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ORIGINAL ARTICLE

Development of an updated PCR assay for detection of African swine fever virus

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Abstract Due to the current unavailability of vaccines or treatments for African swine fever (ASF), which is caused by African swine fever virus (ASFV), rapid and reliable detection of the virus is essential for timely implementation of emergency control measures and differentiation of ASF from other swine diseases with similar clinical presentations. Here, an improved PCR assay was developed and evaluated for sensitive and universal detection of ASFV. Primers specific for ASFV were designed based on the highly conserved region of the vp72 gene sequences of all ASFV strains available in GenBank, and the PCR assay was established and compared with two OIE-validated PCR tests. The analytic detection limit of the PCR assay was 60 DNA copies per reaction. No amplification signal was observed for several other porcine viruses. The novel PCR assay was more sensitive than two OIE-validated PCR assays when testing 14 strains of ASFV representing four genotypes (I, V, VIII and IX) from diverse geographical areas. A total of 62 clinical swine blood samples collected from Uganda were examined by the novel PCR, giving a high agreement (59/62) with a superior sensitive universal probe library-based real-time PCR. Eight out of 62 samples tested positive, and three samples with higher Ct values (39.15, 38.39 and 37.41) in the real-time PCR were negative for ASFV in the novel PCR. In contrast, one (with a Ct value of 29.75 by the real-time PCR) and two (with Ct values of 29.75 and 33.12) ASFV-positive samples were not identified by the two OIE-validated PCR assays, respectively. Taken together, these data show that the novel PCR assay is specific, sensitive, and applicable for molecular diagnosis and surveillance of ASF.

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Introduction

African swine fever (ASF) is an OIE (World Organization for Animal Health)-listed, highly contagious and devastating disease of domestic pigs and wild boar that causes significant economic losses to the pig industry in affected countries [1]. The disease is caused by African swine fever virus (ASFV), which is a large, enveloped, double-stranded DNA virus belonging to the genus *Asfivirus* of the family *Asfarviridae* [2]. The virus typically causes hemorrhagic fever with high mortality in domestic pigs and wild boar, whereas infections in African wild suids, such as warthogs and bushpigs, run a nonpathogenic course [3]. Soft ticks of

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the genus *Ornithodoros* are transmission vectors of ASFV and natural reservoir of the virus.

After a few episodes of long-distance spread from the African continent to Europe and the Americas in the 1960s, 1970s and 1980s, ASF remained restricted to Africa, except for Sardinia in Italy, for almost three decades until 2007, when it was reported in Georgia [1, 4]. It rapidly spread across the Caucasus and into the Russian Federation and may have then spread further to Belarus and Ukraine, and eventually reached the European Union (EU) in 2014. The disease is now endemic in the wild boar populations of a number of Eastern European countries (Latvia, Lithuania, Estonia and Poland), where the virus is spreading, causing large-scale epidemics in domestic pig and wild boar populations in two endemic zones in central and southern Russia. It is spreading widely in the Russian Federation and Belarus, posing a continuing threat to the EU and neighboring countries [5–9]. The strains circulating in these areas belong to vp72 genotype II. Considering the various well-known risk factors for ASF spread and the recent economic and trade expansion of China, the risk of introduction of the disease into China, the largest pig producer in the world, cannot be ignored.

As there is no vaccine or treatment available for ASF, a rapid and reliable diagnosis is essential for timely implementation of control measures to reduce the spread of this devastating disease. Moreover, considering the similarities in clinical signs between ASF and other swine diseases, such as classical swine fever (CSF), and the potential appearance of nonspecific clinical signs, the early and accurate detection of ASFV is vital for rapid decision-making [10]. For virological detection, the OIE-recommended tests include virus isolation, fluorescent antibody test (FAT), and conventional and real-time PCR assays [7, 10–13]. Virus isolation is considered the gold standard for ASF diagnosis. However, as the process is labor-intensive and time-consuming, it is normally used for confirmatory diagnosis and molecular study [10]. PCR assays are widely used due to their rapidity and high sensitivity and specificity, and they are especially applicable for tissues that are unsuitable for virus isolation. The OIE-recommend PCR assays have been adopted for routine diagnosis in reference laboratories [11–13]. Real-time PCR assays developed in recent years have higher diagnostic sensitivity [12, 14, 15], one of which is based on a universal probe library (UPL) showing superior sensitivity when detecting experimental and field samples [14, 15]. Conventional PCR assays are useful and more generally applicable, especially in less-equipped laboratories that do not have real-time PCR facilities. However, the OIE-recommended conventional PCR showed reduced sensitivity when testing field and experimental samples infected with vp72 genotype II strains, which is likely due to a nucleotide mismatch between the primer and the viral target gene [15]. Therefore, it is necessary to update the current PCR assay to allow detection of various genotypes of ASFV that are currently circulating.

