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A Universal Real-Time Fluorescence qPCR Method for Identifying Epidemic Strains of African Swine Fever Virus

Meihui Lv[1,2], Qiuyue Zheng[1], Lili Yang[1], Lin Wang[3], Lili Chen[4], Aifu Yang[5], Jijuan Cao[1*]

[1]Key Laboratory of Biotechnology and Bioresources Utilization of Ministry of Education, Dalian Minzu University, Dalian, China
[2]Dalian Polytechnic University, Dalian, China
[3]Beijing Animal Disease Prevention and Control Center, Beijing, China
[4]Zhonglianrui (Beijing) Biotechnology Co., Ltd., Beijing, China
[5]Dalian Customs Technology Center, Dalian, China
Email: *caojijuan@dlnu.edu.cn

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Abstract

Objective Establishing a highly sensitive real-time fluorescence quantitative PCR (qPCR) method for universal testing of epidemic African swine fever virus (ASFV) strains. Methods The ASFV p72 gene was targeted to design primer probes covering 24 p72 genotypes. The optimal amount of dimethylsulphoxide (DMSO) for qPCR amplification was determined, Various sensitivity and limit of detection (LOD) tests were performed, and clinical samples from China and imported goods were tested. Results The optimal primer-probe combination could specifically detect ASFV, 1.5% DMSO was optimal for qPCR, and LOD reached 3.2 copies/μL with good reproducibility (n = 20, p = 0.369). The method was employed to test 142 clinically suspected samples, of which 30 pig blood and 37 pig tissue samples were ASFV-positive. Moreover, the positive testing rate for ASFV was higher than for the standard qPCR method recommended by the Office International Des Epizooties (OIE), and for the commercially available kit. Thus, our method is superior for testing weakly positive samples with low virus titre, and epidemic strains present in imported goods. Conclusion Our method could be employed for universal testing of epidemic ASFV strains worldwide, ensuring wider coverage of hosts and ASFV strains/endemic strains, reducing false negatives, and benefitting early diagnosis.

Keywords

African Swine Fever Virus, Real-Time Fluorescence qPCR, Epidemic Strain, Virus Detection, DMSO, ASFV Testing

1. Introduction

African swine fever (ASF) is a febrile, acute, and subacute highly contagious infectious disease caused by ASF virus (ASFV) [1]. The virus is highly infectious, and the morbidity and mortality of sick pigs can be as high as 100% [2]. The disease first broke out in Kenya in 1921, then spread to many countries in Europe, Central America, Africa and Asia. Since 2018, it has spread to Mongolia, Vietnam, Cambodia, Laos, North Korea, Philippines, Myanmar, South Korea, Indonesia and other Asia-Pacific countries [3]. On August 3, 2018, it was confirmed by the China Animal Health and Epidemiology Center that ASF was first discovered in Shenyang, China [4]. Subsequently, it also broke out in Jiangsu, Zhejiang, and other provinces in China, and it is still spreading [5]. ASF is the most serious disease in the pig industry. It is listed as a notified disease by the Office International Des Epizooties (OIE), and as a type of infectious disease in China [6] [7]. Public health incidents caused by ASF have brought huge economic and social burdens to the affected countries. The infection mechanism of ASFV is extremely complicated and there are many genotypes, hence an effective vaccine has not yet been developed. The only way to control the spread of disease is to kill diseased pigs, treat the source of the disease, and cut off the source of infection [8] [9].

Detection of ASFV is an arduous task because pig muscle, spleen, liver, lymph, blood, feed and other base materials are infectious if they carry trace amounts of virus [10], and detection is subject to the intervention of complex base materials. Existing testing methods have played a vital role in the prevention of ASFV, including testing methods recommended by the OIE involving virus isolation, fluorescent antibody detection, conventional and real-time PCR testing [11], the ASF detection and diagnosis manual [12], a qPCR method for simultaneous differential diagnosis of classical swine fever virus (CSFV) and ASFV established [13], and a fluorescent PCR method for direct testing of ASFV in samples without DNA extraction [14]. ASFV circulating in China is a virulent strain of genotype II [15]. ASFV epidemic strains vary in different countries, and the virus poses a great risk of spreading through cross-border trade of pig products [16], hence universal testing of epidemic strains of ASFV is important. Therefore, in this study, based on the 24 genotype epidemic strains of ASFV, a specific primer-probe combination was designed to optimise the amount of DMSO to improve the efficiency of qPCR amplification. A highly sensitive and specific real-time fluorescence qPCR testing method was established for universal testing of ASFV epidemic strains.

2. Materials and Methods

2.1. Reagents

Probe qPCR Mix (Cat. No. Code391A) was purchased from Takara (Dalian, China). A magnetic bead-based virus DNA/RNA extraction kit (Cat. No. DP438-T2K) was purchased from Tiangen Biotech (Beijing) Co., Ltd. (Beijing, Chian). Dime-

thylsulphoxide (DMSO) was purchased from Tianjin Kemiou Chemical Reagent Co., Ltd. (Tianjin, China).

2.2. Virus and Samples

ASFV nucleic acid standard (GBW(E)091034, 3.2×10^4 copies/μl) was purchased from Beijing Tianzhitai Biotechnology Co., Ltd. (Beijing, China). Inactivated nucleic acid samples of CFSV, porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 1 (PCV-1), porcine parvovirus (PPV), and porcine pseudorabies virus (PRV) were purchased from Beijing Laboratory Biology Technology Co. Ltd. (Beijing, China).

