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ORIGINAL ARTICLE

Molecular Diagnosis of African Swine Fever by a New Real-Time PCR Using Universal Probe Library

J. Fernández-Pinero[1], C. Gallardo[1], M. Elizalde[1], A. Robles[1], C. Gómez[1], R. Bishop[2], L. Heath[3], E. Couacy-Hymann[4], F. O. Fasina[5], V. Pelayo[1], A. Soler[1] and M. Arias[1]

[1] Centro de Investigación en Sanidad Animal (CISA-INIA), Madrid, Spain
[2] International Livestock Research Institute (ILRI), Nairobi, Kenya
[3] ARC-Onderstepoort Veterinary Institute, Transboundary Animal Diseases Programme, Pretoria, South Africa
[4] LANADA-LCPA Laboratoire de Virologie, Rue du Jardin Botanique BP 206, Bingerville, Ivory Coast
[5] Department of Production Animal Studies, University of Pretoria and National Veterinary Research Institute, Vom, Nigeria

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Correspondence:
J. Fernández-Pinero, Centro de Investigación en Sanidad Animal (CISA-INIA), Valdeolmos, 28130 Madrid, Spain.
Tel.: +34 91 6202300;
Fax: +34 91 6202247;
E-mail: fpinero@inia.es

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Summary

A highly sensitive and specific real-time PCR method was developed for the reliable and rapid detection of African swine fever virus (ASFV). The method uses a commercial Universal Probe Library (UPL) probe combined with a specifically designed primer set to amplify an ASFV DNA fragment within the VP72 coding genome region. The detection range of the optimized UPL PCR technique was confirmed by analysis of a large panel (n = 46) of ASFV isolates, belonging to 19 of the 22 viral p72 genotypes described. No amplification signal was observed when closely clinically related viruses, such as classical swine fever, or other porcine pathogens were tested by this assay. The detection limit of the UPL PCR method was established below 18 DNA copies. Validation experiments using an extensive collection of field porcine and tick samples (n = 260), coming from Eastern and Western African regions affected by ASF, demonstrated that the UPL PCR technique was able to detect over 10% more positive samples than the real-time TaqMan PCR test recommended in the OIE manual, confirming its superior diagnostic sensitivity. Clinical material collected during experimental infections with different ASFV p72 genotypes was useful for assuring both the capacity of the UPL PCR for an early viral DNA detection and the competence of the technique to be applied in any ASF diagnostic target sample. The reliability and robustness of the UPL PCR was finally verified with a panel of ASFV-infected clinical samples which was repeatedly tested at different times. Additionally, an internal control PCR assay was also developed and standardized using UPL probes within the endogenous β-actin gene. Finally, the complete study offers a new validated real-time PCR technique, by means of a standardized commercial probe, providing a simple, rapid and affordable test, which is ready for application in the routine diagnosis of ASF.

Introduction

African swine fever (ASF) is a highly contagious infectious disease affecting *Suidae*, caused by a complex DNA virus of the *Asfarviridae* family (Dixon et al., 2005). It is a notifiable disease, with a serious social and economic impact constraining trade of swine and pig products and affecting food security. African swine fever virus is currently endemic in large parts of sub-Saharan Africa and Sardinia, producing great economic losses (Penrith and Vosloo, 2009). In 2007, ASF emerged in the Caucasus for the first time (Rowlands et al., 2008) and has since spread to several countries in the region. Nowadays, the potential distribution of the ASF virus (ASFV) infection is transcontinental, and it may become a major animal health problem.

A vaccine is not yet available for ASF, and there is no prospect of it being likely in the near future. Therefore, efforts should be concentrated on early detection and appropriate control and eradication programmes. To achieve this, laboratory diagnosis is essential. A wide variety of laboratory tests are available for the detection of ASFV or the specific antibodies produced in infected animals (Arias and Sánchez-Vizcaíno, 2002; OIE, 2008). These techniques allow a rapid and accurate laboratory detection of ASFV-positive and carrier animals. At present, the most convenient, safe and frequently used techniques for ASFV detection are direct immunofluorescence (Bool et al., 1969), hemadsorption (HA) test (Malmquist and Hay, 1960) and polymerase chain reaction (PCR). PCR has been demonstrated to be a consistent, specific and sensitive tool for ASF diagnosis (Agüero et al., 2003; King et al., 2003). Moreover, Because ASFV infection induces a long-term viraemia in the infected pigs, the PCR test has been shown to be an excellent and rapid technique that can be used as a routine diagnostic method for ASF in either surveillance, control or eradication programmes.

Although different molecular techniques have been described for ASFV detection (Steiger et al., 1992; Agüero et al., 2004; Hjertner et al., 2005; Basto et al., 2006; Giammarioli et al., 2008; James et al., 2010; McKillen et al., 2010; Ronish et al., 2011), the molecular diagnosis of ASF still relies on a restricted number of conventional and real-time PCR methods (Agüero et al., 2003; King et al., 2003; Zsak et al., 2005), mainly those recommended by the World Organization for Animal Health (OIE, 2008) in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Primer sets and probes used in the molecular techniques are repeatedly designed within the VP72 coding region, a well-characterized and highly conserved region of the ASFV genome (Yu et al., 1996). Specifically, the OIE-recommended PCR tests have been validated to identify ASFV isolates from all the 22 known p72 virus genotypes (Bastos et al., 2003), including both non-hemadsorbing and low virulent virus isolates.

