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Research article

Enhanced detection of African swine fever virus in samples with low viral load using digital PCR technology

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ABSTRACT

Detection of low viral load samples has long been a challenge for African swine fever (ASF) prevention and control. This study aimed to compare the detection efficacy of droplet digital PCR (ddPCR) and quantitative PCR(qPCR) for African swine fever virus (ASFV) at different viral loads, with a focus on assessing the accuracy of ddPCR in detecting low viral load samples. The results revealed that ddPCR had a detection limit of 1.97 (95% CI 1.48 – 4.12) copies/reaction and was 18.99 times more sensitive than qPCR (detection limit: 37.42, 95% CI 29.56 – 69.87 copies/reaction). In the quantification of high, medium, and low viral load samples, ddPCR showed superior stability with lower intra- (2.06% – 7.58%) and inter-assay (3.83% – 7.50%) coefficients of variation than those of qPCR (intra-assay: 8.08%–29.86%; inter-assay: 9.27%–34.58%). Bland-Altman analysis indicated acceptable consistency between ddPCR and qPCR for high and medium viral load samples; however, discrepancies were observed for low viral load samples, where two samples (2/24, 8.33%) exhibited deviations beyond the acceptable range (−46.18 copies/reaction). Moreover, ddPCR demonstrated better performance in detecting ASFV in clinical samples from asymptomatic pigs and environmental samples, with qPCR showing false negative rates of 7.69% (2/26) and 27.27% (12/44), respectively. McNemar analysis revealed significant differences between the two methods ($P = 0.000$) for samples with a viral load <100 copies/reaction. The results of this study demonstrate that ddPCR has better detection limits and adaptability than qPCR, allowing for a more accurate detection of ASFV in early-stage infections and low-concentration environmental samples. These findings highlight the potential of ddPCR in the prevention and control of ASF.

1. Introduction

ASF is a severe acute disease caused by the ASFV that often leads to significant mortality in domestic and wild pigs, resulting in substantial economic losses to the swine industry in affected countries [1]. Recognising its enormous impact, the World Organization for Animal Health (WOAH) has classified ASF as a notifiable disease and an important transboundary animal disease. This disease was

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initially reported in Kenya and Africa in 1914 [2], and spread to Europe in 1957 [3]. In 2018, it spread to Asia, with China, Vietnam, Cambodia, Laos, and North Korea (the Democratic People's Republic of Korea) as the major affected regions [1]. China, the world's largest producer and consumer of pork, first detected ASFV in Liaoning Province in 2018, and ASFV has since become prevalent [4]. This has severely affected China's swine industry, causing significant global economic losses.

ASFV is a large, enveloped, double-stranded DNA virus belonging to the *Asfivirus* genus of the *Asfarviridae* family. It is the only known DNA virus that can replicate in arthropods and infect vertebrates [5]. ASFV has a complex structure and genome composition that allows it to remain stable in the environment [6,7]. Infected pigs excrete the virus through blood, faeces, saliva, and aerosols. Healthy pigs can contract ASF by coming into contact with ASFV in the environment, leading to its rapid spread within the pig population [8]. After infection with ASFV, pigs typically exhibit early symptoms such as high fever and anorexia, while later stages of infection can lead to systemic organ haemorrhage and acute mortality. Highly virulent ASFV strains can result in a mortality rate as high as 100% [9].

Effective vaccines are considered to be the most efficient approaches for managing animal diseases. However, the development of effective ASF vaccines remains highly challenging. In the case of ASFV, inactivated, DNA, and subunit vaccines have been unable to effectively protect pig populations. Additionally, attenuated ASFV vaccines with artificial deletions of specific genes face challenges such as the risk of virulence reversion and inadequate cross-protection against various viral strains. As a result, the development of an effective ASF vaccine remains a formidable task [10,11]. Currently, timely detection and culling of infected pigs continue to be the primary means of controlling ASF in farms [4]. Therefore, ASFV detection methods are of paramount importance for ASF prevention and control. qPCR is considered the gold standard for detecting ASFV and is widely used in clinical sample testing. However, owing to inherent limitations, qPCR may yield false negative results when detecting samples with low viral loads, leading to failure of promptly interrupting the transmission source, and resulting in further dissemination of ASFV. However, ddPCR, a third-generation PCR technology, has several advantages over qPCR, including absolute quantification unaffected by amplification efficiency, reduced susceptibility to sample inhibition, and improved performance in detecting samples with low copy numbers [12,13]. It has already found application in the detection of animal diseases. Nevertheless, there have been reports demonstrating significant discrepancies between ddPCR and qPCR results in clinical sample testing [14,15]. Therefore, whether ddPCR and qPCR differ in the clinical detection of ASFV remains uncertain. Are ddPCR and qPCR results consistent for clinical samples with different viral loads? To the best of our knowledge, this question has not been addressed in the literature; however it holds tremendous practical importance in ASFV prevention and control. Consequently, in this study, we conducted a comprehensive comparison of the detection efficacies of ddPCR and qPCR in clinical samples to provide novel insights for veterinary professionals in their efforts to control ASF.

