ORIGINAL ARTICLE

Insulated Isothermal Reverse Transcriptase PCR (iiRT-PCR) for Rapid and Sensitive Detection of Classical Swine Fever Virus

O. Lung[1], J. Pasick[2], M. Fisher[2], C. Buchanan[1], A. Erickson[1] and A. Ambagala[1]

[1] National Centres for Animal Disease, Lethbridge Laboratory, Canadian Food Inspection Agency, Lethbridge, AB, Canada
[2] National Centres for Animal Disease, Winnipeg Laboratory, Canadian Food Inspection Agency, Winnipeg, MB, Canada

Summary

Classical swine fever (CSF) is an OIE-listed disease that can have a severe impact on the swine industry. User-friendly, sensitive, rapid diagnostic tests that utilize low-cost field-deployable instruments for CSF diagnosis can be useful for disease surveillance and outbreak monitoring. In this study, we describe validation of a new probe-based insulated isothermal reverse transcriptase PCR (iiRT-PCR) assay for rapid detection of classical swine fever virus (CSFV) on a compact, user-friendly device (POCKIT™ Nucleic Acid Analyzer) that does not need data interpretation by the user. The assay accurately detected CSFV RNA from a diverse panel of 33 CSFV strains representing all three genotypes plus an additional *in vitro*-transcribed RNA from cloned sequences representing a vaccine strain. No cross-reactivity was observed with a panel of 18 viruses associated with livestock including eight other pestivirus strains (bovine viral diarrhoea virus type 1 and type 2, border disease virus, HoBi atypical pestivirus), African swine fever virus, swine vesicular disease virus, swine influenza virus, porcine respiratory and reproductive syndrome virus, porcine circovirus 1, porcine circovirus 2, porcine respiratory coronavirus, vesicular exanthema of swine virus, bovine herpes virus type 1 and vesicular stomatitis virus. The iiRT-PCR assay accurately detected CSFV as early as 2 days post-inoculation in RNA extracted from serum samples of experimentally infected pigs, before appearance of clinical signs. The limit of detection ($LOD_{95\%}$) calculated by probit regression analysis was 23 copies per reaction. The assay has a sample to answer turnaround time of less than an hour using extracted RNA or diluted or low volume of neat serum. The user-friendly, compact device that automatically analyses and displays results could potentially be a useful tool for surveillance and monitoring of CSF in a disease outbreak.

Introduction

Classical swine fever virus (CSFV) is a member of the genus *Pestivirus* of the family Flaviviridae (Wengler 1991). The virus causes disease that ranges from mild to severe in its natural hosts, domestic pigs and wild boars. Classical swine fever virus is an enveloped virus with a positive-sense, single-stranded RNA genome of approximately 12 300 nucleotides. The genome encodes a single open reading frame that is flanked by two untranslated regions (UTRs) at the 5′ and 3′ ends. Classical swine fever virus is currently categorized into three genotypes: 1, 2 and 3 that can be further

divided into 11 subtypes: 1.1, 1.2, 1.3, 1.4, 2.1, 2.2, 2.3, 3.1, 3.2, 3.3 and 3.4 (Lowings et al., 1996; Paton et al., 2000; Postel et al., 2013). Other pestiviruses that infect livestock are bovine viral diarrhoea virus type 1 (BVDV-1) and type 2 (BVDV-2), border disease virus (BDV) of sheep, the recently described atypical pestiviruses (Schirrmeier et al., 2004; Ståhl et al., 2010) and Bungowannah virus (Kirkland et al., 2007). Classical swine fever (CSF) is an OIE-listed notifiable disease that can have an enormous effect on the livestock industry and on trade. For instance, the direct costs of the 1997–1998 epizootic in the Netherlands, excluding loss of exports, amounted to $2 billion (U.S.)

and the slaughter of approximately 10 million pigs (Terpstra and de Smit, 2000). While CSFV has been eradicated from regions such as North America, Australasia and parts of northern Europe, it remains widespread in regions that include South America, eastern Europe and South-East Asia (Vargas et al., 2004).

Pestiviruses are closely related genetically and antigenically. While infections in swine with pestiviruses other than CSFV are usually benign, they do present diagnostic problems: they cause serological cross-reactions in antibody tests (Colijn et al., 1997; Langedijk et al., 2001). Virus isolation is the method of choice for discovering infected herds at the early stage of infection (Terpstra and de Smit, 2000). However, rapid and sensitive tests for early detection of CSFV are desirable for containing outbreaks as conventional virus isolation procedures are time-consuming and require cell culture infrastructure and expertise. Several conventional RT-PCR and real-time RT-PCR assays have been described for detection of CSFV and other pestiviruses (Handel et al., 2004; Hoffmann et al., 2005, 2011; Risatti et al., 2005; Wernike et al., 2013). However, the high cost of current real-time PCR devices, and the need for post-amplification processing with conventional PCR may be a barrier to the widespread use of these technologies in field applications or in developing countries. Thus, low-cost, sensitive and user-friendly methods that can detect CSFV rapidly are still needed.

