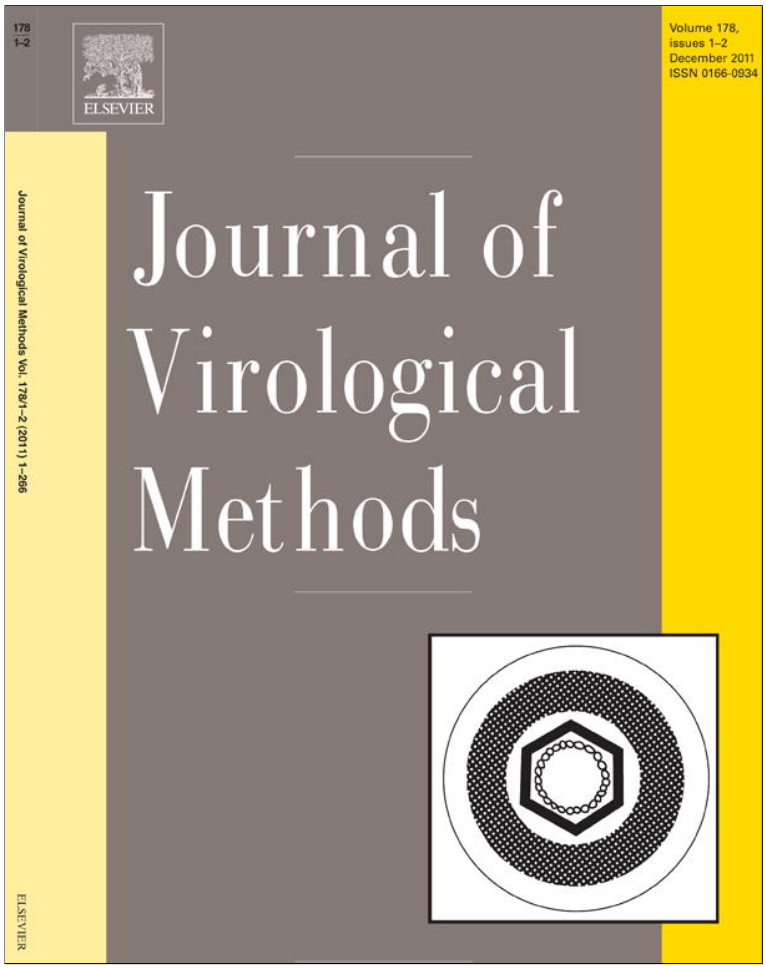

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Development and inter-laboratory validation study of an improved new real-time PCR assay with internal control for detection and laboratory diagnosis of African swine fever virus

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A B S T R A C T

A real-time polymerase chain reaction (PCR) assay for the rapid detection of African swine fever virus (ASFV), multiplexed for simultaneous detection of swine beta-actin as an endogenous control, has been developed and validated by four National Reference Laboratories of the European Union for African swine fever (ASF) including the European Union Reference Laboratory. Primers and a TaqMan® probe specific for ASFV were selected from conserved regions of the p72 gene. The limit of detection of the new real-time PCR assay is 5.7–57 copies of the ASFV genome. High accuracy, reproducibility and robustness of the PCR assay (CV ranging from 0.7 to 5.4%) were demonstrated both within and between laboratories using different real-time PCR equipments. The specificity of virus detection was validated using a panel of 44 isolates collected over many years in various geographical locations in Europe, Africa and America, including recent isolates from the Caucasus region, Sardinia, East and West Africa. Compared to the OIE-prescribed conventional and real-time PCR assays, the sensitivity of the new assay with internal control was improved, as demonstrated by testing 281 field samples collected in recent outbreaks and surveillance areas in Europe and Africa (170 samples) together with samples obtained through experimental infections (111 samples). This is particularly evident in the early days following experimental infection and during the course of the disease in pigs sub-clinically infected with strains of low virulence (from 35 up to 70 dpi). The specificity of the assay was also confirmed on 150 samples from uninfected pigs and wild boar from ASF-free areas. Measured on the total of 431 tested samples, the positive deviation of the new assay reaches 21% or 26% compared to PCR and real-time PCR methods recommended by OIE.

This improved and rigorously validated real-time PCR assay with internal control will provide a rapid, sensitive and reliable molecular tool for ASFV detection in pigs in newly infected areas, control in endemic areas and surveillance in ASF-free areas.

1. Introduction

African swine fever (ASF) is a complex viral disease and one of the most important problems for the pig industry in affected countries. It is classified as a notifiable disease of domestic pigs and wild boar by the World Organisation for Animal Health (OIE). The disease is characterized by a high rate of morbidity and mortality in domestic pigs. The agent responsible for ASF is the African swine fever virus (ASFV), the only known representative of the *Asfarviridae* family. The virus consists of a linear double stranded DNA molecule of 170–193 kb encoding 151–167 genes, depending on the specific isolate (Chapman et al., 2008; De Villiers et al., 2010). ASFV infects members of the vertebrate family *Suidae* including domestic and feral pigs, wild boar, African wild suidae and the argasid tick

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vector (*Ornithodoros* spp.). The epidemiological characteristics of the disease include the potential for rapid spread through direct and indirect contact, as well as a natural transmission by the arthropod vector and wild suidae through a sylvatic cycle, maintained due to viral persistence in argasid ticks (Penrith and Vosloo, 2009).

ASF was indigenous originally to the African continent where the disease is endemic currently in most of sub-Saharan Africa. It was detected for the first time outside the African continent in Portugal in 1957, and subsequently in Spain, remaining endemic in the Iberian Peninsula until the mid 1990s. It also spread to several European countries, including France, Italy, Belgium, the Netherlands, as well as Cuba and Central and South America (Arias and Sánchez-Vizcaíno, 2002). Except for Sardinia, where the disease has remained endemic since its introduction in 1978, all of these countries eradicated ASF successfully by slaughter of infected and carrier pigs combined with implementation of strict biosafety and sanitary measures.

In 2007, further transcontinental spread of ASF occurred with the introduction of the virus to Georgia in the Caucasus region. Delays in recognizing the disease resulted in widespread distribution of the virus to neighbouring countries, including Armenia, Azerbaijan and several territories in the Russian Federation. Infected wild boars have also been reported in the region (FAO, 2008; Rowlands et al., 2008). The significance of the occurrence and risk of endemicity of ASF in the countries neighbouring the European Union (EU) has been evaluated by EFSA experts (EFSA, 2010).

