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Protocols

Design and verification of a highly reliable Linear-After-The-Exponential PCR (LATE-PCR) assay for the detection of African swine fever virus

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A B S T R A C T

African swine fever virus (ASFV) is a highly pathogenic DNA virus that is the causative agent of African swine fever (ASF), an infectious disease of domestic and wild pigs of all breeds and ages, causing a range of syndromes. Acute disease is characterized by high fever, haemorrhages in the reticuloendothelial system, and a high mortality rate. A powerful novel diagnostic assay based on the Linear-After-The-Exponential-PCR (LATE-PCR) principle was developed to detect ASFV. LATE-PCR is an advanced form of asymmetric PCR which results in direct amplification of large amount of single-stranded DNA. Fluorescent readings are acquired using endpoint analysis after PCR amplification. Amplification of the correct product is verified by melting curve analysis. The assay was designed to amplify the VP72 gene of ASFV genome. Nineteen ASFV DNA cell culture virus strains and three tissue samples (spleen, tonsil, and liver) from infected experimental pigs were tested. Virus was detected in all of the cell culture and tissue samples. None of five ASFV-related viruses tested produced a positive signal, demonstrating the high specificity of the assay. The sensitivity of the LATE-PCR assay was determined in two separate real-time monoplex reactions using samples of synthetic ASFV and synthetic control-DNA targets that were diluted serially from 10^9 to 1 initial copies per reaction. The detection limit was 1 and 10 copies/reaction, respectively. The sensitivity of the assay was also tested in a duplex end-point reactions comprised of a constant level of 150 copies of synthetic control-DNA and a clinical sample of spleen tissue diluted serially from 10^{-1} to 10^{-5}. The detection limit was 10^{-5} dilution which corresponds to approximately 1 copy/reaction. Since the assay is designed to be used in either laboratory settings or in a portable PCR machine (Bio-Seeq Portable Veterinary Diagnostics Laboratory; Smiths Detection, Watford UK), the LATE-PCR provides a robust and novel tool for the diagnosis of ASF both in the laboratory and in the field.

1. Introduction

African swine fever (ASF) is a highly lethal and an important disease economically of domestic pigs which is listed as notifiable to the OIE (World Organization for Animal Health; www.oie.int). It is caused by African swine fever virus (ASFV), which was first regarded as an iridovirus on the basis of its morphology, but is now classified as the sole member of the family *Asfarviridae* (genus *Asfivirus*). ASFV is a cytoplasmic, double-stranded DNA virus with a linear, non-segmented genome 170–190 kb in length (Blasco et al., 1989a). It contains 151–165 open reading frames, depending on the strain (Blasco et al., 1989b; Kleiboeker and Scoles, 2001).

The mortality rate from virulent hemorrhagic strains of ASFV often approaches 100% in domestic pigs, whereas infection with less virulent strains may result in sub-acute or chronic infections with lower mortality (Boinas et al. 2004; Dixon et al., 2004; ICTVdB, 2006). Currently there is no vaccine, treatment or cure for ASF, and infected animals, as well as those suspected of infection, are slaughtered. The clinical picture of ASF is virtually indistinguishable from that of classical swine fever, CSF, another OIE notifiable disease of swine. Diagnosis of both ASF and CSF therefore relies heavily on sophisticated testing (Agüero et al., 2004; Rodriguez-Sanchez et al., 2008).

The first documented outbreak of ASF occurred in Kenya in 1921 (Montgomery, 1921) and since then ASF has been reported in most countries of Sub-Saharan Africa, where the virus is maintained either through a sylvatic cycle involving warthogs and/or bush pigs, and soft ticks in the genus *Ornithodoros*, or in a domestic cycle that involves pigs of local breeds, with or without tick involvement (Anderson et al., 1998; Oura et al., 1998; Kleiboeker

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and Scoles, 2001; Boinas et al., 2004). Since there is no available control measure other than diagnosis and slaughter, the disease poses a serious constraint to the development of both smallholder and industrial pig farms in Africa. The disease poses a continuous threat to countries outside the African continent, as shown by major outbreaks in Portugal and Spain in the past, and the ongoing devastating epizootic in the Caucasus region which started in Georgia in 2007, and has since spread to Russia, Armenia and Azerbaijan (Rowlands et al., 2007). In the fall of 2009 the deadly disease of ASF moved 2,000 kilometers from the Caucasus region to St. Petersburg in north-western Russia (FAO report, http://www.fao.org/news/story/en/item/36622/icode/).

The ASFV genome is relatively conserved from strain to strain, with three main variable regions: a central region (CVR) of relatively high variability (Nix et al., 2006) and two terminal variable regions (Blasco et al., 1989a,b; Sumption et al., 1990). One gene in particular, the B646L gene which encodes the major capsid protein p72 (aka VP72) is very highly conserved across all strains (Bastos et al., 2003; Bastos et al., 2004). Current pan-detection assays for ASFV target the VP72 gene based on its high level of conservation.

Several methods for the detection of ASFV have been described previously (Malmquist and Hay, 1960; Alcaraz et al., 1990; Steiger et al., 1992; King et al., 2003; Agüero et al., 2004; Zsak et al., 2005; Hutchings and Ferris, 2006; McKillen et al., 2007; Giammarioli et al., 2008). Most of these assays produce accurate results within 24 h, including sample preparation and virus detection. The assay recommended currently by EU and OIE reference laboratories is a closed-tube TaqMan® PCR assay developed by King et al. (2003) to detect a portion of the VP72 gene. This assay provides detection of ASFV DNA within 24 h of sample receipt with an analytical sensitivity between 100 and 10 copies (King et al., 2003).

