Development of a novel real-time RT-PCR assay to detect Seneca Valley virus associated with emerging cases of vesicular disease in pigs

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Abstract

Seneca Valley virus (SVV) can cause vesicular disease that is clinically indistinguishable from foot-and-mouth disease, vesicular stomatitis and swine vesicular disease. SVV-associated disease has been identified in pigs in several countries, namely USA, Canada, Brazil and China. Diagnostic tests are required to reliably detect this emerging virus, and this report describes the development and evaluation of a novel real-time reverse-transcription (RT) PCR assay (rRT-PCR), targeting the viral polymerase gene (3D) of SVV. This new assay detected all historical and contemporary SVV-1 isolates examined (n=8), while no cross-reactivity was observed with nucleic acid template prepared from other vesicular disease viruses or common swine pathogens. The analytical sensitivity of the rRT-PCR was 0.79 TCID₅₀/ml and the limit of detection was similar using two different RT-PCR master-mixes. The performance of the test was further evaluated using pig nasal (n=25) and rectal swab samples (n=25), where concordant results compared to virus sequencing were generated for 43/50 samples. The availability of this assay, will enable laboratories to rapidly detect SVV in cases of vesicular disease in pigs, negated for notifiable diseases, and could enable existing knowledge gaps to be investigated surrounding the natural epidemiology of SVV.

Keywords: Seneca Valley virus; *Senecavirus A*; Swine; Veterinary Diagnostics; rRT-PCR; real-time

Text

Seneca Valley virus (SVV) is a non-enveloped, single-stranded, positive-sense RNA virus belonging to the species *Senecavirus A* (genus *Senecavirus*, family *Picornaviridae*) (Knowles et al., 2012; Adams et al., 2015). The complete genome sequence was first described by Knowles and Hallenbeck (2005) and shown to be related to, but distinct from, the cardioviruses. Initially isolated from pigs in 1988 (Knowles et al., 2006) and then later confirmed as an extraneous agent in PER.C6 cells (Hales et al., 2008), SVV has been linked to idiopathic vesicular disease in pigs in the USA, Canada, Brazil and China (Pasma et al., 2008; Singh et al., 2012; Leme et al., 2015; Vannucci et al., 2015; Hause et al., 2016; Guo et al., 2016; Wang et al., 2016; Wu et al., 2016). Since its emergence in Brazil, it is estimated that around 80% of swine farms have been affected, with rapid spread across the country over a period of months (Leme et al., 2015; Vannucci et al., 2015). Recently, SVV has been experimentally confirmed to cause vesicular disease in piglets using a contemporary 2015 SVV field isolate from the USA (Chen et al., 2016; Montiel et al., 2016). The clinical signs associated with the vesicular manifestation of SVV infection are indistinguishable from those caused by foot-and-mouth disease (FMD), swine vesicular disease (SVD) and vesicular stomatitis (VS) with pigs presenting with vesicles on the snouts and coronary bands, lameness, anorexia, lethargy, and fever and exclusion of FMD, SVD or VS on affected farms is required by the animal health authorities. The importance of investigating possible notifiable disease in SVV cases is evident, however it results in disruption and additional costs. There is also some concern in affected countries that the emergence of vesicular disease-associated SVV could adversely affect prompt reporting of FMD in pigs.

Current laboratory methods developed for the diagnosis of SVV include virus isolation, virus neutralisation, competitive ELISA, conventional reverse transcriptase (RT) PCR and a SYBR Green-based real-time RT-PCR assay (rRT-PCR) (Knowles et al., 2005; Yang et al., 2011; Leme et al., 2015; Bracht et al., 2016). The rRT-PCR assay developed by Bracht et al. (2016) targets the variable VP1-coding region, and its design was based on a limited number of sequences available in GenBank. However, the increasing availability of full genome sequences has now made it possible to design TaqMan® based rRT-PCR assays based on highly conserved

regions of the SVV genome that complement the front-line rRT-PCR tests used for other vesicular diseases (Reid et al., 2004; Shaw et al., 2007; Reid et al., 2009; Wilson et al., 2009).

