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Development and validation of a droplet digital PCR assay for Nipah virus quantitation

Jiangbing Shuai[1], Kexin Chen[1,2], Xiao Han[1,4], Ruoxue Zeng[1], Houhui Song[2], Linglin Fu[3] and Xiaofeng Zhang[1*]

Abstract

Background Nipah virus (NiV) is a zoonotic pathogen that poses a significant threat because of its wide host range, multiple transmission modes, high transmissibility, and high mortality rates, affecting both human health and animal husbandry. In this study, we developed a one-step reverse transcription droplet digital PCR (RT-ddPCR) assay that targets the *N* gene of NiV.

Results Our RT-ddPCR assay exhibited remarkable sensitivity, with a lower limit of detection of 6.91 copies/reaction. Importantly, it displayed no cross-reactivity with the other 13 common viruses and consistently delivered reliable results with a coefficient of variation below 10% across different concentrations. To validate the effectiveness of our RT-ddPCR assay, we detected 75 NiV armored RNA virus samples, mimicking real-world conditions, and negative control samples, and the RT-ddPCR results perfectly matched the simulated results. Furthermore, compared with a standard quantitative real-time PCR (qPCR) assay, our RT-ddPCR assay demonstrated greater stability when handling complex matrices with low viral loads.

Conclusions These findings show that our NiV RT-ddPCR assay is exceptionally sensitive and provides a robust tool for quantitatively detecting NiV, particularly in stimulated field samples with low viral loads or complex matrices. This advancement has significant implications for early NiV monitoring, safeguarding human health and safety, and advancing animal husbandry practices.

Keywords Droplet digital PCR, Nipah virus, Quantitation assay, Stimulated field samples

Introduction

Nipah virus (NiV) is a widely prevalent zoonotic virus with high mortality rates,, that belongs to the genus *Henipavirus* of the *Paramyxoviridae* family [1]. It is a spherical enveloped, single-stranded, negative-sense RNA virus with a size of approximately 150 nm and a full genome length of approximately 18.2 kb [2]. NiV was first identified in pigs in Malaysia in 1998, and infections by this virus have been discovered in recent years [3, 4]. Flying foxes are considered natural hosts of NiV with spillover crossing the species barriers to susceptible hosts, including humans; pigs act as amplifying intermediate hosts [5, 6]. NiV epidemics broke out Bangladesh in 2001. In the same year, NiV infections were recorded in India.

*Correspondence:
Xiaofeng Zhang
zxf@zaiq.org.cn
[1]Zhejiang Academy of Science and Technology for Inspection and Quarantine, Hangzhou 310016, China
[2]College of Animal Science and Technology, Zhejiang Agriculture & Forestry University, Hangzhou 311300, China
[3]School of Food Science and Biotechnology, Zhejiang Gongshang University, Hangzhou 310018, China
[4]Zhejiang University Institute of Preventive Veterinary Medicine & Zhejiang Provincial Key Laboratory of Preventive Veterinary Medicine, Hangzhou, Zhejiang 310058, China

Studies conducted by Govindakarnavar Arunkumar et al. revealed that vertical bat-to-human infections and horizontal human-to-human transmission occurred [7]. In pigs infected with NiV, shortness of breath progresses to respiratory distress accompanied by involuntary coughing [8, 9]. The fatality rate of NiV ranges from 40 to 75%, depending on the epidemiological control measures and clinical management capabilities present in outbreak areas.

NiV has a wide host range, multiple routes of transmission, strong transmissibility and high mortality. In 2018, the World Health Organization placed Nipah virus disease (NVD) on a restricted list of diseases that pose a serious threat to public health [10]. To date, NVD epidemics have occurred in Southeast Asian and South Asian countries, including Malaysia, Singapore, Bangladesh, Philippines, and India. Notably, Bangladesh has witnessed the highest frequency of NVD outbreaks of any country [11]. In January 2023, Bangladesh experienced a new outbreak of NVD with the highest number of cases and fatalities recorded since 2015 [12]. The transmission of NiV occurs primarily through the consumption of contaminated food or contact with infected animals or bodily fluids. Risk factors include proximity, contact, feeding, virus carriers, or aerosols [13, 14], and ecological, environmental, and anthropogenic factors could play a significant role in influencing NiV outbreaks [15, 16]. Following the expansion of international trade and increased immigration and trade relationships between China and countries in Southeast Asia and South Asia, the threat of NiV to China's border regions has become more evident. During an outbreak, there is a risk of the virus being introduced to China, which could have profound implications for the country's livestock industry and pose significant threats to public health and safety if left unchecked. There are no licenced treatments for NiV, and management is limited to supportive care and symptomatic treatment [17]. Therefore, it is very important to perform early monitoring and prevention of NiV and expand the NiV detection assays for trace virus-contaminated samples.

NiV infection has symptoms that are similar to those of swine influenza virus (SIV), porcine reproductive and respiratory syndrome virus (PRRSV), pseudorabies virus (PRV), and it is difficult to diagnose clinically; laboratory testing is currently the most effective surveillance method [18]. The isolation and culture of the virus requires biosafety level four (BSL-4) laboratory conditions [19]. The detection of viral nucleic acids does not require live virus samples and can be performed in BSL-2 laboratories to assure the sample does not represent a threat. Detection of NiV by quantitative real-time PCR (qPCR) is the general approach for this virus. For the detection of some complex matrix samples or some samples with low viral loads at import and export, qPCR still has limitations. Droplet digital PCR (RT-ddPCR) is a third-generation PCR nucleic acid detection method that emerged after qPCR. Unlike traditional PCR reactions, the PCR reaction of digital PCR is carried out directly in the production droplets. The initial copy number of the target nucleic acid in the system is then calculated via statistical analysis of the number of positive droplets, and the virus content in the sample is directly quantified. It has the advantages of high sensitivity, strong anti-interference, and the ability to provide absolute quantification [20].

In this study, we developed an RT-ddPCR quantitative detection assay for NiV targeting the NiV *N* gene, thereby providing a new detection method for early identification and quantitative monitoring of NiV in samples with low viral loads and complex matrices.