Insulated isothermal PCR (iiPCR) utilizes a single heat source below the reaction vessel to generate temperature gradients by Rayleigh–Benard convection (Krishnan et al., 2002). The denaturation, annealing and extension steps of a PCR occur in different zones in the reaction vessel (Krishnan et al., 2002). The iiRT-PCR assay used in this study utilizes a simple, compact POCKIT™ nucleic acid analyzer that detects optical signals from a target-specific fluorescent-labelled probe for highly sensitive and specific detection of the target virus. Rapid assays using this platform have been used for the detection of pathogens such as white spot syndrome virus (Tsai et al., 2014) and *Salmonella* in chicken meat (Tsen et al., 2013).

In this study, the validation of a simple iiRT-PCR assay for detection of CSFV is described. The assay uses lyophilized reagents and can be performed on a simple portable instrument that provides automated data analysis and results readout within 1 h of addition of purified RNA or a low volume of unextracted serum.

Materials and Methods

Samples and nucleic acid extraction

RNA from a panel of 30 CSFV strains and isolates (Table 1, strains without the superscript 'a') used in this study was obtained from Dr. Irene Greiser-Wilke (EU

Table 1. CSFV strains/isolates used in this study

CSFV strain/isolate	Year	Country	Genotype
Koslov	NA	Czech Republic	1.1
759/Ru	1999	Russia	1.1
Alfort187	1968	France	1.1
Eystrup	1964	Germany	1.1
Peru L8[a]	2008	Peru	1.1
Peru LL28[a]	2008	Peru	1.1
3795/96	1996	Czech Republic	1.2
Brescia	NA	Italy	1.2
VRI 4167	1986	Malaysia	1.3
97-9346/#17	1996	Hungary	1.3
Guatemala HC/#4409	NA	Guatemala	1.3
Honduras 97[a]	1997	Honduras	1.3
NL B64	1997	Spain	2.1
South Africa	2005	South Africa	2.1
V1240/97	1997	Germany	2.1
5119 VA/97	1997	Italy	2.2
Parma98	1998	Italy	2.2
Vi 3295/4/89	1989	Germany	2.2
Bergen	NA	the Netherlands	2.2
V 750	1984	Germany	2.3
Hun 1043/92	1992	Hungary	2.3
Diepholz 1/Han94	1994	Germany	2.3
Visbek/Han95	1995	Germany	2.3
V 487/93	1993	Germany	2.3
Vi 2781-82	1993	Germany	2.3
D4889 I/82/NA	1982	Italy	2.3
2/4	NA	Poland	2.3
EWS3326/97	1997	Germany	2.3
S 310	1997	Germany	2.3
VI 3837/38	1999	Germany	2.3
Spante	1998	Germany	2.3
Congenital Tremor	1964	Great Britain	3.1
Kanagawa (Tap 3)	1974	Japan	3.4

CSFV, classical swine fever virus; NA, information not available.
[a]Tested with clinical samples from experimentally infected pigs, but not laboratory-amplified viruses.

Reference Laboratory for FMD, Hanover, Germany). Archived viral nucleic acid from 18 laboratory-amplified non-CSF viruses including eight other pestiviruses (BVDV 1-Hastings, Singer and NY1 strains; BVDV 2-Ames 125c, 890, 24515 strains; BDV-Coos Bay; HoBi atypical pestivirus), African swine fever virus-Lisbon, swine vesicular disease virus-ITL 19/92, porcine respiratory and reproductive syndrome virus-YNL, swine influenza virus (H3N2), porcine circovirus 1 (PCV1, derived from infectious clone based on GenBank accession no. AY184287), porcine circovirus 2 (PCV2, derived from infectious clone based on GenBank accession no. EF394779), porcine respiratory coronavirus-ISU, vesicular exanthema of swine virus, bovine herpesvirus 1-Edmonton 5 and vesicular stomatitis virus-Ind 1 strain were used to determine the specificity of the iiRT-PCR.

