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Development of a real-time PCR assay based on primer-probe energy transfer for the detection of swine vesicular disease virus*

M. Hakhverdyan[1], **T. B. Rasmussen**[2], **P. Thorén**[1], **Å. Uttenthal**[2], and **S. Belák**[1]

[1]Joint Research and Development Division, Department of Virology, The National Veterinary Institute and the Swedish University of Agricultural Sciences, Uppsala, Sweden
[2]Department of Virology, Danish Institute for Food and Veterinary Research, Lindholm, Denmark

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Summary. A real-time PCR assay based on primer-probe energy transfer (PriProET) was developed to detect swine vesicular disease virus (SVDV). Specificity tests of SVDV and heterologous virus showed specific amplification of SVDV strains only. The amplification plot for the closely related Coxsackievirus B5 remained negative. The sensitivity of assay was five copies of viral genome equivalents. A key point of the assay is tolerance toward mutations in the probe region. Melting curve analysis directly after PCR, with determination of probe melting point, confirmed specific hybridisation of the SVDV strains. Eight of twenty SVDV strains tested, revealed shifted melting points that indicated mutations in the probe region. All predicted mutations were confirmed by nucleotide sequencing. With the PriProET system there is a chance to identify phylogenetically divergent strains of SVDV, which may appear negative in other probe-based real-time PCR assays. At the same time, any difference in melting points may provide an indication of divergence in the probe region. The high sensitivity, specificity, and tolerance toward mutations in the probe region of the SVDV PriProET assay may improve the early and rapid detection of a wide range of SVDV strains, allowing reduced turnaround time and the use of high-throughput, automated technology.

*Nucleotide sequence data reported are available in the GenBank database under the accession numbers DQ250542–DQ250562.

Introduction

Swine vesicular disease (SVD) is a contagious and economically important infectious disease of pigs. SVD was first observed in Italy in 1966 [20]. Subsequent outbreaks have occurred periodically in European and Far East countries [3, 5, 8, 12, 18]. SVD is characterised by the appearance of vesicles on the tongue, in the mouth, and on the feet and hocks. Swine vesicular disease virus (SVDV) strains may vary in virulence, and the disease may be subclinical, mild, or severe, but complete recovery may occur in 2–3 weeks [10]. According to the Office International des Epizooties (OIE), the main importance of the disease is that the clinical signs produced by the SVDV are indistinguishable from those caused by foot-and-mouth disease virus (FMDV) and vesicular stomatitis virus (VSV) (http://www.oie.int). This means that all outbreaks of vesicular disease in pigs must be carefully investigated.

SVDV belongs to the genus *Enterovirus*, in the family *Picornaviridae* [20]. The virus has a single-stranded positive-sense RNA genome with a length of 7.4 kb. Only one serotype of SVDV has been described, and at least seven phylogenetically distinct groups have been identified by comparing the nucleotide sequences of the VP1 or 3BC genes [16, 30]. SVDV is antigenically closely related to the human pathogen Coxsackievirus B5 (CV-B5) [9, 11, 13, 29]. It can be distinguished from CV-B5 by cross-neutralisation, immunodiffusion, and IgM ELISA tests [4, 5, 31].

The conventional laboratory diagnosis of SVD is based on various methods, such as virus isolation, antigen and antibody ELISA, immunohistochemistry, in-situ hybridisation, RT-PCR-ELISA, RT-PCR, and nested RT-PCR [2, 6, 7, 16, 17, 19, 21, 22, 28]. Although promising results have been obtained with conventional PCR assays, these methods are time-consuming and laborious and have high risk of cross-contamination. Real-time PCR opens new possibilities to overcome these problems and further increases the sensitivity and speed of the diagnosis and differentiation from other vesicular diseases. The need for rapid and sensitive SVDV detection and virus discrimination from FMDV and VSV have accelerated the development of real-time PCR [14, 25–27]. However, many real-time PCR systems are sensitive to genetic mutations [23], hence possibly hindering the detection of some variants of the virus. The real-time PCR system described in the paper is able to overcome this problem.