2. Materials and methods

2.1. Plasmid and viral genome

A standard plasmid containing the reference *ASFV B646L* gene sequence (NCBI: MN172368) was synthesized and cloned into avector by Shanghai Bioengineering Co., Ltd., The *HLJ/18-ASFV* genome was generously provided by the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

2.2. Primers and probes

The nucleotide sequences of the primers and probes used for detecting the *ASFV pB646L* gene were obtained using the qPCR method established by King et al. [16], as described in the "Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2022" published by the WOAH. The probe was labelled with FAM fluorescence at the 5′ end and BHQ1 quencher at the 3′ end. All primers and probes were synthesized by Sangon Biotech (Shanghai, China). Primer F sequence: 5′-CTG CTC ATG GTA TCA ATC TTA TCG A-3'; Primer R sequence: 5′-GAT ACC ACA AGA TCR GCC GT-3'. Fluorescence-labelled hydrolysis probe 5′-FAM- CCA CGG GAG GAA TAC CAA CCC

Table 1
Sample sources and test results.

Sample Type	Source	Count	qPCR$^+$	ddPCR$^+$	DSC	Consistency
blood sample	clinically symptomatic pigs	116	52	52	0	100 %
	clinically asymptomatic pigs	96	24	26	2	92.31 %
	Total	212	76	78	2	97.44 %
environmental sample	pig barn	108	30	35	5	85.71 %
	feed warehouse	18	0	0	0	100 %
	cafeteria	12	0	0	0	100 %
	staff dormitory	12	0	2	2	0
	livestock caretaker	30	0	2	2	0
	vehicle	18	2	5	3	40.00 %
	Total	198	32	44	12	72.73 %
–	Total (all sample)	410	108	122	14	88.52 %

qPCR$^+$: qPCR Positive Count; ddPCR$^+$: ddPCR Positive Count.
DSC: differentiated sample count.

AGT G-3′-BHQ1.

2.3. Sample collection

A total of 212 blood samples and 198 environmental samples were collected from four ASF-affected farms, as shown in Table 1. All samples were collected by professional veterinarians employed at the respective pig farms. The sampling protocol was supervised and approved by the Chongqing Academy of Animal Science Ethics Committee (file number: XKY - 20220306). Blood samples were obtained from live pigs, and prior to sampling, professional veterinarians completed a "Sample ID and Clinical Symptoms Reference Chart". Briefly, 2 mL of blood was collected via the anterior vena cava and stored in 5-mL sterile tubes containing EDTA as an anticoagulant. Among the 212 blood samples collected, 116 were from asymptomatic pigs, and 96 were from symptomatic pigs exhibiting clinical symptoms, including elevated body temperature, anorexia, sluggishness, and rapid breathing. Environmental samples were obtained from various locations, including pig pens, transport vehicles for feed delivery, feed storage areas, dormitories, dining areas, and farm personnel. A sterile moistened cotton swab was used to collect all environmental samples, with each sample placed in an individual sample collection bag and sealed. All samples were stored at 4 °C and transported to the laboratory for genomic extraction within 24 h.