Individual ASFV isolates have been reported to induce a range of clinical symptoms varying from peracute to chronic disease to apparently healthy virus carriers, in parallel with distinct lesions (Martins and Leitão, 1994). In contrast, ASFV usually induces a silent infection in wild African pigs, which act as a reservoir for virus transmission. The clinical features and post-mortem findings may be indistinguishable from those seen during other pig diseases, particularly classical swine fever (CSF) infection (Kleiboeker, 2002).

The current lack of a vaccine increases the importance of reliable and differential ASF laboratory diagnosis for implementation of specific control measures and eradication programs. Laboratory examination is essential to establish a definitive diagnosis of ASF, provide relevant information about the time of infection and support successfully control and eradication programs (OIE, 2008). Virus isolation and the haemadsorption test (Malmquist and Hay, 1960) are specific and sensitive but also too laborious and time-consuming to be employed for routine or rapid diagnosis. The polymerase chain reaction (PCR) provides a sensitive, specific and more rapid alternative to virus isolation for the detection of ASFV, allowing amplification of highly conserved regions of the genome using specific primers. Several PCR and real-time PCR assays have been described for detection or genotype characterization of ASFV (Agüero et al., 2003, 2004; Bastos et al., 2003; Giammarioli et al., 2008; King et al., 2003; McKillen et al., 2007, 2010; Michaud et al., 2007; Steiger et al., 1992; Zsak et al., 2005), as well as isothermal amplification assays (Hjertner et al., 2005; James et al., 2010) and Linear-After-The-Exponential PCR (LATE-PCR) assay (Ronish et al., 2010). Three of these are included as sensitive techniques for diagnostic purposes in the OIE manual: a highly sensitive gel-based PCR assay (Agüero et al., 2003), a PCR test that allows the differentiation of ASFV from CSFV (Agüero et al., 2004) and one real-time PCR assay (King et al., 2003). A wide range of isolates belonging to all the 22 known virus genotypes (Boshoff et al., 2007), including both non-haemadsorbing viruses and isolates of low virulence, can be detected with PCR assays, even in inactivated or degraded samples. However, false negative PCR results due to the presence of inhibitors or damaged nucleic acids cannot be excluded, particularly when working with degraded samples (Belák and Thorén, 2001).

The aim of the present study was to improve ASFV PCR detection by development of a sensitive and specific real-time PCR TaqMan® assay. To prevent false negative results that may occur in the presence of PCR inhibitors, an internal endogenous control targeting the swine beta-actin gene has been included for the first time in an ASF PCR assay. Four National Reference Laboratories for ASF of the EU, including the European Union Reference Laboratory (URL-ASF), have been involved in the characterization and validation of the assay, as well as for assessment of linearity, sensitivity, specificity and robustness.

2. Materials and methods

2.1. Reference viruses and cells

The ASFVs analysed in this study are shown in Table 1. A total of 44 reference ASFV representative of P72 genotypes I, II, V, VIII, IX and X, collected from different outbreaks, were included in this study. ASFV strains were cultured on primary leukocyte cultures following the procedure described by Malmquist and Hay (1960), except for strain Ba71V, which is adapted to Vero cells (Salas and Vinuela, 1986). Strains were available in the ASFV collections of the URL-ASF (CISA-INIA), or in those of the Italian Reference Laboratory (IZSUM). ASFV "Madagascar" isolate was kindly provided by Dr. E. Albina from CIRAD (Montpellier, France). In addition, DNA and RNA from a range of other porcine viruses, representatives of classical swine fever virus (CSFV), Aujeszky's disease virus (ADV), porcine circovirus type 2 (PCV2), porcine reproductive and respiratory syndrome virus (PRRSV), encephalomyocarditis virus (EMCV) and swine vesicular disease virus (SVDV) from the Belgian (VAR) and French (Anses) Reference Laboratories were selected for cross reactivity testing.

2.2. ASF experimental samples

Serum samples were collected at regular intervals until the end of the studies from three independent experimental infections performed at the BSL-3 animal facilities at CISA-INIA, with ASFV strains belonging to P72 genotypes I, IX and X. Two additional experiments were carried out at Anses with highly virulent ASFV isolates belonging to P72 genotypes I and II. A short description of the experimental designs is presented below.

2.2.1. Experimental infections with ASFV P72-genotype I

Two Landrace × Large White pigs were inoculated experimentally by the intramuscular route with 10^2 50% tissue culture infectious doses per ml ($TCID_{50}$/ml) of the attenuated ASFV strain E75 CV1-4. Animal #1 was killed humanely at 21 days post infection (dpi). Animal #2 was re-inoculated at 30 dpi with 10^3 50% haemadsorbing doses per ml (HAD_{50}/ml) of the homologous virulent E75L8 strain and then given a third challenge at 45 dpi with the heterologous virulent strain E70 (10^3 HAD_{50}/ml). The animal was killed humanely at 51 dpi.

In another experiment 5 Large White pigs were inoculated by the intramuscular route with 10^4 HAD_{50}/ml of the ASFV virulent West African isolate Benin 97/1 (Chapman et al., 2008). The pigs developed rapidly severe ASF symptoms. One died at 4 dpi, the others were slaughtered at 5 dpi. Blood samples were taken on 0, 3 and 5 dpi.

2.2.2. Experimental infections with ASFV P72-genotype II

Six SPF pigs vaccinated against classical swine fever and treated with antibiotics were infected experimentally with an inoculum

Table 1
Details of representative ASFV viruses used in the study.