In this study, a novel PCR assay was developed for detection of ASFV and compared with two OIE-validated conventional PCR tests [11, 13, 16] and one UPL-based real-time PCR [14].

Materials and methods

Viruses and cells

It is not possible to import viable ASFV into China due to biosecurity restrictions. The genomic DNA of 14 ASFV strains representing genotypes I, V, VIII and IX originating from diverse regions of the world was kept at the National Veterinary Institute (SVA), Uppsala, Sweden (Table 1). Other clinically related swine viruses were included in the specificity evaluation for the PCR in this study, including the highly pathogenic HuN4 strain and the classical CH-1a strain of porcine reproductive and respiratory syndrome virus (PRRSV), the porcine circovirus type 2 (PCV2) JXL strain, the classical swine fever virus (CSFV) Shimen strain (genotype 1.1), HLJ strain (genotype 2.1) and HCLV vaccine strain (genotype 1.1), the pseudorabies virus (PRV) variant TJ strain and classical SC strain, and the porcine parvovirus (PPV) BQ strain. PRRSV was propagated in MARC-145 cells, and CSFV, PRV, PPV and PCV2 were propagated in PK-15 cells.

Primers

The forward primer P72-F (5′-GGT TGG TAT TCC TCC CGT G-3′, nt 261–279) and the reverse primer P72-R (5′-GAT TGG CAC AAG TTC GGA C-3′, nt 568–586) were designed based on the alignment of 35 complete (Fig. 1) and 158 partial (data not shown) coding sequences (CDS) of the ASFV vp72 gene available in the GenBank database. The primers were chosen in highly conserved regions of the targeted sequences (Fig. 1), and the expected size of the PCR product was 326 bp. *In silico* analysis was also performed for the primers validated by OIE (Fig. 1).

DNA/RNA extraction and cDNA synthesis

DNA was extracted from cell cultures using a DNeasy Blood & Tissue Kit (QIAGEN, Germany) according to the manufacturer's instructions. For RNA virus included in the specificity test, RNA was extracted from cell cultures using a QIAamp Viral RNA Kit (QIAGEN, Germany) according to the manufacturer's manual. Reverse transcription of RNA was performed as described previously [17].

Table 1 Viruses included in the specificity test of the novel PCR assay for ASFV

Virus	Country of origin	Isolate	Genotype
ASFV	Mozambique	Mozambique 1964	V
	Angola	Angola 1972	I
	Malawi	Chalaswa 1983	VIII
	Cape Verde	Cape Verde 1997	I
	Uganda	Hoima 2003	IX
	Kenya	Kenya 2006	IX
	Kenya	Kenya 2007	IX
	Burkina Faso	Burkina Faso 2007	I
	Spain	Pontevedra 1970	I
	Spain	Badajoz 1971	I
	Portugal	Lisbon 60	I
	Spain	E75	I
	Italy	Sardinia 1988	I
	Haiti	Port-au-Prince 81	I
CSFV	China	Shimen	1.1
	China	HCLV	1.1
	China	HLJ	2.1
PRRSV	China	HuN4	Highly pathogenic
	China	CH-1a	Classical
PCV2	China	JXL	
PRV	China	TJ	Variant
	China	SC	Classical
PPV	China	BQ	