A total of 142 suspected clinical samples comprising pig blood and tissue samples were obtained from different provinces in China and imported goods, including 74 whole blood samples and 68 tissue samples including pig spleen, liver and lymph, all of which were collected and stored by the Veterinary Diagnosis Room of the Animal Disease Prevention and Control Center, the Veterinary Diagnosis Room of the Beijing Animal Disease Prevention and Control Center, the Animal Quarantine Room of the Dalian Customs Technology Center, and the Animal Quarantine Room of the Qingdao Customs Technology Center. This study was conducted according to the Guidelines for the Laboratory Animal Use and Care Committee of the Ministry of Health, China, and the Ethics Committee on Animal Research of Dalian Minzu University (No. 2019MDLL03).

2.2.1. Target Sequence Homology Analysis by DNAStar Software

Capsid protein p72 gene is an important component of virus particles and one of the main antigens detected in infected pigs. P72 gene with high conservation can be selected as the gene for detecting ASFV. According to the genetic information for the 24 ASFV isolates with different genotypes in GenBank (**Table 1**), homology analysis of 24 ASFV epidemic virulent strains was performed by DNAStar software (DNAStar, Madison, WI) using the p72 gene of the ASFV genotype II virulent strain (Georgia2007 virulent strain) as the target gene.

2.2.2. Primer and Probe Design

In order to ensure the conservation and specificity of primers and probes, we analysed the previously reported ASFV gene sequence, selected the conserved region of p72 gene as the target fragment, and designed three sets of primer probes using Primer Premier 6.0 for all different genotypes of the 24 ASFV isolates (**Table 2**). The corresponding primer probes listed in **Table 2** were selected in accordance with the general rule of degenerate bases to achieve a combination of degenerate primers and probes (**Table 3**). It can be seen in **Table 2** that the first, second and third sets of primer probes did not cover all genotypes, but after mixing the first and second sets, and the first and third sets, all 24 genotypes were covered. Therefore, the five sets of primer probes listed in **Table 3** were used for subsequent screening. The 5' end was labelled with 6-carboxyfluorescein (FAM)

reporter dye and the 3' end was labelled with quencher fluorophore black hole quencher (BHQ 1) reporter dye. All primer probes were synthesised by Takara (Dalian, China).

2.3. PCR System and Reaction Conditions

The total volume of the optimised reaction system was 25 μl, including 15 μl PCR reaction solution, 1 μl enzyme mixture, 1 μl (0.4 μM) forward primer, 1 μl (0.4 μM) reverse primer, 1 μl (0.4 μM) probe, 0.375 μl DMSO, 2 μl sample, and the volume was brought to 25 μl with RNase-free water.

The optimised PCR conditions involved 15 cycles at 50˚C for 10 s, followed by 35 cycles at 95˚C for 1 min, 95˚C for 10 S, and a final extension at 55˚C for 10 min.

Table 1. Twenty-four strains of African swine fever virus with different genotypes.

Virulent strain	Genotype	Genbank accession number	Isolation location
Kongo73	I	KJ671545	Congo
Georgia2007	II	AM999764	Georgia
BOT/1/99	III	AF504886	Mozambique
RSA/1/99/W	IV	AF449477	South Africa
Tengani	V	AF301541	Malawi
SPEC265	VI	AF270710	Mozambique
RSA/1/98	VII	AF302818	South Africa
Malawi/1978	VIII	AF270707	Malawi
Ken07.Eld1	IX	FJ154441	Spain
MWHOG/1	X	AY351548	East Africa
KAB/62	XI	AY351522	East Africa
MFUE6/1	XII	AY351561	East Africa
SUM/1411	XIII	AY351542	East Africa
NYA/12	XIV	AY351555	East Africa
TAN/1/01	XV	AY494552	East Africa
TAN/2003/1	XVI	AY494550	East Africa
ZIM/92/1	XVII	DQ250119	South Africa
NAM/1/95	XVIII	DQ250122	Namibia
SPEC/125	XIX	DQ250112.1	South Africa
RSA/1/95	XX	DQ250123	South Africa
SPEC/53	XXV	DQ250111	South Africa
SPEC/245	XXII	DQ250117	South Africa
ETH/1	XXIII	KT795354	Ethiopia
MOZ-10/2006	XXIV	KY353989	Mozambique

Table 2. Sets of primers/probes designed for specific amplification of the p72 gene of ASFV epidemic strains.

Group	Primer/probe	Primer/probe sequence (5'-3')	Length (bp)	Covered genotype	Uncovered genotype
Set 1	p72-1F	ATCCGATCACATTACCTA	108	I, II, VII, VIII, XI, XII, XIII, XIV, XV, XVI, XVII, XVIII, XXI, XXII	III, IV, V, VI, IX, X, XIX, XX, XXIII, XXIV
	p72-1R	AGTGGAAGGGTATGTAAG			
	p72-1P	CCGTAACTGCTCATGGTATCAATCT			
Set 2	p72-2F	TCTGCAGCTCTTACATAC	162	III, IV, V, VI, IX, XIX, XX, XXIII, XXIV	I, II, VII, VIII, XI, XII, XIII, XIV, XV, XVI, XVII, XVIII, XXI, XXII
	p72-2R	CCCAACTAATATAAAATTCTCTTG			
	p72-2P	CCACTACGGAGGCAATGCGA			
	p72-2R1	CCCAGCTAATATAAAACTCTCTTG		X	
	p72-2P1	TCACTACGGAGGCAATTCGA			
Set 3	p72-3F	GCGATGATGATTACCTTTG	97	III, IV, V, VI, XIX, XX, XXIII, XXIV, IX	I, II, VII, VIII, XI, XII, XIII, XIV, XV, XVI, XVII, XVIII, XXI, XXII
	p72-3R	CCCAACTAATATAAAATTCTCTTG			
	p72-3P	AGCCACGGGAGGAATACCAAC			
	p72-3R1	CCCAGCTAATATAAAATTCTCTTG		IX	
	p72-3R2	CCCAGCTAATATAAAACTCTCTTG		X	
	p72-3P1	AACCACGGGAGGAATACCAAC		IX, X	

Table 3. Sequences of primers/probes for specific amplification of the p72 gene of ASFV epidemic strains.

