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SHORT COMMUNICATION

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Development and application of a SYBR Green I fluorescent PCR assay for the differentiation of genotypes I and II African swine fever viruses

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Abstract

African swine fever (ASF) is a highly fatal hemorrhagic disease affecting domestic pigs caused by African swine fever virus (ASFV). Genetic analysis of ASFV isolates to date has identified 24 geographically related genotypes with various subgroups, but only genotype I and II ASFVs have been reported outside Africa. ASFV genotype II and genotype I viruses were reported in China in 2018 and 2021, respectively. In this study, unique and highly conserved noncoding regions were found between *MGF_505-9R* and *MGF_505-10R* in the 188 genomes of ASFV genotypes I and II. A pair of primers was designed on the basis of this region. By optimizing the reaction system and conditions, a SYBR Green I fluorescence PCR assay that can distinguish between ASFV genotypes I and II was established, and the sensitivity, reproducibility and specificity were evaluated. The detection limit was 1 $TCID_{50}$/0.1 mL for both genotypes, with no cross-reactivity observed with other common pig pathogens. The intra- and interbatch variation coefficients were both less than 1.2%. Clinical sample detection analysis revealed 47 positive cases out of 100, including 3 for genotype I and 44 for genotype II, aligning with results from the WOAH-recommended and national standard methods. The method developed in this study allows for the differentiation of ASFV genotypes I and II without the need for genome sequencing, offering a convenient and rapid approach for ASFV detection and genotype identification.

Keywords African swine fever virus, Genotype II, Genotype I, SYBR Green I fluorescent PCR, Diagnosis

Introduction

African swine fever (ASF), caused by African swine fever virus (ASFV), is a highly destructive viral disease that affects pigs, leading to considerable economic damage, disruptions in international trade, and significant sociophysical impacts (De la Torre et al. 2015; Gaudreault et al. 2020; Wang et al. 2019). It is listed as a "notifiable disease" by the World Organization for Animal Health (WOAH). ASF was first reported in Kenya in the 1910s and has traditionally been present on the African continent. In 1978, the disease was introduced to Sardinia, Italy, where it has since become endemic (African swine fever: detection and diagnosis – A manual for

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veterinarians, 2017; Wang et al. 2019). Since January 2020, ASF has been reported in 35 countries, affecting more than 1,000,000 domestic pigs and over 30,000 wild boars, with losses exceeding 1,700,000 animals (African Swine Fever – Situation Report 5 2022; Global Control of African swine fever2020). ASFV has been classified into 24 genotypes on the basis of the B646L gene sequence encoding the p72 capsid protein (De la Torre et al. 2015; African swine fever: detection and diagnosis – A manual for veterinarians, 2017; Global Control of African swine fever: A GF-TADs Initiative, 2020), and eight serogroups have been identified through hemadsorption inhibition assays (Malogolovkin et al. 2015).

To date, only ASFV genotypes I and II have been reported outside of Africa. ASFV was first reported in China in August 2018 and belongs to the *B646L* (p72) genotype II with *EP402R* (CD2v)-based serogroup 8 (Ge et al. 2018). In August 2021, a *B646L* (p72) genotype I strain with *EP402R* (CD2v)-based serogroup 4 was subsequently reported in China (Sun et al. 2021a). The emergence of these variant strains has complicated the molecular and serological diagnosis and control of ASFV, highlighting the urgent need for a method capable of differentiating ASFV genotypes I and II before the implementation of control measures.

Determination of the optimal reaction conditions

SYBR Green I fluorescent PCR was performed after the annealing temperatures and primer concentrations were optimized. The reaction mixture, with a final volume of 20 µL, included 10 µL of 2×SYBR Green Mix, 0.45 µM of each primer, and 5 µL of sample DNA. The amplification conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 61.4°C for 30 s. The fluorescence was recorded after each cycle, and a Ct value of ≤40 was considered positive. The PCR products of the ASFV genotypes I and II samples were visualized *via* 1.5% gel electrophoresis of the 241 bp bands and 331 bp bands, respectively.