Materials and methods

Virus and nucleic acid preparation

Adenovirus particles containing the full length of African swine fever virus (ASFV) *p72* gene were purchased from Sangon Biotech (Shanghai, China). Inactivated vaccines against classic swine fever virus (CSFV, C strain) and SIV were purchased from Wuhan Keqian Biology (Wuhan, China). The foot-and-mouth disease virus (FMDV) inactivated vaccine was obtained from Jinyu Biotechnology (Inner Mongolia, China). The PRRSV, PRV (Bartha strain), porcine epidemic diarrhoea virus (PEDV, CV777 strain), porcine delta coronavirus (PDCoV) and porcine circovirus 2 (PCV2, JH SRJ strain) were generously provided by Zhejiang University (Hangzhou, China). Porcine bocavirus (PBoV) and Japanese encephalitis virus (JEV) were isolated and preserved in our laboratory.

Nucleic acid extraction of all the viruses was performed via a the CqEx-DNA/RNA Virus (CDC) nucleic acid extraction kit (Tianlong Technology, Xi'an, China) according to the manufacturer's instructions, and the extracted viral DNA or RNA was stored at -20 ℃ for subsequent use.

Primers and probe

The gene sequences of the NiV strains published in GenBank were compared and analyzed, the conserved regions were selected, and the primers and probe were designed using Primer3plus according to the recommended conditions range of primers and probes applicable to the one-step RT-ddPCR probe method. The sequences were entered into NCBI (http://blast.ncbi.nlm.nih.gov/blast.cgi) for specific comparisons, perform sequence alignment with other paramyxoviruses to confirm the gene specificity of the selected gene sequence. The amplification primers and probe sequences were synthesised by Sangon Biotech (Shanghai, China) and are listed in Table 1.

Table 1 Sequences of the primers and probe

Primers and probe	Sequence (5′-3′)	Position
NiV-FP	CTAAAGGCAGAGCAGTAG	843–860
NiV-RP	GATACCTTGTCTCCAACC	933–950
NiV-Probe-FAM	FAM-TGGCAGGATTCTTCGCAACC ATCA-BHQ1	906–926

Standard plasmid construction

We used the NiV *N*gene sequence (GenBank: NC_002728.1) as a template and referred to the primer amplification fragment. The recombinant plasmid was constructed by Sangon Biotech (Shanghai, China) and the initial concentrations using a NanoDrop spectrophotometer (Thermo Fisher, Waltham, MA, USA). The concentrations was calculated as follows: plasmid copy number (copies/reaction) = (plasmid concentration × 10^{-9}×6.02×10^{23}) × System volume/(660 Dalton/bases × DNA length). This plasmid was serially diluted with using nuclease-free water according to predetermined concentration gradients (10^8 copies/reaction), was subsequently stored at -20 °C, and then was used in downstream analyses.

In vitro-transcribed RNA

Using linearised plasmid containing the target sequence, RNA was transcribed in vitro with the Riboprobe(r) System-SP6/T7 (Promega, USA) according to the manufacturer's instructions. The T7-transcribed positive control was digested with DNase and purified with the RNeasy Kit (Qiagen, USA). The stock solutions of the in vitro-transcribed RNA were stored at-70 °C, and the diluted working solutions were stored at -20 °C. For in vitro-transcribed RNA, carry out ten replicates at various doses; compute the mean and coefficient of variation (CV%); compare the outcomes with armored RNA.

NiV armored RNA virus preparation

The plasmid containing the complete gene sequence of the NiV *N*gene was inserted into the phage MS2-pET-28b vector via a Seamless Cloning Kit (Jinbaxter Biology, Beijing, China). The recombinant plasmid was then transformed into *E. coli* BL21DE3 competent cells, and the clones were verified by sequencing at Sangon Biotech (Shanghai, China). The single clone with correct sequencing was used for strain activation and expanded culture, the positive close was confirmed by sequencing. After 20 h of incubation with isopropyl-β-D-thiogalactoside (IPTG), the cells were disrupted and digested with RNase A and DNase I, and the product was purified to yield the NiV phage-armored RNA virus. The concentration was measured by standard, and the virus were stored at -20 ℃ for later use. The prepared NiV armored RNA virus was subjected to nucleic acid extraction, and the extracted product was amplified and sequencing confirm by 3 different companies (Sangon, Tsingke, HZYKang Biotech) for sequencing. The sequencing results were compared to ensure the accuracy armored RNA was correct.

Optimisation of RT-ddPCR

The RT-ddPCR assay was optimised in 20 μL using the One-step RT-ddPCR advanced kit for probe (Bio-Rad). The assay master mix contained 5 μL of Supermix, 2 μL reverse transcriptase, 1 μL 300 mM DTT, 2 μL RNA template or standard plasmid (10^3 copies/reaction), and the primers (final concentration at 100 nM to 1 100 nM) and probe (100 nM to 700 nM), according to the manufacturer's instructions. The mixture reaction was analysed in the QX 600™ Droplet Digital PCR system (Bio-Rad). The annealing temperature was optimised from 55℃ to 62℃. The RT-ddPCR results were analysed and optimised according to the degree of dispersion.

Standard curves

The 10^1-10^6 copies/reaction NiV armored RNA virus, which was diluted 10 times was used as a template to perform the RT-ddPCR reaction. Each concentration included 16 replicates. Based on the actual detection concentration, the standard curve was generated using the detection value for each concentration by GraphPad and compared the results with those obtained using the Nipah virus qPCR assay recommended by the World Organization for Animal Health (WOAH) (Manual of Terrestrial Animal Diagnostic Tests and Vaccine. 2021 Edition, Chap. 3.1.14). After determining the Ct values, we calculated the correlation coefficient R^2 of the two assays and evaluated the amplification efficiency (E) of the each system.