This paper therefore describes the development and evaluation of a novel TaqMan® rRT-PCR assay, targeting the conserved viral polymerase 3D region for the detection of SVV. This new assay was validated at both Kansas State Veterinary Diagnostic Laboratory (KSVDL) and the Pirbright Institute (TPI) using established rRT-PCR reagents and equipment at each location. Ten published SVV full genomes (DQ641257, KC667560, KR063107, KR063108, KR063109, KT757280, KT757281, KT757282, KT321458 and KX349733) and seven unpublished SVV partial genome sequences (EU271766-EU271772) (Table 1) were used for the design of the primers; SVV3D-F1: 5'-AGAATTTGGAAGCCATGCTCT-3'; SVV3D-R1: 5'-GAGCCAACATAGARACAGATTGC-3' and the probe, SVV3D-Pr1: 5'-FAM-TTCAAACCAGGAACACTACTCGAGA-BHQ1-3'. The forward primer and probe have 100% match with all 17 aligned sequences (Table 1). The reverse primer has one mismatch with one isolate NJ-90-10324 and thus the reverse primer was designed with a degenerate base (C/T=Y) at nucleotide location 7089 (Table 1).

SVV RNA was extracted from a range of cell culture supernatants and clinical samples (Table 2) using the KingFisher Flex automated extraction platform (ThermoFisher Scientific, UK) and the MagMAX™-96 Viral RNA Isolation Kit (ThermoFisher Scientific, UK) following manufacturer guidelines. The fluorogenic rRT-PCR was performed using either Path-ID™ Multiplex One-Step Kit (Applied Biosystems, UK) and/or SuperScript® III Platinum® One-Step qRT-PCR Kit (Invitrogen, UK). The Path-ID™ Multiplex One-Step Kit reaction mix was employed at KSVDL and was prepared using 10 µl 2X buffer and 1× reaction mix: 0.8 µl (10 µM) forward primer, 0.8 µl (10 µM) reverse primer, 0.4 µl (10 µM) probe, 1 µl nuclease-free water and 2 µl of enzyme mix. Amplification conditions consisted of reverse-transcription (RT) performed at 48°C for 10 min followed by 95°C for 10 min and then 45 cycles of PCR, with each cycle consisting of 95°C for 15 s and 60°C for 50 s. The SuperScript® III Platinum® One-Step qRT-PCR Kit reaction mix was employed at TPI and was prepared using 12.5 µl 2X buffer and 1× reaction mix: 2 µl (10 µM) forward primer, 2 µl (10 µM) reverse primer, 1.5 µl

(5 µM) probe, 1 µl molecular biology grade water, 0.5 µl ROX (diluted 1/10 in nuclease-free water) and 0.5 µl of enzyme mix. In all cases, 5 µl of extracted RNA was used per reaction. Amplification conditions consisted of RT performed at 60°C for 30 min, followed by 95°C for 10 min and then 50 cycles of PCR, with each cycle consisting of 95°C for 15 s and 60°C for 60 s. All rRT-PCR reactions were performed using an Applied Biosystems 7500 Fast real-time PCR instrument (Applied Biosystems, UK) for assays were performed at TPI or Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (Hercules, CA, USA) for assays performed at KSVDL.