DNA standards

A partial vp72 gene fragment of ASFV was amplified by PCR from the genomic DNA of the ASFV E75 strain using the following pair of primers: forward primer (P72-Sd-F), 5′-GAA GAA GAA GAA AGT TAA TAG-3′ (nt 36–56), reverse primer (P72-Sd-R), 5′-CAT TAT ATA TGG CAT CAG GAG-3′ (nt 1929–1949). PCR was performed in a 50-μL reaction volume containing 1× Ex Taq buffer, 2.5 U of Ex Taq Hot-Start DNA polymerase (TaKaRa, Japan), 0.4 μM each primer, 0.2 mM each deoxynucleoside triphosphate (dNTP) and 3 μL of the genomic DNA. The PCR profile consisted of an initial denaturation at 95 °C for 5 min, followed by 35 amplification cycles (95 °C for 30 s, 51 °C for 1 min and 72 °C for 2 min) and a final extension step at 72 °C for 10 min using a TaKaRa PCR thermal cycler. The PCR product was cloned into the pMD-18T vector (TaKaRa, Japan) to generate the recombinant plasmid pMD-vp72. The nucleotide sequences of the construct were determined using an automated sequencer (ABI PRISM 3100 Genetic Analyzer, USA). The plasmid was quantified using the following formula: Y (copies/μL) = (6.02×10^{23} copies) × (plasmid concentration g/μL)/[(number of bases) × (660 daltons/base)] [18].

Optimization of the novel PCR assay

To develop a sensitive PCR assay, the parameters of the assay were optimized, including annealing temperature and concentrations of PCR components (primers, dNTPs and Mg^{2+}, etc.). Optimal conditions for the novel assay were established as follows: a 25-μL PCR mixture containing 1 × Ex Taq buffer, 1.25 U of Ex Taq Hot-Start DNA polymerase (TaKaRa, Japan), 0.4 μM each primer, 0.25 mM each dNTP, 2 mM $MgCl_2$ and 3 μL of the DNA. The PCR cycling consisted of an initial denaturation at 95 °C for 5 min, followed by 40 amplification cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s, with a final extension step at 72 °C for 10 min. The PCR products were subjected to electrophoresis through a 2 % agarose gel in TAE buffer (40 mM Tris-acetate, pH 8.0, and 1 mM EDTA) and stained with ethidium bromide for visualization using a Gel Doc XR + System (Bio-Rad, USA).

Specificity test of the novel PCR assay

The specificity of the novel PCR was evaluated by testing other porcine viruses, including CSFV of subgenotypes 1.1 and 2.1, classical and highly pathogenic PRRSV, and