2.4. DNA extraction

For blood samples, 200 μL of whole blood was aspirated for genomic extraction using the QIAampMinElute Virus Spin Kit (QIAGEN, Germany). For environmental samples, environmental swabs were cut using scissors and placed into 1.5 mL centrifuge tubes. Next, 500 μL of ultrapure water was added, and the tubes were vortexed for approximately 15 s to dissolve the collected particles into the liquid. Subsequently, the samples were centrifuged at 10,000×*g* for 10 min to remove the cotton swabs, which were then discarded. After another 10-s vortex, the liquid and sediment were thoroughly mixed, and 200 μL of the liquid was used for genomic extraction. Genomic extraction of all environmental samples was performed using the QIAamp PowerFecal DNA Kit (QIAGEN, Germany). All genomic extracts were stored at −80 °C upon completion.

2.5. Reaction system and amplification conditions

ddPCR was performed according to the protocol recommended by Bio-Rad Corporation. Each reaction system had a volume of 20 μL, comprising 10 μL of 2 × ddPCR master mix (Bio-Rad), 0.5 μL each of forward and reverse primer (10 nmol/μL), 0.5 μL of probe (10 nmol/μL), 2 μL of the reaction template, and 5 μL of nuclease-free water. The prepared 20 μL reaction mixture was transferred to DG8™ Cartridges, and using the QX-200 Droplet Generator (Bio-Rad), it was automatically emulsified with 70 μL of droplet generation oil. Next, 40 μL of the emulsion was transferred to a 96-well reaction plate, heated, and sealed at 180 °C for 5 s, and then subjected to PCR.

The specific PCR program was as follows: pre-denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 2 min, and a final enzyme deactivation cycle at 98 °C for 10 min. After amplification, the reaction plate was placed in a QX-200 Droplet Reader (Bio-Rad, USA) for data analysis.

The qPCR was performed using a LightCycler96 instrument (ROCHE, Switzerland). The reaction system was consistent with ddPCR using the QIAGEN 2 × qPCR master mix. Reaction conditions were as described by King et al. [16].

2.6. Evaluation of the linear relationship for ddPCR and qPCR

The plasmid containing the target fragment was digested into linear fragments by *Hin*dIII. The concentration of digested DNA was measured using a NanoDrop One microvolume UV–Vis spectrophotometre (Thermo Fisher Scientific, USA) for three replicates. Subsequently, 10-fold serial dilutions of the standard linear fragment were prepared as templates to evaluate the linearity relationship (repeated three times for each template) and detection limit range for both ddPCR and qPCR assays. The standard plasmid content was calculated using the Avogadro constant formula as follows:

$$\text{copy number (copies / μL)} = \left(\text{A260 (ng / μL)} \times 10^{-9} \times 6.02 \times 10^{23}\right) / \left(\text{DNA length (bp)} \times 660\right)$$

2.7. Evaluation of the detection limits of ddPCR and qPCR

A series of two-fold serial dilutions of low-concentration standard linear plasmids were prepared as templates and diluted below the minimum detection range of both ddPCR and qPCR. Each concentration was tested in eight replicates for detection. Probit regression analysis was performed using the IBM SPSS software (IBM, USA) to determine the limit of detection (LoD) for both methods at a 95% reproducible probability.

2.8. ddPCR and qPCR reproducibility assessment

The ddPCR and qPCR methods were assessed for intra- and inter-assay repeatability and consistency of quantitative detection results using high, medium, low, and extremely low concentrations of linear plasmids as templates. The coefficients of variation (CV) were calculated to compare the repeatability of the two methods. Furthermore, Bland-Altman analysis was performed using GraphPad

Prism 8.0 (GraphPad Software,USA) to compare the consistency of the quantitative detection results for samples with different concentrations between the two detection methods.

2.9. Detection of clinical samples by ddPCR and qPCR

A total of 410 clinical samples were tested using ddPCR and qPCR, with genomic DNA from clinical samples, the HLJ/18 - ASFV genome (positive control), and water (negative control) serving as templates. The detection performances of ddPCR and qPCR in clinical samples were compared. In case of inconsistent results between the two methods for a particular sample, the sample was subjected to one repeat test to determine the final detection result. McNemar test (IBM SPSS, USA) was used to compare the consistency of the detection results between ddPCR and qPCR for clinical samples.