Primer-probe energy transfer (PriProET) has been successfully applied for the detection of porcine circovirus type 2 (PCV2), FMDV, and VSV [15, 23, 24]. The basic principle of the assay relies on fluorescence resonance energy transfer (FRET) from donor to reporter fluorophore. Briefly, one of the two primers is labelled with a donor fluorescence dye (FAM) at the 5′-end, in addition to the hybridisation probe, which is labelled with an reporter fluorescence dye (Texas Red or Cy5) at the 3′-end. During the PCR cycles, when the probe anneals to the extended fluorescent primer, energy is transferred from the donor to the reporter due to their close proximity. The reporter emits fluorescence that is monitored and quantified. A detailed description of this real-time detection system is given in our recent publication [23].

In this paper, we report the application of PriProET real-time PCR assay for the rapid, highly specific and sensitive detection of SVDV. The paper continues a series of new real-time PCR assays recently developed for the vesicular cluster of viruses using the PriProET system [23, 24]. The system is robust and it provides a powerful diagnostic tool. Besides being sensitive and specific, it tolerates mismatches in the probe region, and therefore even viruses that generate mutations in the genome during the course of evolution are detected.

Materials and methods

Viruses

SVDV strains; FMDV O and C; VSV New Jersey and Indiana; human CV-B5 strain Faulkner used in this study were obtained from CISA-INIA (Valdeolmos, Madrid, Spain) and IAH (Pirbright, UK) and are listed in Table 1.

RNA extraction and preparation of cDNA

Samples from CISA-INIA: Initial steps of total RNA extraction from blood, epithelial cells, and faeces from experimentally infected animals at CISA-INIA were performed using Tripure isolation reagent (Roche Molecular Biochemicals, Germany) [1]. RNA was precipitated in a mix containing ethanol and sodium acetate and supplied to the National Veterinary Institute (SVA, Uppsala, Sweden). The final extraction steps – washing with 80% alcohol, drying, and resuspension in a final volume of 50 µl of DMPC (dimethylpyrocarbonate) water were performed at SVA.

Samples from IAH: Samples have a cell culture origin. Total RNA inactivated in TRIZOL (Sigma, St. Louis, Mo, USA) were supplied to the SVA and extracted according to the manufacturer's instructions. RNA was recovered in 50 µl of sterile, nuclease-free DMPC water and either used immediately or stored at −70 °C until further analysis.

cDNA was produced in 25 µl reaction volume using 5 µl RNA, 1 µl random hexamers (pdN_6, 0.02U, Amersham, Uppsala, Sweden), 2.5 µl dNTPs (2 mM), 5 µl 5X First Strand Buffer, 1 µl RNAguard (1000 U, Amersham Bioscience, USA), and 1 µl M-MLV reverse transcriptase (200 U, Ambion, Austin, Texas, USA). The reaction was incubated for 5 min at 22 °C, followed by 90 min at 37 °C, and the enzyme was finally inactivated by heating for 5 min at 95 °C.

Primers and probe design

The SVDV oligonucleotide primers and probe were designed from the published sequence of the 3D gene of the NET/1/92 strain (GenBank accession no. AF268065). Group-specific alignments of DNA sequences were performed using Lasergene software (DNASTAR, Inc., version 5, Madison, WI, USA). Primer and probe sequences were selected from a highly conserved region of the 3D gene using Primer Express™ software (version 1.0, Applied Biosystems, Foster City, CA, USA). A compatible primer set was selected to ensure efficient amplification and detection of most strains of SVDV, but to exclude closely related viruses like CV-B5 (Table 2). Finally, specificity was tested using blast search at NCBI (www.ncbi.nlm.gov).