Isolate	Country of origin	Town/Province	Species of origin	Year of outbreak	P72 genotype	Reference
European						
E70	Spain	Pontevedra	Pig	1970	I	Zsak et al. (2005)
Ba71V	Spain	Badajoz	Vero cell adapted	1971	I	Salas and Vinuela (1986)
E75	Spain	Lerida	Pig	1975	I	Zsak et al. (2005)
B74	Spain	Barcelona	Pig	1985	I	Gallardo et al. (2009)
ZAR/85	Spain	Zaragoza	Pig	1985	I	Bastos et al. (2003)
Hu90	Spain	Huelva	Pig	1990	I	Gallardo et al. (2009)
BEL/85	Belgium	West Flanders	Pig	1985	I	Bastos et al. (2003)
Ss88	Italy	Sardinia	Pig	1988	I	Gallardo et al. (2009)
748 (Ca04.1)	Italy	Sardinia	Pig	2004	I	Giammarioli et al. (2011)
19376 (Nu04.3)	Italy	Sardinia	Pig	2004	I	Giammarioli et al. (2011)
20052 (Nu04.4)	Italy	Sardinia	Pig	2004	I	Giammarioli et al. (2011)
36774 (Nu04.6a)	Italy	Sardinia	Pig	2004	I	Giammarioli et al. (2011)
66505 (Ss04.10)	Italy	Sardinia	Pig	2004	I	Giammarioli et al. (2011)
10428 (Ss05.3a)	Italy	Sardinia	Pig	2005	I	Giammarioli et al. (2011)
11429 (Ss05.3b)	Italy	Sardinia	Pig	2005	I	Giammarioli et al. (2011)
Lisbon/60	Portugal	Lisbon	Pig	1960	I	Bastos et al. (2003)
Arm07	Armenia	Dilijan town	Pig	2007	II	Unpublished CISA
Abk07	Georgia	Abkhazia Republic Gulripish	Pig	2007	II	Unpublished CISA
Che07	Russia	Chechnya Republic Shatoysky	Wild boar	2007	II	Unpublished CISA
Ing08	Russia	Ingushetia Republic Sunzhensky	Wild boar	2008	II	Unpublished CISA
Oren08	Russia	Orenburg Chernorechye	Pig	2008	II	Unpublished CISA
NO08/Av	Russia	Republic North Osetia Vladikawkaz	Pig	2008	II	Unpublished CISA
NO08/Ap	Russia	Republic North Osetia Prigorodni	Pig	2008	II	Unpublished CISA
Az08D	Azerbaijan	Kibale	Pig	2008	II	Unpublished CISA
Az08B	Azerbaijan	Kibale	Pig	2008	II	Unpublished CISA
American						
Brazil78	Brasil	Rio Jaineiro	Pig	1978	I	Gallardo et al. (2009)
Haiti	Haiti	Port-au-Prince	Pig	1981	I	Gallardo et al. (2009)
African						
Moz64	Mozambique		Pig	1964	V	Gallardo et al. (2009)
Ug64	Uganda		Pig	1964	X	Gallardo et al. (2009)
Kat67	Zaire	Katanga	Pig	1967	I	Gallardo et al. (2009)
Ang72	Angola		Pig	1972	I	Gallardo et al. (2009)
IC96	Ivory Coast		Pig	1996	I	Gallardo et al. (2009)
CV97	Cape Verde		Pig	1997	I	Gallardo et al. (2009)
MwLil 20/1	Malawi	Chalaswa	Tick	1983	VIII	Kutish and Rock, unpublished
Ben/1/97	Benin		Pig	1997	I	Bastos et al. (2003)
Nig01	Nigeria	Ibadan	Pig	2001	I	Gallardo et al. (2009)
Ug03H.1	Uganda	Hoima	Pig	2003	IX	Gallardo et al. (2009)
Ken06.Bus	Kenya	Busia	Pig	2006	IX	Gallardo et al. (2009)
Ken07.Eld1	Kenya	Eldoret	Pig	2007	IX	Gallardo et al. (2009)
BF07/IpTC	Burkina Faso	Ipelce	Pig	2007	I	Unpublished CISA
Ken05/Tk1	Kenya	Machakos	*Tick* (*O. porcinus porcinus*)	2005	X	Gallardo et al. (2011)
Ken08Tk.2/1	Kenya	Machakos	*Tick* (*O. porcinus porcinus*)	2008	X	Gallardo et al. (2011)
Ken08Tk.2/3	Kenya	Machakos	*Tick* (*O. porcinus porcinus*)	2008	X	Gallardo et al. (2011)
Madagascar	Madagascar		Pigs	1998–2001	II	Unpublished CIRAD

made with a mix of supernatant of crushed organs collected on pigs during ASF outbreaks in Madagascar between 1998 and 2001. Four pigs developed hyperthermia on day 3 pi and all on day 4 when they were slaughtered. Blood was collected at necropsy from the six animals, as well as organs (tonsil, spleen, submandibular and ileocecal lymph nodes) from only two of them.

2.2.3. Experimental infections with ASFV P72-genotype IX

Four pigs (C1–C4) were inoculated by the intramuscular route with 10 HAD_{50}/ml of the ASFV virulent strain Ken06.Bus (Gallardo et al., 2009) and placed in contact with two naive pigs (CC5 and CC6) being housed in the same box. All the pigs developed acute form of the disease and died, or were slaughtered, between 8 up to 17 dpi.

2.2.4. Experimental infections with ASFV P72-genotype X

Four pigs (C7–C10) were inoculated by the intramuscular route with 10 HAD_{50}/ml of the moderately virulent ASFV strain Ken05/Tk1 (Gallardo et al., 2011) and placed in contact with two naive pigs (CC11 and CC12) being housed in the same box. Four pigs developed acute to subacute form of the disease and died, or were slaughtered, between 11 up to 23 dpi. Survival pigs, C7 and C8, were re-inoculated with a high dose (10^4 HAU) of the homologous strain and followed up for 24 additional days (70 dpi).

2.3. Field sample panels

2.3.1. ASFV-infected areas

A total of 170 field samples were collected from domestic pigs, wild boar, bush pigs and ticks in different geographical localities

Table 2
Panel of field samples collected in ASFV-infected areas.

Country	Town/district	Sampling	Date sampled	Species origin	No. of samples analysed			
					Tissue	Blood	Serum	Pooled ticks
Azerbaijan	Qebele	ASF outbreak	January 2008	Domestic pig	2	–	–	–
Burkina Faso	Bazega	ASF outbreak	April 2007	Domestic pig	11	–	5	–
Burkina Faso	Koulpelogo	ASF outbreak	June 2007	Domestic pig	6	4	–	–
Nigeria	Ibadan	ASF outbreak	July–October 2001	Domestic pig	6	–	–	–
Uganda	Wakiso	ASF outbreak	August 2007	Domestic pig	1	2	–	–
Uganda	Mukono	ASF outbreak	August 2007	Domestic pig	–	1	–	–
Uganda	Nakasongola	ASF outbreak	August 2007	Domestic pig	–	2	–	–
Kenya	Busia	ASF outbreak	May 2006	Domestic pig	10	–	3	–
Kenya	Busia	ASF outbreak	October 2006	Domestic pig	4	–	–	–
Kenya	Kisumu	ASF outbreak	November 2006	Domestic pig	–	–	1	–
Kenya	Kiambu	ASF outbreak	January 2007	Domestic pig	3	–	–	–
Kenya	Eldoret	ASF outbreak	January 2007	Domestic pig	3	–	1	–
Kenya	Nakuru	ASF outbreak	January 2007	Domestic pig	1	–	1	–
Kenya	Machakos	Warthog's burrows	June 2005	*Ornithodorus porcinus*	–	–	–	3
Kenya	Machakos	Warthog's burrows	August 2008	*Ornithodorus porcinus*	–	–	–	7
Kenya	Ruma National Park	ASF surveillance	April 2008	Bush pig	6	–	–	–
Kenya	Homa Bay	ASF surveillance	April 2008	Domestic pig	14	–	–	–
Italy	Sardinia	ASF outbreak	1997–2009	Domestic pig	21	8	–	–
Italy	Sardinia	ASF surveillance	1997–2009	Domestic pig	38	–	–	–
Italy	Sardinia	ASF surveillance	1997–2009	Wild boar	1	5	–	–

