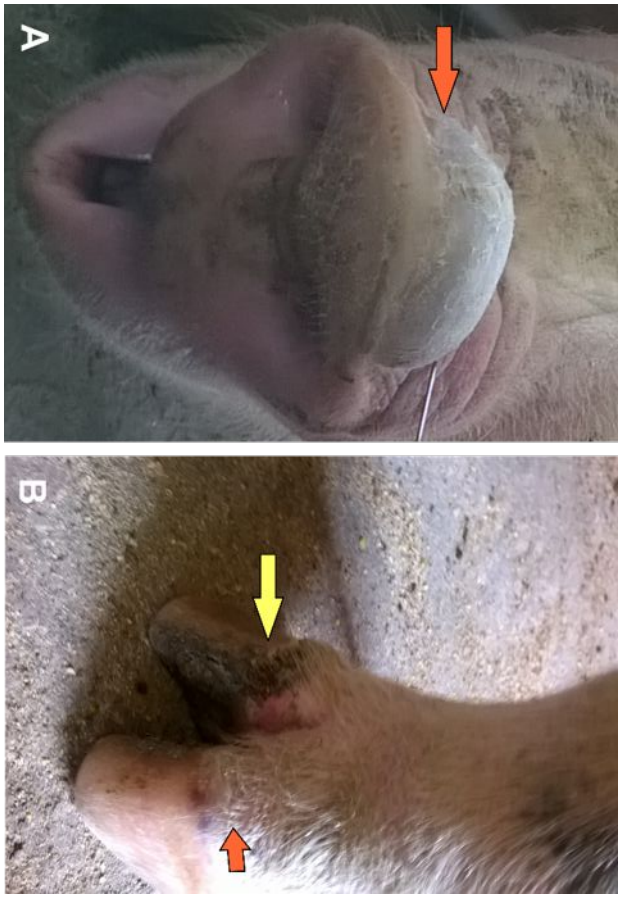
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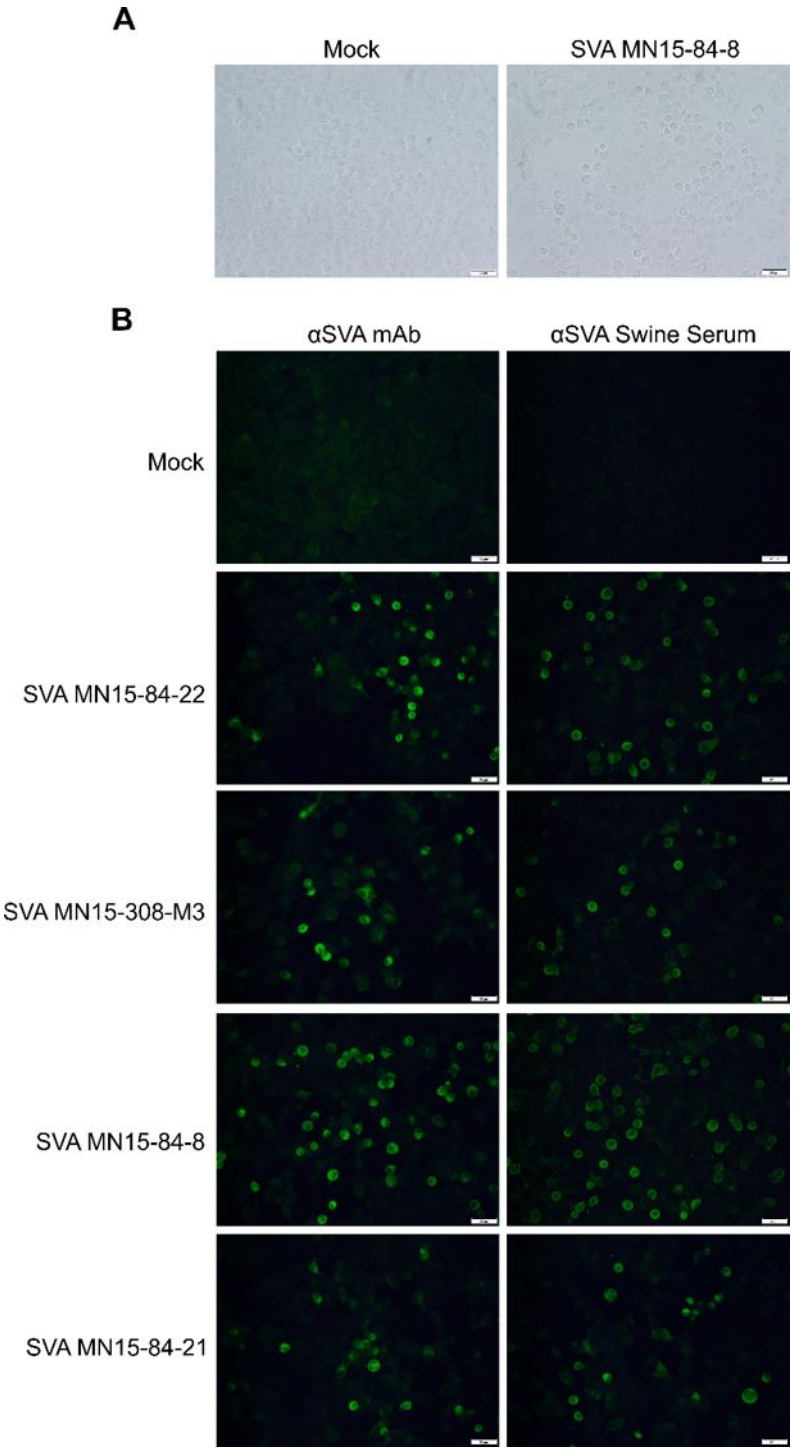