Nevertheless, Reference Laboratories have shown interest in additional PCR methods that are ready to use for ASF diagnosis, which may improve the reference ones, preferably using commercial reagents, which allow for better and easier standardization, validation and harmonization within diagnostic laboratories. Universal Probe Library (UPL; Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) comprises a collection of 165 pre-synthesized fluorogenic hydrolysis locked nucleic acid (LNA) probes, originally designed for gene expression analysis and offered as a universal detection system. These probes are 8–9 LNA residues in length, labelled with FAM dye at the 5′ and a dark quencher dye at the 3′. The main advantages of commercial UPL probes for their potential application in the diagnosis of infectious diseases are reasonably low cost, short delivery time and ready-to-use presentation, being an easy and rapid system for developing and carrying out an advanced molecular technique in the laboratory (Wenzel et al., 2009).

This work presents the development, standardization and validation of a real-time PCR system by using a specific commercial UPL probe, which improves the detection of ASFV. The combination of a specifically designed primer set and an appropriate UPL probe will allow the specific and sensitive detection of ASFV by real-time PCR at a comparably lower cost. In addition, a second real-time PCR test was developed and standardized, taking advantage of UPL probes, for the detection of an endogenous porcine gene (β-actin) to be used as a simple internal control assay in clinical material. Laboratories, mainly those from countries where ASFV is circulating, could benefit from this cost-efficient and simple way of acquiring and using UPL probes.

Materials and Methods

Viruses

Two panels of 20 ASFV isolates belonging to the virus collection of the European Union Reference Laboratory (URL) for ASF, CISA-INIA, Valdeolmos, Spain, and 26 ASFV isolates received from the OIE Reference Laboratory for ASF, OVI, Onderstepoort, South Africa, were used throughout this study (Table 1).

The following porcine viruses were employed in the specificity assays: classical swine fever virus (CSFV) strain Alfort187, American and European serotypes of porcine respiratory and reproductive syndrome virus (PRRSV), porcine circovirus types I and II (PCV-I and PCV-II), Aujeszky's disease virus (ADV) strain Phylaxia, foot-and-mouth disease virus (FMDV) serotype O, swine vesicular disease virus (SVDV) strain UKG/27/72, vesicular stomatitis virus (VSV) serotype Indiana. All the porcine viruses described were obtained from the virus collection held in reserve at CISA-INIA, Valdeolmos, Spain.

Experimental samples

All the *in vivo* experiments were performed in the BSL-3 animal facilities at CISA-INIA, Valdeolmos, Spain. Different groups of Large White × Landrace pigs were inoculated with different ASFV isolates for diverse research purposes, following the established animal welfare principles. For this particular study, experimental clinical samples were obtained as follows:

EDTA-blood and serum samples were collected at 0, 1, 2, 3 and 4 days post-inoculation (dpi) from an 1-year-old

Table 1. African swine fever virus (ASFV) isolates used in the study

ASFV isolate	Country of origin	Town/Province	Year of outbreak	Host species	P72 genotype	ASFV UPL PCR (C_t value)	ASFV Ref PCR (C_t value)
Lisbon60	Portugal	Lisbon	1960	Pig	I	28.25	30.16
E70	Spain	Pontevedra	1970	Pig	I	26.40	28.31
Ba71V	Spain	Badajoz	1971	Vero cell adapted pig isolate	I	24.00	25.41
E75	Spain	Lerida	1975	Pig	I	27.07	28.97
Ss88	Italy	Sardinia	1988	Pig	I	26.87	28.18
Haiti	Haiti	Port-au-Prince	1981	Pig	I	22.80	24.60
Kat67	Zaire	Katanga	1967	Pig	I	21.32	21.97
Ang72	Angola	NK	1972	Pig	I	29.06	30.95
CV97	Cape Verde	NK	1997	Pig	I	31.17	34.84
Nig08/LaOK1	Nigeria	Lagos	2008	Pig	I	21.41	22.04
BF07/IpTC	Burkina Faso	Ipelce	2007	Pig	I	28.94	31.13
Tog09/P2	Togo	Togoville	2009	Pig	I	21.91	21.23
Arm07	Armenia	Dilijan	2007	Pig	II	18.39	20.20
Moz64	Mozambique	NK	1964	Pig	V	32.85	35.56
MwLil 20/1	Malawi	Chalaswa	1983	Tick	VIII	29.96	30.87
Ug64	Uganda	NK	1964	Pig	IX	22.35	22.79
Ug03H.1	Uganda	Hoima	2003	Pig	IX	32.37	35.78
Ken06.B1	Kenya	Busia	2006	Pig	IX	31.27	33.88
Ken07.Eld1	Kenya	Eldoret	2007	Pig	IX	25.04	26.21
Ken05/Tk1	Kenya	Machakos	2005	Tick	X	19.89	18.79
CAM 1/86	Cameroon	NK	1986	Pig	I	27.72	28.03
CAM 1/89	Cameroon	NK	1989	Pig	I	24.92	25.23
IC 1/96	Cote d'Ivoire	NK	1996	Pig	I	24.48	24.67
IC 3/96	Cote d'Ivoire	NK	1996	Pig	I	20.08	20.02
ZAM/2001/6	Zambia	Lusaka	2001	Pig	I	23.05	24.62
Mauritius 2008/1	Mauritius	NK	2008	Pig	II	25.37	25.38
RSA/2008/1	South Africa	Gravelotte	2008	Pig	III	27.08	27.56
RSA/W/1/99/+	South Africa	Soutpansberg	1999	Warthog	IV	23.37	24.34
Spec 265	Mozambique	Maputo	1994	Pig	VI	24.31	24.82
RSA 2003/7	South Africa	Ellisras	2003	Wild pig	VII	25.66	27.39
KEN/2001/1	Kenya	NK	2001	Pig	IX	25.03	26.66
TAN 2005/3	Tanzania	Mwanza City	2005	Pig	IX	25.53	26.57
BUR 84/1	Burundi	Mushasagitega	1984	Pig	X	25.11	25.68
BUR 90/1	Burundi	Muyinga	1990	Pig	X	24.62	24.94
KAB6/2	Zambia	Livingstone Game Park	1983	Tick (Warthog)	XI	26.74	27.58
MZI 92/1	Malawi	Euthini, Mzinda district	1992	Pig	XII	26.29	26.49
SUM 14/11	Zambia	Sumbu National Park	1983	Tick (Warthog)	XIII	29.16	29.68
NYA 1/2	Zambia	Kalumo	1988	Tick (Warthog)	XIV	26.15	26.92
Tan/2008/1	Tanzania	NK	2008	Pig	XV	24.29	24.91
Tan 2003/2	Tanzania	Arusha	2003	Pig	XVI	24.06	25.39
NAM/P/1/95/+	Namibia	Windhoek (Meentkamp)	1995	Pig	XVII	25.84	25.41
RSA/P/1/95/+	South Africa	Hoedspruit	1995	Pig	XX	23.77	24.52
RSA/2008/2	South Africa	Kwalata, Hammanskraal, Gauteng	2008	Tick	XXII	23.37	25.52
RSA 2003/2	South Africa	NK	2003		NK	23.93	25.27
CAM 2009/01	Cameroon	NK	2009	Pig	NK	25.94	25.97
Nam 2009/01	Namibia	NK	2009	Pig	NK	24.44	25.16