The nucleotide sequence of the forward primer SVDV-3D-6511-F was 5′-(FAM)-TCAA CCCGGGCATCGTTAC, the reverse primer SVDV-3D-6617-R was 5′-TGAATAGTCAAAC GCTATGAGATGTC and the probe SVDV-3D-6553-P 5′-GGGTCACACCCAACGGCGCT-(Texas Red)-3′. Forward primer was labelled with a donor fluorophore (FAM, 6-carboxy-

Table 1. Viruses tested in the study. The specific melting temperature (T_m) of the probe for each SVDV strain is displayed together with corresponding number of mutations in the probe region

No.	Virus	Strain	Country of origin	T_m °C	No. of mutations	Institute provider
1	SVDV	ITL 1/66*	Italy	57.5	2	CISA-INIA
2	SVDV	ITL 1/66	Italy	57.5	2	IAH
3	SVDV	ITL 72	Italy	70.5	–	CISA-INIA
4	SVDV	ITL 1/91	Italy	65.2	1	IAH
5	SVDV	ITL 1/92	Italy	65.3	1	IAH
6	SVDV	ITL 19/92	Italy	70.3	–	IAH
7	SVDV	ITL 8/93	Italy	70.5	–	IAH
8	SVDV	ITL 8/94	Italy	70.3	–	IAH
9	SVDV	ITL 3/97	Italy	70.3	–	IAH
10	SVDV	HKN 11/72	Hong Kong	70.5	–	CISA-INIA
11	SVDV	HKN 8/73	Hong Kong	65.0	1	IAH
12	SVDV	HKN 1/80	Hong Kong	58.7	2	IAH
13	SVDV	HKN 11/81	Hong Kong	65.1	1	IAH
14	SVDV	HKN 1/89	Hong Kong	59.5	2	IAH
15	SVDV	UKG 27/72	UK	70.4	–	CISA-INIA
16	SVDV	SPA 1/93	Spain	70.5	–	CISA-INIA
17	SVDV	POR 1/03	Portugal	70.4	–	IAH
18	SVDV	NET 3/92	Netherlands	70.3	–	IAH
19	SVDV	GRE 1/79	Greece	70.3	–	IAH
20	SVDV	SWI 1/74	Switzerland	70.3	–	IAH
21	SVDV	FRA 1/73	France	65.5	1	IAH
22	CV-B5	Faulkner	USA	–	–	CISA-INIA
23	FMDV	C, PHI 9/94	Philippines	–	–	IAH
24	FMDV	O, NET 3/2001	Netherlands	–	–	IAH
25	VSV	New Jersey	USA, 1984	–	–	IAH
26	VSV	New Jersey	Colombia, 1964	–	–	CISA-INIA
27	VSV	Indiana-1	USA, 1942	–	–	CISA-INIA
28	VSV	Indiana-2	Argentina, 1963	–	–	IAH
29	VSV	Indiana-3	Brazil, 1964	–	–	IAH

* ITL 1/66 strain was provided from two different institutes: CISA-INIA (Centro de Investigación en Sanidad Animal, del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Valdeolmos, Madrid, Spain) and IAH (Institute for Animal Health, Pirbright, UK)

fluorescein) at the 5′-end, whereas the probe was labelled with a reporter fluorophore (Texas Red) at the 3′-end. Primers were synthesized at DNA Technology A/S (Aarhus, Denmark) and the probe at TIB MOLBIOL (Berlin, Germany). The resulting amplicon had a length of 107 bases, and the distance between the FAM and the Texas Red fluorophores was 22 bases.

Precautions to reduce contamination

Three separate localities were used in order to prevent contamination: a first room (pre-PCR) to prepare PCR reaction mix; a second room (PCR-1) to add template to the reaction tubes; and

Table 2. Conservative regions and nucleotide mismatches at primers/probe sites of 26 SVDV (GenBank and sequencing of PCR products) and three CV-B5 3D-gene sequences