Viral RNA for PCV1 and 2 was provided by Dr. Marcus Czub (University of Calgary). RNA was extracted from a total of 91 sera samples obtained from 12 healthy pigs and 12 pigs inoculated with four CSFV strains. Briefly, 7 to 8-week-old pigs were inoculated oronasally with the Peru LL28/2008 La Libertad-Trujillo ($n = 4$), Peru L8/2008 Lima-Villa El Salvador ($n = 4$), Honduras 1997 ($n = 2$) and Diepholz 1/Han94 ($n = 2$) strains. Serum samples that were tested fresh without RNA extraction were from pigs inoculated with the Koslov strain using the same method. Animals were anesthetized with isoflurane by face mask and 1.5–2.0 ml of inoculum containing 10^6 50% tissue culture infectious doses ($TCID_{50}$) of each virus was administered per animal. Sera were collected before inoculation and also on various days post-infection (dpi) for 4 or 5 weeks, or until the animal died or was euthanized. Viral RNA was extracted from serum samples using the MagMAX-96 AI/ND Viral Isolation Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions using the MagMAX Express 96 Processor (Life Technologies) and eluted with 50 μl of elution buffer. All laboratory-amplified CSFV strains, BVDV 1-Singer, BDV-Coos Bay, ASFV-Lisbon, SVDV-ITL 19/92 and VSV-Ind 1 were assayed at 1/50 dilutions of nucleic acid extracted from cell culture supernatant, while the remaining non-CSF viruses were assayed at neat quantities. Extracted nucleic acid from all serum samples were also assayed at neat quantities.

All procedures dealing with animal inoculation and care complied with the guidelines of the Canadian Council on Animal Care and were approved by the institutional animal care committee.

In vitro-transcribed RNA

In vitro-transcribed RNA was generated from a plasmid containing a fragment of the 5′UTR of CSFV strain HCLV (GenBank accession no. AF531433) using the MAXIscript T7 kit (Ambion, Austin, TX, USA). Residual DNA was removed using the Ambion Turbo DNA-free kit (Life Technologies). The concentration of RNA was measured by a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Serial dilutions of in vitro-transcribed RNA were made in 40 ng/μl yeast tRNA to determine the limit of detection. Single-use aliquots were stored at −80°C until use.

CSFV iiRT-PCR assay

The CSFV iiRT-PCR assay was designed and manufactured by GeneReach USA (Lexington, MA, USA) on the basis of the probe hydrolysis-based POCKIT™ method

described previously (Tsai et al., 2012). Briefly, 763 nucleotide sequences of the CSFV 5′UTR region were collected from the CSF Database, EU and OIE Reference Laboratory for CSF (http://viro60.tiho-hannover.de/eg/csf/index.php) and aligned to identify conserved regions that can be used for the design of iiRT-PCR primers and probes. Classical swine fever virus 5′UTR-specific primer and probe set were designed according to the recommended principles for iiPCR (http://www.iipcr.com/eweb/uploadfile/20130522114104277.pdf). The recommended length of the primers was between 18 and 30 bases, have a GC content of 45–60% and a T_m of 56–60°C. Repeat sequences, four or more consecutive Gs or Cs and hairpins and primer dimers were avoided. The probe had a length of <30 bases, a GC content between 30% and 80% and a T_m that was 10°C above the T_m of the primers. Runs of a single nucleotide and four or more consecutive Gs or a G at the 5′ end of the probe were avoided. The amplicon was approximately 100 bp, within the recommended range of 70–150 bp. No major secondary structures were found in the amplicon, based on prediction made by the MFOLD program (http://mfold.rna.albany.edu/?q=mfold). Lyophilized iiRT-PCR reagent was rehydrated with 50 μl of Premix buffer (GeneReach USA), and 5 μl of sample was added to the mixture. For laboratory-cultured virus samples, 4 μl of dH$_2$O was added to 1 μl of RNA or DNA, while 5 μl of neat extracted RNA, various volumes of neat unextracted serum and unextracted serum diluted 1 : 2, 1 : 5 or 1 : 10 in dH$_2$O were used for serum samples. Both fresh and archived serum from CSFV infected pigs were used as indicated. Subsequently, 50 μl of the final mixture was transferred to an R-tube™ (GeneReach USA), which was spun briefly in a cubee™ mini centrifuge (GeneReach USA). The R-tubes™ were placed into the reaction chamber of the POCKIT™ Nucleic Acid Analyzer and a run was initiated. The POCKIT™ Nucleic Acid Analyzer collected optical signals through an integrated circuits controller-regulated (CMOS) sensor, and signal-to-noise (S/N) ratios were automatically calculated by dividing light signals collected after iiRT-PCR by those from before iiRT-PCR (Tsai et al., 2012). According to default S/N thresholds, results were converted automatically to '+', '−', or '?' and displayed on the screen at the end of the run. All samples tested gave either '+' or '−' result, with the exception of 5 μl of undiluted and unextracted serum, which all gave '?' results.