3. Results

3.1. Evaluation results of the linear relationship for ddPCR and qPCR

According to King et al. [16], the linear relationship between qPCR and ASFV detection can be evaluated using standard linear plasmids at concentrations ranging from 1.09×10^6 to 1.09×10^0copies/reaction. For ddPCR, the maximum detection limit for the target gene does not exceed 1.32×10^5 copies/reaction. Therefore, standard linear plasmids with concentrations ranging from 1.09×10^5 to 1.09×10^{-1} copies/reaction were used to assess the linear relationship of ddPCR for ASFV detection. The results demonstrated that both methods exhibited excellent linearity. For qPCR, the standard curve is automatically generated after amplification (Fig. 1CD), and the regression equation was $Cq = -3.5433\text{lgx} + 42.33$, with an efficiency (E) of 91.51% and an R^2 value of 0.99. The detection limit ranged from 1.09×10^2 to 1.09×10^1copies/reaction. For ddPCR, the amplification results were analysed using the QX Manager software (Bio-Rad, USA) (Fig. 1A and B), and the linear results were further analysed using SPSS software. The R^2 value was 1.0, and the detection limit ranged from 1.09×10^1 to 1.09×10^0copies/reaction.

3.2. Evaluationof the detection limits for ddPCR and qPCR

Standard linear plasmids with concentrations of 10.92, 5.46, 2.73, 1.36, 0.68, and 0.34 copies/reaction were used as templates for ddPCR detection. Standard linear plasmids with concentrations of 109.20, 54.60, 27.30, 13.65, and 6.83, and 3.43 copies/reaction were used as templates for qPCR detection. Eight replicates were performed foreach concentration and four replicates were performed for the blank controls. The results are presented in Tables 2 and 3. Probit regression analysis revealed that the LoD for ddPCR at a 95%

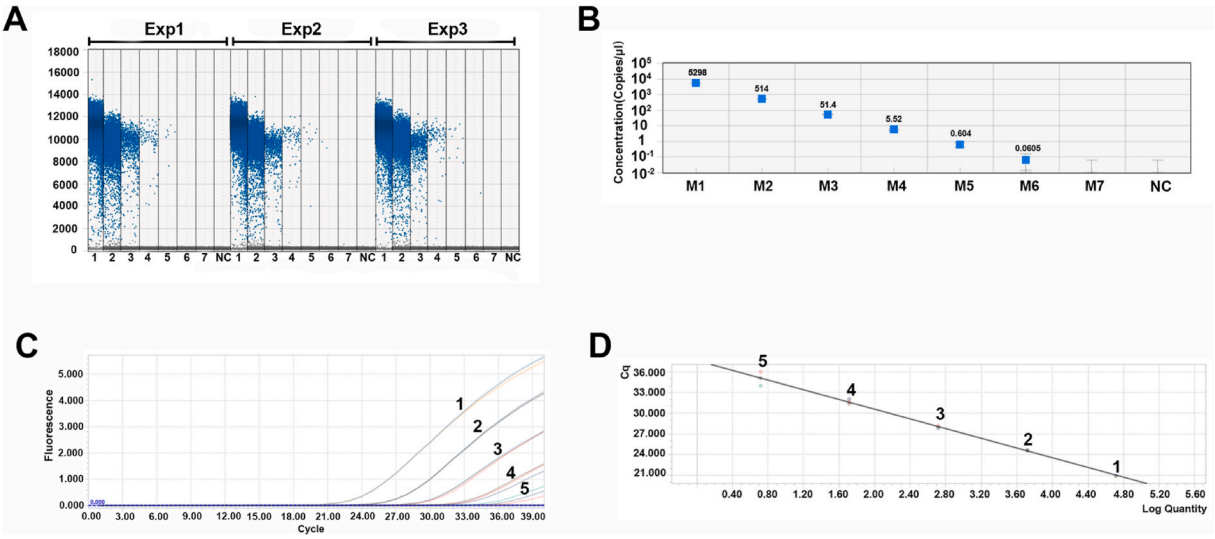

Fig. 1. Assessment of ddPCR and qPCR Linearity Relationship A. Numbers 1–7 represent linear plasmids with concentrations ranging from 1.09×10^5 to 1.09×10^{-1} copies/reaction, and NC represents the negative control. Exp 1–3 indicate three replicates for each experiment. Each point in the graph represents a droplet, with blue points representing positive droplets and gray points representing negative droplets; B. Numbers M1-M7 represent linear plasmids with concentrations ranging from 1.09×10^5 to 1.09×10^{-1} copies/reaction. Each blue square represents the average of three measurements of standard linear plasmids, and the numbers on the blue squares indicate the content of standard linear plasmids per microliter of reaction solution in the 20 μL reaction system; C. Numbers 1–5 represent linear plasmids with concentrations ranging from 1.09×10^6 to 1.09×10^2 copies/reaction. Each curve represents one reaction; D. Numbers 1–5 represent linear plasmids with concentrations ranging from 1.09×10^6 to 1.09×10^2 copies/reaction. Each point represents one reaction. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