Group	Primers/probes	Sequences (5'-3')
Group 1	P72-F1	ATCCGATCACATTACCTA
	P72-R1	AGTGGAAGGGTATGTAAG
	P72-P1	CCGTAACTGCTCATGGTATCAATCT
Group 2	P72-F2	TCTGCAGCTCTTACATAC
	P72-R2	CCCARCTAATATAAAAYTCTCTTG
	P72-P2	YCACTACGGAGGCAATKCGA
Group 3	P72-F3	GCGATGATGATTACCTTTG
	P72-R3	CCCARCTAATATAAAAYTCTCTTG
	P72-P3	ARCCACGGGAGGAATACCAAC
Group 4	Combination of the first and second sets of primers/probes	
Group 5	Combination of the first and third sets of primers/probes	

2.4. Primer Probe Screening and Specificity Analysis Test

In order to verify the specificity of the designed primers/probes, nucleic acid samples from CFSV, PRRSV, PCV, PPV and PRV were used as test materials, ASFV standard served as the positive control, and nucleic acids from uninfected ASFV pig tissue served as negative controls. qPCR was performed on a Bio-Rad CFX96 Touch Real-Time PCR Detection System, fluorescence amplification

curves were plotted, and the cycle threshold (Ct) value was recorded.

2.5. Effect of DMSO on the Efficiency of qPCR Amplification

Using different concentrations of ASFV plasmid as test material, qPCR samples contained different volumes of DMSO (0 as control, 1%, 1.5%, and 2% of the total volume). The effect of DMSO on the amplification efficiency of qPCR was compared, and the optimal DMSO concentration was determined.

2.6. Analysis of Sensitivity and Limit of Detection (LOD) for Different Methods

ASFV nucleic acid standard was used as the test material to carry out sensitivity tests, and diethypyrocarbonate (DEPC) water was used to dilute this 10-fold. Testing was carried out according to the qPCR reaction system and conditions. Fluorescence amplification curves were plotted, the Ct value was recorded, and the linear relationship of the standard curve was analysed.

Additionally, using 10-fold dilutions of ASFV nucleic acid standard, qPCR was performed 20 times using the developed method, a commercially available kit, and the protocol recommended by the OIE. The reproducibility of the three methods at the lowest detection level was determined, and LOD was compared.

2.7. Application to Actual Clinical Samples

Blood samples were used directly for virus DNA/RNA extraction using a magnetic bead-based virus DNA/RNA extraction kit according to manufacturer's instructions. Other samples including pig spleen, liver, lymph, faeces and feed were collected, ground in phosphate-buffered saline (PBS) for 45 s with shaking at 6000 g to homogenise, then centrifuged at 5000 g for 5 min. The supernatant was used for virus DNA/RNA extraction. All 142 clinically suspected samples were tested using the developed method, and compared with the qPCR method recommended by the OIE and a commercially available ASFV detection kit to verify the practicability and consistency of the results.

3. Results

3.1. Homology Analysis of the Target Sequence by DNAStar

The ASFV gene type II Georgia2007 strain (accession number: AM999764) p72 gene was used as the target sequence, and homology analysis was carried out against the other 23 representative ASFV epidemic strains in GenBank using DNAStar software (Figure 1). The results showed that the target sequence shared 93.9100% homology with the other 23 representative ASFV strains in GenBank, and the lowest homology (93.9%) was with strain MWHOG/1.

3.2. Optimisation of Primer Probes

The ASFV nucleic acid standard (3.2×10^3 copies/μl) was used as a control, and the five sets of primer probes listed in Table 3 were used for real-time fluores-

cence qPCR (Table 4, Figure 2 and Figure 3). The results showed that all five sets of primer probes yielded a typical fluorescence amplification curve, and the negative control did not generate such as curve, as expected. In addition, primer/probe sets 4 and 5 displayed stronger fluorescent signals than the other three sets (Figure 2 and Figure 3) hence they were more likely to achieve positive results. Use of mixed primers and probes with the same fluorescent label was clearly beneficial or enhancing the positive fluorescence signal. Comparison revealed that the Ct value (28.09) of primer/probe set 5 was the lowest, and the difference was significant ($p < 0.05$). Therefore, we selected primer/probe set 5 for subsequent specificity, sensitivity and reproducibility testing, and for actual clinical sample detection.

Table 4. qPCR screening optimisation of the five sets of primers/probes.

Serial Number	Sample Name	Primer/probe set	Ct value		Ct mean ± SD
			Sample 1	Sample 2	
1	ASFV nucleic acid standard material (3.2×10^3 copies/μl)	Group 1	34.99	35.27	35.13 ± 0.19^a
2		Group 2	31.98	32.38	32.18 ± 0.28^b
3		Group 3	32.64	32.89	32.76 ± 0.17^b
4		Group 4	30.84	30.29	30.56 ± 0.38^c
5		Group 5	27.96	28.49	28.09 ± 0.26^d

Note: Different letters in the same column of data indicate significant differences ($p < 0.05$).