Construction of standard plasmids

The noncoding regions of the *MGF_505-9R* and *MGF_505-10R* genes of ASFV genotypes I and II were amplified *via* PCR, and the resulting products were used to construct standard control plasmids. The concentrations of these plasmid constructs, named pMD-18 T-ASFV I (76.956 ng/µL) and pMD-18 T-ASFV II (81.722 ng/µL), were determined to be 2.393×10^{10} and 2.466×10^{10} copies/µL (original concentration), respectively. The plasmid constructs were then diluted to 1×10^{10} copies/µL and stored at -80°C until use.

Standard curve of the SYBR Green I fluorescent PCR

A standard curve for SYBR Green I fluorescent PCR was established via a tenfold serial dilution series, ranging from 1×10^{6} to 10^{2} copies of standard DNA per reaction. The regression formula for the pMD-18 T-ASFV I standard curve was y = -3.460x + 40.080 (R^2 = 0.999) (Fig. 1A). The regression formula for the pMD-18 T-ASFV II standard curve was y = -3.379x + 40.006 (R^2 = 0.997) (Fig. 1B). The SYBR Green I fluorescent PCR exhibited amplification efficiencies of 94.6% and 97.7% for the standard positive plasmids of genotypes I and II, respectively. These results indicate the strong performance and efficiency of the developed SYBR Green I fluorescent PCR assay.

Specificity of the SYBR Green I fluorescent PCR

The specificity of the assay was assessed via the use of DNA/cDNA from ASFV genotypes I and II, as well as from Pseudorabies virus (PRV), porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV), porcine circovirus 2 (PCV2), porcine circovirus 3 (PCV3), porcine parvovirus (PPV), porcine epidemic diarrhea virus (PEDV), and Getah virus (GETV), along with nuclease-free water. The results revealed amplification only for ASFV genotypes I and II, with no amplification for the other tested viruses, confirming the assay's high specificity and absence of cross-reactivity.

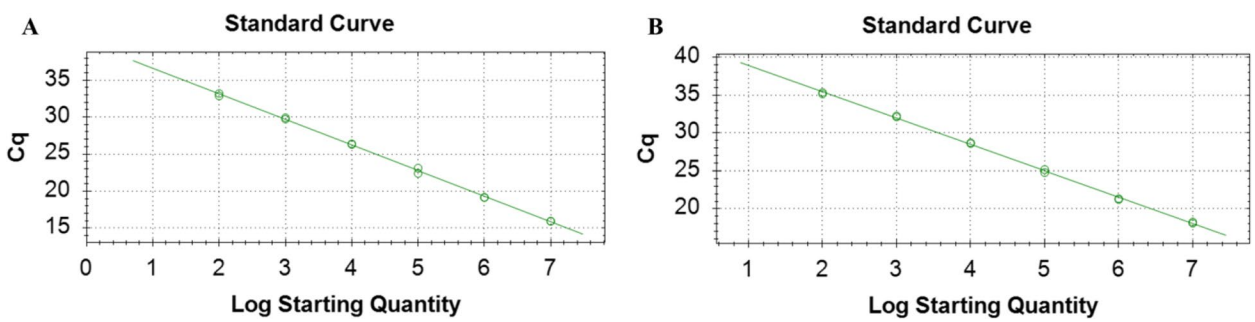

Fig. 1 Standard curves of the SYBR Green I fluorescent PCR assay. **A** The standard plasmid for pMD-18 T-ASFV I and (**B**) the standard plasmid for pMD-18 T-ASFV II

Sensitivity of the SYBR Green I fluorescent PCR

Standard plasmids with concentrations ranging from 1×10^7 to 0.1 copies/μL were used to assess the sensitivity of the assay. In the SYBR Green I fluorescence PCR assay, tenfold serial dilutions of the standard plasmids were simultaneously detected. The findings demonstrated that the assay could reliably detect ASFV genotypes I (Fig. 2A) and II (Fig. 2C) at a concentration of 1 copy/μL. The PCR products of the genotype I and II ASFV samples were visualized via 1.5% gel electrophoresis of the 241 bp bands (Fig. 2B) and 331 bp bands (Fig. 2D), respectively.