Assay specificity and sensitivity of the NiV RT-ddPCR

Since other paramyxoviruses typically require BSL4 laboratory conditions for experimentation or were difficult to obtain viral strains, we selected viruses that could potentially co-infect with NiV or present similar symptoms, and that can be handled within our lab's capabilities, for specificity testing of our method. Assay specificity was evaluated via RT-ddPCR performance using nucleic acids, ASFV, CSFV, PCV 2, PEDV, PDCoV, FMDV, PRRSV, PRV, SIV, PBoV, JEV, PPV, and ddH_2O as negative controls.

The assay sensitivity of this RT-ddPCR assay was estimated by NiV armored RNA viruses with theoretical concentrations of 10^3 copies/reaction, 10^2 copies/reaction, 10 copies/reaction, 7.5 copies/reaction, 5 copies/reaction, 2.5 copies/reaction, and 1 copy/reaction. Each concentration was performed for 30 replicates. The mean measure concentration, standard deviation (SD), and relative standard deviation (RSD) were calculated for

each dilution. Concurrently, qPCR was also run alongside according to standard operating procedure. The lowest concentration, 30 replicates can detect and the RSD≤25% was identified, as the assay's limit of quantification (LoQ) for NiV. To more accurately determine the limit of detection (LoD) of the RT-ddPCR system, the LoD within the 95% confidence interval (CI) was calculated via a 95% probit regression model to assess the sensitivity of this method.

Repeatability of the NiV RT-ddPCR

NiV armored RNA viruses (10^1-10^5 copies/reaction) were diluted for RT-ddPCR, including 16 replicates per dilution. The CV for each dilution to assess the intra-repeatability of the assay. Moreover, the above virues were tested four separate times in four replicates of each dilution, and the CVs of each dilution of these four independent batches were calculated to evaluate the inter-batch repeatability.

Detection of NiV simulated samples

Due to the high risk associated with testing for NiV, which requires BSL-4 laboratory conditions, and its classification as an exotic virus, it is challenging to find actual clinical samples in China. To validate the usability of our method, we utilized pseudo viruses to simulate potential testing scenarios.

A total of 75 porcine serum, tissue (brain tissue, spleen tissue and liver tissue) and pet food feed (pelleted) samples were verified to be free of NiV by standard methods, and all the samples were collected from pig farms or markets. The NiV armored RNA virus was separately spiked into three matrices: NiV-free sera, tissues, and feeds, to simulate samples of at various concentrations. The NiV armored RNA virus with an initial concentration of 1.19×10^6 copies/reaction was serially diluted 10 times with healthy sera to prepare serum samples containing different virus concentrations to simulate the detection of serum samples at various concentration gradients within the quantitative range. Simultaneously, the tissue and feed samples were incubated with the NiV armored RNA virus (1.19×10^5 to 1.19×10^1 copies/reaction, prepared in nuclease-free H_2O). Five grams of the simulated sample was thoroughly ground, mixed with 25 mL of 1 mol/L phosphate-buffered saline (PBS, pH 7.2), homogenised, and subjected to three freeze-thaw cycles. The mixture was then centrifuged at 10 000×*g* for 5 min, and the supernatant collected. 3 replicates were prepared for each concentration gradient of each matrix sample, totaling 45 positive samples. Additionally, we prepared 10 NiV-free samples as negative control for each matrix.

Nucleic acids were extracted with the CDC nucleic acid extraction and purification kit (Tianlong Technology, Xi'an, China) and ddPCR conducted using the conditions optimised earlier. Each sample was repeated three times and the mean, SD and RSD values calculated at each concentration.

Results

Optimisation of NiV RT-ddPCR

We used checkboard analyses to optimise primers/probe concentrations, respectively 100 nmol/L to 1 100 nmol/Land 100 nmol/L to 700 nmol/L. The width of the bands usually was related to the probe binding products, and the narrower the width is, the more stable the probe binding is, and the more stable the system reaction is. As shown in Fig. 1A and C, according to the larger the interval between the negative and positive areas is, and the fewer the intermediate dispersion scattering points are, the better the system's amplification efficiency and the environment of the reaction system and the fewer erro the accurate values are, the optimal concentrations of the primers and probe were 700 nM and 100 nM, respectively. Annealing temperature for RT-ddPCR was subsequently optimized from 55 °C to 63 °C, and the RT-ddPCR results revealed that the optimal annealing temperature was found to be 57 °C (Fig. 2A and C). Therefore, the optimal reaction system and procedure for the established RT-ddPCR assay were as follows: Supermix 5.0 μL, reverse transcriptase, 2.0 μL; 300 mM DTT, 1.0 μL; primers (700 nmol/L), 1.4 μL; probe (100 nmol/L), 0.2 μL; ddH_2O, 8.0 μL; and template 1.0 μL. The reaction conditions were as follows: 50 ℃ 20 min; 95 ℃ 10 min; 94 ℃ 30 s, 57 ℃ 1 min, 40 cycles; 98 ℃ 10 min; 4 ℃ ∞. The heating and cooling rates of all reactions were 2 °C/s.

Comparison of in vitro-transcribed RNA with NiV armored RNA virus

We conducted method validation experiments with reverse-transcribed RNA and compared the results with those from armored RNA virus. The results showed consistency between the armored RNA virus and in vitro-transcribed RNA. To more closely reflect real testing conditions, we chose to present the system validation results using armored RNA virus. The data for this part of the experiment can be found in Suppl. Table 1.

Standard curve

Using the established RT-ddPCR assay, we constructed a 10-fold dilution series of the NiV armored RNA virus and compared the results with those of qPCR. The standard curves revealed that the R^2 for RT-ddPCR was 0.9988, with an E of 104.81% (Fig. 3A), and the qPCR assay yielded an R^2 of 0.9975 with an E of 106.52% (Fig. 3B). The results suggest that both etc. exhibited the expected linear consistency and amplification efficiency.