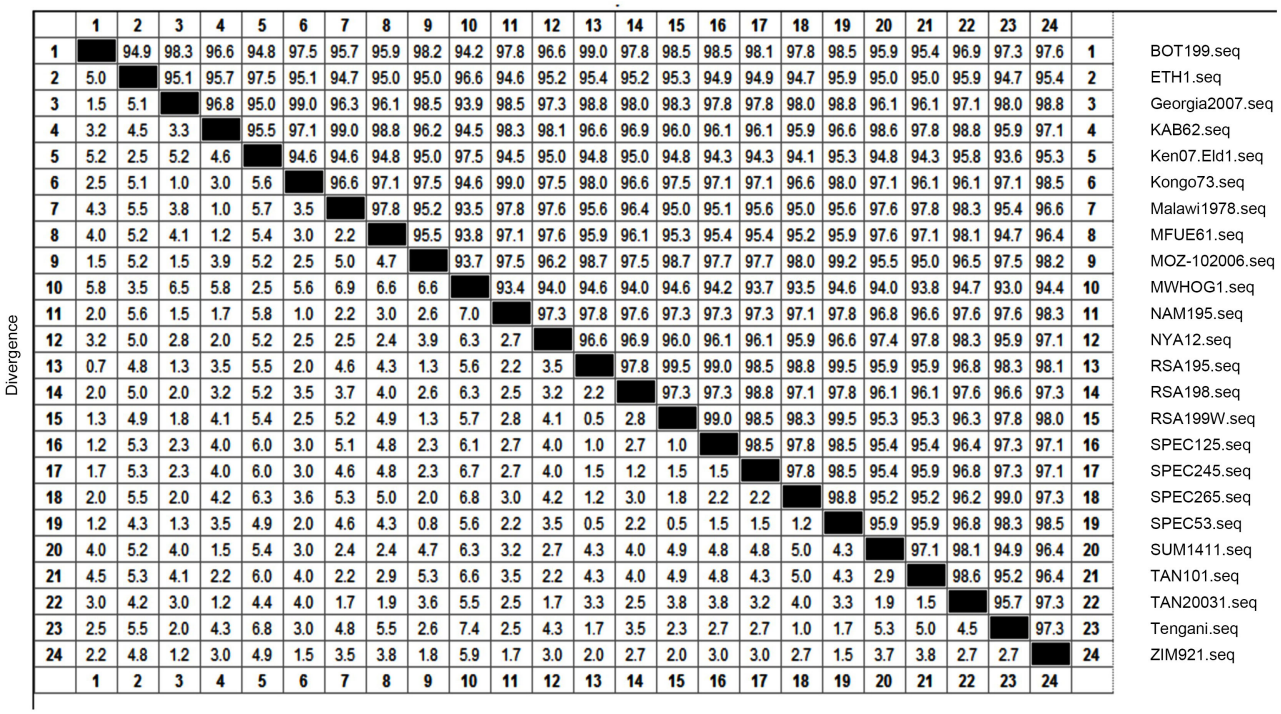

Figure 1. DNAStar homology comparison of the p72 gene target sequence of 24 ASFV strains.

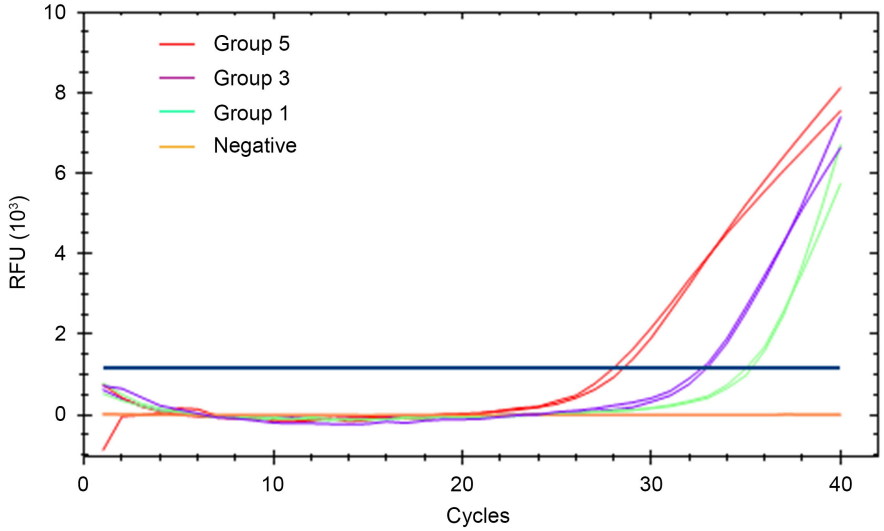

Figure 2. Real-time fluorescence qPCR testing of ASFV using primer probe sets 1, 3 and 5.

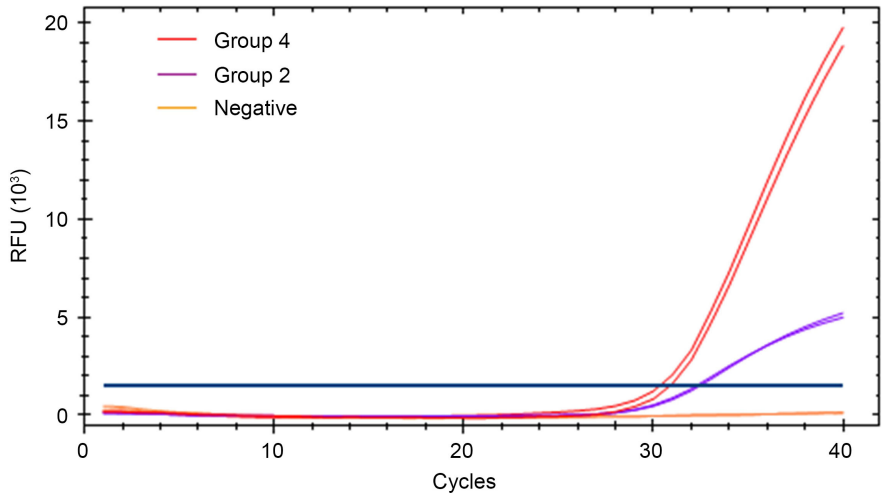

Figure 3. Real-time fluorescence qPCR testing of ASFV using primer probe sets 2 and 4.