where the disease is present. Field samples collected included blood, serum, tissues such as tonsils, spleen, kidney, lung, heart, lymph nodes, and ticks. The Sardinian field samples were provided by IZSUM; the remaining ones originated from the collection of CISA-INIA, through their collaborations with the specific countries and with the International Livestock Research Institute (ILRI). The origin and description of field samples are presented in Table 2.

2.3.2. ASFV-free areas

One hundred negative field samples from domestic pigs (Landrace × Large White pig and Kahyb Hungarian landrace) and fifty from wild boar were collected during surveillance programs in Belgium and France. Samples included blood, serum and tissues like tonsils, spleen or lymph nodes.

2.4. DNA isolation

Laboratories performed viral DNA extraction from stock viruses, experimental and field samples (serum, blood or 10% suspensions of homogenised tissues and ticks) with nucleic acid extraction kits from three different commercial suppliers. The Nucleospin nucleic acid extraction kit (Machery-Nagel) was used at CISA-INIA, the QIAamp DNA kit (Qiagen) at VAR and IZSUM and the DNeasy Mini kit (Qiagen) at Anses, all according to the manufacturers' instructions.

2.5. Primers, TaqMan® probes and ASFV P72 real-time PCR assay

The PCR primers and probe were designed in order to amplify all 22 known ASFV genotypes so far described (Bastos et al., 2003; Boshoff et al., 2007; Lubisi et al., 2005). To this end, more than 180 nucleotide sequences of 536 base pairs (bp) available in the GenBank, which encode the C-terminal end of the ASFV p72 gene, were aligned using the BioEdit sequence alignment editor (Hall, 1999). ASFV specific forward (5′-TGCTCATGGTATCAATCTTATCG-3′) and reverse (5′-CCACTGGGTTGGTATTCCTC-3′) primers were designed from conserved regions using Primer3 software (Rozen and Skaletsky, 2000) generating an amplicon of 159 bp. The TaqMan® probe (5′ FAM-TTCCATCAAAGTTCTGCAGCTCTT-TAMRA 3′) was labelled with the 5′ reporter dye 6-carboxy-fluorescein (FAM) and the 3′ quencher dye 6-carboxy-tetramethylrhodamin (TAMRA).

Forward (5′-CTCGATCATGAAGTGCGACGT-3′) and reverse (5′-GTGATCTCCTTCTGCATCCTGTC-3′) primers and probe (5′-TET-ATCAGGAAGGACCTCTACGCCAACACGG-BHQ1-3′) targeting a 114 bp region of the swine beta-actin gene (ACTB) were included in the assay as an endogenous control (Duvigneau et al., 2005).

Real-time PCR was performed using the commercially available QuantiTect™ Multiplex PCR Kit (Qiagen) in a total volume of 25 μl. Briefly, 2.5 μl DNase RNase-free water, 12.5 μl 2× QuantiTect™ Multiplex PCR master mix, 2.5 μl ASF-specific primer-probe mix 10× (6 μM each of ASF-P72-F and ASF-P72-R primers, 3 μM ASF-P72-FAM-TAMRA probe) and 2.5 μl endogenous control-specific primer-probe mix 10× (6 μM each of ACTB-F and ACTB-R primers, 2 μM ACTB TET-BHQ1 probe) were pooled as a master mix. Finally, a 5 μl aliquot of DNA extracted from the sample was added to 20 μl of PCR master mix. The cycling protocol was as follows: 1 cycle of 50 °C for 2 min, 1 cycle of 95 °C for 10 min followed by 45 cycles consisting of denaturation for 1 min at 95 °C and annealing for 1 min at 60 °C. Assays were performed on different real-time PCR thermocyclers in the four laboratories (ABI 7500 FAST at VAR, Stratagene Mx3005P at CISA-INIA, Biorad Chromo4 at Anses and ABI 7900 HT at IZSUM). Fluorescence values were collected during the annealing step and analysed with the dedicated software. After amplification, a cycle threshold (Ct) value was assigned to each PCR reaction.

To check the size and consistency of the amplicons, PCR products were fractionated by electrophoresis in 3% agarose gels and further sequenced.

2.6. PCR and real-time PCR standard procedures

The presence of the ASFV genome was also confirmed by OIE-prescribed conventional and real-time PCR assays according to Agüero et al. (2003) and King et al. (2003), respectively.

2.7. Analysis of sensitivity, linear dynamic range and precision of ASFV P72 real-time PCR assay

The p72 gene was amplified from the Lisbon60 isolate and cloned in pCRII (Invitrogen) using the TA cloning kit (Invitrogen). The recombinant plasmid was amplified in *Escherichia coli* and purified with a plasmid purification kit (Plasmid midi kit, Qiagen). The presence of the insert was checked after restriction digestion by agarose gel electrophoresis and further confirmed by sequencing. The plasmid was linearised by digestion with the restriction

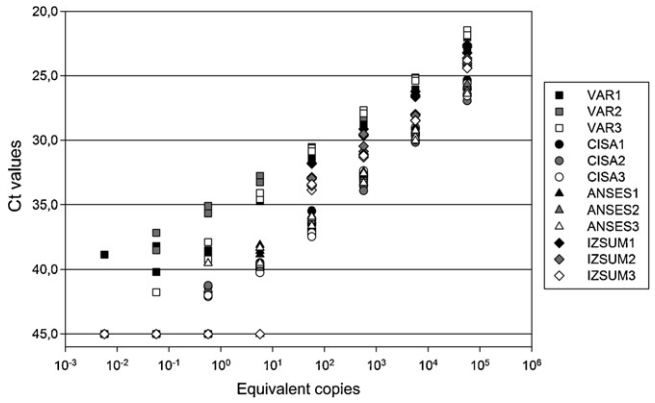

Fig. 1. Quantity vs. Ct values for the new ASFV P72 real-time PCR assay with dilutions of standard DNA from 5.7×10^4 to 5.7×10^{-2}.

enzyme *BamH*I and distributed to the four laboratories. Based on the size of the insert and on the average molecular weight of a base pair, the number of copies of the amplicon was estimated at 5.7×10^9 copies/5 μl after spectrophotometric measurement.