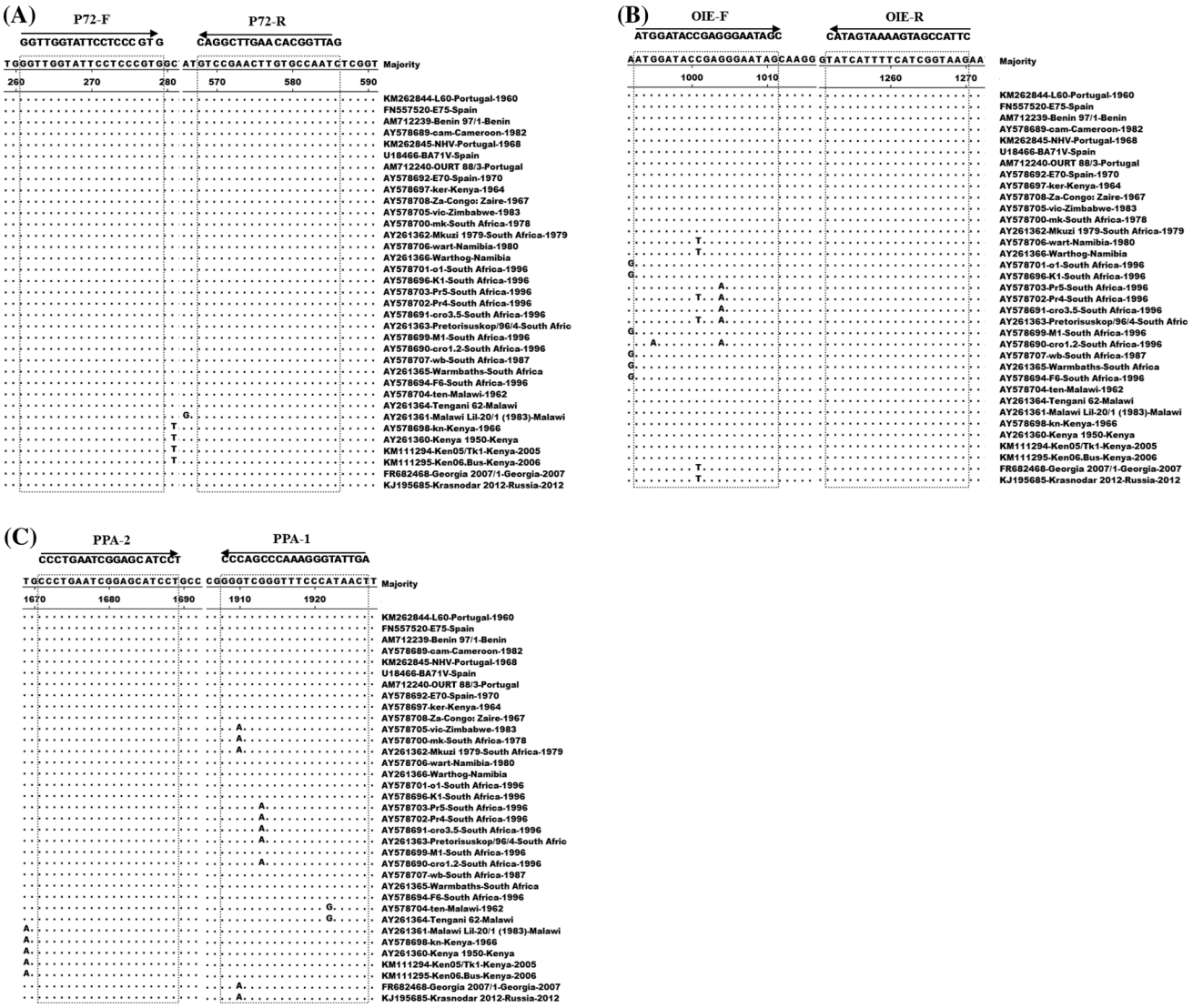

Fig. 1 Alignment of 35 complete coding sequences of the ASFV vp72 gene available in GenBank and the locations of the primer target sequences. (A) Locations of the target sequences of the primers P72-F/P72-R designed in the present study. (B) Locations of the target sites of the primers OIE-F/OIE-R for the previous OIE PCR assay [16]. (C) Locations of the target sites of the primers PPA-1/PPA-2 for the PCR currently described in OIE manual [11, 13]. Dots (·) indicate identical bases. The primer-binding sequences are boxed

classical and variant PRV, PCV2 and PPV, in parallel with 14 ASFV strains from different geographical regions. The PCR products of the expected size were sequenced to confirm the specificity of the assay.

Analytic sensitivity test of the novel PCR assay

In order to determine the detection limit of the test, the PCR assay was performed on serial tenfold dilutions of the prepared ASFV genomic DNA standard (from 2.0×10^4 copies/μL to 2.0×10^{-2} copies/μL). The novel PCR assay was further examined by comparison with two OIE PCR tests [11, 13, 16] following the procedures as described previously. Briefly, PCR was performed in a 25-μL reaction volume containing 1× PCR buffer II (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 2 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 μM each primer, 0.625 U of Taq Gold polymerase (Applied Biosystems) and 3 μL of sample DNA. When the PCR currently described in the OIE manual (PPA-1/2 primer set) was performed, the PCR reaction was carried out as follows: (i) incubation for 10 min at 95 °C; (ii) 40 cycles of 15 s at 95 °C, 30 s at 62 °C, and 30 s at 72 °C; and (iii) incubation for 7 min at 72 °C. When the previous OIE PCR (OIE-F/R primer set) was used, the assay was conducted as follows: (i) initial denaturation for 10 min at 95 °C; (ii) 35 cycles of 30 s at 94 °C, 30 s at 53 °C, and 30 s at 72 °C; and (iii) incubation for 7 min at 72 °C. Amplification products were analyzed

by electrophoresis on a 2 % agarose gel containing ethidium bromide as described above.