NK, not known; UPL, Universal Probe Library.

The first 20 viral isolates were obtained from the virus collection of the European Union Reference Laboratory for ASF, CISA-INIA, Valdeolmos, Spain; the remaining 26 ones were received from the OIE Reference Laboratory for ASF, OVI, Onderstepoort, South Africa. C_t values obtained running in parallel the UPL PCR developed in this study and the reference TaqMan PCR method (Ref PCR) are shown.

pig inoculated by intramuscular route with 10^5 50% hemadsorption units ($HADU_{50}$) of the virulent ASFV Spain 70 strain (p72 genotype I). The animal was humanely killed at 4 dpi, and a spleen sample taken during the necropsy was also used in the study.

Four pigs were inoculated by intramuscular route with 10 $HADU_{50}$ of the ASFV isolate Arm07 (p72 genotype II) and two untreated pigs were kept in contact, being housed in the same box as the inoculated animals. EDTA-blood and serum samples collected at 0, 5, 9, 12 and 15 dpi from survival animals were employed in this study. Organs comprising spleen, heart, liver, lung, kidney, tonsils, ear skin and lymph nodes were obtained from the necropsy.

Serum samples were collected at regular intervals until the end of the studies from domestic pigs intramuscularly inoculated with either 10 $HADU_{50}$ of the ASFV virulent strain Ken06.Bus (p72 genotype IX) or 10 $HADU_{50}$ of the moderately virulent ASFV strain Ken05.Tk1 (p72 genotype X). Two contact animals housed in the same box of inoculated pigs were included in each group. Spleen and liver samples collected from two dead animals infected with ASFV Kenya06.Bus or Kenya05.Tk1 isolates, respectively, were also used in the study.

Finally, EDTA-blood, serum, liver and spleen samples were collected from a healthy non-infected donor pig to be used in the specificity tests and for the preparation of diluted material.

Field samples

A panel of 246 field porcine EDTA-blood, serum and tissue homogenates and a set of 14 *Ornithodoros* tick homogenates samples, available at the URL for ASF, CISA-INIA, Valdeolmos, Spain, were used in the validation studies. Specifically, clinical samples were collected from recent ASF outbreaks occurring between 2001 and 2010 in Eastern and Western African countries: Kenya, Uganda, Tanzania, Republic of the Congo, Nigeria, Burkina Faso, Togo, Ghana and Ivory Coast. All the samples had been previously reported as positive (porcine samples $n = 216$, tick samples $n = 10$) or negative (porcine samples $n = 30$, tick samples $n = 4$) for ASFV using the OIE-prescribed diagnostic techniques for virus detection, including virus isolation and conventional PCR (OIE, 2008).

Nucleic acids extraction

Total nucleic acids were extracted from 200 μl of sample (cell cultures supernatants, EDTA-blood, serum, tick homogenates and tissue homogenates 10% in PBS) using commercial High Pure PCR Template Preparation Kit, following the manufacturer's instructions (Roche Diagnostics GmbH). In the final step, the elution of the nucleic acids was achieved in 50 μl of sterile distilled water. DNA of ASFV isolates received from OIE Reference Laboratory for ASF, OVI, Onderstepoort, South Africa, was obtained using the same procedure, but DNA elution was completed in 100 μl of elution buffer.