The linear dynamic range and sensitivity of the new assay were determined individually in the four laboratories by testing serial dilutions of the quantified plasmid material ranging from 5.7×10^4 to 5.7×10^{-5} copies/5 μl in DNAse-free water. The serial dilutions were examined in triplicate (repetitions) in three independent assays (reproductions) in each laboratory. The value of 45.01 was assigned to Ct values greater than 45 cycles.

Repeatability and reproducibility have been defined in the ISO-5725 guideline (precision of test methods – determination of repeatability and reproducibility for a standard test method by inter-laboratory tests) and were assessed using the previous data by measurement of the coefficient of variation between results with the ANOVA test as implemented in SAS© 9.2 analysis software (SAS Institute Inc.).

3. Results

3.1. Design of ASFV-specific P72 real-time PCR

The real-time PCR protocol specific for detection of the viral genome of the 22 ASFV known genotypes was developed based on the amplification of 159 bp within the ASFV p72 gene. The primers and probes were designed based on homologous regions from a multiple sequence alignment of the available nucleotide sequences derived from the C-terminal end of the ASFV p72 gene deposited in GenBank.

The target regions in the real-time PCR assay described previously (King et al., 2003) and in the present assay are similar. The forward primer is two bases shorter than that of King's assay (nucleotides 2–24) and the reverse primer covers nucleotides 7–25 of the King's assay probe. The primers and probes were analysed for specificity with BLAST analysis to determine potential cross hybridisation with other nucleotide sequences. The primers demonstrated a complete homology with more than 200 sequences present in the GenBank database (http://www.ncbi.nlm.nih.gov/). No significant identity with other porcine pathogens was detected.

3.2. Linear dynamic range, limit of detection and precision of the ASFV-specific P72 real-time PCR with internal control (IC)

The linearity of the real-time PCR assay was demonstrated by titration of recombinant plasmid DNA in the range of 5.7×10^4 to 57 copies/5 μl (Fig. 1). In this range, the efficacy was evaluated to 105% on basis of linear regression of the data from all the laboratories ($y = -3.207x + 39.883$), with an average efficacy of 121% for VAR, 92% for INIA-CISA, 97% for Anses and 115% for IZSUM. The limit of detection differed between 5.7 and 57 copies per reaction among the laboratories.

The complete set of data, including the Ct values obtained for 10 dilution point in three repetitions on three independent reproductions performed in each of the four partner laboratories, were compiled for statistical comparison. Intra-laboratory precision was evaluated for each lab, using the data from the 3 repetitions and 3 reproductions. At the intra-laboratory level, the mean coefficient of variation (CV) was 1.8% (range 0.7–2.9%) within runs and 1.6% (range 0.7–3.3%) between runs for the four laboratories. The inter-laboratory precision, evaluating the "laboratory effect", was obtained by comparison of the four independent reproductions, corresponding to the four participating labs, and taking into account the data obtained by each lab in the nine sets of repetition and reproduction. Inter-laboratory repeatability and reproducibility CV were 2.6% and 5.4% respectively. The robustness compiled all the reproductions performed by the four laboratories as independent tests and give CV value of 2.2 and 5.0% for repeatability and reproducibility, respectively.

3.3. Analytical specificity

A collection of 44 ASFV reference isolates representative of historical and currently circulating strains, different geographical origins and six different ASF genotypes, including genotype II that is currently circulating in the Caucasus and Russian Federation, was tested to evaluate the specificity of the assay (Table 1). All ASFVs were detected with Ct values ranging from 15 to 30. All amplified products were confirmed as specific viral targets by visualization of the appropriate bands of 159 bp on agarose gel. A 114 bp ACTB DNA was amplified in parallel for all samples (data not shown).

In addition, no amplification curves were detected using nucleic acids extracted from CSFV, ADV, PCV-2, EMCV, PRRSV and SVDV, validating the absence of cross-reactivity with other porcine viruses (data not shown).

3.4. Diagnostic sensitivity and specificity

The assay with endogenous control was evaluated for diagnostic purposes using samples collected during animal experiments and under field conditions in ASF endemic and outbreak areas, as well as in ASF-free areas. The positive and negative deviations and relative specificity and sensitivity were evaluated on the same samples by the OIE-referenced detection techniques of conventional and real-time PCR assays (OIE, 2008).

3.4.1. Samples from experimentally infected animals

The ability of the new assay to detect ASFV during acute and chronic infection was assessed in independent experimental infections with ASFV isolates belonging to P72 genotypes I, II, IX and X as described in Section 2. The results were compared with those obtained using the OIE-PCR prescribed procedures (Agüero et al., 2003; King et al., 2003).

In the experimental inoculations performed with highly virulent isolates of genotypes I and II, the first blood samples were collected shortly after the infection. The pigs inoculated with ASFV virulent strain of genotype I were positive with the present P72 PCR assay since the first day of bleeding at 3 dpi and at slaughter day (5 dpi). Average Ct values were 21.41 (±2.03) at 3 dpi and 18.78 (±1.51) at 5 dpi. In the case of infection of pigs with the 'Madagascar' inoculum (genotype II), viral genome was detected on blood and organ samples from infected pigs slaughtered at 4 dpi. Average Ct values were 19.30 (±0.37) for blood samples, 17.09 (±0.40) for