3.3. Analysis of Specificity

Nucleic acid samples from CFSV, PRRSV, PCV, PPV and PRV were used as test materials, the ASFV standard (3.2×10^3 copies/μl) served as a positive control, nucleic acids from pig tissue test material not infected with ASFV served as a negative control, and the results of real-time fluorescence qPCR amplification are shown in **Figure 4**. Only the positive control yielded a typical fluorescence amplification curve, indicating that primer/probe set 5 exhibited good specificity, and could be used for specific detection of ASFV.

3.4. Optimisation of DMSO for qPCR Amplification and Detection of ASFV

DMSO can affect PCR amplification. In this study, different concentrations of ASFV nucleic acid standard were used to compare the effects of adding different

volumes of DMSO on the amplification efficiency of real-time fluorescence qPCR detection. The results showed that adding an appropriate amount of DMSO could improve the amplification efficiency of qPCR, and 1.5% was the optimal amount (Figure 5 and Table 5). Compared with the control group, addition of 1.5% DMSO yielded qPCR Ct values lower than controls for ASFV nucleic acid standard levels at 320 copies ($p = 0.028$), 32 copies ($p = 0.030$) and 3.2 copies ($p = 0.037$), and differences were significant. However, addition of 2% DMSO reduced the amplification efficiency of qPCR. Therefore, adding 1.5% DMSO to the qPCR system could significantly improve the sensitivity of qPCR detection.

Table 5. Effects of different amounts of DMSO additive on the amplification efficiency of qPCR.

ASFV nucleic acid standard concentration	DMSO volume/total reaction volume (%)	Ct value			Ct mean ± SD
		Sample 1	Sample 2	Sample 3	
320 copies/μl	0	25.63	25.37	26.38	25.79 ± 0.52
	1	25.64	25.76	24.83	25.41 ± 0.51
	1.5	24.34	24.99	23.55	24.30 ± 0.72
	2	26.60	25.52	26.02	26.05 ± 0.54
32 copies/μl	0	29.35	28.82	28.81	28.99 ± 0.30
	1	30.76	28.88	29.27	29.63 ± 0.99
	1.5	27.90	28.40	28.27	28.19 ± 0.26
	2	29.81	29.36	31.25	30.14 ± 0.99
3.2 copies/μl	0	33.94	33.39	32.96	33.43 ± 0.50
	1	33.39	34.17	32.88	33.48 ± 0.65
	1.5	32.12	32.68	31.54	32.11 ± 0.57
	2	35.42	34.89	34.87	35.06 ± 0.31

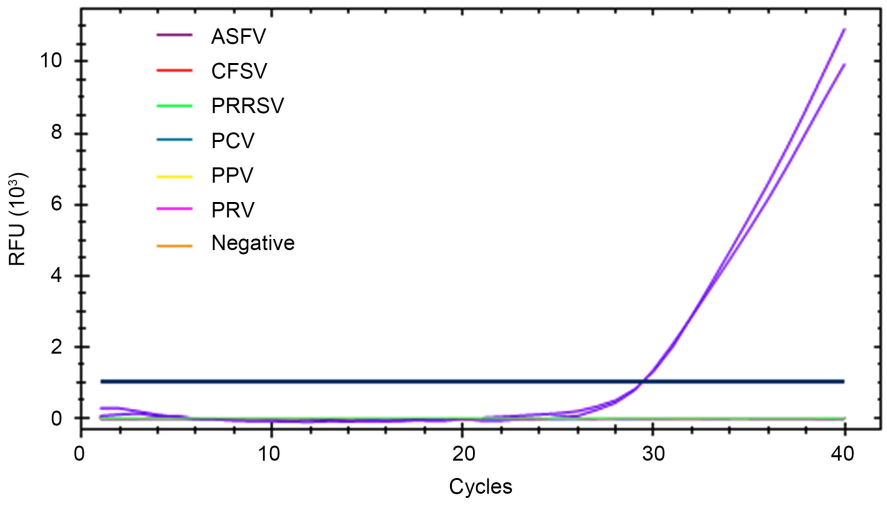

Figure 4. Analysis of the specificity of real-time fluorescence qPCR testing of ASFV.

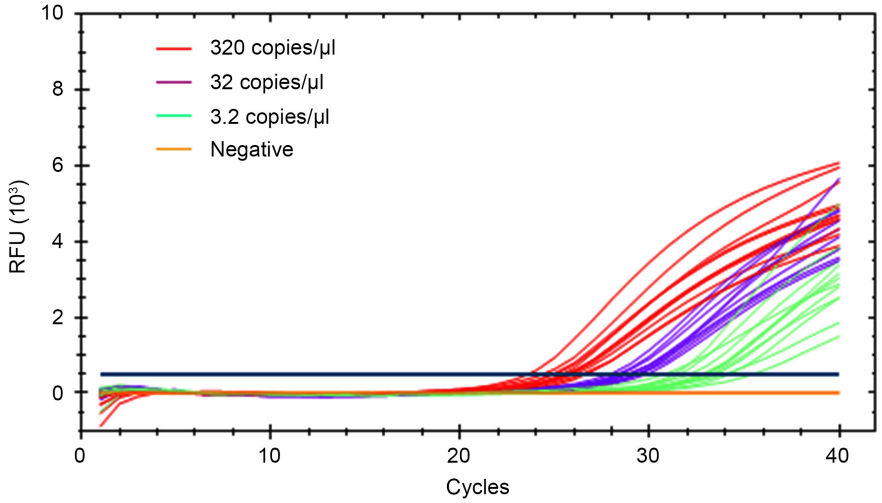

Figure 5. Real-time fluorescence qPCR amplification curves from different concentrations of ASFV nucleic acid standard and different amounts of DMSO additive.