The analytical sensitivity of the rRT-PCR was defined using a 2-fold dilution series of a SVV isolate (KS15-01). Parallel testing by virus isolation showed that the sensitivity of the test was 0.79 TCID₅₀/ml corresponding to a C_T value of 36.8 (Figure 1). Furthermore, the limit of detection (LOD) of the assay, using reference strain IA-89-47552 was equivalent using the Path-ID™ Multiplex One-Step Kit (efficiency of assay was 104.53% calculated by $-1+10^{(-1/\text{slope})}$; endpoint C_T value: 34.71) and SuperScript® III Platinum® One-Step qRT-PCR Kit (efficiency of assay was 110.33% calculated by $1+10^{(-1/\text{slope})}$; endpoint C_T value 37.58) reaction mixes. The rRT-PCR assay detected eight historical and contemporary SVV isolates (NC-88-23626; MN-88-36695; IA-89-47552; NJ-90-10324; IL-92-48963; LA-97-1278, CA-01-131395 and KS15-01) collected over the period 1988-2015 (Table 2). Diagnostic specificity was confirmed using RNA extracted from samples containing vesicular disease viruses (FMD virus, SVD virus and VS virus) and RNA or DNA extracted from other common swine pathogens, including porcine circovirus type 2 (PCV2); porcine parvovirus (PPV); porcine group A rotavirus (GA-rota); porcine group B rotavirus (GB-rota); porcine group C rotavirus (GC-rota); atypical porcine pestivirus (APPV); porcine deltacoronavirus (PDCoV), porcine epidemic diarrhoea virus (PEDV); epizootic hemorrhagic disease virus (EHDV), European type or type 1 porcine reproductive and respiratory syndrome virus (PRRSV-EU); North American type or type 2 porcine reproductive and respiratory syndrome virus (PRRSV-NA); and swine Influenza A virus (SIV) (Table 2).

The performance of the assay was further evaluated with 25 nasal and 25 fecal (rectal) swab pools (5 individual pig swabs/pool) collected in 2015 from North Carolina, USA pigs without clinical signs. Each nasal

sample pool, comprised five nasal swabs which had been eluted into 1ml phosphate buffered saline (PBS). Each fecal sample pool comprised five fecal swabs, each of which had been in contact with 1-2g of pig feces prior to pooling in a sterile collection tube. Unfortunately, it was not possible to pair the nasal and fecal pools to match the same animal groups and therefore the data presented in Figure 2 represent independent results. The presence of SVV in the samples was confirmed by Next-generation sequencing (NGS) using an Illumina MiSeq (Illumina, San Diego, CA) protocol that adopted random primers and was performed as described by Hause et al. (2015). Of these 50 sample pools, SVV was sequenced in 24 of these (Figure 2), while for 25 samples no SVV sequence reads were detected, with one pool unconfirmed by sequencing (pool 23). From these, the rRT-PCR assay produced concordant results for 21/24 positive nasal and fecal sample pools, and 21/25 negative nasal and fecal sample pools. Four samples were positive by rRT-PCR when negative sequencing results had been obtained, and three sample pools were negative by rRT-PCR when positive sequencing results had been obtained (Figure 2). The four samples which were reported positive by rRT-PCR all had $C_T > 37$. The three samples which were rRT-PCR negative but sequence positive only contained 1, 3, and 7 reads, respectively, while the average reads for rRT-PCR positive samples was 1,802 reads per sample. These samples would therefore be considered very weak positives and therefore not be expected to be detected by the rRT-PCR assay, since they are below the determined analytical threshold. In this study, the use of rectal swabs tended to result in more samples with inconsistencies between rRT-PCR and MiSeq results, further work is required to understand this observation should fecal swabs be selected as a sample type for routine surveillance of SVV.

In conclusion, this new rapid and sensitive rRT-PCR could be adopted by reference laboratories to promptly detect SVV in vesicular disease cases that have been negated for notifiable diseases such as FMD. Since the assay design targets the highly conserved 3D region, this SVV rRT-PCR assay is expected to be more robust than other assays targeting more variable regions of the SVV genome. In addition, this assay could be used as a research tool for pathogenesis and epidemiological studies, including investigations into the periods and route of virus excretion, the existence of virus carrier status, and the presence of virus in non-porcine hosts.

Conflict of interest statement

The authors declare that there was no conflict of interest in the preparation of this manuscript.