Primers and probe design for ASFV detection

Based on the DNA sequence of the VP72 coding genome region of ASFV Spain 70 strain (GenBank accession no. S89966), six different combinations of primers and UPL probes were obtained from the ProbeFinder Software (Roche Applied Science). A further multiple-sequence alignment of 38 homologous nucleotide sequences of ASFV isolates belonging to several p72 genotypes, available in GenBank, was generated using ClustalW 2.0 software (European Bioinformatics Institute, Hinxton, Cambridge, UK). Finally, the combination of a modified ASF-VP72-F/ASF-VP72-R primer set, delimiting a DNA fragment of 68 bp, and a UPL#162 probe was selected as the best choice for covering the largest range of ASFV isolates (Table 2). A BLASTn search of the primer sequences confirmed the specificity to ASFV.

Both primers and probe were purchased from Roche Applied Science.

Standard DNA

A plasmid containing the ASFV DNA target fragment of the designed UPL PCR was produced by ATG:biosynthetics GmbH. Specifically, a 74-bp DNA molecule containing the UPL PCR target nucleotide sequence of the reference ASFV Spain 70 strain (GenBank accession no. S89966) was synthesized and cloned into pBSK vector. The plasmid was linearized by *Ban*II restriction enzyme incubation, and from a concentration of 4.68×10^{10} DNA copies/μl, serial dilutions were prepared until 8×10^{-11}. The dilutions containing a range between 4.68×10^7 and 3.74×10^0 DNA copies/μl were stored as standard DNA samples for further use in sensitivity PCR experiments.

African swine fever virus PCR procedures

Real-time PCR tests were performed in 96-well plate MX3005P equipments (Stratagene, Agilent Technologies Inc., Santa Clara, CA, USA). A positive result in real-time PCR was determined by identifying the threshold cycle value (C_t) at which reporter dye emission appeared above background within 40 cycles.

The OIE-recommended real-time PCR method for ASF diagnosis was used as the reference technique in comparative assays (King et al., 2003; OIE, 2008).

Table 2. Primers and probe designed for African swine fever virus (ASFV) and porcine β-actin gene

PCR target	Name	Sequence (5′–3′)	Nucleotide position	ASFV strains homology (%)[a]
ASFV UPL PCR	ASF-VP72-F primer	CCCAGGRGATAAAATGACTG	893–912[b]	100
	ASF-VP72-R primer	CACTRGTTCCCTCCACCGATA	940–960[b]	100
	UPL#162 probe	6FAM-GGCCAGGA-dark quencher dye (Roche cat no. 04694490001)	930–937[b]	100
β-actin UPL PCR	ACT-162-F primer	GGATGCAGAAGGAGATCACG	1022–1041[c]	NA
	ACT-162-R primer	ATCTGCTGGAAGGTGGACAG	1132–1151[c]	NA
	UPL#162 probe	6FAM-GGCCAGGA-dark quencher dye (Roche cat no. 04694490001)	1121–1128[c]	NA

NA, not applicable; UPL, Universal Probe Library.
[a]Nucleotide homology for the 38 ASFV sequences aligned with ClustalW 2.0.
[b]ASFV Spain 70 VP72 gene (GenBank accession no. S89966).
[c]Sus Scrofa β-actin gene (GenBank accession no. AY550069).

UPL PCR

Real-time PCR protocol was optimized using LightCycler 480 Probes Master kit (Roche Applied Science). Briefly, PCR mix was prepared in a final volume of 20 μl containing 2 μl of DNA sample, 1× LC480 Probes Master PCR Mix (containing PCR Buffer, dNTPs and FastStartTaq DNA polymerase), 0.4 μM of each sense and antisense primer and 0.1 μM UPL#162 probe. The incubation profile for ASFV DNA amplification was established as follows: 5 min at 95°C, 45 cycles at 95°C 10 s and 60°C 30 s, with fluorescence acquisition in the FAM channel at the end of each PCR cycle.

Reference real-time PCR

Reference ASFV real-time PCR using primers and TaqMan probe described previously (King et al., 2003), which are located within the VP72 genome region, was performed following the modified fast PCR protocol (Fernández-Pinero et al., 2007) established at the URL for ASF, CISA-INIA, Valdeolmos, Spain, using QuantiFast Probe PCR kit (Qiagen, Hilden, Germany). Briefly, PCR mix was prepared in a volume of 20 μl containing 2 μl of DNA sample, 1× QuantiFast Probe PCR Master Mix (containing QuantiFast Probe PCR Buffer, dNTPs and HotStartTaq Plus DNA polymerase), 0.4 μM of each sense and antisense primer and 0.25 μM of TaqMan probe. African swine fever virus DNA amplification was accomplished with the following incubation programme: 3 min at 95°C, 45 cycles at 95°C 10 s and 58°C 30 s, with fluorescence reading in the FAM channel at the end of each cycle.

Internal control PCR

An internal control real-time PCR assay was developed based on the detection of a porcine DNA fragment within the endogenous β-actin gene. Of the different primers/UPL probe combinations offered by ProbeFinder Software (Roche Applied Science) for the provided porcine β-actin gene sequence (GenBank accession no. AY550069), the specific set that included the UPL#162 probe was selected (Table 2). The assay was designed and optimized to be used in a 'parallel-well' duplex system under the same mix and reaction conditions as the UPL real-time PCR developed for ASFV detection.

Results

Analytical specificity and detection range

Primary UPL real-time PCR assays were carried out to evaluate the competence of the selected primers (ASF-VP72-F/ASF-VP72-R) and UPL probe (UPL#162) for the detection of ASFV. A first panel of 20 reference ASFV isolates, representing viral p72 genotypes I, II, V, VIII, IX and X, was analysed. All of them were properly detected by using the novel UPL real-time PCR, showing in most cases lower C_t values (ranging from 1 to 4 cycles) than those obtained using the protocol of the OIE reference TaqMan real-time PCR established in the laboratory (King et al., 2003) (Table 1).