reproducible probability was 1.97 copies/reaction (95% CI: 1.48 – 4.12), whereas the LoD for qPCR at a 95% reproducible probability was 37.42 copies/reaction (95% CI: 29.56 – 69.87). The LoD for ddPCR was 18.99 times lower than that for qPCR.

3.3. Evaluation results of the reproducibility for ddPCR and qPCR

ddPCR and qPCR were separately used to perform three intra- and inter-assay replicates each on standard linear plasmid samples with concentrations of 1.09×10^5 (high), 1.09×10^3 (medium), 1.09×10^2 (low), and 1.09×10^1 (extremely low) copies/reaction (see Table 4). The results showed that the CVs for both methods were inversely proportional to the template concentration. When the linear plasmid concentration ranged from 1.09×10^5 to 1.09×10^2copies/reaction, the intra-assay CVs for ddPCR and qPCR were 2.06% – 7.58% and 8.08% – 29.86%, respectively; whereas, the inter-assay CVs for ddPCR and qPCR were 3.83% – 7.50% and 9.27% – 34.58%, respectively. These findings demonstrate that ddPCR exhibits significantly better repeatability for sample quantification than qPCR. However, when detecting extremely low-concentration samples, ddPCRshowed relatively larger intra-(CV = 17.78%) and inter-assay differences (CV = 18.98%), whereas qPCR yielded negative results for extremely low-concentration samples.

A total of 24 standard linear plasmid samples with high, medium, and low concentrations were subjected to ddPCR and qPCR detection, and the results were compared using Bland-Altman analysis, as shown in Fig. 2 (A - C). At high and medium concentrations, the average bias between the two methods was −1066 copies/reaction and −39.58 copies/reaction, respectively. The bias for each sample fell within the acceptable range, indicating good agreement between the two methods for quantifying high- and medium-concentration viral templates. However, when samples with low viral concentrations were detected, the average bias was −46.18 copies/reaction, and there were two samples (2/24, 8.33%) with biases exceeding the acceptable range, indicating inconsistency between the two methods in quantifying low-concentration viral templates.

3.4. Application results in clinical samples

A total of 410 samples, including 212 blood samples (116 from clinically symptomatic pigs and 96 from asymptomatic pigs) and 198 environmental samples, were subjected to ddPCR and qPCR detection. The results indicate that ddPCR exhibits superior specificity and sensitivity in the detection of clinical samples (see Table 1, Table 5). There were 108 samples with positive results detected by both qPCR and ddPCR, 288 samples with negative results by both methods, and 14 samples with positive results by ddPCR but negative results by qPCR. Detailed analysis revealed consistent results between the two methods for blood samples from clinically symptomatic pigs, detecting 52 positive samples. However, discrepancies were observed in the detection results for asymptomatic pigs and environmental samples. The number of positive samples detected from the blood samples of asymptomatic pigs using ddPCR and qPCR were 26 and 24, respectively, whereas those from the environmental samples were 44 and 32, respectively. Two blood samples from asymptomatic pigs and 12 environmental samples yielded negative results with qPCR, but positive results with ddPCR.These 14 samples were retested and the results were consistent with the original findings. In this study, the false-negative rates of qPCR for asymptomatic pig and environmental samples were 7.69% (2/26) and 27.27% (12/44), respectively. Further analysis of the viral loads in the samples showed that ddPCR identified 71 samples with viral loads ≥100 copies/reaction and 339 samples with viral loads <100 copies/reaction (including 51 positive and 288 negative samples; Table 6). When the viral load was ≥100 copies/reaction, the qualitative detection results of both methods were completely consistent. However, when the viral load was <100 copies/reaction, McNemar test indicated a highly significant difference ($P = 0.000$) between the qualitative detection results of the two methods, indicating an inconsistency in the detection of low-concentration samples. These findings suggest that ddPCR is more suitable for detecting samples with low viral loads, enabling more accurate detection of ASFV in clinical samples.