GenBank accession	Forward primer (SVDV-3D-6511-F) 19 bp	Probe (SVDV-3D-6553-P)[a] 20bp	Reverse primer (SVDV-3D-6617-R)[a] 26bp
number/strain	TCAACCCGGGCATCGTTAC	AGCGCCGTTGGGTGTGACCC	GACATCTCATAGCGTTTGACTATTCA
AF268065 NET 1/92	. . . . .	. . . . .	. . . . .
AY429470 HK'70	. . . . .	. . . . .	. . . . .
D00435 H/3 '76	. . . . .	. . . . .	. . . . .
D16364 J/1 '73	. . . . .	. . . . .	. . . . .
X54521 UKG 27/72	. . . . .	. . . . .	. . . . .
AJ245863 O '72	. . . . . C	. . . . .	. . . . .
DQ250560 ITL 1/66[b]	. . . . .	. . T . . . . . C	. . . . . T . . . . .
DQ250550 ITL 1/66[c]	. . . . .	. . T . . . . . C	. . . . . T . . . . .
DQ250557 ITL 72	. . . . .	. . . . .	. . . . . T . . . . .
DQ250549 ITL 1/91	. . . . . C . . .	. . . . . C	. G . . . . . A . . . . .
DQ250548 ITL 1/92	. . . . . C . . .	. . . . . C	. G . . . . . A . . . . .
DQ250547 ITL 19/92	. . . . .	. . . . .	. . . . .
DQ250559 ITL 8/93	. . . . .	. . . . .	. . . . . C . . .
DQ250545 ITL 8/94	. . . . .	. . . . .	. . . . . T . . . . .
DQ250546 ITL 3/97	. . . . . T	. . . . .	. . . . . T . . . . .
DQ250561 HKN 11/72	. . . . .	. . . . .	. . . . .
DQ250551 HKN 8/73	. . . . . T	. . . . . C	. . . . .
DQ250553 HKN 1/80	. . . . . A . . . . . T	. . . . . T . . . . . C	. . . . .
DQ250554 HKN 11/81	. . . . .	. . . . . T	. . . . .
DQ250552 HKN 1/89	. . . . .	. . T . . T	. . . . . C . . .
DQ250558 UKG 27/72	. . . . .	. . . . .	. . . . .
DQ250562 SPA 1/93	. . . . . A	. . . . .	. . . . . T . . . . .
DQ250543 POR 1/03	. . . . .	. . . . .	. G . . C . . . . . T . . . . .
DQ250544 NET 3/92	. . . . .	. . . . .	. . . . .
DQ250555 GRE 1/79	. . . . . A	. . . . .	. . . . .
DQ250542 SWI 1/74	. . . . .	. . . . .	. . . . .
DQ250556 FRA 1/73	. . . . .	. . . . . T . .	. . . . . C . . . . .
AF114383 CV-B5[d]	. A . . T . . A . . G . . T . . G . .	. . T . . T	. T . . C . . T . . . . . C . . . . . T . . C . . T
AY875692 CV-B5[e]	. . . . . A . . . . . C . .	. . T . . T . . . . . A . . . . . T . .	. . . . . C . . . . .
X67706 CV-B5[f]	. G . . T . . A . . G . . T . . A . .	. . T . . A . . . . . A . . . . . T . .	. . . . C . . . . . T . . . . . T C

[a]Complementary sequence, 5′-3′
[b]Obtained from CISA-INIA, Valdeolmos, Madrid, Spain
[c]Obtained from IAH, Pirbright, UK
[d]Faulkner strain
[e]2000/CSF/KOR strain
[f]1954/85/UK strain

a third room to carry out real-time PCR. Furthermore, special tube holders and individual tube openers were used to avoid cross-contamination. The inner working surface of the laminar flow cabinet in the PCR-1 room was always wiped with a 10% chlorine solution and constantly exposed by UV-light after finishing the work.

PriProET-PCR assay

PriProET real-time PCR was performed in a total volume of 25 µl in a 96-well plate or in 0.2-ml optical tubes (Applied Biosystems) under the following optimised conditions: each 25-µl reaction contained 2 µl of cDNA, 0.5 µM of forward FAM-primer and 0.2 µM reverse primer, 0.3 µM of the probe, 0.5 mM dNTPs, 2.5 µl of 10X TITANIUM *Taq* PCR buffer (400 mM Tricine-KOH [pH 8.0], 160 mM KCl, 35 mM $MgCl_2$, 37.5 µg/ml BSA), 0.5 µl of 50X TITANIUM *Taq* DNA polymerase (BD Bioscience, Clontech Laboratories), and DMPC-water.