Detection limits of SYBR Green I fluorescent PCR for viruses

To determine the minimum detection limit, DNA samples from ASFV genotypes I and II were tested at concentrations of 1, 10 and 100 $TCID_{50}$/0.1 mL *via* SYBR Green I fluorescent PCR (Fig. 3). The assay's detection limit was 1 TCID50/0.1 mL for both genotypes, demonstrating its high sensitivity.

Reproducibility of the SYBR Green I fluorescent PCR assay

The reproducibility of the assay was evaluated by assessing its ability to distinguish ASFV genotypes I and II across different intra-assay and interassay runs. The variation in Ct values was recorded, and as shown in Table 1, the method displayed high repeatability and stability. The intra-assay CV values for SYBR Green I fluorescence PCR ranged from 0.25% to 0.94% for genotype I and from 0.32% to 0.90% for genotype II. The interassay CV values ranged from 0.85% to 1.13% for genotype I and from 0.74% to 0.93% for genotype II.

Detection results of the clinical samples

Among the 100 clinical samples tested via SYBR Green I fluorescent PCR, 47 were positive for ASFV. The results were consistent with those obtained from the qPCR methods recommended by WOAH and the Chinese National Standard of *Diagnostic Techniques for African Swine Fever* (GB/T 18648–2020) and were consistent with the results of the developed SYBR Green I fluorescent PCR in this study. The established SYBR Green I fluorescent PCR products of 47 positive samples were visualized *via* gel electrophoresis, and three and 44 ASFV genotype I and II samples were detected, respectively. Twenty-two samples with high levels of genomic DNA were successfully sequenced *via* the *B646L* gene sequencing method in 47 positive samples. The sequencing results revealed that two and 20 samples were ASFV genotypes I and II, respectively. The 22 successfully sequenced samples were consistent with the results of the developed SYBR Green I fluorescent PCR in this study (Table 2). The results suggested the high susceptibility of the SYBR Green I fluorescent PCR system.