4. Analysis and discussion

ASF is one of the most devastating infectious diseases in swine, and currently poses a severe threat to the global swine industry. Owing to the lack of effective vaccines and medications, the rapid detection and elimination of infected pigs remains the primary means of controlling ASF on farms. qPCR is currently the most widely used ASFV detection technology; qPCR diagnostic methods for ASFV have been established by Kinget al. [16], Fernández-Pineroet al. [17], and Qi et al. [18] and have been successfully applied in clinical testing. However, qPCR methods have certain limitations, such as imprecise detection of samples with low viral copy numbers, susceptibility to inhibition by various factors, and the need to prepare a standard curve [12,19]. ddPCR, a third-generation PCR

Table 2
Evaluation of the Limit of Detection in ddPCR Assay.

Initial Concentration (copies/reaction)	Count	ddPCR$^+$	ddPCR$^-$	Positive rate
10.92	8	8	0	100%
5.46	8	8	0	100%
2.73	8	8	0	100%
1.37	8	5	3	62.5%
0.68	8	3	5	37.5%
0.34	8	0	8	0
NC	4	0	4	0

ddPCR$^+$: ddPCR Positive Count; ddPCR$^-$: ddPCRNegativeCount; NC: negative control.

Table 3
Evaluation of the Limit of Detection in qPCRAssay.

Initial Concentration (copies/reaction)	Count	$qPCR^{+}$	$qPCR^{-}$	Positive rate
109.20	8	8	0	100%
54.60	8	8	0	100%
27.30	8	5	3	62.5%
13.65	8	1	7	12.5%
6.83	8	0	8	0
3.42	8	0	8	0
NC	4	0	4	0

$qPCR^{+}$: qPCR Positive Count; $qPCR^{-}$: qPCR Negative Count; NC: negative control.

Table 4
Repeatability and Reproducibility of ASFV Detection Using ddPCR and qPCR Assay.

Initial concentration (copies/reaction)	ddPCR				qPCR			
	Repeatability (intra-assay variation)		Reproducibility (inter-assay variation)		Reproducibility (intra-assay variation)		Reproducibility (inter-assay variation)	
	Mean ± SD (copies/reaction)	CV (%)	Mean (copies/reaction)	CV (%)	Mean (copies/reaction)	CV (%)	Mean (copies/reaction)	CV (%)
1.09×10^5	104800 ± 2457.64	2.35	108600 ± 4161.73	3.83	106888 ± 8643.91	8.08	106551 ± 9876.84	9.27
1.09×10^3	1167.33 ± 24.03	2.06	1150.33 ± 47.81	4.16	1004.16 ± 151.34	15.07	1161.21 ± 177.66	15.29
1.09×10^2	110.67 ± 8.39	7.58	107.67 ± 8.08	7.50	103.45 ± 30.70	29.68	97.74 ± 33.80	34.58
1.09×10^1	11.93 ± 2.12	17.78	12.8 ± 2.43	18.98	–	–	–	–

SD: standard deviation; CV: coefficient of variation.