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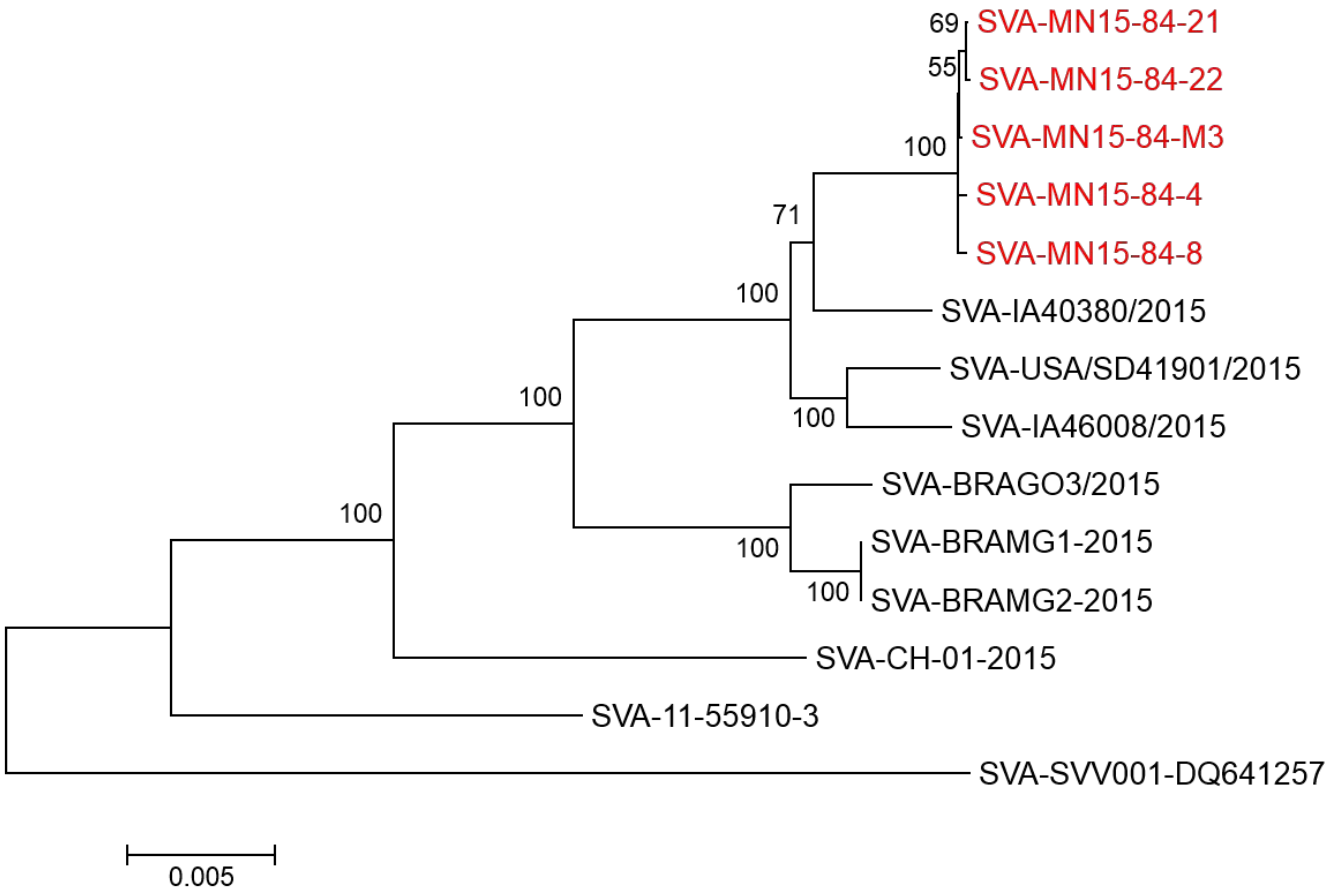