Statistical analysis

Statistical probit analysis, a nonlinear regression model, was performed using commercial software SPSS 14.0 (SPSS Inc., Chicago, IL, USA) to determine limit of detection with 95% confidence ($LOD_{95\%}$).

Real-time RT-PCR

Real-time RT-PCR for CSF was performed with the Cepheid Smart Cycler II (Cepheid, Sunnyvale, CA, USA) for samples from pigs inoculated with the Peru L8/2008 and LL28/2008 strains, or the ABI 7900 HT (Life Technologies) for samples from pigs inoculated with the Diepholz 1/Han 94 and Honduras 97 strains as per the Standard Operating Procedure at the Canadian Reference Laboratory for CSF (Arainga et al., 2010). Each reaction had a total volume of 25 μl and consisted of 9 μl of RNA added to 16 μl of master mix. The master mix for the real-time RT-PCR contains 1× QuantiTect Probe Master Mix (Qiagen, Mississauga, ON, Canada), 0.4 μM of forward primer, 0.9 μM of reverse primer, 0.4 μM of probe, 15 μM each of forward and reverse primer and 10 μM of probe for an armoured dengue RNA internal control and 0.25 μl of QuantiTect RT Enzyme Mix (Qiagen). The primers used were CSF-Taq-5′ For (5′-TCA GTA GTT CGA CGT RRG CAG AA-3′) and CSF-Taq-3′ Rev (5′-GCA TGC CCT CGT CCA CRT-3′). The probe used was CSF-5′UTRMGB-2 Probe (5′-6FAM-CYY RCC TCG AGA TGC-MGBNFQ-3′) (Arainga et al., 2010). Real-time PCR conditions were 50°C for 30 min, 95°C for 15 min, followed by 40 cycles of 94°C for 30 s and 60°C for 1 min. Clinical samples that produced C_t values of ≤35.99 were considered positive, while samples that produced C_t values >36 were considered suspicious.

Results

Testing of the iiRT-PCR assay with laboratory-amplified samples

Nucleic acid from a panel of laboratory-amplified CSFV strains (n = 30, Table 1, strains without superscript 'a') and non-CSFV viruses that affect livestock (n = 18) were used to determine the specificity of the iiRT-PCR assay. The iiRT-PCR utilized a pair of 23-mer primers and a 19-mer probe (Table 2). The iiRT-PCR assay accurately detected all CSFV RNA samples which represented all three genotypes, and eight of 11 subgenotypes that were available for testing and gave negative results for three BVDV type 1 strains, three BVDV type 2 strains, BDV, HoBi atypical pestivirus, ASFV and nine other viruses that affect livestock. Data from replicate assays of strains representing each of the three CSFV genotypes and two non-target pestiviruses performed on two different POC-KIT™ instruments were analysed to evaluate assay reproducibility. All four replicates conducted with each of the three CSFV strains and triplicates of the two non-target viruses gave consistent and expected results. The average and standard deviation of S/N values observed for Alfort (genotype 1), Diepholz (genotype 2), and Kanagawa (genotype 3) were 4.14 ± 0.11, 4.01 ± 0.09 and

Table 2. Primer and probe sequence of CSFV iiRT-PCR

Name	Sequence (5′–3′)	nt[a]	Note
CSF F1	GACGAGGGCATGCCCAAGA	232–250	Forward primer
CSF R1	GGCCTCTGCAGCGCCCTAT	315–333	Reverse primer
CSF-P2r	FAM-CAGGTCGTACTCCCATCAC-MGB.NFQ	296–314	Probe

CSFV, classical swine fever virus.
[a]Nucleotide positions based on GenBank accession no. AF531433.

4.08 ± 0.07, respectively. For the non-target pestiviruses, the average and standard deviation of the S/N ratios were 0.99 ± 0.03 for BVDV type 1-Hastings and 0.97 ± 0.02 for BVDV type 2-890.

Testing of the iiRT-PCR assay with clinical samples

The iiRT-PCR assay was tested with RNA from 12 serum samples of healthy pigs and 79 serum samples from pigs infected with four different CSF strains: Honduras 1997, Diepholz 1/Han94, Peru LL28/2008 and Peru L8/2008 (Table 3). All serum samples taken from the healthy pigs as well as all 12 dpi 0 samples taken before pigs were experimentally infected with CSFV gave negative iiRT-PCR results, indicating 100% specificity for these samples.