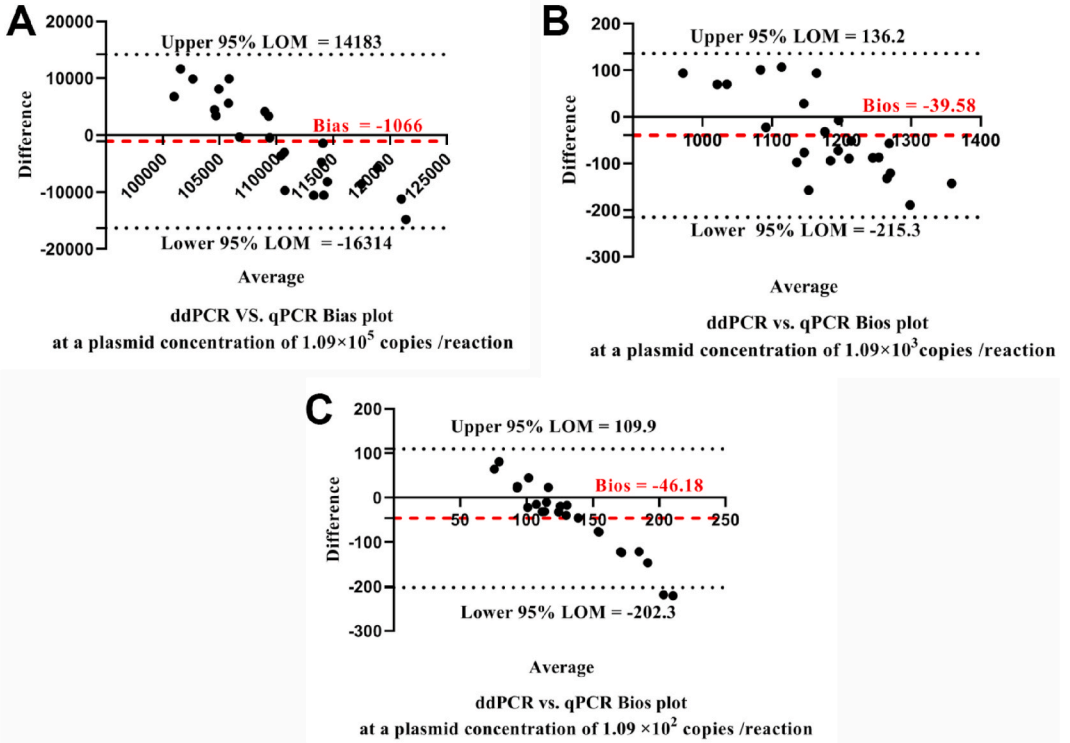

Fig. 2. The Bland-Altman bias plot was employed to evaluate the quantitative detection results of the two methods for different concentration samples The Upper 95% Limit of Agreement (Upper 95% LOM) and Lower 95% Limit of Agreement (Lower 95% LOM) were indicated in the graph, and the numerical values are expressed in units of copies/reaction.

Table 5
Clinical Specificity and Sensitivity Detection Results of PCR and ddPCR.

			Results of ddPCR		Total	Consistency of ddPCR and qPCR
			Positive	Negative		
Results of qPCR	**Positive**	blood sample	76	0	108	88.52% (108/122)
		environmental sample	32	0		
	Negative	blood sample	2	134	302	95.36% (288/302)
		environmental sample	12	154		
	Total		122	288	410	

NOTE: in the 410 clinical samples, qPCR detected 108 positive samples and 302 negative samples, while ddPCR detected 122 positive samples and 288 negative samples. The consistency of positive clinical samples between the two methods was 88.52% (108/122), indicating that all samples detected as positive by qPCR were also positive by ddPCR. For negative clinical samples, the consistency between the two methods was 95.36% (288/302), with 14 samples showing negative results in qPCR but positive results in ddPCR.

Table 6
Detection Results of ddPCR and qPCR Assays for **Low Viral Load Samples (<100 copies/reaction)**.

ddPCR	qPCR	Count
+	+	37
+	–	14
–	+	0
–	–	288

+: Positive;-: negative.

technology, eliminates these drawbacks. First, it employs the limiting dilution method to randomly distribute the samples into over 10,000 droplets, with each droplet equivalent to one PCR. This essentially transforms one PCR into >10,000 individual PCRs, theoretically achieving single-molecule amplification. Second, ddPCR measures fluorescence signals at the endpoint, independent of *Ct* values, thus effectively overcoming the influence of PCR inhibitors. Finally, ddPCR utilises a Poisson distribution to calculate the original concentration of samples, allowing direct determination of sample concentration without the need for standard curves and achieving true absolute quantification [20]. Recently, ddPCR has been used to detect ASFV. Wu et al. [18] developed a ddPCR detection method targeting the ASFV *K205R* gene, which exhibited a sensitivity 10 times higher than that of real-time PCR. Zhu et al. [21] established a ddPCR method capable of simultaneously detecting ASFV *B646L* and *EP402R* to distinguish between virulent and gene-deleted strains. However, these reports lack a comprehensive evaluation of the detection efficacy of ddPCR, leaving veterinary professionals uncertain when to employ ddPCR effectively, hindering its practical application in ASFV detection. In this study, using WOAH-recommended primers and probes, we conducted a detailed assessment of ddPCR and qPCR detection efficacy in samples with low viral content, laying the foundation for the widespread use of ddPCR in clinical detection.