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References

- Adams, M.J., Lefkowitz, E.J., King, A.M.Q., Bamford, D.H., Breitbart, M., Davison, A.J., Ghabrial, S.A., Gorbalenya, A.E., Knowles, N.J., Krell, P., Lavigne, R., Prangishvili, D., Sanfaçon, H., Siddell, S.G., Simmonds, P., Carstens, E.B., 2015. Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2015). *Arch. Virol.* 160, 1837–1850.
- Bracht, A. J., O’Hearn, E. S., Fabian, A. W., Barrette, R. W., & Sayed, A. (2016). Real-Time Reverse Transcription PCR Assay for Detection of Senecavirus A in Swine Vesicular Diagnostic Specimens. *PLoS ONE*, 11(1), e0146211. <http://doi.org/10.1371/journal.pone.0146211>
- Chen, Z., Yuan, F., Li, Y., Shang, P., Schroeder, R., Lechtenberg, K., Henningson, J., Hause, B., Bai, J., Rowland, R., Clavijo, A., Fang, Y. 2016. Construction and characterization of a full-length cDNA infectious clone of newly emerging porcine Seneca Valley virus. *Virology*. In press.

Guo B, Piñeyro PE, Rademacher CJ, Zheng Y, Li G, Yuan J, Hoang H, Gauger PC, Madson DM, Schwartz KJ, Canning PE, Arruda BL, Cooper VL, Baum DH, Linhares DC, Main RG, Yoon KJ. 2016. Novel Senecavirus A in Swine with Vesicular Disease, United States, July 2015. *Emerg Infect Dis.* 22(7):1325-7.

Hales, L.M., Knowles, N.J., Reddy, P.S., Xu, L., Hay, C., Hallenbeck, PL., 2008. Complete genome sequence analysis of Seneca Valley virus-001, a novel oncolytic picornavirus. *J. Gen. Virol.* 89, 1265–1275.

Hause, B.M., Collin, E.A., Anderson, J., Hesse, R.A. and Anderson, G., 2015. Bovine rhinitis viruses are common in U.S. cattle with bovine respiratory disease. *PLoS ONE* 10, e0121998.

Hause, B. M., Myers O., Duff, J., Hesse, R. A. 2016. Senecavirus A in Pigs, United States, 2015. *Emerging Infectious Diseases.* 22, 1323-25.

Knowles, N.J., Hovi, T., Hyypiä, T., King, A.M.Q., Lindberg, A.M., Pallansch, M.A., Palmenberg, A.C., Simmonds, P., Skern, T., Stanway, G., Yamashita, T., Zell, R. (2012). *Picornaviridae*. In: *Virus Taxonomy: Classification and Nomenclature of Viruses: Ninth Report of the International Committee on Taxonomy of Viruses*. Ed: King, A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J. San Diego: Elsevier, pp 855-880.

Knowles, N.J., Hallenbeck, P. 2005. A new picornavirus is most closely related to cardioviruses. EUROPIIC 2005: XIIIth Meeting of the European Study Group on the Molecular Biology of Picornaviruses, Lunteren, The Netherlands, 23rd-29th May 2005. Abstract A14.

Knowles, N.J., Hales, L.M., Jones, B.H., Landgraf, J.G., House, J.A., Skele, K.L., Burroughs, K.D., Hallenbeck, P.L. 2006. Epidemiology of Seneca Valley virus: identification and characterization of isolates from pigs in the United States. Northern Lights EUROPIIC 2006: XIVth Meeting of the European Study Group on the Molecular Biology of Picornaviruses, Saariselkä, Inari, Finland, 26th November-1st December 2006. Abstract G2.

Leme, R.A., Zotti, E., Alcantara, B.K., Oliveira, M.V., Freitas, L.A., Alfieri, A.F., Alfieri, A.A. 2015 Senecavirus A: an emerging vesicular infection in Brazilian pig herds. *Transbound. Emerg. Dis.* 62, 603-611.

Montiel, N., Buckley, A., Guo, B., Kulshreshtha, V., VanGeelen, A., Hoang, H., Rademacher, C., Yoon, K.J., Lager, K. 2016. Vesicular disease in 9-week-old pigs experimentally infected with Senecavirus A. *Emerg. Infect. Dis.* 22, 1246-1248.

Pasma, T., Davidson, S., Shaw, S.L. 2008. Idiopathic vesicular disease in swine in Manitoba. *Can. Vet. J.* 49, 84-85.