The earliest dpi samples that generated positive iiRT-PCR results were dpi 2 samples from pigs infected with the Peru LL28 strain (one of two pigs), dpi 3 samples from Peru L8/2008 and 1997 Honduras strain infected pigs (four out of four pigs) and dpi 5 samples from pigs infected with the Diepholz strain (two of two pigs) (Table 3). The Diepholz 1/Han94 strain was also tested with laboratory-amplified material (Table 1); however, the Honduras 1997, Peru LL28/2008 and Peru L8/2008 strains were new strains that were only tested with clinical material. Thus, the total number of CSFV strains detectable by the iiRT-PCR assay is 34 (33 virus strains plus *in vitro*-transcribed RNA from synthetic CSFV-HCLV sequence used in the limit of detection analysis). All samples from the 12 experimentally infected pigs from time points later than the earliest dpi that gave positive results by iiRT-PCR were positive. The last time points tested in this study were dpi 15 for Diepholz 1 Han/94, 11 for Honduras 1997, 30 for Peru LL28/2008 and 12 for Peru L8/2008 (Table 3).

Comparison of the iiRT-PCR results with real-time RT-PCR

RNA from a total of 78 serum samples was tested with both the iiRT-PCR and real-time RT-PCR (Table 3). All 12 dpi

Table 3. Comparison of iiRT-PCR and real-time RT-PCR results

Strain	No. samples	Dpi tested	Earliest '+' dpi (No. pigs)	
			iiPCR	Real-time PCR
Diepholz 1/Han94	15	0, 1, 3, 5, 7, 9, 11, 15	5 (2/2)	5 (2/2)
Honduras 97	14	0, 1, 3, 5, 7, 9, 11	3 (2/2)	3 (0/2)
			5 (2/2)	5 (2/2)
Peru L8/2008	22	0, 1, 2, 3, 4, 5, 9, 10, 11, 12	3 (2/2)	3 (0/2)[a]
			4 (2/2)	4 (1/2)[a]
Peru LL28/2008	27	0, 1, 2, 3, 4, 5, 6, 7, 12, 14, 21, 28	2 (1/2)	2 (0/2)
		30[b]	3 (2/2)	3 (2/2)

iiPCR, insulated isothermal PCR.
[a]Non-positive samples had C_t values greater than the cut-off for positivity and are considered 'suspicious'.
[b]The dpi 30 sample was only tested with iiRT-PCR, but not tested by real-time RT-PCR.

0 samples taken before the experimental inoculations were negative by both iiRT-PCR and real-time RT-PCR. Of the remaining 66 samples, 61 gave identical results by both methods. One dpi 2 sample from a pig inoculated with the Peru LL28 strain and two dpi 3 samples from pigs inoculated with the Honduras 1997 strain were positive by iiRT-PCR, but negative by real-time RT-PCR. In addition, two dpi 3 and one dpi 4 sample from pigs inoculated with Peru L8/2008 strain were positive by iiRT-PCR, but had real-time RT-PCR C_t values that were greater than the cut-off for positivity (Table 3). Samples taken at all later time points were positive by both assays. These results suggest that iiRT-PCR may be more sensitive than the real-time RT-PCR assay for certain strains.

Analytical sensitivity of CSFV iiRT-PCR

Serial dilutions of *in vitro*-transcribed RNA were used to evaluate the analytical sensitivity of the optimized CSFV iiRT-PCR and real-time RT-PCR. The percentages of positive results were 100.0% (10/10), 100.0% (20/20), 85% (17/20) and 0% (0/15) for 100, 50, 20 and 0 copies of the *in vitro*-transcribed RNA, respectively. The LOD95% calculated by probit regression analysis was 23 copies per reaction. These results were comparable to the results obtained with the real-time RT-PCR using both Smart Cycler II and ABI 7900HT instrument platforms. The lowest copy detected by the Smart Cycler II was 28 copies (average C_t value for three replicates was 33.57), while the lowest copy detectable by the ABI 7900HT instrument was 31.4 copies (average C_t value for three replicates was 37.006).

Testing of CSFV iiRT-PCR with unextracted serum

The ability to directly detect virus in clinical samples without nucleic acid extraction can simplify an assay and shorten the turnaround time needed to obtain results. The iiRT-PCR successfully detected CSFV RNA when up to 4 μl

of neat, fresh (unfrozen), unextracted dpi 10 serum from pigs inoculated with the Koslov strain was used (Table 4). Positive results were also obtained with fresh unextracted serum samples that were diluted 1 : 2 and 1 : 5, and with archived (frozen) serum samples that were thawed and diluted 1 : 2, 1 : 5 and 1 : 10 (Table 4).