The results of this study demonstrate that ddPCR exhibits significantly higher sensitivity than qPCR, with a detection limit of 1.97 (95% CI 1.48 – 4.12) copies/reaction, making it 18.99 times more sensitive than qPCR. Furthermore, ddPCR showed superior repeatability over qPCR, with both inter- and intra-assay CVs notably lower than those of qPCR. Bland-Altman analysis comparing the quantitative detection results of the two methods revealed that they were in agreement when testing high and medium viral concentration samples. However, ddPCR performed better for quantitative detection when testing low-concentration samples. In addition, we compared the detection efficacies of ddPCR and qPCR in clinical samples. The results show that both methods provided consistent detection results in pigs with clinical symptoms. However, qPCR yielded false-negative results for asymptomatic pigs and environmental samples with false-negative rates of 7.69% (2/26) and 27.27% (12/44), respectively. The false-negative rate in environmental samples was significantly higher than that in tissue samples, indicating that while a low viral load is the primary reason for false negatives in qPCR, the complex composition of environmental samples also has a substantial impact on the sensitivity of qPCR.Further analysis using the McNemar method indicated that when the viral load was< 100 copies/reaction, there was a significant difference in the qualitative detection results between the two methods ($P = 0.000$). Collectively, these findings suggest that qPCR cannot accurately detect samples with low viral loads, whereas ddPCR is more suitable for detecting samples with low viral content. These results emphasise the superiority of ddPCR for detecting low viral load samples and its potential as a valuable tool for the clinical detection of ASF.

The transmission of ASFV in pigs and its contamination in the environment are critical factors in the spread of ASF in pig farms. Despite the implementation of strict biosecurity measures in most pig farms, the efficacy of these measures needs to be accurately assessed, necessitating more precise detection methods. The results of this study indicate that qPCR is not suitable for monitoring samples with low viral loads, whereas ddPCR can effectively address this issue. While the price of ddPCR equipment and consumables remains considerably expensive, its exorbitant costs are beyond the means of most pig farms, making the comprehensive substitution of qPCR with ddPCR for farm testing impractical. Nonetheless, ddPCR exhibits exceptional performance in detecting samples with low viral loads. Therefore, we believe that ddPCR technology is advantageous for ASF prevention and control in the following three scenarios: 1. during the early stages of ASF outbreaks in pig farms, ddPCR is preferred for detection when testing asymptomatic pigs

and environmental samples; 2. in the event of an ASF outbreak in a pig farm, after disinfection of pig sheds, ddPCR can be used to detect ASFV in environmental samples to verify the effectiveness of the disinfection process; and 3. in pig farms with a history of ASF, ddPCR can be used to comprehensively test the environment before resuming pig rearing, ensuring that there is no residual ASFV contamination. The use of ddPCR in these scenarios could facilitate more effective ASF prevention and control strategies. It offers improved sensitivity and accuracy in detecting low viral load samples, thus serving as a valuable tool for confirming biosecurity measures and monitoring ASF outbreaks.

In conclusion, ddPCR is a specific, sensitive, and reliable method that can effectively detect samples with low viral loads. This serves as a valuable alternative to qPCR and can be widely promoted for ASF prevention and control.

Ethics approval and consent to participate

The experimental protocol and sampling scheme of this manuscript obtained supervision and approval from the Ethics Committee of Chongqing Livestock Science Academy (File Number: XKY-20220306).

Data availability statement

The corresponding author has provided all the data for this manuscript in the supplementary materials.

CRediT authorship contribution statement

R. Yang: Writing – original draft, Resources, Methodology, Data curation. **W.-G. Fu:** Writing – original draft, Resources, Investigation, Data curation. **J. Zhou:** Visualization, Methodology. **Y.-F. Zhang:** Resources, Investigation. **L. Yang:** Resources, Methodology, Investigation. **H.-B. Yang:** Investigation, Data curation. **L.-Z. Fu:** Writing – original draft, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28426.

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