The real-time PCR thermodynamic conditions were 95 °C for 2 min, followed by 55 cycles of amplification: 95 °C for 15 sec, 60 °C for 15 sec, 72 °C for 15 sec. This was immediately followed by a melting point analysis: [95 °C for 15 sec], 96 cycles of [50 °C for 10 sec with

auto-increments of 0.5 °C]. Data were collected at 60 °C, and the entire melting curve analysis was used to confirm specific amplification of the target virus. Ct was determined during the exponential phase of PCR and defined as the cycle at which the fluorescence exceeded the baseline fluorescence. The baseline was set to the mean value of TexasRed fluorescence in cycles 5–15 plus 3 times the standard deviation in the same cycles. An ABI PRISM 7700 Sequence Detector was used for the experiments (Applied Biosystems, Foster City, CA, USA). Fluorescent data were analysed using an in-house pre-programmed Excel datasheet [23], supplemented by a programme for drawing of melting peaks (Adept Scientific, DK).

Sensitivity determination and PCR efficiency

The sensitivity of PriProET real-time PCR was determined using a 107-bp synthetic DNA template ordered from DNA Technology. The sequence of the template was based on the exact match of the 3D gene fragment used for the primers/probe design (NET/1/92 strain, GenBank accession no. AF268065). The number of copies in the stock solution was determined using the molarity of the template and Avogadro's formula. Ten-fold dilutions series from 10^9 to 1 copy were prepared in TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) containing 10 μg/ml salmon sperm DNA (Ambion, Austin, TX, USA) to assure a constant amount of nucleic acids in the diluted samples. A dilution containing five copies of synthetic amplicon was included in the sensitivity test as well. A standard curve was generated, and PCR efficiency was calculated using formula $E = 10^{(-1/a)} - 1$, where E is the PCR amplification efficiency and a is the slope value, which was obtained after the generation of standard curve. All tests were performed in triplicate.

Specificity tests

Specificity was tested using all available SVDV strains obtained from CISA-INIA (Madrid, Spain) and IAH (Pirbright, UK). Heterologous virus strains were included as well (Table 1). All 20 SVDV strains were detected, while CV-B5, FMDV, and VSV were negative. PCR products were tested on a 2% agarose gel to confirm predicted product size of amplified SVDV.

Direct sequencing of PCR products

In order to reveal a correlation between the melting temperature and the number of mutations in the probe region, PCR products of 20 SVDV strains (Table 1) were purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and subsequently sequenced on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the corresponding flanking primers (5′-3′): SVDV-3D-6407-F CGACAAAGTGGC CAAGGGAAA (forward) and SVDV-3D-6932-R ATCGAT(G/A)GGCCACGGGTATGAA (reverse). The flanking primers were designed using Lasergene software.

Results

Assay optimisation

The initial test of the PriProET assay using UKG 27/72 and ITL 8/93 strains showed specific amplification of viral nucleic acids, but with an early lag phase in the amplification plot and decreased endpoint fluorescence. The melting point also rapidly decreased due to competition between hybridisation of the probe and re-hybridisation of the two strands of the amplicon. In order to circumvent this

problem, a primer titration experiment was performed. Optimisation of primer concentration showed that the assay worked most efficiently in asymmetric PCR when forward FAM-primer was added in excess (0.5 μM) and reverse non-labelled primer in 0.2 μM. Probe titration, performed with the optimal primer ratios, showed that 0.3 μM was superior.

The next step was optimisation of the assay at different annealing temperatures ($T_a = 50$, 55, 60 °C) using 10-fold dilutions of the UKG 27/72 strain (from undiluted sample to 10^{-6}, in triplicates). The results showed that $T_a = 60$ °C gave higher specificity and sensitivity. The detection limit was 10^{-6} at $T_a = 60$ °C, while PCRs at $T_a = 50$ and 55 °C did not detect 10^{-6} dilutions. However, $T_a = 55$ °C gave 1–2 cycle lower Ct value compared with $T_a = 50$ and 60 °C. As this is a diagnostic assay, the higher sensitivity is much more important than the lower Ct-value. Thus, $T_a = 60$ °C was chosen as an optimal temperature and used in the further SVDV PriProET evaluation.