Comparisons of different PCR assays

A total of 14 ASFV strains representing four genotypes (I, V, VIII and IX) (Table 1) from diverse regions of the world were diluted tenfold and tested using the novel assay, in parallel with the two OIE-validated PCR assays as well as a real-time PCR for ASFV developed by Haines et al. [19], with some modifications, to compare the detection limit of the assays. The real-time PCR was performed using the primers and probes described by Haines et al. [19] with an Ag Path-ID one-step RT-PCR kit (Life Technologies). The reaction contained 1× PCR buffer, 0.8 μM ASFV primers, 0.2 μM Cy5-labelled ASFV probe, 1 μL of enzyme mix, and 3 μL of viral nucleic acid. Cycling steps for the assay included activation of DNA polymerase at 95 °C for 10 min, 48 cycles of 45 °C for 15 s and 60 °C for 45 s.

Detection of ASFV in clinical samples

A total of 62 whole blood samples were collected from clinically affected or apparently healthy domestic pigs from farms investigated for ASF in Uganda between 2010 and 2015 and delivered to the National Animal Disease Diagnostics and Epidemiology Center (NADDEC), Entebbe, Uganda. Nucleic acid was extracted from 50 μL of each blood sample using a MagMAX^{TM}-96 DNA Multi-Sample Kit according to manufacturer's instructions (Life Technologies). The ASFV DNA preparations were transported to SVA, Uppsala, Sweden. A small aliquot of DNA samples was further transferred from SVA to HVRI, Harbin, China, and all samples were tested in parallel using the novel PCR assay, two OIE-validated PCR assays [11, 13, 16] and a UPL-based real-time PCR [14] as described previously.

Results

Conservation of the target sequence recognized by the selected primers

The primers P72-F/P72-R, which were based on vp72 gene sequences of ASFV from the GenBank database, corresponded to sites that were more conserved than those used in the two OIE PCR tests [11, 13, 16] (Fig. 1).

Specificity of the novel PCR assay

To confirm the specificity of the novel PCR, other swine viruses were tested with the assay. As expected, the new PCR assay amplified all 14 ASFV DNA preparations and did not give any positive results when several non-ASFV swine viruses were examined, including CSFV, PRRSV, PRV, PCV2 and PPV (partial results shown in Fig. 2). The specificity was also confirmed by sequencing the PCR products of the expected size.

Sensitivity of the novel PCR assay

The novel PCR assay detected a minimum of 60 DNA copies of the ASFV vp72 DNA standard (Fig. 3). Comparison tests with two OIE PCR assays using PPA-1/2 primers [11, 13] or OIE-F/R primers [16] showed that the novel PCR yielded stronger bands than the others (Fig. 3).

Evaluation of the novel PCR assay for detection of diverse ASFV strains

As shown in Table 2, the novel PCR had an equal or 10 times lower detection limit (10^{-3} and 10^{-5} for isolates Burkina Faso 2007 and Port-au-Prince 81, respectively)

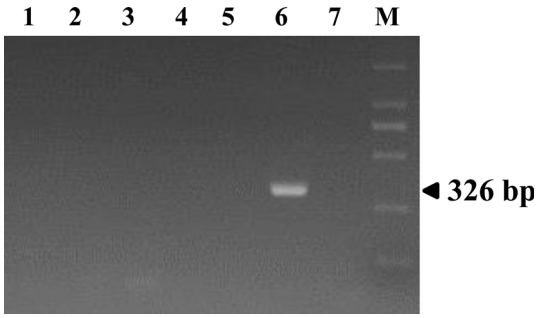

Fig. 2 Specificity of the novel ASFV PCR assay. Related swine viruses were tested in this study to evaluate the specificity of the assay. Lane 1, CSFV Shimen strain; lane 2, PRRSV HuN4 strain; lane 3, PPV BQ strain; lane 4, PRV TJ strain; lane 5, PCV2 JXL; lane 6, ASFV E75 strain; lane 7, negative control (distilled water); lane M, DNA molecular weight marker