Specificity of the ASFV UPL PCR was subsequently evaluated testing closely clinically related CSFV, as well as other viruses affecting swine: PRRSV, PCV-I and PCV-II, ADV, FMDV, SVDV and VSV, and clinical material (EDTA-blood, serum, liver and spleen) obtained from a non-infected donor pig. Fluorescence signal was observed exclusively in ASFV-positive control (data not shown).

Finally, a comprehensive study to assure the detection range of the developed UPL PCR was carried out with the analysis of a second collection of 26 ASFV isolates, belonging to 17 of the 22 reported p72 genotypes. All the viral DNAs were tested in parallel by the reference TaqMan PCR in a comparative study, all of them giving a

Table 3. Analytical sensitivity of African swine fever virus Universal Probe Library (UPL) real-time PCR

Standard DNA copies	UPL PCR (C_t)
4.68×10^7	16.54 (SD 0.48)
4.68×10^6	19.46 (SD 0.10)
4.68×10^5	22.73 (SD 0.25)
4.68×10^4	25.84 (SD 0.13)
4.68×10^3	28.88 (SD 0.12)
4.68×10^2	31.63 (SD 0.27)
9.36×10^1	34.26 (SD 0.03)
1.87×10^1	36.50 (SD 0.42)
3.74×10^0	38.35[a]/No C_t

Serial DNA dilutions of quantified plasmid containing VP72 target fragment were tested in triplicates. Mean C_t values and standard deviation (SD) obtained are shown.
[a]Mean C_t value of two replicates, the third one was negative (No C_t).

strong positive fluorescent amplification reaction by both techniques. Overall, the C_t values reported by the UPL PCR were lower than those obtained by the reference TaqMan PCR method (Table 1).

Analytical sensitivity

Initially, PCR experiments were carried out testing duplicates of 10-fold serial dilutions of several ASFV isolates representing different p72 viral genotypes, namely CV97, Nig08/LaOK1, Ss88 (all belonging to p72 genotype I), MwLil20/1 (most divergent, p72 genotype VIII) and Ken07.Eld1 (p72 genotype IX). The results obtained from parallel PCR runs showed UPL PCR giving a sensitivity 10 times higher than that provided by the established reference TaqMan PCR for each ASFV isolate analysed (data not shown).

Further analytical sensitivity assays to set up the detection limit of the UPL PCR were performed, analysing triplicates of serial dilutions of quantified DNA plasmid containing the VP72 PCR target fragment. The detection limit of the UPL PCR proved to be between 4 and 18 DNA copies (Table 3). A standard curve was constructed from the amplification plots, and a linear dynamic range was observed across seven orders of magnitude tested, ranging from 4.68×10^7 to 1.87×10^1 DNA copies per reaction (Fig. 1).

Diagnostic sensitivity and specificity

The first diagnostic sensitivity tests of the developed ASFV UPL PCR were carried out with the analysis of EDTA-blood and serum samples collected daily from a pig, experimentally infected with ASFV Spain 70 strain (p72 genotype I). African swine fever virus DNA was consistently detected as early as from the 1st (EDTA-blood) and 3rd (serum) day post-inoculation (dpi), confirming the competence of the new UPL PCR for the ASFV detection at very early stages of infection (data not shown).

Additionally, a collection of samples obtained from an experimental inoculation assay with the ASFV isolate Arm07 was tested to further prove the competence of the UPL PCR detecting the p72 genotype II currently circulating in the Caucasus region. EDTA-blood and serum samples were reported as ASFV positive from the first dpi collected (5 dpi) in four inoculated pigs and from 12 dpi in the two contact animals. Strong fluorescence signal was also observed in all organs collected at necropsy from the six pigs, comprising spleen, heart, liver, lung, kidney, tonsils, ear skin and lymph nodes (data not shown).

In a comparative study, serum samples collected at different dpi from domestic pigs experimentally inoculated with either ASFV Ken06.Bus (p72 genotype IX) or Ken05.Tk1 (p72 genotype X) isolates, as well as from pigs kept in contact with them, were analysed at the same time by both UPL and reference TaqMan PCR techniques. The UPL PCR was able to detect the presence of the virus between 3 days to more than 1 week before to the reference method in four of the six inoculated animals and in all four contact pigs, demonstrating its capacity for detecting low levels of viraemia in the animals. Also, lower C_t values were mostly reported by the new technique (Table 4).

Finally, an extensive panel of field samples originating from recent ASF outbreaks occurring over the last 10 years in different Eastern and Western African countries was analysed to assure the validity of the UPL PCR for diagnostic purposes. Specifically, 246 porcine EDTA-blood, serum and tissue homogenates, as well as 14 *Ornithodoros* tick homogenates, were tested in a comparative study by the new UPL and the reference TaqMan PCR methods. Fluorescent amplification signal was produced by the UPL PCR in all porcine ($n = 216$) and tick ($n = 10$) samples previously identified as ASFV positive by performing the OIE-prescribed viral diagnostic tests, including conventional PCR and virus isolation. Comparatively, more than 10% of these 226 ASFV-positive samples (porcine samples $n = 28$, tick samples $n = 1$) remained undetected when the reference TaqMan PCR was applied. In more detail, 54% ($n = 122$) and 30% ($n = 68$) of the entire positive samples gave similar or lower C_t values, respectively, using the UPL PCR compared with the results offered in parallel by the reference TaqMan PCR (meaning a C_t value difference between the two tests below or >1, respectively). On the other hand, the amplification profile of all the ASFV-negative samples tested ($n = 34$) remained below the background