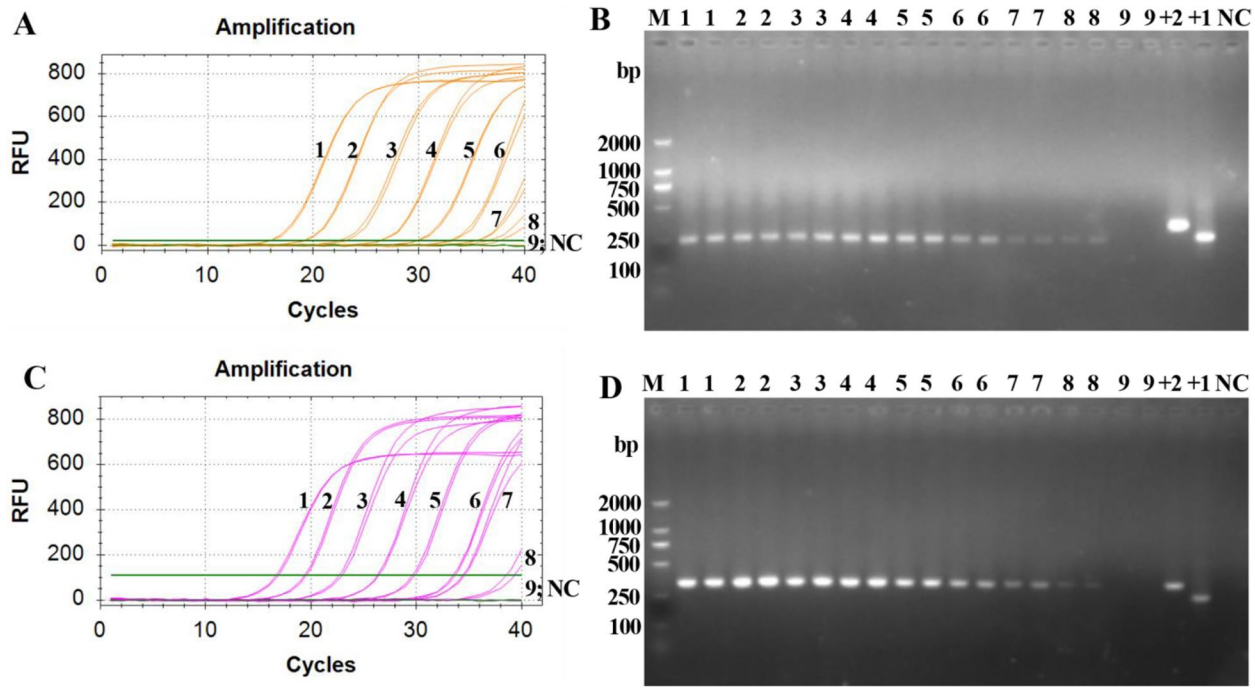

Fig. 2 Sensitivity test of SYBR Green I fluorescent PCR. Note that 1×10^7 to 0.1 copies/μL standard plasmids were used as templates for pMD-18 T-ASFV I (**A**: orange lines; **B**) and pMD-18 T-ASFV II (**C**: purple lines; **D**) detection. M: DNA Marker 2000; 1–9:10^7–0.1 copies/μL standard template; + : positive control; NC: nuclease-free water

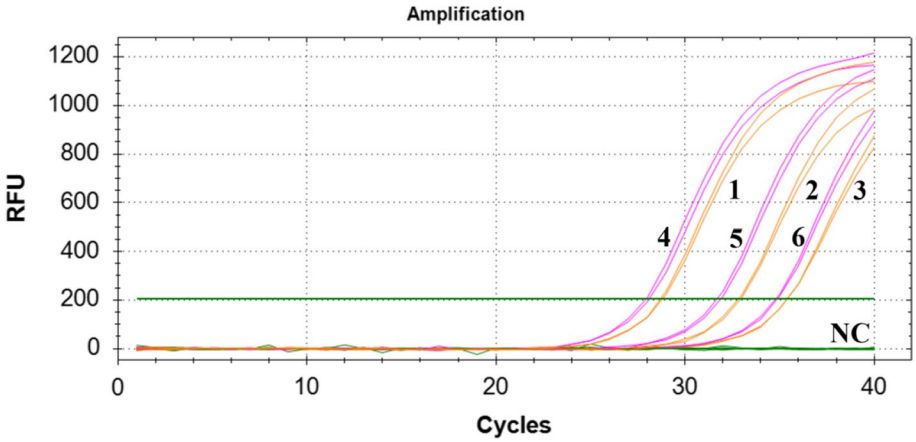

Fig. 3 Detection limits of SYBR Green I fluorescent PCR for viruses. Note that 100 to 1 $TCID_{50}$/0.1 mL viruses were used as templates for genotype I ASFV (orange lines) and genotype II ASFV (purple lines) detection. 1–3: 100 to 1 $TCID_{50}$/0.1 mL for genotype I viruses; 4–6: 100 to 1 $TCID_{50}$/0.1 mL for genotype II viruses; NC: nuclease-free water

Table 1 Repeatability evaluation of the SYBR Green I fluorescent PCR for intra-assay and interassay variation

Dilution (copies/μL)	pMD-18 T-ASFV I				pMD-18 T-ASFV II			
	Intra-assay variation[a]		Interassay variation[b]		Intra-assay variation[a]		Interassay variation[b]	
	(Ct)[c]	CV (%)	(Ct)[c]	CV (%)	(Ct)[c]	CV (%)	(Ct)[c]	CV (%)
1	19.14 ± 0.05	0.25	19.00 ± 0.26	0.85	18.97 ± 0.06	0.32	18.04 ± 0.13	0.74
2	26.69 ± 0.19	0.71	28.54 ± 0.90	0.90	27.32 ± 0.11	0.39	28.77 ± 0.25	0.86
3	34.87 ± 0.33	0.94	34.33 ± 0.39	1.13	33.60 ± 0.30	0.90	32.62 ± 0.30	0.93

CV coefficient of variation

[a] Intra-assay variation was determined on three replicates of recombinant plasmid dilutions analyzed in the same PCR run