A new assay for ASFV detection based on the Linear-After-The-Exponential (LATE) PCR (Pierce and Wangh, 2007) is described. The assay is an advanced form of asymmetric PCR which allows for rapid and sensitive detection at the endpoint, together with PrimeSafe™II (Rice et al., 2007), a PCR additive that maintains the fidelity of amplification over a broad range of target concentrations by suppressing mis-priming throughout the reaction. LATE-PCR assays generate abundant quantities of single-stranded amplicons that can be detected in real-time, or can be characterized at endpoint using sequence-specific probes. The assay functions as a duplex with an internal control-DNA. The detection limit of the duplex assay was determined to be approximately one genome copy per microliter with both synthetic target and clinical samples. It gave a positive signal for 14 different ASFV strains, as well as three clinical samples. It was also specific to ASFV, testing negative against similar viruses.

The severe disease manifestations of ASF infection combined with the lack of treatment or vaccination options means that early detection is necessary to prevent serious consequences for farming areas, countries, and entire regions. Current detection methods require the use of expensive laboratory equipment and are often time consuming. The LATE-PCR assay described below can be used with standard laboratory equipment and in the Bio-Seeq Portable Veterinary Diagnostics Laboratory, a portable sample preparation and PCR instrument built by Smiths Detection. This device is specifically engineered for use in the field with a minimum of operator training. It includes an automated unit that carries out sample preparation and LATE-PCR analysis on site in a matter of hours (http://www.smithsdetection.com/eng/1025_4312.php). Individual sample preparation units for the Bio-Seeq as well as the entire machine can be immersed in disinfectants (Virkon or Fam30) so as to ensure that virus is not transported away from the site of field testing.

2. Materials and methods

2.1. LATE-PCR assay

The assay was designed for the amplification of the VP72 gene of ASFV based on an alignment of 32 sequences from GenBank using ClustalW alignment software (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and takes advantage of the fact that LATE-PCR amplification produces large quantities of single-stranded DNA which can be probed once amplification is complete by dropping the temperature of the reaction below the annealing temperature. Probes used in this manner hybridize to a target sequence over a broad temperature range and the intensities of the resulting signals reflect number of target molecules present in the sample. The duplex assay described below includes an innocuous internal control-DNA, which is a synthetic target of 85 bp of no known function. Primer and probe design for both ASFV and the control-DNA followed the criteria of LATE-PCR outlined by Sanchez et al. (2004), and Pierce et al. (2005). Fluorescent reads are acquired using endpoint analysis after PCR amplification. Amplification of the correct product was verified by melting curve analysis.

2.2. Design of primers, probes, and targets

The ASFV Limiting primer (LP), Excess primer (XP) and the fluorescent probe were designed to amplify and detect a 247 bp region of pathogenic isolate E70 (GenBank accession AY578692) using LATE-PCR design criteria. Briefly, the LP was designed to have a melting temperature (T_m) higher than the XP, resulting in efficient exponential amplification of a double-stranded amplicon followed by an abrupt switch to linear amplification of a single-strand when the LP runs out. The probe had a melting temperature of 55.5 °C guaranteeing that it does not interfere with primer binding and extension during the 58.0 °C annealing step of the thermal cycle, but does bind at endpoint when the temperature is dropped below the annealing temperature (Sanchez et al., 2004). The sequence of the control-DNA target was a modified version of the *Xist* gene expressed in female mouse embryos (Hartshorn et al., 2007). The primers were modified to match LATE-PCR primer criteria with melting temperatures close to the ASFV primer sequences. The control-DNA probe is a 21 bp long sequence which is also designed to fit LATE-PCR probe criteria. ASFV and control-DNA probes are low-temperature molecular beacons with a short loop and a stem of two nucleotides. Fluorophores are attached to the 5′ end and Black Hole Quenchers to the 3′ end. All sequences are shown in Table 1. The ASFV probe was designed with a single G/T mismatch to the original target to reduce the effects of a hairpin in the probe structure. Nonspecific interactions were avoided based on Visual OMP (version 6.6.0) software (DNA Software, Inc., Ann Arbor, MI). This program was also used to calculate melting temperatures all primers and probes at their initial concentrations.

The assay was tested initially against a truncated, synthetic single-stranded DNA target. The duplex reaction included the synthetic single-stranded control-DNA target. All synthetic targets and primers were ordered either from Sigma-Aldrich (St. Louis, MO, USA), DNA Technology A/S (Aarhus, Denmark) or from CyberGene (Stockholm, Sweden). The sequences for the synthesized oligonucleotide targets are listed in Table 1.

2.3. Assay composition

Each duplex reaction was run in a final volume of 25 μl and contained the following reagents: 1× Platinum® *Tfi Reaction* Buffer (Invitrogen), 3 mM $MgCl_2$, 250 μM dNTPs, 50 nM ASFV Limiting Primer, 1 μM ASFV Excess Primer, 50 nM Control-DNA Limiting Primer, 1 μM Control-DNA Excess Primer, 100 nM ASFV Probe with

Table 1
Sequences and melting temperatures of ASFV and control-DNA Primers[a], Probes and Synthetic Test Targets.[b]

Name	Sequence (5′ → 3′)	Modification	T_m[c]
ASFV LP	CTGATACGTGTCCATAAAACGCAGGTGAC	None	69.3 °C
ASFV XP	CTGGAAGAGCTGTATCTCTATCCTG	None	67.1 °C
ASFV Probe	AACGAGATTGGCATAAGTTCTT	5′ Quasar 670; 3′ BHQ2	55.5 °C
Control-DNA LP	CGTTAACTTGTCGAGCCTACGTGTTCTACTCC	None	71.1 °C
Control-DNA XP	GAGCTGAACACCTACTACTTGATCT	None	67.4 °C
Control-DNA Probe	AACGACTCTTAATCACAGCTT	5′ Cal Orange 560; 3′ BHQ1	57.2 °C
ASFV Target	CTGGAAGAGCTGTATCTCTATCCTGAAAGCTTACATGTCCGAACTTGTGC CAATCTCGGTGTTGAGGTGTGGGTCACCTGCGTTT-TATGGACACGTATCAG	None	83.2 °C
Control-DNA Target	GAGCTGAACACCTACTACTTGATCTTACTTGCTGTGATTAAGAGTCGAAC ATGGGAGTAGAACACGTAGGCTCGACAAGTTAACG	None	79.0 °C