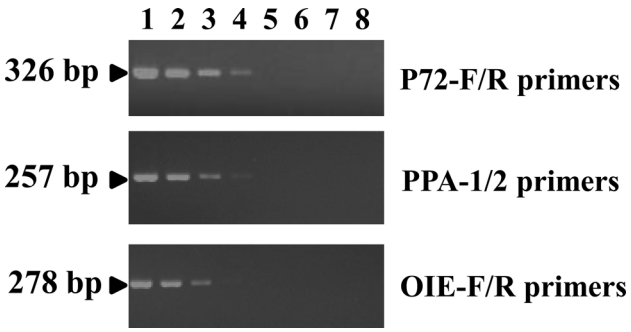

Fig. 3 Sensitivity of the novel ASFV PCR assay with the primers P72-F/P72-R compared with the two OIE-validated PCR assays performed with primers PPA-1/2 or OIE-F/R [11, 13, 16]. Lanes 1–7, tenfold serial dilutions of the ASFV vp72 gene standard from 6.0×10^{4} to 6.0×10^{-2} copies; lane 8, negative control (distilled water)

Table 2 Detection limit of different PCR assays for detection of 14 ASFV strains isolated from diverse geographical regions

Isolate	Country of origin	Novel PCR in this study	PCR currently described in the OIE manual	Previous OIE PCR	Real-time PCR (Ct value) (Haines et al., 2013)
AFRICAN					
Mozambique 1964	Mozambique	10^{-2}	10^{-1}	10^{-2}	10^{-2} (35.73)
Angola 1972	Angola	10^{-3}	10^{-3}	10^{-3}	10^{-4} (36.53)
Chalaswa 1983	Malawi	10^{-3}	10^{-3}	10^{-3}	10^{-3} (33.35)
Cape Verde 1997	Cape Verde	10^{-2}	10^{-2}	10^{-2}	10^{-2} (35.65)
Hoima 2003	Uganda	10^{-2}	10^{-2}	10^{-2}	10^{-2} (31.70)
Kenya 2006	Kenya	10^{0}	10^{0}	10^{0}	10^{-2} (37.09)
Kenya 2007	Kenya	10^{-2}	10^{-2}	10^{-2}	10^{-3} (39.50)
Burkina Faso 2007	Burkina Faso	10^{-3}	10^{-1}	10^{-2}	10^{-4} (37.87)
EUROPEAN					
Pontevedra 1970	Spain	10^{-3}	10^{-2}	10^{-3}	10^{-5} (38.72)
Badajoz 1971	Spain	10^{-5}	10^{-4}	10^{-5}	10^{-5} (34.92)
Lisbon 60	Portugal	10^{-4}	10^{-4}	10^{-4}	10^{-5} (34.52)
E75	Spain	10^{-3}	10^{-3}	10^{-3}	10^{-2} (32.50)
Sardinia 1988	Italy	10^{-2}	10^{-1}	10^{-2}	10^{-3} (36.28)
AMERICAN					
Port-au-Prince 81	Haiti	10^{-5}	10^{-3}	10^{-4}	10^{-6} (35.65)

Note: the detection limit is given as the lowest detectable dilution of the DNA

when compared with that of the previous OIE PCR (10^{-2} and 10^{-4}, respectively). In addition, the novel PCR had an equal, 100 times lower (isolates Burkina Faso 2007 and Port-au-Prince 81) or 10 times lower detection limit (isolates Mozambique 1964, Pontevedra 1970, Badajoz 1971 and Sardinia 1988) when compared with the PCR assay currently described in the OIE manual [11, 13].

Detection of ASFV in clinical samples by the novel PCR assay

Eight out of 62 samples tested positive for ASFV with the novel assay, consistent with the UPL-based real-time PCR [14] in which the positive samples had Ct values of 20.33–33.12. Three samples with higher Ct values (39.15, 38.39 and 37.41) tested negative in the novel PCR. The remaining 51 samples tested negative in both assays. Interestingly, one ASFV-positive sample was found to be negative using the PCR assay currently described in the OIE manual, and two were negative in the previous OIE PCR [11, 13, 16] (Table 3).