[b] Interassay variation was calculated on the basis of the results from three independent PCR runs

[c] Mean ± standard deviation (SD)

Table 2 Information on the 47 clinical ASFV-positive samples obtained via the *B646L* gene sequencing method and SYBR Green I fluorescent PCR used in this study

Method		*B646L* gene sequencing method			
		Genotypes I	Genotypes II	Sequencing failed	Total
SYBR Green I fluorescent PCR	Genotypes I	2	0	1	3
	Genotypes II	0	20	24	44
	Total	2	20	25	47

Discussion

ASF is an infectious disease listed by the World Organization for Animal Health (WOAH) that continues to circulate in many countries, posing a severe threat to the global swine industry (Blome et al. 2020; Danzetta et al. 2020). With no effective vaccines or treatments currently available, accurate detection of ASF is essential for its control and prevention (Li et al. 2022b). Rapid and sensitive diagnostic methods are crucial for the successful implementation of all preventive and control measures. ASFV genotypes I and II are the only strains reported outside Africa, with genotype II ASFV being predominant in China since 2018 (Ge et al. 2018). Genotype I ASFV strains emerged in Shandong and Henan Provinces and displayed relatively low pathogenicity but relatively high transmissibility (Sun et al. 2021a). The emergence of these variants has introduced new challenges for ASFV diagnosis and control,

necessitating the development of a method to distinguish between genotypes I and II before any interventions are applied.

Currently, various real-time PCR assays are employed for ASFV diagnosis (Pikalo et al. 2022), targeting genes such as B646L (P72), EP402R (CD2v), and MGF_360, which can distinguish wild-type strains from gene-deleted strains (Guo et al. 2020; Yang et al. 2022). Madden et al. developed a chromatographic lateral flow immunoassay (LFIA) using monoclonal antibodies against the viral p30 protein to detect ASFV genotypes I and II antigens (Madden et al. 2022). However, these methods cannot differentiate ASFV genotypes I and II. Cao used a pair of primers and two probes targeting the *B646L* gene to differentiate ASFV genotypes I and II, achieving sensitivities of 10 copies/reaction for genotype I and 100 copies/reaction for genotype II (Cao et al. 2022). Gao used two pairs of primers and two probes targeting the *B646L* gene of genotypes I and II or the *E183L* gene of genotype I for the differentiation of genotypes I and II ASFV, with sensitivities of 1.07×10^2 copies/μL for genotype I and 3.13×10^4 copies/μL for genotype II (Gao et al. 2022). Qian used primers and probes targeting the *B646L*, *F1055L* and *E183L* genes to develop triplex real-time qPCR, achieving detection limits of 399.647, 374.409 and 355.083 copies/reaction, respectively (Qian et al. 2023). Li and Song independently developed duplex isothermal and real-time PCR methods for ASFV genotypes I and II differentiation on the basis of the *E296R* gene; these methods are capable of detecting 10 and 20 copies per reaction of standard plasmid DNA for both genotypes (Li et al. 2022a; Song et al. 2022). However, these methods are complex and lack sensitivity validation on the basis of viral samples.

In this study, a comparison was made among 188 whole-genome sequences, including all the reported whole-genome sequences in China. This includes the genotype II ASFV reported in 2018 (Ge et al. 2018), the genotype I ASFV reported in 2021 (Sun et al. 2021a), the lower virulent genotype II ASFV reported in 2021 (Sun et al. 2021b), and the recombinant ASFV reported in 2023 (Zhao et al. 2023). A pair of primers targeting the nondoding regions of the *MGF_505-9R* and *MGF_505-10R* genes were utilized to establish a method capable of simultaneous detection of both genotype I and II ASFVs, which can detect 1 copy/μL for both genotype I and II ASFVs. The detection limits of the PCR were 1 $TCID_{50}$/0.1 mL for ASFV genotypes I and II, respectively. The PCR products of the genotype I and II ASFV samples were visualized via gel electrophoresis of the 241 bp bands and 331 bp bands, respectively. These results indicated that it can differentiate ASFV genotypes I and II on the basis of the band size of the PCR products. This research has not been carried out with other similar methods.