Discussion

In this report, a highly sensitive and specific iiRT-PCR for the rapid detection of CSFV is described. The assay detected RNA from a broad range of CSFV strains ($n = 34$) representing all three genotypes and did not show cross-reactivity with other pestiviruses and other livestock-associated viruses. For the testing of serum samples from experimentally infected pigs, less RNA (5 μl) was used for the iiRT-PCR than the real-time RT-PCR (9 μl) routinely

Table 4. Testing of unextracted serum with the iiRT-PCR assay

Sample[a,b]	No. replicates	Result	Average S/N $\pm$ SD
Fresh unextracted neat serum			
4 μl	5	+	2.43 $\pm$ 0.41
2.5 μl	1	+	3.01
Fresh unextracted diluted serum			
1 : 2 dilution	3	+	3.03 $\pm$ 0.08
1 : 5 dilution	5	+	3.48 $\pm$ 0.08
Thawed unextracted diluted serum			
1 : 2 dilution	5	+	2.14 $\pm$ 0.24
1 : 5 dilution	5	+	3.01 $\pm$ 0.11
1 : 10 dilution	4	+	3.56 $\pm$ 0.24

[a]Thawed serum tested were dpi 11 samples from pigs infected with the Honduras strain.
[b]Fresh serum tested were dpi 10 samples from pigs infected with the Koslov strain.

employed at the Canadian Reference Laboratory for CSF, and the iiRT-PCR detected Honduras 1997, Peru L8/2008 and LL28/2008 RNA in serum samples that were negative by the 40 cycle real-time RT-PCR assay. These results suggest that the iiRT-PCR may be slightly more sensitive than the real-time RT-PCR at least for certain strains. The sensitivity of the real-time RT-PCR may be improved by increasing the cycle number; however, this will also increase the time required to complete the assay. Real-time PCR will allow simultaneous amplification and quantitative real-time detection of amplification, while iiRT-PCR is an end-point assay. The manufacturer set threshold for positivity was used in this study for the iiRT-PCR assay, but it is possible for the user to adjust the cut-off for positivity with custom programs. Not including the time needed to extract RNA, the iiRT-PCR took less than an hour to complete, much less than the approximately 2–2.5 h needed for the real-time RT-PCR used in this study.

The observation that iiRT-PCR can detect CSFV RNA in up to 4 μl of fresh unextracted serum suggests this assay may be used for direct detection of CSFV in serum with a total turnaround time of approximately an hour. An inverse correlation was observed between the S/N value and the volume and concentration of unextracted serum used in the iiRT-PCR. These results suggest that 4 μl of neat unprocessed serum and 5 μl of 1 : 2 diluted unprocessed serum may be close to the maximum amount of unextracted material that can be used in the CSF iiRT-PCR assay. Whether other types of unextracted clinical material and the minimum amount of processing that can be successfully used in iiPCRs remain to be determined.

Loop-mediated isothermal amplification (LAMP) methods that do not require expensive instrumentation have been suggested as suitable platforms for field applications (Notomi et al., 2000). Several in-house LAMP assays have been developed for the detection of CSFV nucleic acids and tested with regional isolates or strains representing a small number of CSFV subgenotypes, and few if any non-CSFV pestiviruses (Chen et al., 2009; Yin et al., 2010; Zhang et al., 2010). In contrast to earlier CSFV-specific LAMP assays which utilized agarose gel electrophoresis or visualization with the naked eye for interpretation of results, a LAMP assay combined with a lateral flow dipstick (LFD) that was validated with a large panel of pestivirus strains was recently described (*Chowdry* et al.*, 2014). In this study, seven (1.1, 1.2, 1.3, 2.1, 2.2, 2.3 and 3.1) of eight subgenotypes tested were detected, but the assay failed to detect the genotype 3.4 Kanagawa strain, perhaps due to mismatches with the primers used in the assay (Chowdry et al., 2014). The LAMP–LFD assay had an analytical sensitivity of approximately 100 copies per reaction on the two genotypes tested. In contrast, the iiRT-PCR described in this study detected strains representing all

eight subgenotypes tested, including the Kanagawa strain, and had a limit of detection of about 23 copies, suggesting the iiRT-PCR assay has broader coverage and may be more sensitive than the LAMP–LFD assay. Both the RT-LAMP and iiRT-PCRs are simple to perform and can be completed in approximately an hour. RT-LAMP assays can be performed with just a water bath or heat block and results can be visualized without instrumentation. Thus, LAMP assays that do not require a signal detector may be less costly to perform than iiRT-PCR if cost, and not sensitivity is the primary concern. However, genetic variations of target viruses can have a big impact on the sensitivity and reliability of a LAMP assay due to the use of 4–6 primers. Thus, use of LAMP assays where broader genetic variation of CSFV may be encountered will require further validation with more genetically diverse CSFV and non-CSFV samples. The iiRT-PCR assay for CSFV reported here is commercially available and utilizes lyophilized reagents, while the LAMP assays for CSFV detection reported to date are in-house assays using wet reagents. Thus, further development and validation of the LAMP assays using lyophilized reagents are desirable for on-farm detection.