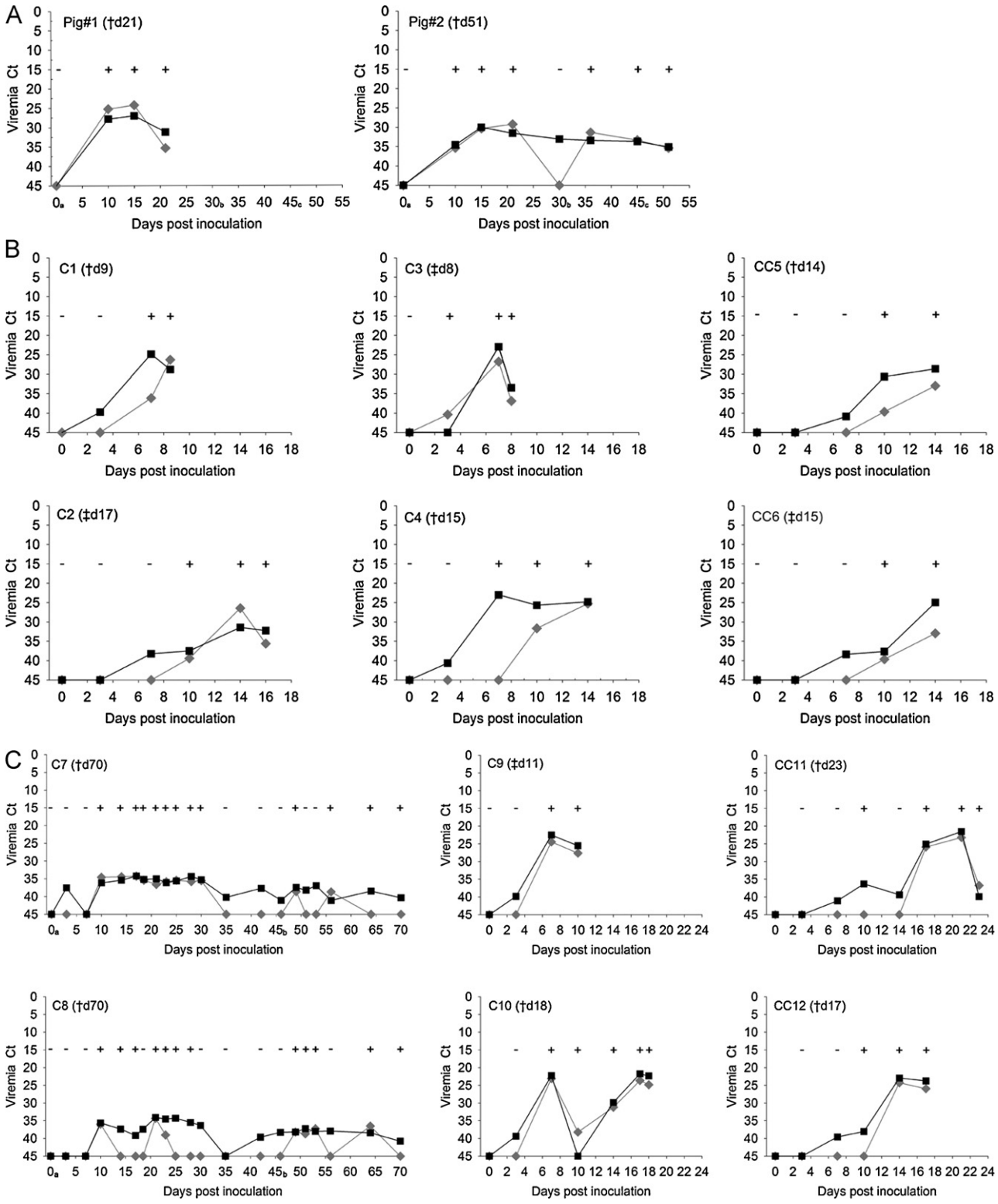

Fig. 2. Viremia (PCR results and Ct values) in serum samples after experimental infections with ASFV isolate of genotype I (A), IX (B) and X (C). Positive and negative results obtained with conventional PCR (Agüero et al., 2003) are indicated by + and − signs; cycle threshold values (Ct) obtained with the OIE-referred real-time PCR assay (King et al., 2003) are represented in grey diamonds and with the present P72 assay in black squares. Slaughtering (†) or death (‡) dates are indicated. In (A), date of infection with E75 CV1-4 strain is noticed by "a", with E75L8 strain by "b" and with E70 strain by "c". In (C), date of first infection with Ken05/Tk1 strain is noticed by "a", and boost infection at 46 dpi by "b".

spleen, 22.26 (±0.66) for tonsils, 22.71 (±1.02) and 22.83 (±1.36) for submandibular and ileocecal lymph nodes, respectively.

In the experimental inoculation of two pigs with ASFV attenuated strain of genotype I, viremia was detected from the first day of bleeding at 10 dpi up to the latest 21 dpi in pig #1 using all PCR procedures (Fig. 2A). Using the new assay, the viremia was also detected in pig #2 from the first day of bleeding (10 dpi), and was maintained along the experimental infection up to 51 dpi. Using

the two OIE-prescribed PCR assays a negative result was obtained on serum sample collected at 30 dpi, before the challenge with the homologous virulent strain.

In the experimental direct inoculation of pigs and infection of contact pigs with ASFV genotypes IX and X, viremia was detected generally from the first day of bleeding, at 3 or 7 dpi with the newly developed real-time PCR assay (Fig. 2B and C). The two exceptions were observed in pig C3 on 3 dpi and pig C10 on 10 dpi where viral DNA was not detected. In surviving infected pigs, considered to have a carrier animal status (C7 and C8), viremia was detected consistently up to 70 dpi using the new assay, including during the 24 days following boosting with the homologous strain. However, virus detection in experimental surviving pigs, emulating a carrier pig status, was not always achieved with either the prescribed conventional or real-time PCR assays, in contrast with the positive reaction using the new assay.

3.4.2. Field-exposed animal samples

To evaluate the applicability of the test as a diagnostic and surveillance method for the screening of field isolates, 170 samples collected during ASF outbreaks in Sardinia, Kenya, Uganda, Burkina Faso and Azerbaijan, as well as in surveillance studies performed in Sardinia and Kenya, were analysed retrospectively (Table 2). All 170 samples had been analysed previously for the presence of the ASFV genome using OIE-prescribed conventional and real-time PCR procedures (Agüero et al., 2003; King et al., 2003).

Among the panel, 94 samples were confirmed as ASFV-positive with both Agüero and King assays. ASFV genomic DNA was also amplified with the Agüero assay in four additional samples; three of these were tick-derived samples, obtained in the surveillance program performed in Central Kenya during 2008, that were negative with the King assay. The new assay detected all the 94 positive samples, except the same four samples which were previously negative with the King assay. All the positive samples of Sardinian origin were confirmed with the new assay.