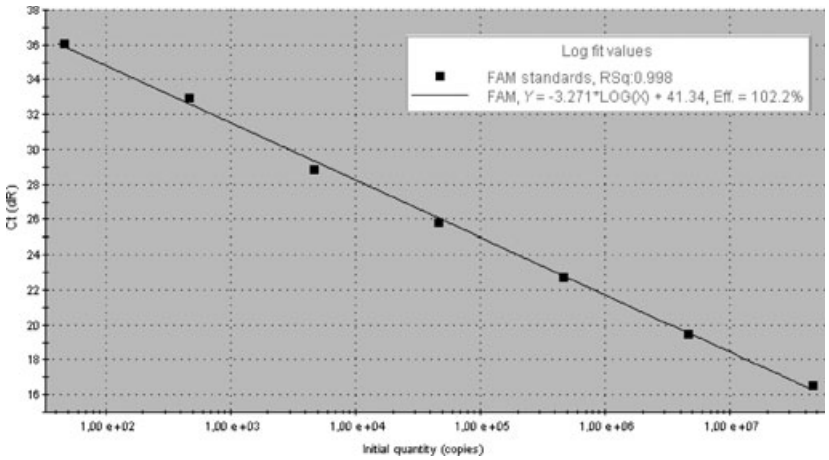

Fig. 1. Standard curve of the African swine fever virus Universal Probe Library PCR. The curve was generated by analysis of triplicates of 10-fold serial dilutions of the quantified DNA plasmid used as synthetic positive control. Each ■ corresponds to the mean value of three replicates.

Table 4. Detection of African swine fever virus (ASFV) in serum samples from experimental infection assays

	0 dpi		3 dpi		7 dpi		9–10 dpi		14 dpi		16 dpi		18 dpi		21 dpi	
Pig no.	Ref PCR	UPL PCR	Ref PCR	UPL PCR	Ref PCR	UPL PCR	Ref PCR	UPL PCR	Ref PCR	UPL PCR	Ref PCR	UPL PCR	Ref PCR	UPL PCR	Ref PCR	UPL PCR
C1	No C_t	No C_t	No C_t	No C_t	32.27	20.02	26.31	20.45	†							
C2	No C_t	No C_t	No C_t	No C_t	No C_t	39.33	No C_t	34.44	23.96	23.67	28.17	21.61	†			
C3	No C_t	No C_t	No C_t	No C_t	22.56	21.12	†									
C4	No C_t	No C_t	No C_t	37.59	23.35	19.20	26.73	19.67	†							
CC5	No C_t	No C_t	No C_t	No C_t	No C_t	39.85	30.39	27.61	25.26	23.93	†					
CC6	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	36.06	28.19	20.52	†					
C9	No C_t	No C_t	No C_t	39.90	24.21	21.71	26.36	23.23	†							
C10	No C_t	No C_t	No C_t	39.87	21.67	20.44	No C_t	No C_t	29.22	29.71	NS	NS	23.63	20.48	†	
CC11	No C_t	No C_t	No C_t	No C_t	No C_t	39.20	No C_t	34.89	No C_t	38.84	NS	NS	23.40	23.79	24.62	23.85
CC12	No C_t	No C_t	No C_t	No C_t	No C_t	39.44	No C_t	38.09	22.94	21.42	NS	NS	25.48	21.49	†	

dpi, day post-inoculation; NS, no sample; UPL, Universal Probe Library.
Pigs no. C1–C4 and C9–C10 were intramuscularly inoculated with 10 $HADU_{50}$ of the ASFV virulent strain Ken06.Bus (p72 genotype IX) or 10 $HADU_{50}$ of the moderately virulent ASFV strain Ken05.Tk1 (p72 genotype X), respectively. Animals no. CC5–CC6 and CC11–CC12 were kept in contact with each inoculated group. Comparison of C_t values obtained running in parallel the UPL PCR developed in this study and the reference TaqMan PCR method (Ref PCR).
†Died animal.

fluorescence level in both the UPL and TaqMan PCR tests (data not shown).

Repeatability assays

Two ASFV-positive experimental samples were selected to perform an initial repeatability assay. Particularly, EDTA-blood collected at 1 dpi from a pig inoculated with ASFV Spain 70 strain and serum obtained at 5 dpi from a pig inoculated with ASFV Arm07 isolate, representing in this order a weak and a strong ASFV-positive samples, were used at this time. Ten replicates of each extracted viral DNA were incorporated into one run of the UPL PCR, giving mean C_t values of 33.24 and 24.98, with a standard deviation of 0.28 and 0.17, respectively.

Subsequently, a collection of 27 samples was used to further check the repeatability of the UPL PCR. Clinical ASFV-infected samples or ASF viral suspensions were diluted in healthy donor pig material to prepare the sample panel. The collection finally comprised 23 porcine serum, blood and tissue samples containing different amounts of very recent or reference ASFV isolates, representing four p72 genotypes: I, II, IX and X, as well as one CSFV isolate and three virus-negative samples (Table 5).