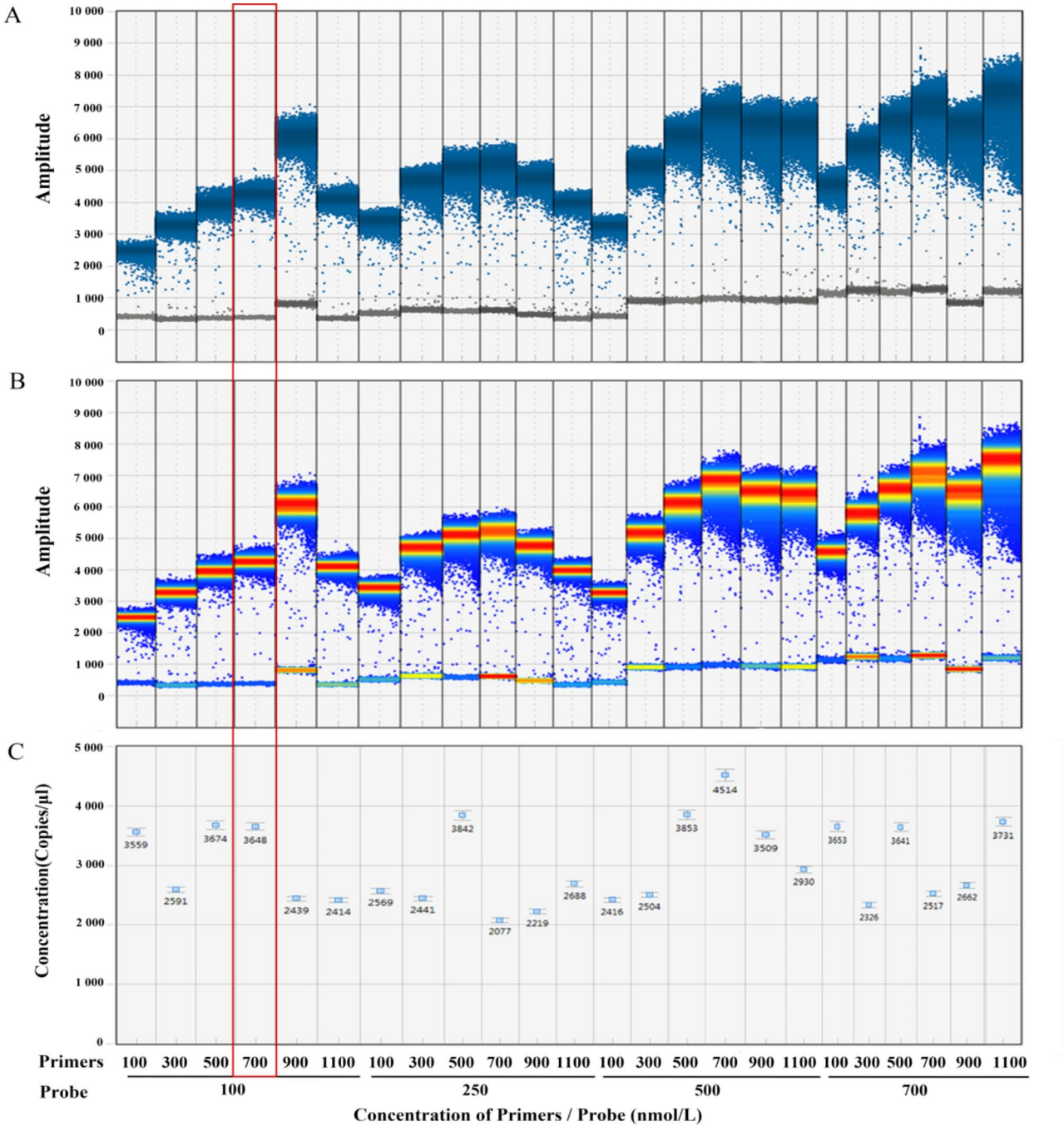

Fig. 1 Optimization of primers and probe concentration. Primers (100 nM to 1100 nM) and probe (100 nM to 700 nM) concentrations were optimized by the checkerboard method. The results were analyzed by positive (**A**) dots, (**B**) heat map, and (**C**) error of measured concentration

Analytical specificity and sensitivity of the NiV RT-ddPCR

The gene fragments selected for amplification exhibit differences from the gene sequences of other paramyxoviruses, providing specificity (Appendix 1). NiV and 13 common virus genomes were used to verify the analytical specificity. As shown in Fig. 4, only NiV RNA exhibited specific amplification, without nonspecific amplification for other viruses, indicating that this RT-ddPCR is highly specific.

As shown in Table 2, the sensitivity results showed that the RT-ddPCR could detect all 30 replicates at a concentration of 7.5 copies/reaction, and the RSD was <25%; therefor the LoQ of NiV RT-ddPCR was 7.5 copies/reaction. To further determine the LoD of this RT-ddPCR, a probit regression model with a repeatability probability of