Assay specificity and sensitivity

A specificity test of SVDV and heterologous virus (listed in Table 1) showed specific amplification with SVDV strains only. The amplification plot for the closely related CV-B5 remained below the threshold, confirming the high specificity of the assay. Also, the melting profile of CV-B5 was negative (results not shown). The eight SVDV strains out of twenty had low endpoint fluorescence, with melting profiles reflecting several mutations causing change in T_m (for T_m value see Table 1). For example, both ITL 1/66 strains (obtained from Spain and UK) had shifted melting points with $T_m = 57.5$ °C, while HKN 11/81 had 65.0 °C, compared to the UKG 27/72 strain, with a perfect probe match at $T_m = 70.4$ °C (Fig. 1). Gel electrophoresis confirmed the predicted product size (107 bp), including with strains with shifted melting points (Fig. 2).

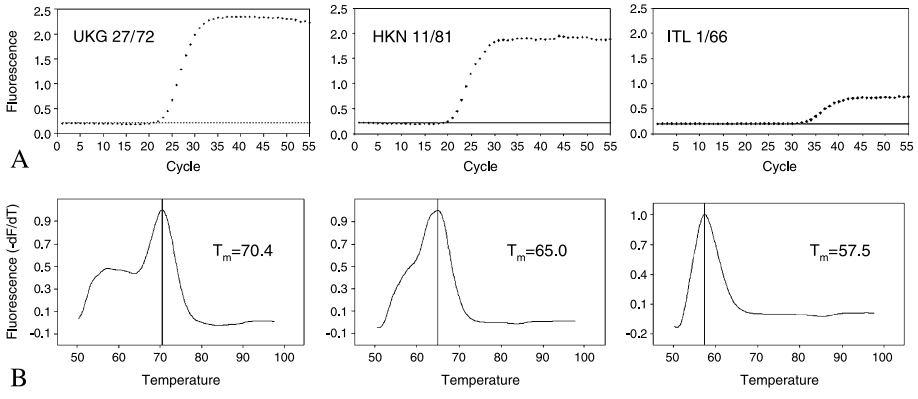

Fig. 1. Amplification plots (**A**) and melting peaks (**B**) of three SVDV isolates: UKG 27/72 with perfect probe match, HKN 11/81 with one mutation, and ITL 1/66 with two mutations in the probe region. ITL 1/66 has low endpoint fluorescence and a drastically shifted melting point compared to UKG 27/72 and HKN 11/81

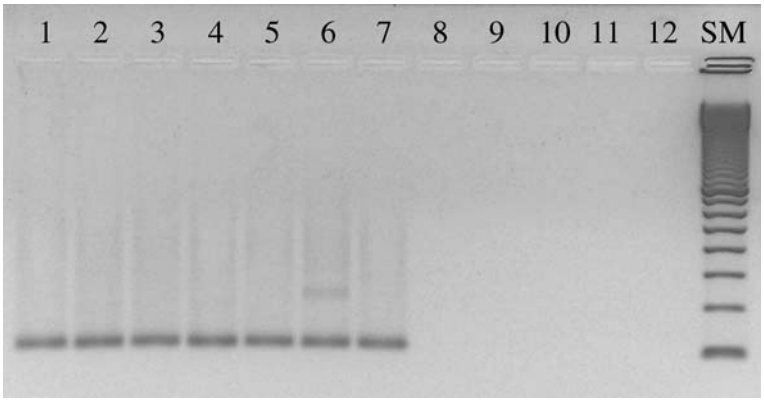

Fig. 2. Specificity test. 2% agarose gel electrophoresis of PCR products of the SVDV PriProET assay. Selected strains: *1* ITL 1/66; *2* ITL 72; *3* HKN 11/72; *4* UKG 27/72; *5* SPA 1/93; *6* ITL 8/93; *7* ITL 3/97; *8* CV-B5; *9* FMDV – C, PHI 19/94; *10* VSV Indiana-1, Colorado 1942; *11* VSV New Jersey, Colorado 1984; *12* PCR-negative control, water; *SM* size marker, 100-base. ITL 1/66 (No. 1) with two mutations in the probe region has the same product size (107 bp) as the rest of the SVDV strains. All heterologous strains including PCR negative control (No. 12) are negative