LP – Limiting Primer.
XP – Excess Primer.
[a] The control-DNA primer pair was designed to have a T_m within one degree of the respective ASFV primers ($\Delta T_m^{XP} < 1$ °C, $\Delta T_m^{LP} < 1$ °C). The ASFV probe was modified with a 5′ Quasar 670 fluor (QSR670) and a 3′ Black Hole Quencher 2 (BHQ2). The control-DNA probe was modified with a Cal Orange 560 fluor and a BHQ1. All probes are low-temperature molecular beacons with a short loop and a stem of two nucleotides. All melting temperatures were calculated by Visual OMP.
[b] Synthetic test targets were manufactured as single-stranded molecules.
[c] T_m – melting temperature of primers and probes at the starting concentration. The double-stranded synthetic test targets melting temperatures were calculated by Visual OMP. The real viral test target for ASFV is 247 bp.

a 5′ Quasar 670 (QSR670) fluor and a 3′ Black Hole Quencher 2, 100 nM Control-DNA Probe with a 5′ Cal Orange 560 (CO560) fluor and a 3′ Black Hole Quencher 1 (Biosearch Technologies, Novato, CA, USA), 300 nM PrimeSafe™II (Rice et al., 2007) and 2,5 units of antibody-complexed Platinum® *Tfi* Exo(-) DNA Polymerase (Invitrogen, Carlsbad, CA, Cat. No: 60684-050).

The monoplex reaction consisted of the same components described above except that the water was added to the reaction mixture instead of the control-DNA primers, probe (CO560) and corresponding target.

2.4. Samples

Nineteen ASFV DNA virus strains from cultured cells and three tissue samples from the spleen, tonsil, and liver of a pig infected deliberately were kindly provided by Dr. Carmina Gallardo and Dr. Jovita Fernandez Pinero of the European Community Reference Laboratory (CRL) for ASF, CISA-INIA (Centro de Investigación en Sanidad Animal, del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria), Valdeolmos, Madrid, Spain (Tables 2 and 3). Fourteen of the 19 samples were used in the development and evaluation of the ASFV LATE-PCR assay, while 6 samples were used in an experiment comparing the LATE-PCR assay to the CRL recommended PCR assay routinely employed at the National Veterinary Institute (SVA) (King et al., 2003). ASFV strain E70 (Spain) was used in both the evaluation and comparison experiments. The DNA from virus strains was extracted directly from primary cell cultures (leukocytes and/or alveolar macrophages) using a nucleic acid extraction kit (Nucleospin/Machery-Nagel–Cultek) following the manufacturer's procedures. The DNA was then concentrated by ethanol precipitation: 1/10 volume of 3 M NaOAc and 3 volumes ethanol were added to the DNA solution then left overnight at −70 °C. The solution was spun in a microcentrifuge for 10 min to pellet the DNA, then washed with 70% ethanol and spun for another 10 min. The DNA was air-dried and resuspended in a final volume of 100 μl of distillate RNAse-free water. Each sample was diluted 1:10 in water before testing. Testing on the viral DNA samples was performed at SVA and the Swedish University of Agricultural Sciences (SLU) in Uppsala, Sweden.

The DNA was isolated from the tissue samples (Table 3) by a Magnatrix 8000 extraction robot and MagAttract Virus Mini Kit protocol (Qiagen), according to the manufacturer's instructions. The nucleic acid from each sample was eluted in 100 μl of elution buffer and was stored at −20 °C. The samples, together with two ASFV positive control samples and an ASFV negative control sample of porcine DNA, were tested in monoplex format, in the Rotor-Gene 3000 (Qiagen/Corbett Research, Australia). The thermal profile is given below.

Table 2
ASFV strains tested in the study.

Place and date of isolation	Name of the strain	Origin/source	Country
Mozambique 1964	Moz64	Pig	Mozambique
Angola 1972	Ang72	Pig	Angola
Chalaswa 1983	MwLil 20/1	Tick, pig pen	Malawi
Cape Verde 1997	CV97	Pig	Cape Verde
Hoima 2003	Ug03H	Pig	Uganda
Kenya 2006	Ken06.B1	Pig	Kenya
Kenya 2007	Ken07.Eld1	Pig	Kenya
Burkina Faso 2007	BF07	Pig	Burkina Faso
Badajoz 1971	Ba71V	Vero cell adapted pig isolate	Spain
Lérida 1975	E75	Pig	Spain
Lisbon 1960	L60	Pig	Portugal
Sardinia 1988	Ss88	Pig	Italy
Port-au-Prince 1981	Haiti	Pig	Haiti
Pontevedra 1970	E70*	Pig	Spain
Togolese Republic 2009	Tog09/P1*	Pig	Togolese Republic
Kenya 2005	Ken05.Tk1*	Tick	Kenya
Kenya 2006	Ken06.Bus*	Pig	Kenya
Armenia 2007	Arm07*	Pig	Armenia
Nigeria 2008	Nig08 La/Ok*	Pig	Nigeria

Samples were provided by Centro de Investigación en Sanidad Animal, del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (CISA-INIA), Madrid, Valdeolmos, Spain.
* Used in comparison experiment. See Table 4 for results.

Table 3
ASFV clinical samples used in the study.