3.5. Analysis of Sensitivity and LOD for Different Methods

3.5.1. Analysis of Sensitivity

The ASFV standard (3.2×10^4 copies/μl) was used as test material for 10-fold serial dilution, and this method was used for sensitivity analysis of real-time fluorescence qPCR. It can be seen from **Figure 6** and **Table 6** that when the minimum concentration of ASFV standard was 3.2 copies/μl, there was an obvious fluorescence amplification curve (average Ct = 35.56). Taking the log value of the ASFV standard concentration as the abscissa and the Ct value as the ordinate, a standard curve was drawn (**Figure 7**), for which the standard curve equation was $y = 37.26 - 3.433 \log_{10}X$, and the correlation coefficient R^2 was 0.9993. The results indicate a good fit and a linear relationship ($R^2 > 0.99$). Therefore, the testing sensitivity of the method established in this study could reach 3.2 copies/μl.

3.5.2. Analysis of LOD

The ASFV nucleic acid standard was used as test material to compare LOD values from our method, the qPCR method recommended by OIE, and a commercially available kit. Four concentrations of ASFV standard were used to perform 20 real-time fluorescent qPCR replicates (n = 20), and the results are summarised in **Table 7**. The analysis showed that at the lowest ASFV nucleic acid standard concentration (3.2 copies/μl), LOD detected by our method achieved the best reproducibility (p = 0.369), with a positive rate of 100% (**Figure 8**), and the average Ct of LOD was significantly lower than for the qPCR method recommended by OIE and the commercially available kit. The LOD value was significantly different from that determined by the qPCR method recommended by OIE and the commercially available kit, with positive rates of 65% and 75%, respectively. The LOD of our method reached 3.2 copies/μl, indicating high detection sensitivity and good reproducibility, which is more conducive for detecting ASFV in weakly positive samples with low virus titre, hence it could replace the established qPCR method recommended by the OIE.

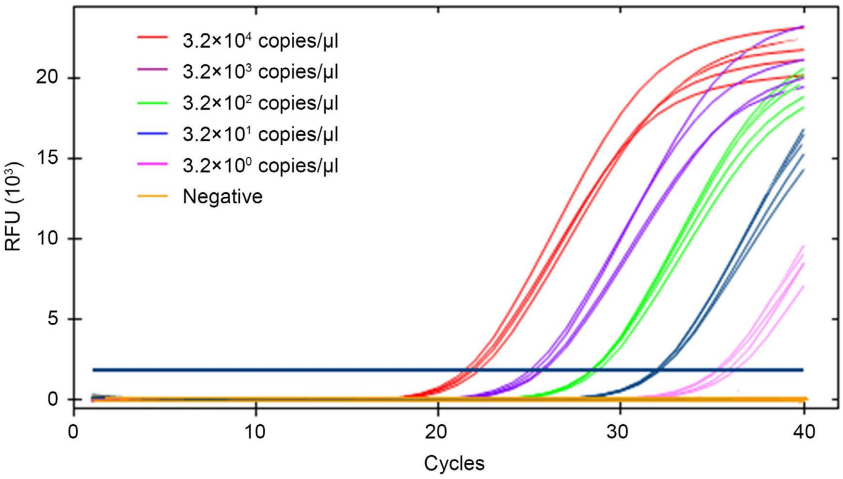

Figure 6. Graph of qPCR sensitivity for the detection of ASFV using the developed method.

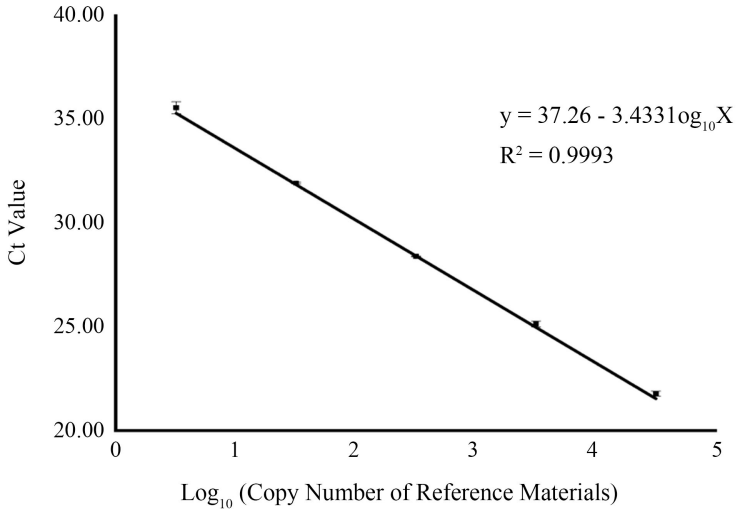

Figure 7. qPCR sensitivity based on standard curves for the detection of ASFV using the developed method.