Out of the 170 samples, 72 were classified as negative by conventional PCR and by the real-time PCR described by King et al. (2003). However, using the new assay, positive results were obtained from 19 previously negative samples, with Ct values ranging from 35.4 to 41.2. This result allowed to establish the limit of positive detection in 41.2 Ct. The discrepant samples originated from the outbreaks that occurs in Burkina Faso, Uganda and Kenya between 2006 and 2007 and also from the surveillance study performed in Kenya on domestic and bush pigs. The amplified PCR products were fractionated on agarose gels to verify the specificity of the amplicons and further sequencing confirmed homology with genotypes known to be circulating in different geographical regions according to previous studies. P72 genotype I was identify in samples from Burkina Faso, genotype IX in samples from the Kenyan and Ugandan outbreaks and genotype X in samples collected in the surveillance study in Kenya. No discrepant results were detected in the negative field samples from Italy.

3.4.3. Field animal samples from ASF-free areas

Negative field samples from domestic pigs and wild boar, collected in ASFV-free regions (Belgium and France), did not present an amplification signal for ASFV P72 and were detected positive for ACTB by the real-time PCR assay, demonstrating the specificity of the test (data not shown).

3.4.4. Positive and negative deviations, relative specificity and sensitivity

Compared to other OIE-prescribed conventional and real-time PCR, the new P72 PCR assay detected as positive 22 and 33 experimental samples out of the 111 tested and identified previously as negative (positive deviation of 26% and 38%, respectively). Similarly, 19 additional positive samples were detected in the field-exposed animals panel, corresponding to 17% of positive deviation. No variation was observed in the analysis of the samples from the ASF-free areas. Measured on the cumulated total of 431 tested samples, the positive deviation reaches 21% compared to Agüero's assay and 26% compared to King's assay. Only 6 positives (including 3 pools of ticks) among the three panels were not detected with the new assay indicating a negative deviation of 3 and 1% compared to Agüero and King assays, respectively.

The relative specificity and sensitivity of the new assay are evaluated respectively to 96% and 85% compared to the conventional assay, and 99% and 82% in regard to King's assay.

4. Discussion

African swine fever is a transboundary animal disease of high socio-economic importance, with a high mortality rate, that spreads in domestic pig herds and wild suidae. In the absence of vaccination or therapeutics, accurate differential diagnosis of ASF, based on rapid, sensitive and reliable laboratory tests to confirm clinical cases is critical for the introduction of control measures by the competent authorities (EFSA, 2010). For this purpose, PCR assays have been recommended as the methods of choice for the detection of economically important viruses, including those validated for African swine fever (OIE, 2008).

The ASFV full length genome sequence has only been determined for a restricted number of isolates (11 at this date) due to its large size between approximately 170 and 193 kbp. This number is not even representative of the all genotypic diversity characterized up to now (De Villiers et al., 2010). Length variation in the virus genome are induced by loss and gain of copies within multigene families, as well as by variation in number of tandem repeat sequences located within coding and intergenic regions (review in Giammarioli et al., 2011). Three genes, p72, p54 and pB602L (central variable region), have been studied more largely for classification of major genotypes (Bastos et al., 2003; Boshoff et al., 2007; Gallardo et al., 2009; Lubisi et al., 2005). With the highest level of sequence conservation and the larger representation of the existing viral diversity, p72 gene was designed as target for PCR assay.

The novelty of the present assay compared to other ASFV TaqMan® assays is the improvement of sensitivity and the multiplexing with an endogenous control reaction targeting the ACTB of suidae. The same conserved region of p72 gene was already targeted by the assay described by King et al. (2003) but the PCR efficiency has been improved notably due to factor like the length of the amplicon (reduced from 250 to 159 bp) and modification of primers and probe (Wong and Medrano, 2005) or improvement of manufactured PCR reagents. Additionally, the use of an internal control is an important aspect of quality control. It provides the insurance of an efficient DNA extraction and confirms the absence of PCR-inhibitors in each sample, therefore avoiding false negative results (Belák and Thorén, 2001).

Characterization and validation of the assay including the endogenous control as an efficient and reliable molecular diagnostic test for virus detection in tissue and blood samples of domestic pigs, wild boar and wild African suidae was performed with the participation of the EU Reference Laboratories for ASF in Spain, together with the National Reference Laboratories of Italy, France and Belgium.

The analytical specificity and sensitivity of the present TaqMan® assay are comparable to the performance of the established for the validated diagnostic assays (Agüero et al., 2004; King et al., 2003) and others assays published more recently (Giammarioli et al., 2008; McKillen et al., 2010). The assay detected a large set of 44 representative ASFV isolates collected over many years in various geographical locations in Europe (Iberian Peninsula, Italy, Belgium

and Caucasus region), America (Brazil and Haiti) and Africa (East, West and Central Africa and Madagascar), including currently circulating isolates present in Sardinia, Caucasus region, Madagascar, East and West Africa. No cross-reactivity with other porcine viruses has been observed.

The assay was also sensitive with a detection limit between 5.7 and 57 ASFV genomes per reaction. The CVs measured within and between runs in the different laboratories are comparable to those described in other published real-time PCR assay for detection of porcine circovirus (Pal et al., 2008) or bovine herpesvirus (Wang et al., 2007). Importantly transposition of the assay to different laboratories had only a limited impact on the accuracy of the data as demonstrated by CVs for inter-laboratory precision and robustness remaining low (2.6 and 2.2% for repeatability and 5.4 and 5.0% for reproducibility) despite the use of thermocycler equipments from different companies (ABI 7500 FAST and 7900 from Applied Biosystems, Mx3005P from Stratagene and Chromo 4 from Biorad) in the four laboratories. This strongly suggests that the PCR assay could be transferred to additional laboratories without significant loss of performance, whatever the available equipment is.

Accuracy related to the extraction method has not been quantified between laboratories in the present study. However, different extraction methods (Nucleospin nucleic acid extraction kit from Machery-Nagel, QIAamp DNA and DNeasy Mini kit from Qiagen) have been used successfully by the different laboratories on tissues, blood and sera for viral DNA extraction and amplification with the P72 assay.

The diagnostic sensitivity and specificity of the assay was demonstrated using 431 positive and negative samples from field-exposed and experimentally infected animals as well as animals from ASF-free areas.