Reid, S.M., Ebert, K., Bachanek-Bankowska, K., Batten, C., Sanders, A., Wright, C., Shaw, A.E., Ryan, E.D., Hutchings, G. H., Ferris, N.P., Paton, D.J. and King, D.P. 2009. Performance of real-time RT-PCR for the detection of foot-and-mouth disease virus during field outbreaks in the United Kingdom in 2007. *J. Vet. Diagn. Inves.* 21 (3): 321-330.

Reid, S.M., Ferris, N.P., Hutchings, G.H., King, D.P. and Alexandersen, S. 2004. Evaluation of real-time reverse transcription polymerase chain reaction assays for the detection of swine vesicular disease virus. *J. Virol. Meth.* 116 (2): 169-176.

Shaw, A.E., Reid, S.M., Ebert, K., Hutchings, G.H., Ferris, N.P. and King, D.P. 2007. Implementation of a one-step real-time RT-PCR protocol for diagnosis of foot-and-mouth disease. *J. Virol. Meth.* 143 (1): 81-85.

Singh, K., Corner, S., Clark, S.G., Scherba, G., Fredrickson, R., 2012. Seneca Valley virus and vesicular lesions in a pig with idiopathic vesicular disease. *J. Vet. Sci. Technol.* 3, 123.

Vannucci, F.A., Linhares, D.C., Barcellos, D.E., Lam, H.C., Collins, J., Marthaler, D., 2015. Identification and complete genome of Seneca Valley Virus in vesicular fluid and sera of pigs affected with idiopathic vesicular disease, Brazil. *Transbound. Emerg. Dis.* 62, 589-593.

Wang L, Prarat M, Hayes J, Zhang Y. 2016. Detection and Genomic Characterization of Senecavirus A, Ohio, USA, 2015. *Emerg Infect Dis.* 22(7):1321-3.

Wilson, W.C., Letchworth, G.J., Jiménez, C., Herrero, M.V., Navarro, R., Paz, P., Cornish, T.E., Smoliga, G., Pauszek, S.J., Dornak, C., George, M., Rodriguez, L.L., 2009. Field evaluation of a multiplex real-time reverse transcription polymerase chain reaction assay for detection of Vesicular stomatitis virus. *J Vet Diagn Invest.* Mar;21(2):179-86.

Wu, Q., Zhao, X., Chen, Y., He, X., Zhang, G., Ma, J. 2016. Complete genome sequence of Seneca Valley Virus CH-01-2015 identified in China. *Genome Announc.* 4(1). pii: e01509-15.

Yang, M., van Bruggen, R., Xu, W. 2012. Generation and diagnostic application of monoclonal antibodies against Seneca Valley virus. *J. Vet. Diagn. Invest.* 24, 42-50.

Table 1.

Alignment of SVV rRT-PCR primers and probe against 17 field isolate 3D sequences (nt 7024-7102 on DQ641257).

SSene3D-F1	AGAATTGGAAGCCATGCTCT	
SSene3D-R1		GCAATCTGTCTCTATGTTGGCTC
SSene3D-Pr1	TTCAAACCAGGAACACTACTCGAGA	
NC-88-23626	AGAATTGGAAGCCATGCTCTCTCTACTTCAAACCAGGAACACTACTCGAGAAGCTGCAATCTGTTTCTATGTTGGCTC	
MN-88-36695	-----	
IA-89-47552	-----	
NJ-90-10324	-----C-----	
IL-92-48963	-----	
LA-97-1278	-----	
CA-01-131395	-----	
SVV-001	-----	
CAN_11-55910-3	-----	
BRA/MG1/2015	-----	
BRA/MG2/2015	-----	
BRA/GO3/2015	-----	
USA/IA40380/2015	-----	
USA/SD41901/2015	-----	
USA/IA46008/2015	-----	
CH-01-2015	-----	
KS15-01/2015	-----	

-, identical to the sequence of NC-88-23626. nt: nucleotide.