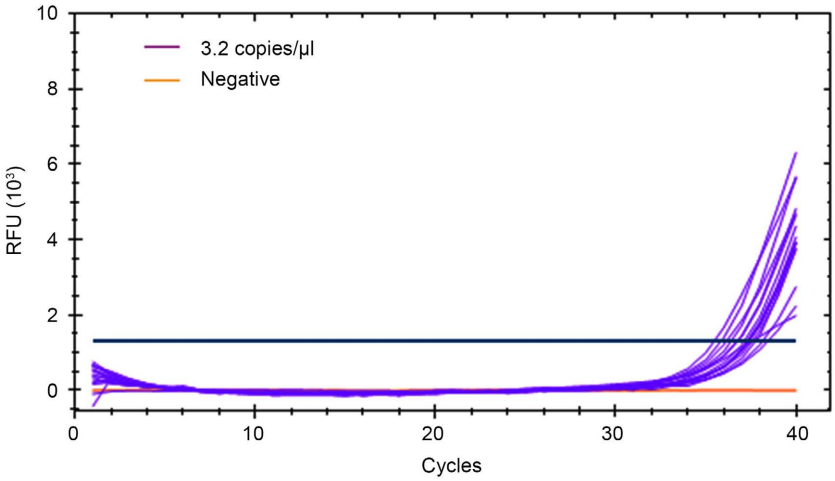

Figure 8. qPCR repeatability for determining LOD (n = 20).

Table 6. qPCR sensitivity of ASFV detection by the developed method.

Serial Number	Sample Name	Concentration (copies/μl)	Ct value					Ct mean
			Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	
1	ASFV Nucleic acid standard substance	3.2×10^4	21.68	21.89	21.41	22.20	21.59	21.79
2		3.2×10^3	25.27	25.02	25.57	25.71	ND	25.39
3		3.2×10^2	28.39	28.42	28.33	28.71	28.45	28.46
4		3.2×10	31.97	31.89	32.12	32.11	31.99	32.02
5		3.2	35.32	35.82	35.19	36.24	35.26	35.56
6	Negative control	0	ND	ND	ND	ND	ND	ND

Note: ND, not detected.

Table 7. Comparison of LOD values detected by different methods.

Methods	Positive rate for different concentrations (copies/μl; number of positive results/number of total samples, %)				Ct average of the lowest concentration
	3.2 × 103	3.2 × 102	3.2 × 10	3.2	
Our method	20/20 (100%)	20/20 (100%)	20/20 (100%)	20/20 (100%)	36.23
OIE recommended method	20/20 (100%)	20/20 (100%)	18/20 (90%)	15/20 (75%)	38.63
Commercially available kit	20/20 (100%)	20/20 (100%)	18/20 (90%)	13/20 (65%)	39.16

3.6. Application to Clinical Samples

Our method was used to test 142 samples from clinically suspected cases in the veterinary diagnosis room of the China Animal Disease Prevention and Control Center and the Beijing Animal Disease Prevention and Control Center. The results were compared with those obtained using the qPCR method recommended by the OIE and a commercially available kit. Among the 142 clinically suspected cases, 74 were pig blood samples and 68 were pig tissues (spleen, liver and lymph). The test results for the three methods are summarised in Table 8. After testing, 30 pig blood and 37 pig tissue samples were ASFV-positive according to our method, with positive testing rates of 40.5% and 54.4%, respectively. The results of pig blood sample detected by the qPCR method recommended by OIE and commercially available test kit were 100% consistent with our method. Furthermore, the positive detection rates for ASFV-positive pig tissue samples were 52.9% and 51.5%, respectively, which were lower than the positive testing rate of our method.

The significance of the differences in positive detection rate for pig tissue samples was analysed, and all three methods showed differences when testing weakly positive pig tissue samples with Ct values between 35 and 40 (Table 9). Among them, samples No. 2 and No. 4 tested positive by our method and the OIE method, but negative according to the commercially available kit. Meanwhile, sample No. 3 tested positive by our method and the commercially available kit, but negative using the OIE recommended method. These three samples

Table 8. Detection of 142 clinically suspected pig cases using the three methods.

Methods	Pig blood samples		Pig tissue samples	
	Positive number/total number of samples	Positive rate (%)	Positive number/total number of samples	Positive rate (%)
Our method	30/74	40.5%	37/68	54.4%
OIE recommended method	30/74	40.5%	36/68	52.9%
Commercially available kit	30/74	40.5%	35/68	51.5%

Table 9. Detection and analysis of five weakly positive pig tissue samples using the three methods.

Sample Number	Our method (Ct value)		OIE recommended method (Ct value)		Commercial kit (Ct value)	
	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
No.1	35.37	35.41	38.91	38.62	39.12	38.93
No.2	37.92	37.14	38.56	ND	ND	ND
No.3	37.14	37.52	ND	ND	39.11	38.43
No.4	38.53	38.76	ND	38.92	ND	ND
No.5	37.56	37.53	39.12	39.14	38.42	39.10

Note: ND, not detected.

were from imported goods, which may differ from epidemic strains in China, explaining why the qPCR method recommended by the OIE and the commercially available kit failed to give positive detection. It can also be seen that our method proved highly sensitive for detecting weakly positive samples with low virus titre, and the results were stable and reliable. Thus, our method could detect all 24 genotype strains/isolates of ASFV, with good practicability.

4. Discussion

This research explored the universal testing of ASFV epidemic strains circulating worldwide, and established a highly sensitive testing method for samples with low virus titres. Compared with the qPCR method recommended by the OIE and a commercially available kit in China, it was superior for early diagnosis of ASFV.