Table 5. Repeatability assay of the African swine fever virus (ASFV) UPL PCR using a panel of 27 samples

ASFV isolate	p72 genotype	Original sample	Sample dilution	Mean C_t value[a]	SD
E70	I	Porcine spleen homogenate, 4 dpi	Neat	23.78	0.43
			10^{-1}	26.43	0.39
			10^{-2}	30.07	0.33
			10^{-3}	32.82	0.5
			10^{-4}	36.29	1.27
Tog09	I	Porcine sera spiked with viral suspension	Neat	22.62	0.49
			10^{-4}	34.44	0.62
			10^{-5}	37.56	0.92
Nig08 La/Ok	I	Porcine blood spiked with viral suspension	Neat	21.59	0.28
			10^{-1}	26.26	0.29
			10^{-3}	32.18	0.36
Arm07	II	Porcine sera, 9 dpi	Neat	18.37	0.35
			10^{-3}	27.51	0.17
			10^{-4}	30.84	0.28
			10^{-5}	35.31	1.48
Ken06.Bus	IX	Porcine spleen homogenate, 9 dpi	Neat	19.69	0.32
			10^{-2}	26.51	0.31
			10^{-3}	29.71	0.39
			10^{-4}	33.23	0.77
Ken05.Tk1	X	Porcine liver homogenate, 18 dpi	Neat	19.91	0.26
			10^{-3}	29.96	0.35
			10^{-4}	33.38	0.48
			10^{-5}	37.04	0.75
		Classical swine fever virus		No C_t	
		Water		No C_t	
		Negative porcine blood		No C_t	
		Negative porcine spleen homogenate		No C_t	

dpi, day post-inoculation; SD, standard deviation; UPL, Universal Probe Library.
African swine fever virus-positive samples were prepared from material collected from experimentally infected pigs or using viral suspensions, comprising representative ASFV isolates and p72 genotypes. Diluted clinical material was prepared in the same matrix than the corresponding original sample. Viral DNA was extracted from the 27 samples and tested by the UPL PCR.
[a]Mean C_t value of eight separated UPL PCR runs.

After viral DNA extraction, samples were subjected to UPL PCR analysis in eight independent amplification runs prepared at different times by different technical staff and using randomly two real-time PCR MX3005P equipments (Stratagene). All the ASFV-positive samples were repeatedly detected by the UPL PCR assay in all eight runs performed, while negative material remained undetected. Standard deviation of the C_t values remained below 0.5 in most positive samples, a higher value only being reported in the seven samples containing the lowest viral DNA amount of the different p72 genotypes included in the study (Table 5).

Internal control PCR

The internal control PCR assay developed for β-actin gene detection, which uses a specific primer set ACT-162F/ACT-162R and the UPL#162 probe, was optimized to be carried out under the same mix and reaction conditions as the UPL real-time PCR developed for ASFV detection. Five serum and five EDTA-blood samples collected at different dpi from one ASFV-Arm07 experimentally infected pig, and homogenates of different ASFV target organs – spleen, liver, lung, kidney, tonsils, ear skin and lymph nodes, obtained from the same infected pig and one contact animal – were tested to assure the detection of endogenous internal control in any ASF diagnostic target sample. Additionally, a set of 48 porcine serum, EDTA-blood and tissue homogenates originating from recently ASF-affected African areas were included in the evaluation study. The internal control PCR was able to detect the porcine β-actin gene in all the samples analysed, showing some different C_t values depending on the sample matrix, with standard deviation rates ranging from 0.04 to 2.03 (Table 6).

Discussion

Molecular diagnostic techniques of ASF in the National Reference Laboratories of the EU member states are

Table 6. Evaluation of the internal control PCR for β-actin gene detection in different porcine material

Sample matrix	No. samples	Mean C_t value[a]	SD
EDTA-blood	16	24.38	1.63
Serum	25	33.25	2.03
Spleen	5	26.87	1.21
Liver	5	27.29	1.34
Lung	4	28.37	1.55
Kidney	4	26.60	1.63
Tonsils	3	28.90	0.44
Ear skin	2	33.21	0.04
Lymph nodes	8	27.87	1.23

SD, standard deviation.
[a]Mean C_t value obtained for the no. of samples analysed.

mainly based on two OIE-recommended methods, being a conventional (Agüero et al., 2003) and a real-time PCR techniques (King et al., 2003). These two procedures have been widely validated over time and are useful tools for routine diagnosis of the disease. The conventional PCR requires the manipulation of the amplified DNA products, increasing the contamination risk, and is being broadly replaced by the real-time PCR system. On the other hand, the reference real-time PCR developed some time ago amplifies a viral DNA fragment of 250 bp (King et al., 2003), being considered now as not optimal in size for a real-time PCR procedure. Although this latter mentioned technique gives good sensitivity and specificity rates, the robustness of the method is decreased when weak ASFV-positive samples are analysed. This limitation could be relevant in some epidemiological scenarios, such as African regions where chronically infected animals exhibit a low level but persistent viraemia. In this study, the improvement of the sensitivity and robustness of the real-time PCR technique was considered, and an innovative molecular approach to obtain a new ready-to-use PCR system was applied using commercial reagents, which could be suitable for better standardization, validation and harmonization in the laboratory diagnosis.