The compact POCKIT™ Nucleic Acid Analyzer instrument with its lyophilized reagents and automated data analysis and result readout is easy to use. The requirement for a separate RNA extraction step requiring additional instrumentation and the maximum throughput of eight samples per run for the instrument used in this study may hinder its use as an instrument for high-throughput applications in the laboratory and at the point-of-need. However, an instrument with a throughput of 24 samples per run is available and with the high sensitivity of the iiRT-PCR assay, pooling of samples should be feasible. In combination with existing commercial portable automated nucleic acid extraction systems, the POCKIT™ Nucleic Acid Analyzer, may have potential as a low-throughput automated two-step method for on-site detection. The observation that low volume of serum can be used directly in iiRT-PCR without nucleic acid extraction can potentially simplify the diagnostic workflow considerably in the laboratory and in the field, and shorten the time needed to obtain results from serum samples to <1 h.

Acknowledgements

provided by Drs. Irene Greiser-Wilke (EU Reference Laboratory for CSF), Marcus Czub (University of Calgary) and Susan Detmer (University of Saskatchewan). The authors also acknowledge Prof. Soren Alexandersen and Dr. Noriko Goji for helpful suggestions about experiments and Sandor Dudas and two anonymous reviewers for suggestions regarding the manuscript.

References

Arainga, M., T. Hisanaga, K. Hills, K. Handel, H. Rivera, and J. Pasick, 2010: Phylogenetic analysis of classical swine fever virus isolates from Peru. *Transbound. Emerg. Dis.* 57, 262–270.

Chen, H. T., J. Zhang, L. N. Ma, Y. P. Ma, Y. Z. Ding, X. T. Liu, L. Chen, L. Q. Ma, Y. G. Zhang, and Y. S. Liu, 2009: Rapid pre-clinical detection of classical swine fever by reverse transcription loop-mediated isothermal amplification. *Mol. Cell. Probes* 23, 71–74.

Chowdry, V. K., Y. Luo, F. Widen, H. J. Qiu, H. Shan, S. Belák, and Y. H. Liu, 2014: Development of a loop-mediated isothermal amplification assay combined with a lateral flow dipstick for rapid and simple detection of classical swine fever virus in the field. *J. Virol. Methods* 197, 14–18.

Colijn, E. O., M. Bloemraad, and G. Wensvoort, 1997: An improved ELISA for the detection of serum antibodies directed against classical swine fever virus. *Vet. Microbiol.* 59, 15–25.

Handel, K., H. Kehler, K. Hills, and J. Pasick, 2004: Comparison of reverse transcriptase-polymerase chain reaction, virus isolation, and immunoperoxidase assays for detecting pigs infected with low, moderate, and high virulent strains of classical swine fever virus. *J. Vet. Diagn. Invest.* 16, 132–138.

Hoffmann, B., M. Beer, C. Schelp, H. Shirrmeier, and K. Depner, 2005: Validation of a real-time RT-PCR assay for sensitive and specific detection of classical swine fever. *J. Virol. Methods* 130, 36–44.

Hoffmann, B., S. Blome, P. Bonilauri, J. Fernandez-Pinero, I. Greiser-Wilke, A. Haegeman, M. Isaksson, F. Koenen, N. LeBlanc, I. Leifer, M. F. Le Potier, W. Loeffen, T. B. Rasmussen, T. Stafdejek, K. Stahl, M. Tignon, A. Uttenthal, W. van der Poel, and M. Beer, 2011: Classical swine fever virus detection: results of a real-time reverse transcription polymerase chain reaction ring trial conducted in the framework of the European network of excellence for epizootic disease diagnosis and control. *J. Vet. Diagn. Invest.* 23, 999–1004.

Kirkland, P. D., M. J. Frost, D. S. Finlaison, K. R. King, J. F. Ridpath, and X. Gu, 2007: Identification of a novel virus in pigs-Bungowannah virus: a possible new species of pestivirus. *Virus Res.* 129, 26–34.

Krishnan, M., V. M. Ugaz, and J. A. Burns, 2002: PCR in a Rayleigh-Benard convection cell. *Science* 298, 4.

Langedijk, J. P., W. G. Middel, R. H. Meloen, J. A. Kramps, and J. A. de Smit, 2001: Enzyme-linked immunosorbent assay using a virus type-specific peptide based on a subdomain of envelope protein E^{rns} for serologic diagnosis of pestivirus infections in swine. *J. Clin. Microbiol.* 39, 906–912.