Discussion

ASF is one of the most severe viral diseases of domestic pigs and wild boar. Currently the continuous spread of the disease through Africa, Europe and the Russian Federation keeps the neighboring countries on heightened alert [7–9]. The disease has the potential to spread rapidly to new, uninfected areas, causing great damage to the pig industry and trade restrictions. This emphasizes the need for early and rapid diagnosis of the disease.

Currently, PCR is the most widely used assay with high sensitivity and specificity, and both conventional and real-time PCR assays have been recommended for virological and molecular diagnosis of ASF. Conventional PCR assays are useful and generally more applicable, especially in less-equipped laboratories. So far, several conventional PCR assays have been developed [11, 16, 20]. However, one report showed that the OIE-recommended conventional PCR for ASFV had low sensitivity, most likely due to an imperfect match of the primers with the target sequences of some ASFV genotypes [15]. Therefore, it is necessary to update the current assays, which were developed over 10 years ago, to enable the detection of all possible circulating ASFV strains. In this study, a novel and sensitive PCR test was developed and compared with the two OIE-validated PCR assays.

When designing primers for molecular diagnostic tests, it is important to select regions of the viral genome that are highly conserved to ensure that the assay can detect all known genotypes of the virus. Here, a pair of primers was designed based on the alignment of all vp72 genes sequences of ASFV that are available in GenBank, targeting conserved areas of the gene and making these

Table 3 Comparison of the novel PCR assay with two OIE-validated conventional PCR assays and a real-time PCR assay using a universal probe library for detection of clinical samples from Uganda

Lab ID	Field ID	Novel PCR in this study	PCR currently described in the OIE manual	Previous OIE PCR	Real-time PCR using a universal probe library (Fernández-Pinero et al., 2013) (Ct values)
1	UG1	−	−	−	No Ct
2	UG2	−	−	−	No Ct
3	UG3	+	−	−	**33.12**
4	UG4	−	−	−	No Ct
5	UG5	−	−	−	No Ct
6	UG6	−	−	−	No Ct
7	UG7	−	−	−	No Ct
8	UG8	−	−	−	No Ct
9	UG9	−	−	−	No Ct
10	UG10	−	−	−	No Ct
11	UG11	−	−	−	No Ct
12	UG12	−	−	−	No Ct
13	UG13/2014	−	−	−	No Ct
14	UG14/2014	−	−	−	No Ct
15	UG15/2010	−	−	−	No Ct
16	UG16	−	−	−	No Ct
17	UG17/2011	−	−	−	No Ct
18	UG18/2011	+	+	+	**26.03**
19	UG19/2010	−	−	−	No Ct
20	UG20/2011	−	−	−	No Ct
21	UG21/2011	−	−	−	No Ct
23	UG23/2011	+	+	+	**27.90**
24	UG24/2011	−	−	−	No Ct
25	UG25/2011	−	−	−	No Ct
26	UG26/2011	+	+	+	**22.09**
28	UG28/2010	−	−	−	No Ct
29	UG29/2015	−	−	−	No Ct
30	UG30	−	−	−	No Ct
31	UG31/2010	−	−	−	No Ct
32	UG32/2012	−	−	−	No Ct
33	UG33/2011	−	−	−	No Ct
34	UG34/2011	−	−	−	No Ct
35	UG35/2011	−	−	−	No Ct
36	UG36/2010	−	−	−	No Ct
37	UG37/2010	−	−	−	No Ct
38	UG38/2010	−	−	−	No Ct
39	UG39/2011	−	−	−	37.41
40	UG40/2010	+	+	+	**21.35**
41	UG41/2010	+	+	+	**20.33**
42	UG42/2010	−	−	−	38.39
43	UG43/2010	−	−	−	No Ct
44	UG44/2011	+	+	+	**22.80**
45	UG45	−	−	−	No Ct
46	UG46/2011	−	−	−	No Ct
47	UG47/2010	−	−	−	No Ct
48	UG48	−	−	−	No Ct