Isolate	Tissue	Dpi	Virus titer of inoculum
Ben97/1	Spleen	7	10^4 HAU/ml
Ken06	Tonsil	7	10^5 HAU/ml
E75L2	Liver	10	10 HAU/ml

Abbreviations: HAU – Hemagglutinating unit; Dpi – days post infection.

2.5. Conditions using Stratagene Mx3005P Sequence Detector

LATE-PCR of synthetic targets was initially carried out in a Stratagene Mx3005P Sequence Detector (Stratagene, La Jolla, CA) with the following thermal profile: 1 cycle at 95 °C for 3 min; 50 cycles of 95 °C for 10 s, 58 °C for 15 s, and 72 °C for 30 s; and 1 cycle at 70 °C for 3 min, 50 °C for 3 min, and 35 °C for 3 min with fluorescence acquisition during the last cycle at 70 °C, 50 °C, and 35 °C in the QSR670 and CO560 channels. Experiments were run using endpoint analysis rather than real-time to reduce amplification of nonspecific products. Data analysis was carried out using Microsoft Excel Software.

2.6. Conditions using Corbett Rotor-Gene 3000

LATE-PCR amplification of synthetic targets and viral DNA (cell culture and clinical samples) was carried out in a Rotor-Gene 3000 (Qiagen/Corbett Research, Australia) program with the following thermal profile: 1 cycle at 95 °C for 3 min; 50 cycles of 95 °C for 10 s, 58 °C for 15 s, and 72 °C for 30 s; and 1 cycle at 70 °C for 3 min, 50 °C for 3 min, 40 °C for 3 min, with fluorescence acquisition during the last cycle at 70 °C, 50 °C, and 40 °C in the Cy5 channel (source 625 nm, detector 660 high pass filter nm, gain 5) and JOE channel (source 530 nm, detector 555 nm, gain 10). The lowest detection temperature is 40 °C due to the temperature limitations of the Rotor-Gene thermocycler. Either Cy5 (data acquiring for ASFV target and viral DNA) or JOE (data acquiring for control-DNA) channels with the same thermodynamic profile as above were used when samples tested in monoplex format.

2.7. Sensitivity determination and LATE-PCR efficiency

To determine the assay sensitivity, a series of dilutions of known concentration of the synthetic ASFV target monoplex were tested. Dilutions ranged from 10^9 target copies/reaction to approximately 1 copy/reaction. Target samples 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. The number of copies in the stock solution was determined using the molarity of the template and Avogadro's formula. A standard curve was generated, and LATE-PCR efficiency was calculated using integrated Rotor-Gene 3000 instrument software. Dilutions were tested in real-time format with the following thermal profile: 1 cycle at 95 °C for 3 min; 50 cycles of 95 °C for 10 s, 58 °C for 15 s, 72 °C for 30 s, and 45 °C for 20 s reading at 45 °C. Dilutions were also tested at endpoint.

2.8. Comparison to established method

In order to confirm the efficacy of the LATE-PCR assay, six of the samples (marked with asterisk in Table 2) and dilutions of those samples were tested blindly in monoplex format and compared to the ASFV TaqMan® PCR assay used for routine diagnosis at SVA. The latter is based on the CRL recommended method, King et al. (2003) with minor modifications.

2.9. Range of detection and specificity tests

To determine the range of detection of the assay, 1:10 dilutions of the 14 ASFV DNA samples were tested. To determine the specificity of the assay, it was also tested against five viruses with similar symptoms to ASFV (Table 5), as well as ASFV negative porcine blood and spleen homogenate.

3. Results

3.1. Assay optimization

The LATE-PCR assay was constructed and optimized initially in two separate monoplex reactions using synthetic ASFV and control-DNA targets (Fig. 1a and c, respectively). Optimization included adjustment of annealing temperature, choice of fluorophores for the ASFV and control-DNA probes, time and temperature for data collection at endpoint and PrimeSafe™II titration. The optimization and testing of synthetic targets was carried out in both a Rotor-Gene 3000 thermocycler and a Stratagene Mx3005P Sequence Detector. In order to determine the efficiency and sensitivity of each monoplex, serial dilutions from 10^9 to 1 initial copies/reaction were carried out in triplicate and detected in real-time using a probe that hybridized to the accumulated product during a 45 °C step inserted after the extension step in each thermal cycle. The results show that products were detected in both sets of reactions at every dilution and replicate reactions were highly reproducible (Fig. 1a and c). A probe-target melting curve was constructed at endpoint for the single-strand amplicons generated, and the 1st derivative was taken to determine the T_m of all ASFV and all control-DNA reactions (Fig. 1b and d, respectively). Fig. 1b shows that all ASFV monoplex reactions generate very similar products with a dominant melting peak at 52.5 °C. Fig. 1d shows the melting peak derivative of the control-DNA, which peaks at approximately 55.5 °C. Reaction efficiencies of synthetic ASFV and control-DNA targets based on a standard curve and calculated by Rotor-Gene 3000 instrument software were 93% and 96%, respectively (not shown).

After optimization of the ASFV and control-DNA monoplex reactions, the complete duplex reaction was tested at endpoint (Fig. 2). This reaction was comprised of two ASFV primers, two control-DNA primers, one ASFV probe, one control-DNA probe and 300 nM PrimeSafe™II, to prevent nonspecific interactions during amplification. A serial dilution of the ASFV synthetic target was tested at endpoint after 50 cycles of amplification, reading in the QSR670 channel (Fig. 2a). Each reaction contained 150 target copies of the control-DNA. Fluorescent signal data were normalized by dividing all fluorescence values at each temperature by the fluorescence at 70 °C, a temperature at which probes are not bound to targets. The background level of fluorescence prior to the cycle at which product first appears was then subtracted from the normalized data. The amount of ASFV in a sample was judged by the normalized/background-corrected signal at 35 °C. In the absence of an added template the normalized/background-corrected signal was 0. Based on these empirical observations a signal of greater than 0.2 units was considered positive. This threshold was consistent with the empirical observation that samples having known numbers of ASFV copies, between 1 target/reaction to 10^7 targets/reaction, displayed normalized/background-subtract fluorescent signals in the range 0.6–4.2 fluorescent units.