Since 2020, there have been 2197 ASF epidemics in domestic pigs and 7238 in wild boar in 26 countries and regions around the world, and the epidemic is still characterised by continuous outbreaks [17]. The ASFV genome is large and complex, with obvious genetic diversity [18]. After ASFV infects the host, it is detoxified before obvious clinical symptoms appear, and the virus survives for a long time in the external environment where it is strongly resistant to common disinfectants. China is the world's largest pig-raising country, and its breeding volume accounts for more than 50% of the worldwide total. The disease has had a huge impact on the pig industry in Africa, Europe and Asia, especially China. Part of the reason why there is no applicable ASF vaccine is due to the complexity of the virus, which encodes various proteins related to immune escape [9]

[19]. In addition, insufficient understanding of the determinants of ASFV immune protection also hinders the development and design of vaccines [19]. In the absence of ASF vaccines, reliable laboratory diagnosis and appropriate biosafety measures are essential for disease prevention and control [20].

There are many problems in the detection of ASFV in pig products, pig feed, and the environment. Analysis of pig spleen, liver, lymph and other complex matrices requires extraction and purification of DNA to avoid interference during PCR amplification that may impact detection sensitivity. Different ASFV epidemic strains and regional genetic variation are further challenges to the universal testing of ASFV. In many cases, it is not possible to determine the exact route of introduction into a domestic pig herd, but most introductions are attributed to indirect transmission of the virus, including raw pork, processed pig-derived products, animal feed, and blood-feeding invertebrates carrying the virus [21].

Researchers have investigated the detection of ASFV. Yuzi Luo *et al.* [22] established a PCR method with an LOD of 60 copies; Shaoling Zeng *et al.* [23] established a real-time fluorescence PCR method with an LOD of 10 copies; Yuan Lin *et al.* [24] established a droplet digital PCR method with an LOD of 0.8 copies; Deguo Wang *et al.* [25] established a loop-mediated isothermal amplification (LAMP) method with an LOD of six copies; Xiaoxu Fan *et al.* [26] established recombinase polymerase amplification (RPA) and recombinase aid amplification (RAA) methods with LOD values of 93.4 and 53.6 copies; Yin Wang *et al.* [27] established a fluorescence quantitative PCR detection method based on an ASFV internal reference gene with an LOD of six copies or 0.11 TCID50/ml per reaction; Dongyan Xiong *et al.* [28] proposed a new method for multi-locus sequence typing of ASFV genomes based on alleles.

Rapid and accurate molecular typing during ASFV outbreaks is of great significance for revealing the diversity and source of ASFV. According to the B646L sequence encoding the main capsid protein p72, epidemic strains of ASFV can be divided into 24 genotypes [29]. It is necessary to establish detection methods covering all genotypes of ASFV in order to prevent missed detection, which makes it particularly difficult to establish ASFV detection methods. However, there are few studies on the universality of the testing of the 24 genotypes of ASFV epidemic strains. Varlamov *et al.* [30] and Kim *et al.* [31] reported that additives such as DMSO can enhance the sensitivity and efficiency of PCR detection. Nevertheless, there are few studies on improving the testing efficiency of weakly positive samples containing low ASFV titres. Oluwole *et al.* [32] research show that ASF is still prevalent in Nigerian native pigs, their hybrid pigs and backcross pigs. Its propagation ability is huge, which is particularly important for the detection of ASF.

In the present study, the gene sequences of 24 different genotypes of ASFV epidemic strains in Genbank were assessed. In order to reduce false negatives, a combination of primers and probes covering 24 different genotypes was de-

signed and screened to ensure wider coverage of hosts and ASFV. A real-time fluorescence PCR method for universal testing of ASFV was established, which could detect ASFV strains/isolates more widely than previous methods, and showed good specificity and universality for ASFV testing. The optimal amount of DMSO (1.5%) improved the sensitivity of qPCR detection at low virus concentrations, and the LOD of this method reached 3.2 copies. Compared with the real-time fluorescent PCR method in GB/T 18648-2020 standard, the coincidence rate of the detection sample is 100%. A total of 142 samples from clinically suspected pig cases from all parts of China and imported goods were tested and compared, and the results showed that the positive testing rate for ASFV detection by our method was higher than for both the qPCR method recommended by the OIE and a commercially available kit. This was especially true for weakly positive samples showing stronger fluorescence amplification signals and Ct values, hence our method can be used more widely for detection of ASFV strains/epidemic strains. Therefore, our real-time fluorescence qPCR method for universal testing of ASFV in swine products is of great significance for preventing the risk of ASFV spreading through cross-border trade. However, optimising the testing sensitivity of real-time fluorescence qPCR based on DMSO additives is not the only choice; other methods with higher biological reactivity and improved PCR sensitivity and efficiency will be sought in future work.

Highlights

- A real-time fluorescence qPCR detection method for ASFV strains was established.
- Primer probe sets targeting the ASFV p72 gene in 24 genotypes were prepared.
- The optimal amount of DMSO additive for qPCR amplification was determined.
- The method outperformed an existing qPCR method and a commercially available kit.
- The method could widen coverage, reduce false negatives and benefit early diagnosis.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Abbreviations

African swine fever virus, ASFV; African swine fever, ASF; classical swine fever virus, CSFV; porcine reproductive and respiratory syndrome virus, PRRSV; porcine circovirus type 1, PCV-1; porcine parvovirus, PPV; porcine pseudorabies virus, PRV; dimethylsulphoxide, DMSO; limit of detection, LOD; Office International Des Epizooties, OIE; 6-carboxyfluorescein, FAM; black hole quencher, BHQ; cycle threshold, Ct; diethypyrocarbonate, DEPC; phosphate-buffered saline, PBS; loop-mediated isothermal amplification, LAMP; recombinase polymerase amplification, RPA; recombinase aidamplification, RAA.