Lowings, P., G. Ibata, J. Needham, and D. Paton, 1996: Classical swine fever virus diversity and evolution. *J. Gen. Virol.* 77, 1311–1321.

Notomi, T., H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino, and T. Hase, 2000: Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res.* 28, E63.

Paton, D. J., A. McGoldrick, I. Greiser-Wilke, S. Parchariyanon, J. Y. Song, P. P. Liou, T. Stadejek, J. P. Lowings, H. Björklund, and S. Belák, 2000: Genetic typing of classical swine fever virus. *Vet. Microbiol.* 73, 137–157.

Postel, A., S. Schmeiser, C. L. Perara, L. J. P. Rodriguez, M. T. Frias-Lepoureau, and P. Becher, 2013: Classical swine fever virus isolates from Cuba form a new subgenotype 1.4. *Vet. Microbiol.* 161, 334–338.

Risatti, G. R., L. Holinka, Z. Lu, G. Kutish, J. D. Callahan, W. M. Nelson, E. Brea Tió, and M. V. Borca, 2005: Diagnostic evaluation of a real-time reverse transcriptase PCR assay for detection of classical swine fever virus. *J. Clin. Microbiol.* 43, 468–471.

Schirrmeier, H., G. Strebelow, K. Depner, B. Hoffmann, and M. Beer, 2004: Genetic and antigenic characterization of an atypical pestivirus isolate, a putative member of a novel pestivirus species. *J. Gen. Virol.* 85, 3647–3652.

Ståhl, K., M. Beer, H. Schirrmeier, B. Hoffmann, S. Belák, and S. Alenius, 2010: Atypical 'Hobi'-like pestiviruses— recent findings and implications thereof. *Vet. Microbiol.* 142, 90–93.

Terpstra, C., and A. J. de Smit, 2000: The 1997/1998 epizootic of swine fever in the Netherlands: control strategies under a non-vaccination regimen. *Vet. Microbiol.* 77, 3–15.

Tsai, Y. L., T. H. T. Wang, G. H. F. Chang, C. F. Tsai, C. K. Lin, P. H. Teng, C. Su, C. C. Jeng, and P. Y. Lee, 2012: Development of TaqMan probe-based insulated isothermal PCR (ii-PCR) for sensitive and specific on-site pathogen detection. *PLoS ONE* 7, e45278.

Tsai, Y. L., H. C. Wang, C. F. Lo, K. T. Nelson, D. Lightner, B. R. Ou, A. L. Hour, C. F. Tsai, C. C. Yen, H. F. C. Chang, P. H. Teng, and P. Y. Lee, 2014: Validation of a commercial insulated isothermal PCR based POCKIT test for rapid and easy detection of white spot syndrome virus infection in *Litopenaeus vannamei*. *PLoS ONE* 9, e90545.

Tsen, H. Y., C. M. Shih, P. H. Teng, H. Y. Chen, C. W. Lin, C. S. Chiou, H. T. Wang, H. F. Chang, T. Y. Chung, P. Y. Lee, and Y. C. Chiang, 2013: Detection of *Salmonella* in chicken meat by insulated isothermal PCR. *J. Food Prot.* 76, 1322–1329.

Vargas, T. M., F. N. Calcagno, and J. Lubroth, 2004: Situation of classical swine fever and the epidemiologic and ecologic aspects affecting its distribution in the American continent. *Ann. N. Y. Acad. Sci.* 1026, 54–64.

Wengler, G. 1991: Classification and nomenclature of viruses. Fifth report of the International Committee on Taxonomy of Viruses. In: Francki, R. I. B., C. M. Fauquet, D. L. Knudsen and F. Brown (eds), Archives of Virology (Suppl. 2), pp. 228–229. Springer-Verlag, Wien, New York.

Wernike, K., M. Beer, and B. Hoffmann, 2013: Rapid detection of foot-and-mouth disease virus, influenza A virus and classical swine fever virus by high speed real-time RT-PCR. *J. Virol. Methods* 193, 50–54.

Yin, S. I., Y. Shang, G. Zhou, H. Tian, Y. Liu, X. Cai, and X. Liu, 2010: Development and rapid detection of classical swine fever virus by reverse transcription loop-mediated isothermal amplification (RT-LAMP). *J. Biotechol.* 146, 147–150.

Zhang, X. J., Y. Sun, L. H. Liu, S. Belak, and H. J. Qiu, 2010: Validation of a loop-mediated isothermal amplification assay for visualized detection of wild-type classical swine fever virus. *J. Virol. Methods* 167, 74–78.