Table 2. Viruses used to evaluate the performance of the SVV rRT-PCR

Virus	Virus/sample designation	Location	Country	Sample type	Date collected	Use in study
SVV	NC-88-23626	North Carolina	USA	IB-RS-2	1988	DSe
SVV	MN-88-36695	Minnesota	USA	IB-RS-2	1988	DSe
SVV	IA-89-47552	Iowa	USA	IB-RS-2	1989	DSe/ASe
SVV	NJ-90-10324	New Jersey	USA	IB-RS-2	1990	DSe
SVV	IL-92-48963	Illinois	USA	IB-RS-2	1992	DSe
SVV	LA-97-1278	Louisiana	USA	IB-RS-2	1997	DSe
SVV	CA-01-131395	California	USA	IB-RS-2	2001	DSe
SVV	KS15-01	Kansas	USA	PK-15	2015	ASe
FMDV	O/UAE/2/2003	United Arab Emirates	UAE	BTY1	2003	DSp
SVDV	UKG/155/80	United Kingdom (UK)	UK	OS	1980	DSp
VS NJV	29344/Columbia/2000	Columbia	Columbia	OS	2000	DSp
VS INV	29356/Columbia/2000	Columbia	Columbia	OS	2000	DSp
PCV2	Clinical sample	Iowa	USA	lung	2014	DSp
GA-Rota	Clinical sample, n=2	Kansas	USA	feces	2016	DSp
GB-Rota	Clinical sample, n=2	Kansas	USA	SI, feces	2015	DSp
GC-Rota	Clinical sample, n=2	Kansas	USA	feces	2016	DSp
APPV	Clinical sample, n=2	Kansas	USA	serum	2016	DSp
PDCoV	Clinical sample, n=2	Kansas	USA	feces	2016	DSp
PEDV	Clinical sample, n=2	Kansas	USA	feces	2016	DSp
EHDV	Clinical sample, n=2	Kansas, Missouri	USA	spleen	2015	DSp
PRRSV-EU	Cell cultural isolate (SD01-08)	South Dakota	USA	serum	2001	DSp
PRRSV-NA	Clinical sample, n=2	Kansas, Hawaii	USA	serum	2015, 2016	DSp
SIV	Clinical sample, n=2	Kansas	USA	NS, lung	2012	DSp

IB-RS-2: renal swine cells. BTY: Primary bovine thyroid cells. OS: Original epithelial suspension. SVV: Seneca Valley virus. PK-15: Porcine kidney cells. SI: Small intestine. NS: Nasal swab; FMDV: foot-and-mouth disease virus; VS NJV: vesicular stomatitis New Jersey virus; VS INV: vesicular stomatitis Indiana virus. SVDV: swine vesicular disease virus; PCV2: porcine circovirus type 2; GA-Rota: porcine group A rotavirus; GB-Rota: porcine group B rotavirus; GC-Rota: porcine group C rotavirus; APPV: atypical porcine pestivirus, PDCoV: porcine deltacoronavirus, PEDV: porcine epidemic diarrhea virus, EHDV: epizootic hemorrhagic disease virus, PRRSV-EU: European genotype or type 1 porcine reproductive and respiratory syndrome virus; PRRSV-NA: North American type or type 2 porcine reproductive and respiratory syndrome virus; SIV: swine Influenza A virus. DSe: Diagnostic sensitivity; ASe: Analytical sensitivity; DSp: Diagnostic specificity.

Figure 1. a) Standard curve of the SVV rRT-PCR; b) Analytical sensitivity of the SVV rRT-PCR. Correlation between TCID₅₀ and rRT-PCR threshold cycles (C_T) generated from 10-fold dilution series of KS15-01. Points represent mean of triplicate values with error bars representing the standard deviation above and below the mean, although the max range in C_T between triplicate values was 0.04-0.17.

Figure 2. Concordance between SVV rRT-PCR and MiSeq of a) nasal (●) or b) rectal/fecal (○) swabs collected from North Carolina, USA. Each data point represents a pool containing five individual samples.