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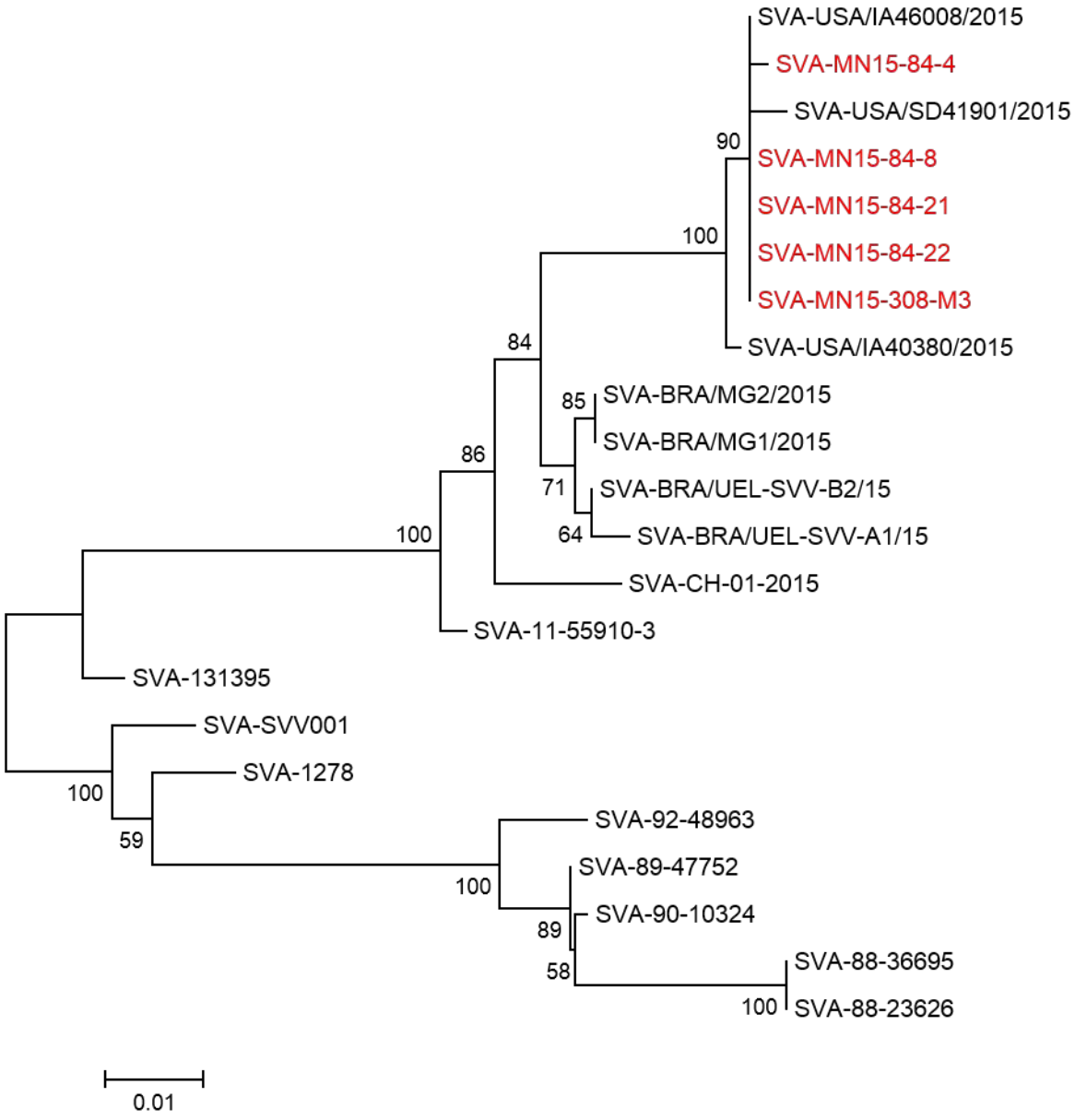

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