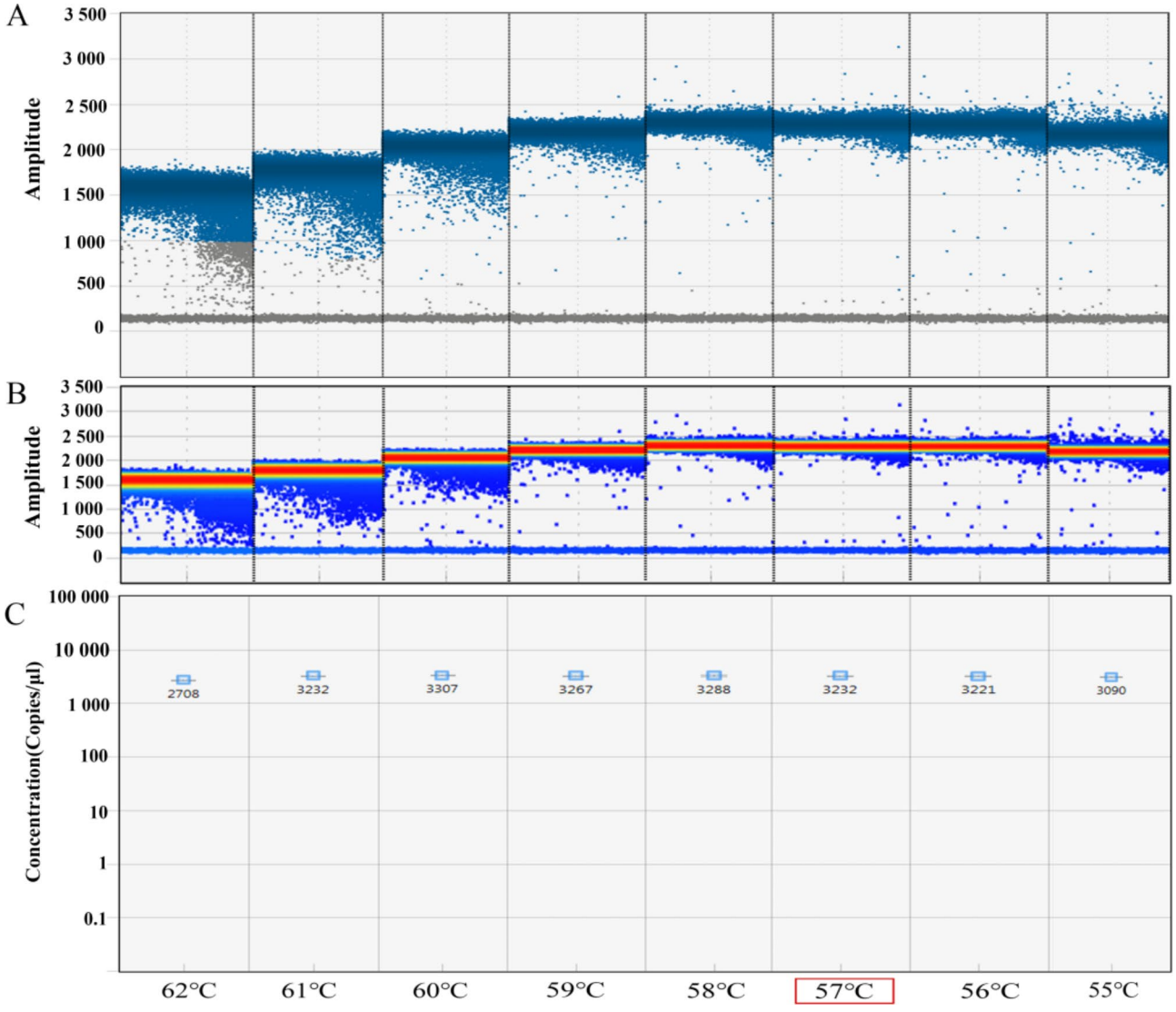

Fig. 2 Optimization of the annealing temperature. The annealing temperature was optimized from 66 ℃ to 55 ℃. The results were analyzed by positive (**A**) dots, (**B**) heat maps, and (**C**) error of measured concentration

95% was developed (Fig. 5), and the RT-ddPCR LoD was 5.76 copies/reaction (95%CI: 4.58–8.43 copies/reaction).

Repeatability

Using the established NiV RT-ddPCR system, the NiV armored RNA viruses (10^1-10^5 copies/reaction) were subjected to sixteen instances of intra-batch repeatability tests, in addition to four separate sessions of inter-batch repeatability experiments, each comprising four repetitions. The results indicated that the CV% values for intra-batch repeatability at the various concentrations were 0.35%, 0.34%, 0.84%, 2.67%, and 7.29%, all of which were below 10% (Fig. 6A). Moreover, the CV% values across the four batches at different concentrations were 0.15%, 0.06%, 0.48%, 1.00%, and 3.49%, all of which were less than 5%, indicating that there was no significant variation (Fig. 6B). Showing high repeatability.

Detection of the simulated samples

45 simulated positive samples and 30 NiV-free negative samples in different matrices were tested for NiV presence via RT-ddPCR assay, and the results were compared with those of qPCR (WOAH). The positive coincidence rates of RT-ddPCR and qPCR were 60% and 53.34%, respectively, and the sensitivity of RT-ddPCR was 1.12 times greater than that of the qPCR (Table 3). Moreover, the diagnostic sensitivities (DSes) were 100% and 88.89% (Table 3), respectively. RT-ddPCR identified all positive simulated samples across different matrices(Fig. 7A), and the RSD values of the detected concentrations between replicates ranged from 0.1 to 8%. Not all reactions were

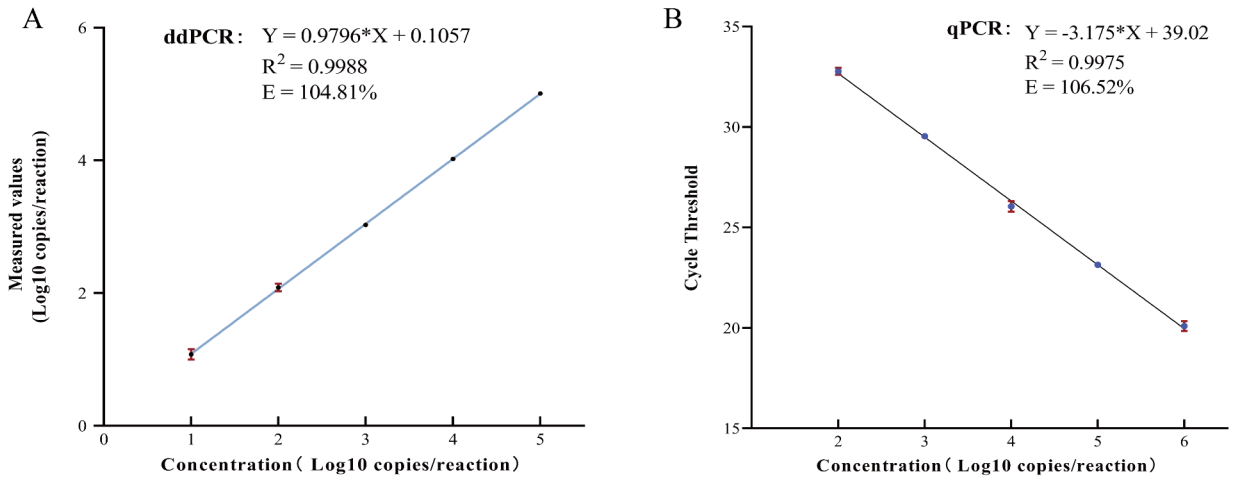

Fig. 3 Standard curve of NiV RT-ddPCR. The NiV RNA genome (duilted from 10^5 to 10^1) were used to create the standard curve of ddPCR (**A**). The qPCR method was used as a reference control (**B**). Each concentration was repeated for 16 times