Design and optimization of new real-time PCR methods are usually time-consuming and very costly for diagnostic laboratories. Additionally, fluorogenic probes generally take the longest delivery time among all PCR reagents, while common primers can be available within 1 or 2 days. Universal Probe Library is a collection of commercial dual-labelled hydrolysis probes for use in real-time PCR assays. Locked nucleic acids are incorporated into the sequence of each UPL probe, being just 8–9 nucleotides in length. Locked nucleic acids are DNA nucleotide analogues with increased binding strengths compared with standard DNA nucleotides, maintaining the high melting temperature required for hybridizing PCR probes (Braasch and Corey, 2001; Costa et al., 2004). The sequence composition of the UPL probes reflects the most frequently encountered octamers and nonamers in sequence data banks (Wenzel et al., 2009). In this case, the PCR specificity is attained by the strict combination of specific forward and reverse primers and the appropriate UPL probe. By using the free online ProbeFinder software, each specific real-time PCR assay is designed by a simple two-step procedure. Then, the acquisition of the selected UPL probe in a ready-to-use presentation takes a short time at a lower cost of around 30% compared with customized standard hydrolysis probes.

Some real-time PCR methods have already been developed or are under development for the detection of pathogens using UPL probes, such as human pandemic H1N1 influenza virus (Wenzel et al., 2009) or human immunodeficiency virus (HIV). This work shows for the first time a real-time PCR method for the detection of a virus affecting livestock using this technology. The method employs an ASFV-specific primer set and an appropriate UPL probe (UPL#162) designed within the highly conserved VP72 coding genome region, which assures a specific amplification result.

Initial experiments were addressed to confirm the specificity of the optimized ASFV PCR test incorporating the selected primer set and UPL probe, by analysis of a panel of porcine viruses, including ASFV, clinically related CSFV and other viruses affecting suidae, and clinical material (EDTA-blood, serum, liver and spleen homogenates) collected from a healthy donor pig, with the only fluorescence signal obtained for the ASFV isolates included in the assays. Further assays were carried out to check the detection competence of the novel method, and a panel of 46 ASFV isolates belonging to 19 of the 22 p72 genotypes, obtained from different years and geographical origins, was subjected to the UPL PCR analysis, all of them being correctly detected, which revealed an excellent analytical specificity of the new ASFV real-time PCR.

Universal Probe Library PCR showed a 10-fold superior analytical sensitivity for the different ASFV isolates tested, representing p72 genotypes I (the one mostly distributed in Sardinia and West Africa), VIII (the most divergent p72 genotype) and IX (representative of East Africa), in comparison with the OIE reference TaqMan PCR (based on King et al., 2003). Subsequently, the detection limit of the UPL PCR was determined between 4 and 18 DNA copies, which is within the range of the sensitivity level expected for a standard TaqMan real-time PCR technique.

The ASFV UPL PCR proved to be sensitive enough for the early detection of viral DNA in infected animals. EDTA-blood and serum samples collected from pigs experimentally inoculated with ASFV isolates of different

virulence (belonging to p72 genotypes I, II, IX and X) were reported as positive within the first week of infection, being detected in some of them up to 1 week before the reference TaqMan PCR test. The ability of the UPL PCR method to detect low levels of viraemia during the initial stages of infection is of great value for rapid detection of infected animals when no antibodies are still present and there are not yet even typical clinical signs. Organs collected from the experimental infections were useful for assuring the competence of the UPL PCR technique to be applied in any ASF diagnostic target sample.

Validation experiments using a collection of 260 field porcine and tick samples, originating from Eastern and Western African regions affected by ASF, demonstrated the detection capacity and value of the novel UPL real-time PCR for routine diagnosis. Compared to the results obtained by performing the reference TaqMan PCR method, the UPL PCR technique was able to detect over 10% more positive samples, confirming an increase in the diagnostic sensitivity of this new assay. Furthermore, the UPL PCR proved to be a truly repeatable and robust system when a panel of ASFV-positive and ASFV-negative samples was repeatedly analysed at separate times by different technical staff. All samples were consistently correctly reported, and even the weakest positive ones with a mean C_t value more than 35 were properly scored in the eight PCR runs performed.

Additionally, an internal control PCR assay was also developed using UPL probes. This test uses the same UPL#162 probe but combined with a primer set specifically designed for the detection of a DNA fragment within the endogenous β-actin gene, frequently selected for designing internal control PCR methods for animal disease diagnosis (Toussaint et al., 2007; Van Borm et al., 2007). This assay can be run in a parallel-well manner under the same reaction conditions as the ASFV UPL PCR. This approach means the availability of an affordable internal control assay avoiding the competition that might arise in duplex PCR systems. The developed β-actin UPL PCR test showed its utility in all porcine samples assayed, showing some different C_t values depending on the sample matrix, which may be primarily due to the different genomic DNA content. The internal control PCR allows checking the status of clinical material and avoids the appearance of false negative results, being particularly useful for samples preserved in inappropriate conditions.

The complete studies showed the developed UPL real-time PCR to be a reliable, sensitive and specific method for detecting the range of ASFV p72 genotypes and a useful tool in clinical specimens, even suitable for tick sample analysis. The simple and rapid procedure means that, under standard laboratory conditions, it is feasible to report a final diagnostic PCR result within 2 h, which is in the range of ELISA tests. In conclusion, the developed UPL PCR method showed itself to be a promising tool for early, rapid and affordable molecular diagnosis of ASF for application in the current epidemiological scenarios of Africa and Europe. Moreover, the convenience of UPL probes is increased by their low cost, simplicity of ordering and use and compatibility with any real-time PCR instrument and reagents, having the potential to be incorporated into diagnostic laboratories for pathogen detection.

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