As expected, the samples containing only the control-DNA did not generate a signal in the QSR670 channel, but did do so in the *CO560* channel (Fig. 2b). The resulting endpoint data are reported as a normalized value at 35 °C. The threshold value for the control-DNA was chosen as 0.2 for a positive signal. All of the control-DNA samples were detected in the CO560 channel with signals ranging from 0.7 to 0.88 normalized fluorescent units.

3.2. Sensitivity and specificity using viral DNA samples

To determine the sensitivity and specificity the monoplex and duplex assays, both were tested on clinical samples. Three samples, Ben97/1 from spleen tissue, Ken06 from tonsil tissue, and E75 from liver tissue, were tested in monoplex format. Two known ASFV standards, and ASFV-free porcine DNA served as positive and neg-

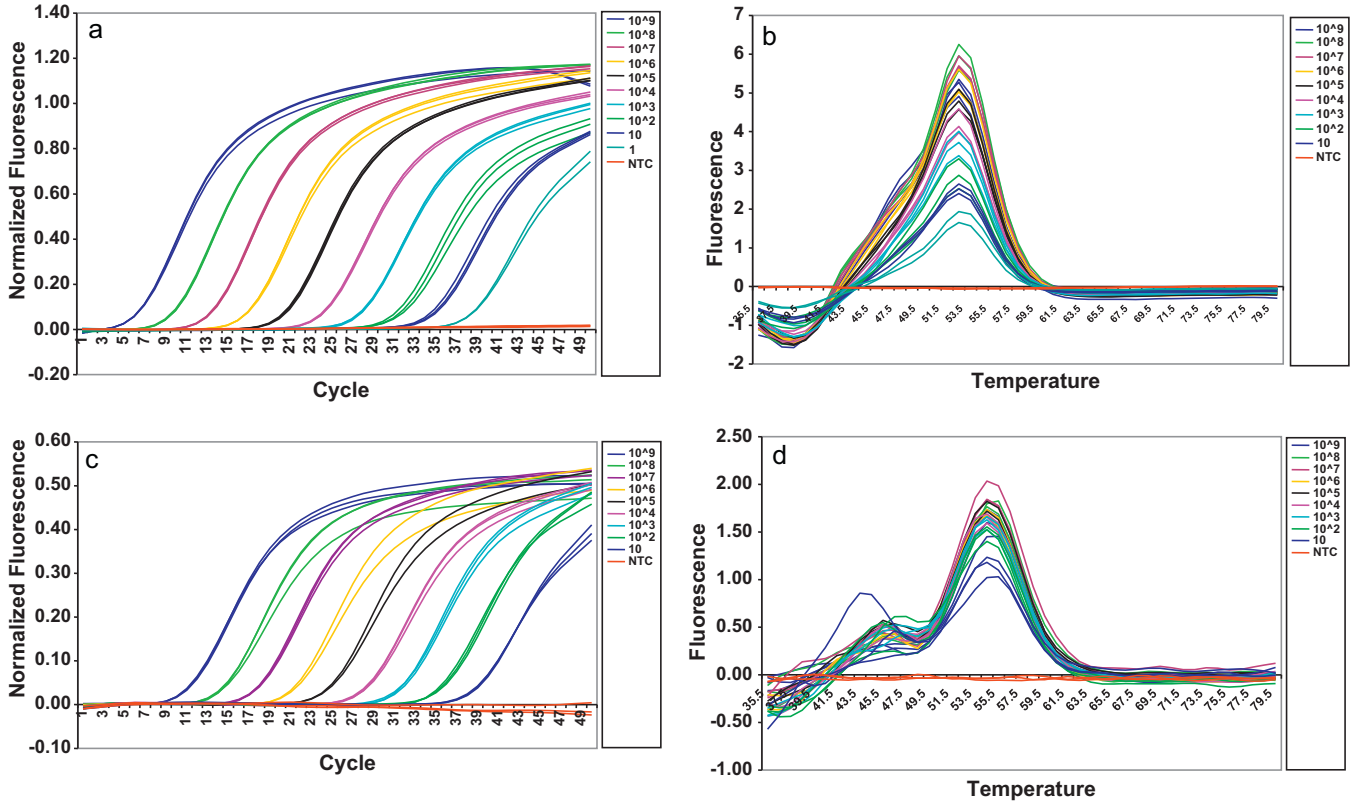

Fig. 1. (a) Quantitation curves of 10-fold dilution series, 10^9 to 1 copy/reaction, tested in triplicate with the ASFV monoplex assay analyzed in real-time using the QSR670 probe. The no template controls (NTC) contained no DNA and remained negative. The threshold value was calculated by the Rotor-Gene algorithm to be 0.02007. One of three samples with 1 copy/reaction was excluded from the analysis due to its large deviation in Ct value. (b) QSR670 probe melting derivatives at endpoint from monoplex dilution series. The T_m for the synthetic ASFV target was calculated by Visual OMP to be 55.5 °C. The observed T_m was approximately 52.5 °C. (c) Quantitation curves of 10-fold dilution series, 10^9 to 1 copy/reaction, tested in triplicate with the control-DNA monoplex assay analyzed in real-time using the CO560 probe. The no template controls (NTC) contained no DNA and remained negative. The threshold value was calculated by the Rotor-Gene algorithm to be 0.0353. One 10^6 and one 10^5 replicate were excluded due to large deviations in their Ct values. (d) CO560 probe melting derivatives at endpoint from monoplex dilution series. The T_m of the control-DNA was calculated using Visual OMP to be 57.2 °C. The melting peak derivative shows a T_m of approximately 55.5 °C.