In order to confirm the correlation between the melting temperature and the number of mutations in the probe region for the strains with a shifted melting point (ITL 1/66, ITL 1/91, ITL 1/92, HKN 8/73, HKN 1/80, HKN 11/81, HKN 1/89 and FRA 1/73; Table 1), cDNA was amplified with flanking primers and sequenced. The sequence analyses showed that the 8 strains with shifted T_m had one or two mutations in the probe region (Table 2), which correlated to the observed shift in T_m's (Table 1).

The analytical sensitivity (detection limit) of the SVDV PriProET real-time PCR assay was tested using ten-fold dilution series of synthetic template with a range from 10^9 to one copy, in triplicate. The template with five copies was included as well to determine more precisely the detection limit. The assay could

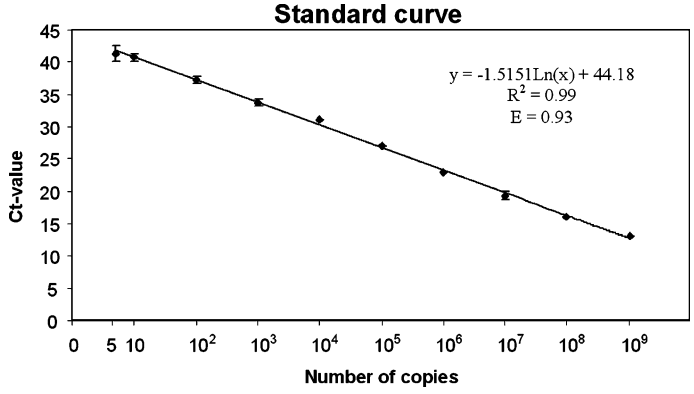

Fig. 3. SVDV standard curve based on 10-fold dilution series of synthetic amplicon (from 10^9 to one copy, three replicates of each dilution, two experiments). Detection limit is five viral genome equivalents. R^2 correlation coefficient; *E* PCR amplification efficiency

detect five copies of viral genome equivalents. The experiments were done in triplicate and average data of cycle threshold was used to design a standard curve and calculate PCR amplification efficiency, which was 93% (Fig. 3).

An SVDV molecular beacon real-time PCR assay based on the same primers and probe used in this study was also tested with SVDV strains listed in Table 1. ITL 1/91 was negative. ITL 1/92, ITL 3/97 and POR 1/03 had Ct over 35 and a very faint band on an agarose gel when tested for the predicted product size. The remaining 16 strains were amplified successfully (data not shown).

Discussion

The development of highly sensitive, specific, and rapid diagnostic systems for the detection of animal pathogens remains an ultimate goal for the high-throughput diagnostic laboratory. SVDV control is vital in all pig-producing countries. Classical methods for SVD diagnosis, such as virus isolation and antigen- or antibody-detection ELISA are good, but conventional methods used for the detection of SVDV are laborious and take many hours or several days. At the same time, the sensitivity of these methods is low compared to different PCR techniques.

The present study was performed to develop a sensitive, specific, and rapid diagnostic system for the detection of SVDV using PriProET real-time PCR. This system has several beneficial features compared to previously developed SVDV real-time PCR assays [25, 27]. It is highly specific, covers all seven recognized genetic groups of SVDV [16, 30], and detected all strains tested in this study. Moreover, the primers are able to differentiate SVDV strains from FMDV, VSV, and even the closely related human pathogen CV-B5. This observation confirmed the high specificity of the primers used for the detection of SVDV (Fig. 2). Furthermore, our study shows that the assay can tolerate two mutations in the probe region and effectively detects those SVDV strains that may rapidly evolve during the course of evolution. The assay is sensitive, since it can detect five copies of viral genome equivalents. Thus, the PriProET system may provide an effective tool to trace SVDV in the early stage of infection during outbreaks.