The new P72 real-time PCR assay improved the ASFV DNA detection compared to the OIE prescribed PCR assays during the entire clinical course of the experimental ASF infections with virulent to subacute strains from genotypes I, II, IX and X. Infection was first detected at 3–7 days post infection and later during the whole time of the infection from 10 up to 70 dpi in experimentally infected pigs presenting a chronic form of the disease. Using the OIE procedures viremia was often detected later after infection and could only be detected intermittently in chronically infected animals. Detection of ASFV positive samples from experimental infections was improved, as indicated by positive deviation from 26% and 38%, compared to PCR assays previously published by Agüero et al. (2003) and King et al. (2003). With the new assay, consistent detection is possible, both early after infection (from 3 dpi) and later in surviving animals.

Experience from the past indicates that changes may occur in the virulence of African swine fever, like decrease of the mortality rate and variation of the clinical picture from acute to chronic, including apparent recovery to normal health. This has been observed in the 80s when the disease was endemic in the Iberian Peninsula. Both subclinical infection and recovery from acute and subacute disease may result in a persistent virus infection in pigs which can appear clinically normal. Incidence of these animals in the pig population has been estimated by serological surveys at that time. Significant proportion of infected inapparent virus carriers were detected as antibodies were present in 0.9% of 25,000 abattoir sera in Portugal and in 0.75% of more than 20,000 sera collected from various areas in Spain, before implementation of eradication programs (Sanchez-Botija, 1982).

The transmission of virus by recovered pigs without clinical signs seemed to be rare as infectious virus levels are very low or undetectable. In these animals, virus levels are sufficient for transmission to domestic pigs through a biological vector where present, but usually not by direct contact between animals (Sánchez-Vizcaíno, 2006). However, virus reactivation characterized by viremia and virus excretion could happen in case of stress, i.e. during transport, bad husbandry or pregnancy (Wilkinson, 1984). Moreover, presence of virus in tissues may still pose a threat for transmission of ASF if uncooked meat from apparently healthy carrier pigs is fed to uninfected pigs. The detection of inapparent carrier animals is of the upmost importance as they can play a potential role in the development of endemicity (EFSA, 2010).

Similar improvement of sensitivity was observed in the panel of field-exposed animals. Nineteen samples among those collected in Africa and identified previously as negative with the prescribed conventional and TaqMan® assays, were positive with the new assay (Ct values >35), which is indicative of an improved sensitivity in detection of animals with low viral load (positive deviation of 17%). The determination of the genotype of these viruses indicated correlation with genetic types known to be present in the region of collection, confirming the specificity and excluding the possibility of cross-contamination in the laboratory. Moreover, these samples allowed to determine a limit Ct value for a positive reaction of 41.2 in the new assay, when field samples are analysed.

Improvement of virus detection influences the success of eradication programs established for control of the disease. Positive samples detected in field panel collected in Sardinia were confirmed by the P72 assay and no additional positive were detected. This result indicated that no inapparent carriers were present in this endemic area. Moreover the sensitivity of the detection method used up to now by the Italian laboratories could not be considered as a critical point in the success of eradication and control programs in domestic and feral pigs (2005/362/CE and 2005/363/CE). Similarly, inapparent carriers have not been detected up to now in samples collected from Caucasus region. In Africa, the situation is different as the new assay detected additional positives with low viral load among samples collected from Burkina Faso (genotype I), Kenya (genotypes IX and X) and Uganda (genotype IX). In those countries inapparent carriers are present both in domestic pig, feral pig and wild suidae populations. They act as reservoir for the virus which spreads via direct and indirect transmission where biological vectors are present (Wilkinson, 1984). When serological testing is not available, or antibodies are not induced (i.e. in some wild African tolerant suidae population) improvement of virus detection sensitivity is of the upmost importance for detection of carrier animals. So, implementation of the new assay could improve the sensitivity of virus detection and, by consequence, the efficacy of contingency plans.

By contrast, failure of detection when compared to the Agüero conventional assay was mainly observed for tick samples. The present assay, similar to that of King et al. (2003), failed to detect ASFV on three occasions in this type of material, despite a positive signal from swine actin. The lower sensitivity observed with these samples might be due to reduced amplification efficacy of real-time PCR assays in presence of tick-specific PCR inhibitors (Basto et al., 2006). Therefore, in addition of the use of the conventional PCR (Agüero et al., 2003), the PCR protocol specifically developed for soft ticks (Basto et al., 2006) and recommended by the OIE should be used for ASFV detection in vectors.

Additionally, the multiplexing with endogenous control has the advantage of validation of the each individual real-time PCR reaction, including the extraction phase and the possible presence of PCR inhibitors at the sample level. The ACTB and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been traditionally used as endogenous controls due to their strong expression in all mammalian tissues (Bustin, 2000; Duvigneau et al., 2005; Nygard et al., 2007; Thellin et al., 1999). In the present assay, the ACTB was detected consistently in various matrices such as blood, sera and tissues, including tonsils, spleen, lymph nodes, liver, kidney, heart and lung. The ACTB primers and probes allowed sensitive and specific detection of the endogen control in domestic pigs, wild

boars and wild African suidae, including also suidae blood meal in ticks.

In conclusion, the present communication reports the development and inter-laboratory validation of an assay suitable for the rapid, reproducible, sensitive and specific detection of ASFV. The assay includes an ACTB endogenous control and presents an improved analytical and diagnostic sensitivity compared to the OIE-prescribed assays, mainly in case of recovered carrier pigs. The recent appearance of ASF in the Caucasus region has increased the risk of emergence of the disease in the European Union. The speed of diagnosis in terms of time of analysis and earliest detection of infection is the key issue for control of the disease. Laboratory detection with a highly sensitive molecular assay, such as the one described in this work, allowing earlier and more consistent detection of acute to low virulent isolates is essential for control of ASF in disease outbreaks in new areas, to achieve eradication in endemic regions, to monitor virus prevalence following outbreaks and for surveillance of the disease in areas thought to be free of ASFV.

Ethical approval

Animal experiments were performed in accordance with the EC Directive 86/609/EEC and following the guidelines for the accommodation and care of animals used for experimental and other scientific purposes as described in the recommendation 2007/526/EC. The experimental protocols were approved previously by the Committee on the Ethics of Animal Experiments of INIA for CISA-INIA (agreement number CBS 2008/020) and by the "Direction des Services Vétérinaires des Côtes d'Armor"for Anses (agreement number 22-17).

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