ative controls, respectively (Fig. 3a). All of the resulting data were normalized and background subtracted. A threshold of 0.2 normalized fluorescent units above the negative control (normalized to 0) was chosen to establish a positive signal. All three clinical samples gave clear, positive signals. One sample, Ben97/1 was tested further in a serial dilution in the duplex assay using endpoint fluorescent signals (Fig. 3b). A detection limit was 10^{-5}-fold dilution, which corresponds to approximately 1 copy/μl. The latter conclusion is based on two experiments of 10-fold serial dilutions of Ben97/1 in real-time PCR format, together with positive standards that allowed quantitation of copy numbers in the tested unknown clinical sample (data not shown).

In order to determine its scope the duplex assay was tested against a total of 14 different viral strains (Table 2) at SVA/SLU. The data were collected at endpoint and were normalized to 70 °C and background subtracted (Fig. 4). The endpoint data show a strong positive signal above the threshold (0.2 normalized fluorescent units) for all 14 of the virus strains. The normalized, background subtracted fluorescent values ranged from 3.8 to 15.1, with the positive control near 15 units.

All viral strains were then used to compare the LATE-PCR assay and the routine SVA diagnostic assay. Table 4 shows that 82.6% (19/23) of the results obtained using the LATE-PCR correlated with those from SVA method. Among the remaining four samples Tog09 strain diluted 10,000-fold was detected using the SVA's method but not detect using the LATE-PCR assay. In contrast, in the three other cases the SVA method was negative in one of the two replicates, while the LATE-PCR assay was positive in both replicates.

The specificity of the LATE-PCR ASFV assay was determined by testing against five ASFV-related viruses that cause similar symptoms to ASF and require the use of laboratory tests to differentiate them (Table 5). None of these viruses generated a positive signal, nor did negative porcine sera or spleen homogenate, indicating that the assay is highly specific for ASFV.

4. Discussion

This paper describes the design and verification of a novel assay for detection of African swine fever virus (ASFV) based on Linear-After-The-Exponential PCR (LATE-PCR). Because of the properties of LATE-PCR, each reaction produces large amount of specific, single-stranded DNA, which can then be probed with a sequence-specific probe. When tested against synthetic targets, the assay proved to be effective even at low target numbers. Indeed, this assay generated robust positive signals down to approximately 1 molecule/reaction. The new duplex assay is also sensitive at low numbers control-DNA copies added to the reaction and the resulting control-DNA signals are only observed in the CO560 channel. This indicates that there are no nonspecific interactions or false positive signal are generated during LATE-PCR amplification.

The current assay used for the detection of ASFV is a symmetric PCR TaqMan® assay developed by King et al. (2003) with an analyt-

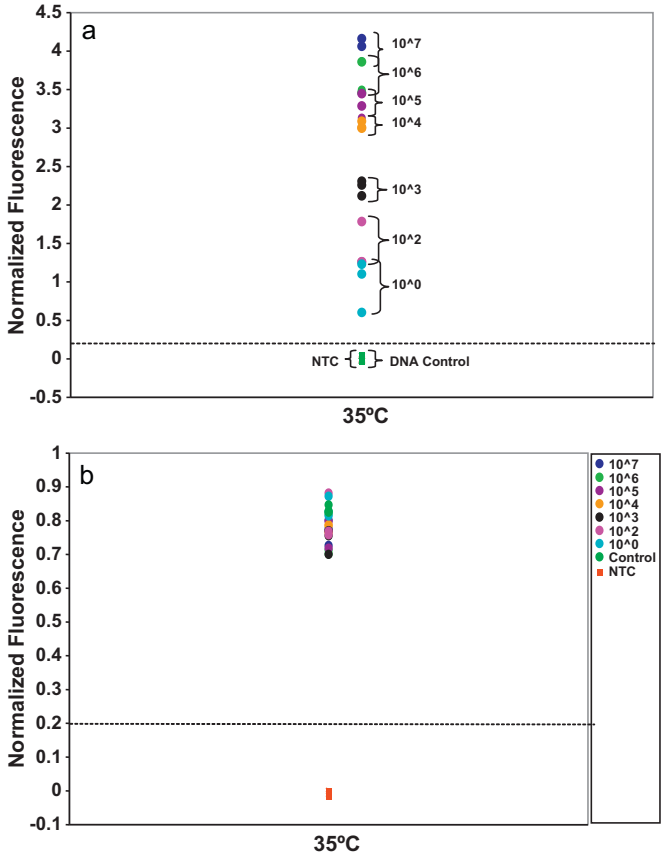

Fig. 2. ASFV duplex assay analyzed at endpoint at 35 °C using the Stratagene MxPro software. The full duplex was tested on a serial dilution of synthetic ASFV targets and 150 copies of the control-DNA. Amplification was carried out for 50 cycles. All values are normalized to 70 °C with the baseline subtracted. (a) Endpoint detection of a serial dilution of ASFV synthetic target in the QSR670 channel. Fluorescence at endpoint is directly related to the starting concentration of target. 10^1 copies/reaction samples are not reported due to an error during setup. Samples are detected down to approximately 1 copy/reaction. (b) Endpoint detection of 150 copies per reaction of the control-DNA in the CO560 channel at each concentration of the ASFV target. Endpoint analysis shows all samples giving similar signals. Samples labeled "control" contained only the control-DNA.