Eight of twenty SVDV strains tested, revealed shifted melting points that indicated mutations in the probe region. All of the predicted mutations were confirmed by nucleotide sequencing. The shift in the melting points is based on mismatches between the probe and the amplicon, and the more mutations in the probe region, the lower the T_m. The melting temperature allows the number of mutations in the probe region to be predicted, i.e. $T_m = 70\,^{\circ}C$ with a range $\pm 2.5\,^{\circ}C$ corresponds to a perfect probe match, while $T_m = 65 \pm 2.5\,^{\circ}C$ and $T_m = 60 \pm 2.5\,^{\circ}C$ correspond to one or two mutations, respectively. Previous studies have shown that virus strains with up to six nucleotide differences in the probe region can be detected by the melting profile, although it cannot be quantified [24]. Further studies are needed to test the maximum limit of mutations that will still give a positive reaction both in the amplification plot and the melting profile.

In comparison to other SVDV real-time PCR assays that were developed recently (TaqMan, HybProbes) [25–27], the PriProET system has several benefits,

allowing it to be used as a primary diagnostic method: a) The assay applies only one primers/probe set in order to detect a diverse range of SVDV strains. In former studies [25] it was necessary to use two primers/probe sets in order to detect different SVDV strains. The PriProET assay can tolerate two mismatches (with a known limit up to six nucleotide difference, see above) in the probe region, as was the case with ITL 1/66, HKN 1/80, HKN 1/89, and the other five SVDV strains (Table 1); b) A melting curve analysis directly after PCR, with determination of probe melting point, can confirm specific hybridisation of SVDV strains with a mutation in the probe region; c) The assay can differentiate human CV-B5 virus from SVDV strains, which are serologically almost identical. This confirms the high specificity of the developed system. In one published assay, CV-B5 produced a strong reaction using both primer/probe sets [25], in the other, CV-B5 was not included in the study [27]; d) The strain collection used in this paper covers all determined antigenically/phylogenetically distinct groups of SVDV [3, 16, 30]. The simultaneous detection of all of these variants further confirms the high specificity and flexibility of the PriProET system.

The results with different SVDV strains and especially those with mutations in the probe region demonstrate the robustness of the PriProET system compared to the other real-time PCR assays (molecular beacon, TaqMan) that usually require a perfect probe match with the target of interest. With the PriProET system there is a chance to identify phylogenetically divergent strains of SVDV, which may appear negative in other probe-based real-time PCR assays. At the same time, any difference in melting points may provide an indication of divergence in the probe region. Initially, the primers and probe used in these experiments were intended for detection by molecular beacons. In spite of the theoretical fitness of the probe, we discovered a reduced sensitivity using molecular beacon compared to PriProET. This discrepancy in the sensitivity is explained by the FRET system. For the PriProET system, even a probe hybridising with low efficiency would bring the reporter fluorophore in proximity to the donor, enabling the release of reporter fluorescence. There is no competition with the stem-loop structure (as in molecular beacons), and there is no need for probe degradation to release fluorescence (as in TaqMan), which impairs detection of strains with mutations in the probe target region. In addition, the PriProET system gives a specific T_m for each of the strains, which can reveal mutations in the target. The observation that PriProET detects more SVDV strains than molecular beacons makes the former superior for analysis of unknown diagnostic specimens.

Besides specificity and sensitivity, the PriProET assay provides all of the benefits of real-time PCR features, such as rapid detection, low risk of contamination, robustness, and high throughput. Furthermore, as other real-time PCR assays, PriProET can easily be included in automated diagnostic systems.

In conclusion, the high sensitivity and specificity of the SVDV PriProET assay may improve the early and rapid detection of a wide range of SVDV strains, allowing reduced turnaround time and the use of high-throughput, automated technology.

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Author's address: M. Hakhverdyan, Department of Virology, The National Veterinary Institute, Ulls väg 2B, 751 89 Uppsala, Sweden; e-mail: mikhayil.hakhverdyan@sva.se