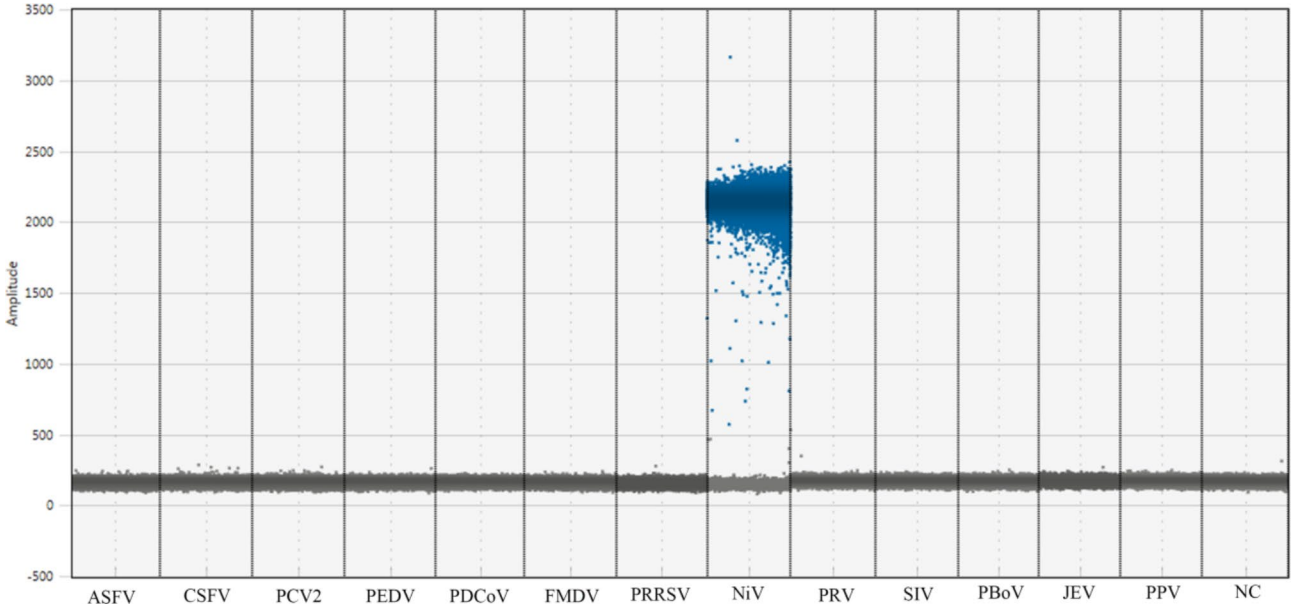

Fig. 4 Analytical specificity of RT-ddPCR for NiV. The nuclear acid of the ASFV, CSFV, PCV2, PEDV, PDCoV, FMDV, PRRSV, NiV, PRV, SIV, PBoV, JEV, and PPV were analyzed by ddPCR, ddH_2O was used as the negative control (NC)

Table 2 Analytical sensitivity of NiV RT-ddPCR

NiV armored RNA virus concentration (copies/reaction)	RT-ddPCR			qPCR		
	Mean values (copies/reaction) ± SD	Positive/ Reactions	RSD	Mean Ct values ± SD	Positive/ Reactions	RSD
1×10^3	1077.20 ± 44.44	30/30	0.56%	29.56 ± 0.07	30/30	0.25%
1×10^2	121.78 ± 13.23	30/30	2.20%	32.63 ± 0.26	30/30	0.78%
1×10^1	10.81 ± 1.20	30/30	4.60%	32.20 ± 10.89	27/30	33.83%
7.5	7.43 ± 0.85	30/30	11.38%	29.47 ± 14.83	17/30	50.30%
5	5.02 ± 1.63	27/30	32.47%	22.46 ± 18.37	25/30	81.78%
2.5	2.06 ± 1.48	21/30	71.71%	18.10 ± 19.36	21/30	106.96%
1	0.99 ± 1.20	17/30	120.47%	/	0/30	/