The SYBR Green PCR technique is widely regarded as suitable for quantitative detection and species discrimination and has been effectively utilized in numerous experiments for species identification, quantification, and differentiation (Shahrajabian and Sun 2024). While both TaqMan qRT–PCR and SYBR Green PCR offer sensitivity and speed, the SYBR Green PCR assay is more cost-effective and simpler, as it does not require a probe (Arya et al. 2005; Shahrajabian and Sun 2024).

For the recombinant ASFV strains reported in 2023 (Zhao et al. 2023), sequence alignment revealed that the genotype results of the method in this study were consistent with those of the *B646L* gene sequencing method. These results suggest that SYBR Green I fluorescent PCR could serve as an initial tool for identifying ASFV recombinant strains. To date, there have been no reports of base mutations or deletions between the ASFV noncoding regions of *MGF_505-9R* and *MGF_505-10R*. It is unclear whether these mutations or deletions will occur in this region in the future. However, this method can accurately differentiate ASFV genotypes I and II, offering clinical significance and serving as a valuable tool for the differential diagnosis of these genotypes.

Conclusion

The SYBR Green I fluorescent PCR method, which targets the noncoding regions of ASFV *MGF_505-9R* and *MGF_505-10R* established in this study, offers a specific, sensitive, and efficient detection approach for all ASFV strains reported in China, making it suitable for clinical applications.

Methods

Facility and ethics statements

All experiments involving live ASFV manipulation were conducted within the Animal Biosafety Level 3 (ABSL-3) Laboratory of Huazhong Agricultural University, adhering to the Standard Technical Specifications for Safe Operation of African Swine Fever Virus Experiments (No. HZAU A3-III-10-C).

Design of primers

Sequence alignment of 188 complete ASFV genomes, including 78 genotype I and 110 genotype II genomes, was conducted *via* DNASTAR software (DNASTAR Inc., Madison, WI, USA). Highly conserved noncoding regions were identified between the *MGF_505-9R* and *MGF_505-10R* genes in both genotypes. The primers designed in these regions—MGF_505-9R-10R-F (5′-CAGGCTAATTGTAAATAGTTG-3′) and MGF_505-9R-10R-R (5′-AACTAATGTATTAGCAGAACT-3′)—are capable of

detecting both the ASFV genotype I and II strains, yielding amplicon lengths of 241 bp for genotype I and 331 bp for genotype II.

Development and optimization of SYBR Green I fluorescent PCR

Genomic DNA was extracted *via* the TGuide S32 Magnetic Tissue DNA Kit (TIANGEN Biotech Co., Ltd., Beijing) according to the manufacturer's instructions. The SYBR Green I fluorescence PCR assay was conducted in a 20 μL reaction volume, consisting of 5 μL of template DNA, 10 μL of 2×SYBR Green Mix (Yeasen Biotechnology Co., Ltd., Shanghai), varying concentrations (0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45 or 0.50 μM) of forward and reverse primers, and nuclease-free water to a final volume of 20 μL. PCR was carried out via the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., China) under the following conditions: initial denaturation at 95°C for 3 min; 40 cycles of 95°C for 10 s; and annealing at various temperatures between 55°C and 65°C for 30 s. Fluorescence signals were recorded at the end of each extension step.

Standard curve generation for SYBR Green I fluorescent PCR

To generate plasmids containing the noncoding regions of the *MGF_505-9R* and *MGF_505-10R* genes from ASFV genotypes I and II, these regions were amplified and subsequently cloned and inserted into the pMD-18 T plasmid. The copy number was calculated via the formula (copy number = $[(6.02\times10^{23})\times([\text{ng}/\mu\text{L}]\times10^{-9})]/[\text{DNA length}\times660]$). The plasmid stock was adjusted to a concentration of 10^7 copies/μL and then subjected to a tenfold serial dilution with nuclease-free water to generate the standard curve.