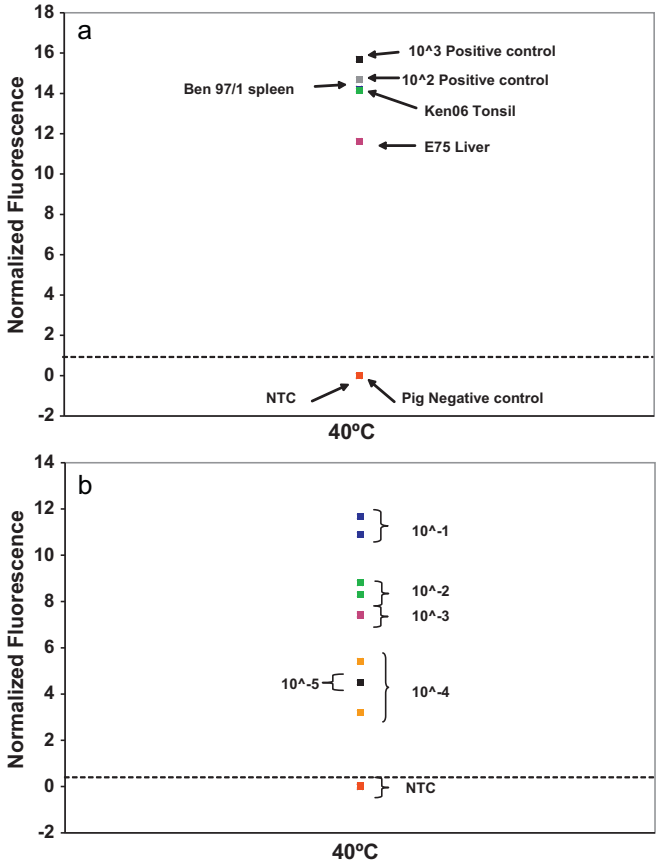

Fig. 3. (a) ASFV monoplex assay of three clinical samples Ben97/1, Ken06 and E75 extracted from spleen, tonsil and liver tissues, respectively. The ASFV monoplex was also tested against a negative control containing only porcine DNA, and two standard ASFV controls. NTCs contained no DNA. The threshold (dotted line) was set at 0.2 normalized fluorescent units. (b) ASFV clinical strain Ben97/1 was serially diluted from 10^{-1} to 10^{-5} and was tested in duplex using the endpoint format. Limit of detection corresponding to approximately 1 copy/μl was reached at a 10^{-5} dilution. All values are normalized to 70 °C with the baseline subtracted.

ical sensitivity between 100 and 10 copies per reaction. However, the strength of the symmetric PCR signal decreases dramatically with decreasing number of targets. This decrease in robustness is most likely due to low levels of mis-priming that are typical of symmetric PCR assays and also account for the scatter among replicate assays as they enter the plateau phase of the reaction. LATE-PCR, in contrast, produces large excess of single-stranded amplicon over double-stranded amplicon and the single-stranded molecules do not interfere with polymerase activity. In addition, PrimeSafe™II acts to reduce mis-priming throughout the reaction, not just prior to the first thermal cycle. LATE-PCR also allows for the use of probes that have melting temperatures below the annealing temperature of amplification. As a consequence, amplification during the reaction proceeds under optimal conditions without interference of probes and with minimum mis-priming. Product detection at endpoint can be qualitative or quantitative and separation of amplification and detection reduces the total run time of the assay.

The melting curve analysis of the assay on both synthetic and viral targets revealed a probe T_m which is a few degrees below the theoretical temperature calculated by Visual OMP software. This difference is most likely due to the Platinum® *Tfi* reaction buffer, which reduces the predicted melting temperature by 1–3 °C.

Testing of viral DNA from three tissue samples from pigs infected deliberately, as well as 19 strains of ASFV extracted from infected cell cultures, was performed at SVA/SLU in Uppsala, Sweden. All three tissue samples produced a very strong positive signal under both the LATE-PCR monoplex and duplex assays. Additional tests with heterologous viruses proved that the LATE-PCR assay is specific to ASFV. In comparison to the CRL method currently used at SVA, the LATE-PCR assay performed as well, showing comparable detection capabilities.

According to King et al. (2003) detection of ASFV by means of the TaqMan® assay requires 24 h from the time the sample is received. All procedures require the use of expensive, non-portable laboratory equipment. The devastation caused by an outbreak of ASF makes early detection a necessity for the prevention of further loss. The combination of a sensitive and reliable LATE-PCR assay with a diagnostic system designed to be used in the field by veterinarians would be ideal. Smiths Detection Diagnostics is currently developing the Bio-Seeq Portable Veterinary Diagnostics Laboratory, a field instrument that aims at bringing the LATE-PCR assay for ASFV to the site of the outbreak, thereby significantly reduce the time required to identify pathogen (Belák et al., 2009). The Bio-Seeq includes five independent thermocyclers, each of which accommodates a Universal Sample Preparation Device (USPD) that processes a biopsy sample all the way through tissue homogenization, nucleic acid extraction and purification, PCR amplification, and data communication with a global command center. The required reagents are