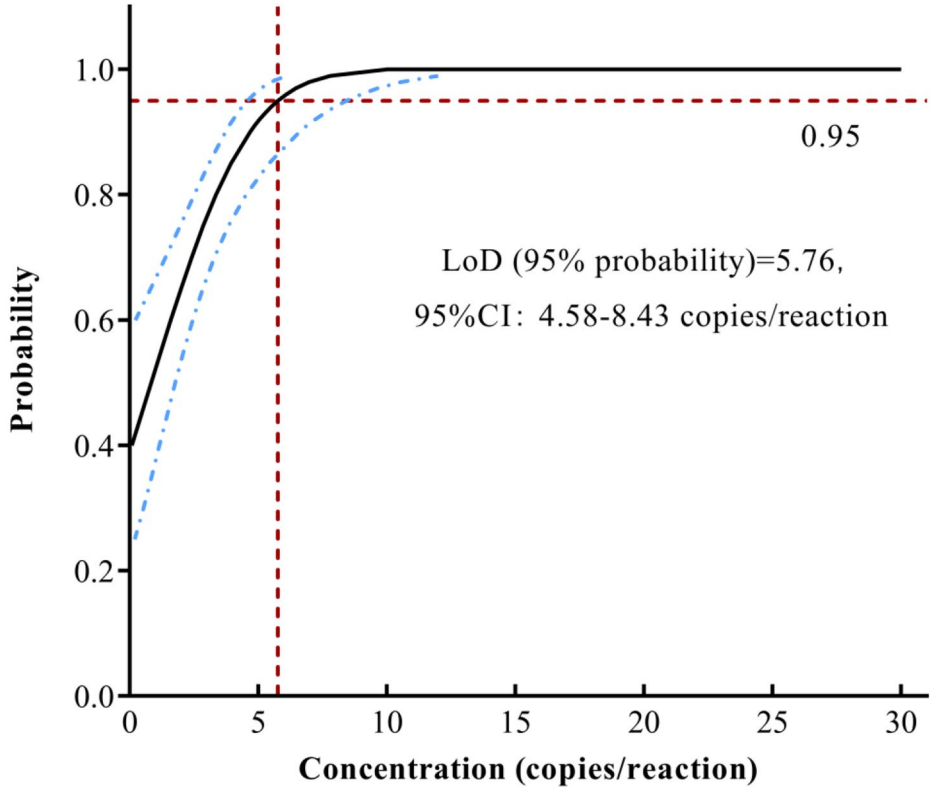

Fig. 5 Limit of detection of the RT-ddPCR. The limit of detection (LoD) of the ddPCR system within the 95% confidence interval (CI) was calculated using a 95% probit regression model, assessing the sensitivity of this method. Data are representative of 16 replicates for each concentration

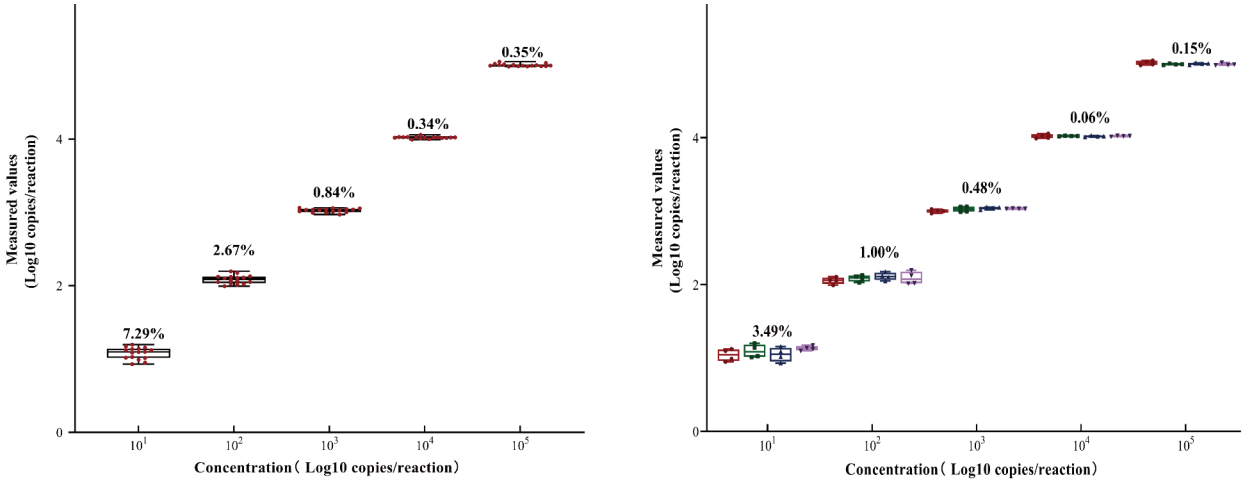

Fig. 6 Intra- and inter-assay RT-ddPCR repeatability. NiV armored RNA viruses (10^1-10^5 copies/reaction) were diluted for RT-ddPCR, with 16 replicates per dilution, to assess the intra-batch repeatability of the assay, and the NiV armored RNA viruses were tested four different times to evaluate the inter-batch repeatability. Intra-assay reproducibility (**A**) and inter-batch repeatability (**B**) of ddPCR

detected by qPCR at 1.19×10^1 copies/reaction (Fig. 7B). (Fig. 7B), indicating that the RT-ddPCR assay is more sensitive than qPCR. These findings indicate that RT-ddPCR can accurately detect low viral loads of NiV in samples with diverse matrices and offers good stability.

Analysis of the RT-ddPCR detected concentration values revealed some discrepancies in the actual detected NiV values across divergent matrices (Fig. 7C). Specifically, the actual detected values in the serum and tissue samples were essentially consistent with the theoretical viral concentrations added. For the feed samples,

Table 3 Diagnostic sensitivity (DSe) and specificity (DSp) of RT-ddPCR and qPCR

		RT-ddPCR			qPCR			Sensitivity (times)
		+	-	Total	+	-	Total	
Simulation situation	+	45	0	45	40	5	45	/
	-	0	30	30	0	30	30	/
	Total	45	30	75	40	35	75	/
Positive rate		60% (45/75)			53.34% (40/75)			1.12
DSe		100% (45/45)			88.89% (40/45)			/
DSp		100% (30/30)			100% (30/30)			/

Fig. 7 Comparison of RT-ddPCR and qPCR for the detection of simulated NiV positive samples. Serum, tissue and feed samples were used to prepare 45 simulated positive samples and 30 NiV-free negative samples. The NiV armored RNA virus (1.19×10^5 to 1.19×10^1 copies/reaction) was separately spiked into three matrices, namely NiV-free serums, tissues, and feeds, to simulate samples of various concentrations. Analysis results of 75 simulated actual samples by ddPCR (**A**), qPCR (**B**) and differential analysis via ddPCR (**C**). Each sample was repeated 3 times

the detection concentration was lower than the added viral concentration; at an added concentration ranging from 1.19×10^3 to 1.19×10^1 copies/reaction, the actual detected values of NiV in the feed samples were extremely significantly different from the serum sample detected values ($P<0.001$) and a significantly different from the tissue sample detected values ($P<0.01$), but all the replicates could still be stably detected. The mean detected concentrations were 0.88×10^3 copies/reaction (RSD 5.41%), 0.99×10^2 copies/reaction (RSD 5.61%) and 0.82×10^1 copies/reaction (RSD 7.11%) respectively, indicating that RT-ddPCR has good performance in the detection of low viral load and complex matrix samples.