Sensitivity and specificity of SYBR Green I fluorescent PCR

The sensitivity of SYBR Green I fluorescent PCR was tested by using tenfold diluted standard positive plasmids ranging from 10^7 to 10^{-1} copies/μL as templates. The threshold cycle (Ct) value of these standard dilutions was plotted against the log value of the copy number. The PCR products were visualized *via* 1.5% gel electrophoresis to verify the presence of the expected 241 bp bands and 331 bp bands. The specificity of SYBR Green I fluorescent PCR was determined by testing DNA samples of ASFV genotypes I and II (during epidemiological surveillance) and the cDNA/DNA of other porcine viruses, including PRV, PRRSV, CSFV, PCV2, PCV3, PPV, PEDV and GETV. Genomic DNA and RNA were extracted via TGuide S32 Magnetic Tissue DNA Kit (TIANGEN Biotech Co., Ltd., Beijing). First-strand cDNA was synthesized from the extracted viral RNA via a PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). The sensitivity of SYBR Green I fluorescent PCR was evaluated via a tenfold serial dilution of standard positive plasmid DNA, ranging from 10^7 to 10^{-1} copies/μL as templates. The threshold cycle (Ct) values for these dilutions were plotted against the logarithm of the copy numbers. The PCR products were analyzed *via* 1.5% agarose gel electrophoresis to confirm the presence of the expected 241 bp and 331 bp bands. The specificity of the assay was assessed by testing DNA samples from ASFV genotypes I and II (collected during epidemiological surveillance) and cDNA/DNA from other porcine viruses, including PRV, PRRSV, CSFV, PCV2, PCV3, PPV, PEDV and GETV. Genomic DNA and RNA were extracted *via* the TGuide S32 Magnetic Tissue DNA Kit (TIANGEN Biotech Co., Ltd., Beijing), and first-strand cDNA was synthesized from the viral RNA *via* the PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China).

Determination of the minimum detection limit for viruses

The minimum detection limit of SYBR Green I fluorescent PCR was evaluated via ASFV DNA samples from epidemiological surveillance, as well as DNA extracted from cultured ASFV genotypes I and II, with concentrations of 100 TCID50/0.1 mL, 10 TCID50/0.1 mL, and 1 TCID50/0.1 mL. Genomic DNA was extracted via the TGuide S32 Magnetic Tissue DNA Kit (TIANGEN Biotech Co., Ltd., Beijing).

Repeatability and reproducibility of the SYBR Green I fluorescent PCR

Repeatability (intra-assay precision) and reproducibility (interassay precision) were assessed via positive DNA samples from ASFV genotypes I and II. Intra-assay variability was evaluated by detecting each DNA sample in triplicate *via* the established SYBR Green I fluorescent PCR in a single run. For interassay variability, each DNA dilution was tested across three independent experiments. The coefficients of variation of the Ct values were calculated from the intra- and interassay results.

Application of SYBR Green I fluorescent PCR

This study utilized 100 ASFV-positive DNA samples collected from April 2020 to December 2022 to distinguish ASFV genotypes through established SYBR Green I fluorescent PCR. These results were compared with those of the qPCR method described by WOAH (King et al., 2003) and the Chinese National Standard (CNS) for *Diagnostic Techniques for African Swine Fever* (GB/T 18648–2020). Positive PCR products were visualized via 1.5% gel electrophoresis to differentiate genotypes I and II, and the results were verified *via* the ASFV *B646L* gene sequencing method. The amplification of the *B646L* genes for sequencing followed the protocol of Bastos (Bastos et al. 2003).

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Authors' contributions
All the authors have read and approved the final version of the manuscript. Designed the experiments: YXX, HQG and LWT. Performed the experiments: YXX, WHW, LDF and CXY. Analyzed the data: YXX, WHW, XQ and ZCJ. Provided reagents and material: XFQ, KXG and HQG. Wrote the paper: YXX. Proofed the manuscript: LWT and HQG.

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Availability of data and materials
Data will be shared upon request by the readers.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors of this article have agreed to publish this article in *Animal Diseases*.

Competing interests
The author declares that he/she has no competing interests. Author Wentao Li was not involved in the journal's review or decisions related to this manuscript.

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