Table 3 continued

Lab ID	Field ID	Novel PCR in this study	PCR currently described in the OIE manual	Previous OIE PCR	Real-time PCR using a universal probe library (Fernández-Pinero et al., 2013) (Ct values)
49	UG49/2011	–	–	–	No Ct
50	UG50/2010	–	–	–	39.15
51	UG51/2011	–	–	–	No Ct
52	UG52/2011	–	–	–	No Ct
53	UG53/2011	+	+	–	**29.75**
54	UG54/2011	–	–	–	No Ct
55	UG55/2011	–	–	–	No Ct
56	UG56/2011	–	–	–	No Ct
57	UG57/2010	–	–	–	No Ct
58	UG58/2011	–	–	–	No Ct
59	UG59	–	–	–	No Ct
60	UG60/2010	–	–	–	No Ct
61	UG61/2011	–	–	–	No Ct
62	UG62/2011	–	–	–	No Ct
63	UG63/2011	–	–	–	No Ct
64	UG64	–	–	–	No Ct

Note: +, positive; –, negative

primers more universally applicable than those used in the previous OIE PCR [16] or the PCR currently described in the OIE manual [11, 13]. Due to the unavailability of genotype II ASFV strains that are currently circulating in some Eastern European countries and the Russian Federation, such strains were not included in the validation of the PCR assay described here. Nevertheless, multiple sequence alignment showed that the primers used in the present study are conserved enough to cover these genotype II strains. BLAST searches of the new ASFV primers confirmed that they target highly conserved regions of the ASFV vp72 gene sequences present in the current NCBI nucleotide sequence collection; no mismatches were observed with the primers used for the novel PCR, while several mismatches were found between the OIE-validated primers and ASFV isolates of genotype II that are currently circulating in some areas, e.g., Georgia 2007/1 and Krasnodar 2012 (Fig. 1), indicating that the novel assay could be used for universal detection of ASFV.

The PCR assay developed in the present study was specific, and no cross-reactions were observed with selected non-ASFV porcine viruses, including CSFV, PRRSV, PRV, PCV2 and PPV. Thus, the assay could effectively differentiate ASF from other pig viral diseases and therefore ensure accurate diagnosis and facilitate timely control of ASF. Moreover, the assay showed a higher diagnostic sensitivity than the two OIE PCR assays when detecting diverse ASFV strains, as demonstrated by its lower detection limit when compared to the latter two PCR assays.

An important route of introduction and spread of ASFV is through transportation of infected pigs or contaminated pork products. Therefore, a highly sensitive diagnostic test is very important to screen for and thus interrupt the potential introduction of the virus. The novel PCR showed a high agreement (59/62) with a previously described UPL-based real-time PCR when testing 62 clinical samples collected in Uganda. Three samples with higher Ct values (39.15, 38.39 and 37.41) in the real-time PCR gave negative results in the novel PCR. Importantly, one (with a Ct value of 29.75) and two (with Ct values of 29.75 and 33.12) ASFV-positive samples were not identified by the two OIE PCR assays performed with the primers PPA1-2 or OIE-F/R. Similarly, a recent study showed that the conventional PCR currently described in the OIE manual failed to give positive results for some samples infected with vp72 genotype II ASFV strains (with Ct values of >30) [15]. The authors proposed that the lower sensitivity of the assay might be due to the presence of a nucleotide mismatch of the primer with the target sequences of some ASFV isolates [15], which is consistent with what we observed in our sequence alignment. As shown in Fig. 1, both OIE primer pairs display mismatches with several isolates, including Georgia 2007/1 (genotype II) (GenBank accession no. FR682468) and Krasnodar 2012 (genotype II) (KJ195685), indicating that the updated PCR developed in this study might be more reliable and applicable for clinical use.

Conclusions

In summary, an improved PCR was developed to enable the detection of all possible circulating ASFV strains based on the primers that target highly conserved regions of the vp72 gene sequences available in GenBank. The novel PCR shows higher sensitivity than the OIE-validated PCR assays, and a high level of agreement with the highly sensitive UPL-based real-time PCR. We believe that the combination of the novel PCR and the reported real-time PCR using a universal probe library could provide reliable diagnosis and surveillance of ASF. Further studies will be required to fully validate the PCR assay by testing field samples from ASF-endemic as well as ASF-free areas.

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Compliance with ethical standards

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Conflict of interest All authors declare that they have no competing interests.

Ethical approval None of the experiments in this study involved human participants.

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