Discussion

The NiV RNA genome encodes for six structural proteins: the fusion protein (F), attachment protein (G), matrix protein (M), nucleocapsid protein (N), large polymerase (L), and phosphoprotein (P) [2]. The *N*gene can be efficiently expressed and is highly conserved, making its use in the diagnosis and epidemiological investigation of NiV infection highly feasible. The terrestrial manual and border industry standards(WOAH) adopt the *N*gene as the target gene for nucleic acid testing methods, which indicate that the sequences of *N*gene is highly important in the development of NiV laboratory testing research.

Current detection technologies for viruses in the application of laboratory differential diagnosis methods mainly include virus isolation, serum neutralisation test, enzyme-linked immunosorbent assay (ELISA), loop-mediated isothermal amplification (LAMP), RT-PCR, and fluorescence quantitative PCR, etc. Due to the high biosafety level associated with NiV, molecular diagnostic techniques with high sensitivity and ease of execution have become essential for NiV detection and risk alerts [14]. In our study, we used the conserved NiV *N*gene sequence as the target gene to design a pair of specific primers and probe. By optimizing the system, the developed RT-ddPCR assay has the potential to show highly sensitive and specific characteristics, without cross-amplifying other common 13 viral nucleic acids. This assay showed strong and efficient amplification, with both intra-assay and inter-assay CV%s being less than 6%. The ddPCR showed highly sensitive, specific and repeatability characteristics. The RT-ddPCR method for NiV detection we developed was highly sensitive, with a LoD as low as 5.76 copies/reaction, ten times lower than the lower detection limit of qPCR, corroborating previous literature [21, 22]. Finally, to further verify the feasibility of the assay in samples, the established RT-ddPCR assay was used to detect NiV armored RNA virus simulated field samples. The virus concentration can be stably detected in the range of 10^1-10^5 copies/reaction with different concentration gradients, with 100% concordance with the simulated conditions and the detected values were basically consistent with the theoretical value. In the low-concentration detection of tissue and feed samples, the detection results of RT-ddPCR are more stable and

reliable than the qPCR results are and can directly reflect the known concentration of the virus. When the theoretical concentration is 1.19×10^1 copies/reaction, the qPCR method suffers from increased RSDs and false negatives. Especially in the analysis of feed samples, qPCR shows obvious instability. This may be due to inhibitory factors (protease, collagen, plant polysaccharides and so on) in the complex matrix that affect the amplification efficiency of qPCR. Analysis of the actual RT-ddPCR values across different matrix samples revealed significant variations, confirming that more complex matrices lead to greater discrepancies between actual and theoretical values and increase the difficulty of detection. However, the positive coincidence rate was 100%, which was was more 1.12 times greater than that of qPCR results. All positive samples were stably analysed through the RT-ddPCR assay, with the RSDs being less than 25%. The recovery rates for the serum and tissue samples were between 80 and 98%, whereas the feed samples had approximately 70% recovery, indicating high sensitivity of the assay. The RT-ddPCR mechanism effectively mitigates the influence of inhibitors and other impurities on amplification efficiency, increasing the stability and sensitivity of the analysis [23], suggesting that the established NiV RT-ddPCR has good application prospects for trace-level NiV sample detection.

Conclusions

Our study developed a NiV RT-ddPCR detection assay with high specificity and sensitivity, stability, and repeatability. This assay enables the absolute quantification of the NiV copy number in samples. In one hand the one-step RT-ddPCR detection system can simplify operational steps and reduce operational errors and consumption. In other hand, to some extent, it can also achieve costs reduction. Moreover, the NiV armored RNA virus prepared in our study boasts high concentration, good purity, and stable preservation advantages, making it suitable as a positive control for NiV detection. Moreover, this assay meets the requirements for both qualitative and quantitative detection of NiV in samples. It provides a new and reliable technical approach for early warning monitoring of NiV infection, differential diagnosis with other common viruses, study of its transmission patterns, and epidemiological risk assessment.

Abbreviations

ASFV	African swine fever virus
BSL-4	Biosafety level four
CI	Confidence interval
CSFV	Classical swine fever virus
CV	Coefficient of variation
ddPCR	Droplet digital polymerase chain reaction
DSe	Diagnostic sensitivity
DSp	Diagnostic specificity
ELISA	Enzyme-linked immunosorbent assay
FMDV	Foot-and-mouth disease virus
JEV	Japanese encephalitis virus
LAMP	Loop-mediated isothermal amplification
LoD	Limit of detection
LoQ	Limit of quantification
NiV	Nipah virus
nM	Nmol/L
NVD	Nipah virus disease
PBoV	Porcine bocavirus
PCV2	Porcine circovirus 2
PDCoV	Porcine delta coronavirus
PEDV	Porcine epidemic diarrhoea virus
PRRSV	Porcine reproductive and respiratory syndrome virus
PRV	Pseudorabies virus
qPCR	Real-time quantitative polymerase chain reaction
RSD	Relative standard deviation
SD	Standard deviation
SIV	Swine influenza virus

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-024-04245-y.

Supplementary Material 1

Supplementary Material 2

Author contributions

SJB: Methodology, Investigation, Formal analysis, Writing-review & editing, Funding acquisition, Supervision. CKX: Methodology, Investigation, Formal analysis, Writing-review & editing. HX: Conceptualization, Methodology, Investigation, Formal analysis, Writing-review & editing. ZRX: Conceptualization, Writing-review & editing. SHH: Writing-review & editing, Supervision. FLL: Writing-review & editing, Supervision. ZXF: Conceptualization, Methodology, Writing – review & editing, Funding acquisition, Supervision.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee of Zhejiang University (Permit Number: ZJU20230399) in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals, and all methods were performed in accordance with relevant guidelines and regulations. Informed consent was obtained from the owners for the inclusion of the animals in this study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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