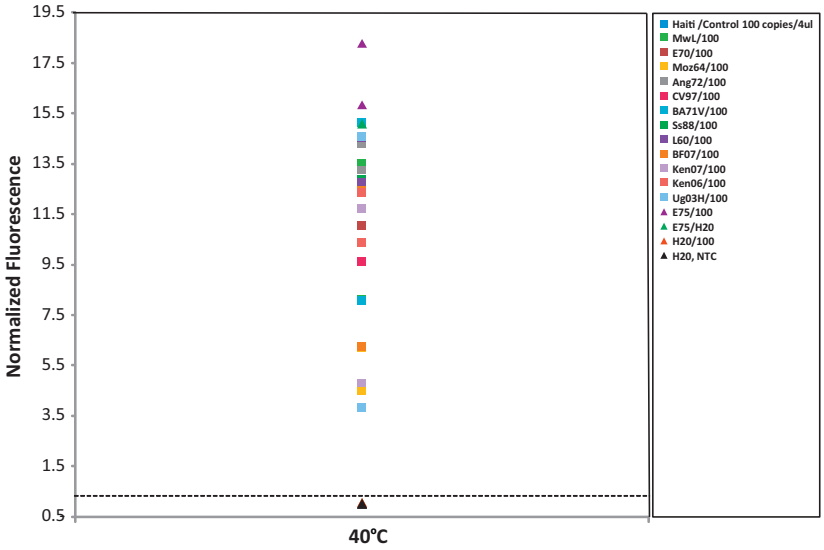

Fig. 4. ASFV duplex assay endpoint detection of 14 strains of ASFV. All samples were run in duplicate and all showed a positive signal at 40 °C. Differences in fluorescence reflect differences in initial target concentration. All data have been normalized to 70 °C and are baseline subtracted.

Table 4
Comparison of the LATE-PCR assay used in this study and the CRL recommended method (ASFV TaqMan®, King et al., 2003) used at the National Veterinary Institute, Uppsala, Sweden.

Sample	CRL-based method		LATE-PCR, this study			
	Mean Ct[#]	Result	Run 1	Run 2	Result	Correlation
E70	25.93	Positive	Reaction	Reaction	Positive	Yes
Dilution 10^{-1}	25.03	Positive	Reaction	Reaction	Positive	Yes
Dilution 10^{-2}	28.27	Positive	Reaction	Reaction	Positive	Yes
Dilution 10^{-3}	31.22	Positive	Reaction	Reaction	Positive	Yes
Dilution 10^{-4}	41.95[*]	Doubtful	Reaction	Reaction	Positive	No
Tog09	21.23	Positive	Reaction	Reaction	Positive	Yes
Dilution 10^{-4}	35.44	Positive	No Reaction	No Reaction	Negative	No
Dilution 10^{-5}	0	Negative	No Reaction	No Reaction	Negative	Yes
Ken05.Tk1	17.77	Positive	Reaction	Reaction	Positive	Yes
Dilution 10^{-3}	28.21	Positive	Reaction	Reaction	Positive	Yes
Dilution 10^{-4}	31.87	Positive	Reaction	Reaction	Positive	Yes
Dilution 10^{-5}	36.37[*]	Doubtful	Reaction	Reaction	Positive	No
Ken06.Bus	18.32	Positive	Reaction	Reaction	Positive	Yes
Dilution 10^{-2}	24.81	Positive	Reaction	Reaction	Positive	Yes
Dilution 10^{-3}	28.34	Positive	Reaction	Reaction	Positive	Yes
Dilution 10^{-4}	32.1	Positive	Reaction	Reaction	Positive	Yes
Arm07	18.64	Positive	Reaction	Reaction	Positive	Yes
Dilution 10^{-3}	31.15	Positive	Reaction	Reaction	Positive	Yes
Dilution 10^{-4}	36.42[*]	Doubtful	Reaction	Reaction	Positive	No
Dilution 10^{-5}	0	Negative	No Reaction	No Reaction	Negative	Yes
Nig08 La/Ok	20.28	Positive	Reaction	Reaction	Positive	Yes
Dilution 10^{-1}	25.39	Positive	Reaction	Reaction	Positive	Yes
Dilution 10^{-3}	30.11	Positive	Reaction	Reaction	Positive	Yes

[#] Mean Ct of two real-time PCR runs.
[*] One replicate failed to produce a positive result.

Table 5
Heterologous viruses used to test specificity of the ASFV assay.

Virus	Strain/serotype	Tissue	Source	Signal
CSFV	Alfort	Cell culture	SVA	Negative
PRRSV	European strain, SP2777	Serum	UCM	Negative
PCV-2	Stoon-1010	Plasmid	SVA	Negative
SVDV	HKN 1/80	Cell culture	IAH	Negative
VSV	Indiana-1 (Indiana C, 1942)	Cell culture	IAH	Negative
VSV	New Jersey, Colorado 1984	Cell culture	IAH	Negative

Abbreviations: CSFV – Classical swine fever virus; PRRSV – Porcine respiratory and reproductive syndrome virus; PCV-2 – Porcine circovirus 2; SVDV – Swine vesicular disease virus; VSV – Vesicular stomatitis virus; SVA – National Veterinary Institute, Uppsala, Sweden; UCM – Universidad Complutense de Madrid, Madrid, Spain; CVI – Central Veterinary Institute, Budapest, Hungary; IAH – Institute for Animal Health, Pirbright, UK.

stored lyophilized in the reagent pack of the USPD which is labeled with a barcode that instructs the thermocycler what protocol of heating and cooling to use. The Bio-Seeq is designed to carry out endpoint analysis, which allows the instrument to make the best use of the unique features of LATE-PCR such as extended temperature spaces and multiplexed detection which are not available in other PCR chemistries.

5. Conclusions

The results show that LATE-PCR assay described above is an efficient, specific and sensitive test. The use of this novel assay allows for endpoint analysis, which provides a quick test with sensitivity below 10 viral genome copies. The LATE-PCR assay has

been optimized to generate reliable and reproducible results in either standard laboratory diagnostic instruments or settings or in a portable instrument now under development. Since the on-site or front-line diagnosis of ASF is very important, LATE-PCR provides a powerful novel tool for the ASF eradication programmes.

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Erratum

Erratum to "Design and verification of a highly reliable Linear-After-The-Exponential PCR (LATE-PCR) assay for the detection of African swine fever virus" [J. Virol. Methods 172 (2011) 8–15]

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