

# Study on the Dynamic Proliferation of JEV in BHK-21 Cells

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## Keywords

Japanese encephalitis virus · Proliferation profile · Proliferation curve · Quantitative real-time PCR

## Abstract

**Introduction:** Epidemic Japanese encephalitis is one of the most important zoonotic diseases that cause central nervous system damage. The vaccination has become the most effective and economical measure for its control. Hence, real-time monitoring of Japanese encephalitis virus (JEV) proliferation is crucial to optimize virus inoculation, culturing conditions, and virus harvest time. **Methods:** The proliferation dynamics of JEV in BHK-21 cells was studied by combining the established quantitative PCR method with the conventional TCID<sub>50</sub> assay in this study. **Results:** The proliferation curve determined by the 2 methods has a definite parallel relationship, but the quantitative real-time PCR method (4 h) is faster and more sensitive than the TCID<sub>50</sub> method (3–4 days). The determination results of TCID<sub>50</sub> showed that the highest viral titer was  $10^{5.44}$  TCID<sub>50</sub>/0.1 mL and  $10^{4.86}$  TCID<sub>50</sub>/0.1 mL in cell suspension and culture supernate, respectively, while the virus RNA copies reached the peak at  $1.0 \times 10^{7.5}$  copies/μL and  $1.0 \times 10^{5.6}$  copies/μL in cell suspension and culture supernate, respectively. **Conclusion:** The comprehensive analysis showed that the best time for JEV proliferation in BHK-21 cell was 60 h post infection.

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## Introduction

Epidemic Japanese encephalitis (JE) is one of the most important zoonotic diseases caused by the transmission of Japanese encephalitis virus (JEV) through mosquitoes and causes central nervous system damage. Approximately 70,000 people are infected with JE every year worldwide, of which 10,000–15,000 die [1, 2]. Survivors of severe cases often leave severe sequelae. JEV is classified within the family Flaviviridae, genus *Flavivirus*. It is an enveloped virus containing a positive-sense single-stranded ribonucleic acid (RNA) genome approximately 11 kilobases in length. The genome encodes 3 structural proteins (capsid, pre-membrane or membrane, and envelope (E)), and 7 non-structural proteins [3–5].

There are no effective drugs for treating JE currently, and vaccination has become the most effective and economical measure for epidemic control. In the production of JEV, BHK-21 and Vero cells are mostly used for the proliferation of the virus [6–8]. As is known to all, the quality and effect of the vaccine are closely associated with the proliferation of the virus in the cell. Hence, real-time monitoring of JEV proliferation is crucial to optimize virus inoculation, culturing conditions, and virus harvest time.

TCID<sub>50</sub> detection is currently used to determine the quantitative assay of JEV [9–11]. However, due to the

**Table 1.** Primers and probes used in this study

Name	Sequences (5'–3')	Amplicon size, bp
JEV-F*	GCTGGATCC CTGAAATGTAGGCTGAAAAT	184
JEV-R*	CCAAGCTT TGAGGCTCGCAAGGAAACA	
JEV-R-F**	CAACCTATGGCATGTGTGA	103
JEV-R-R**	CCATCACTCCCAGAGTAG	
JEV-probe	FAM-CAATGACAACTGTTCCGTGACCA-BHQ	

JEV, Japanese encephalitis virus. Letters in italics indicate the restriction site in the primer. \* Primers used for the construction of the standard plasmids and conventional PCR. \*\* Primers used for qRT-PCR.

complexity of the method, the practical application is restricted by many conditions. Nowadays, qRT-PCR has been widely used for the qualitative and quantitative detection of the virus due to its specificity, sensitivity, and accuracy [12–18], whereas qRT-PCR detects only viral nucleic acids, including inactive viruses. Therefore, the TCID<sub>50</sub> assay is more valuable for evaluating the preparation of live virus particles, theoretically. However, for all this, if a reliable correlation is found between the above 2 methods, qRT-PCR may be possible to replace or partially replace the TCID<sub>50</sub>, which is more conducive to practical applications. In this study, a TaqMan probe-based qRT-PCR analysis method was developed to monitor the proliferation of JEV in BHK-21 cells real timely, and the results of this method were compared with the results of TCID<sub>50</sub> method. This study provides a new approach for studying the in vitro growth laws of JEV and virus detection during vaccine preparation.

## Materials and Methods

### Cells and Viruses

Baby hamster kidney-21 cells (American Type Culture Collection), JEV vaccine strain SA 14-14-2 (SINOLAND), PPV (porcine parvovirus), PCV2 (porcine circovirus type 2), CSFV (classical swine fever virus), PRV (pseudorabies virus), PRRSV (porcine reproductive and respiratory syndrome virus), and TGEV (transmissible gastroenteritis virus) were all kept in the College of Veterinary Medicine, Northwest A & F University. All experiments related to egg drop syndrome virus were done in a P2 biosafety laboratory and strictly carried out according to the Laboratory Biosafety Manual in the College of Veterinary Medicine, Northwest A & F University.

### Primers and Probes Design

Multiple sequence alignments of the E gene of JEV available in GenBank were performed with Clustal-W (DNASTar Inc., Madison, WI, USA), and conserved regions were identified. Primers and probe were designed to target conserved regions of E gene by Primer Premier 5.0 (Primer Biosoft International, Palo Alto, CA, USA). All primers and probe were synthesized by Sangon Biotech. Co., Ltd (Shanghai, China). All data related to the primers and probe are shown in Table 1. A BLAST search was also conducted to verify the specificities of the primers and probe.

### RNA Extraction and Conventional RT-PCR

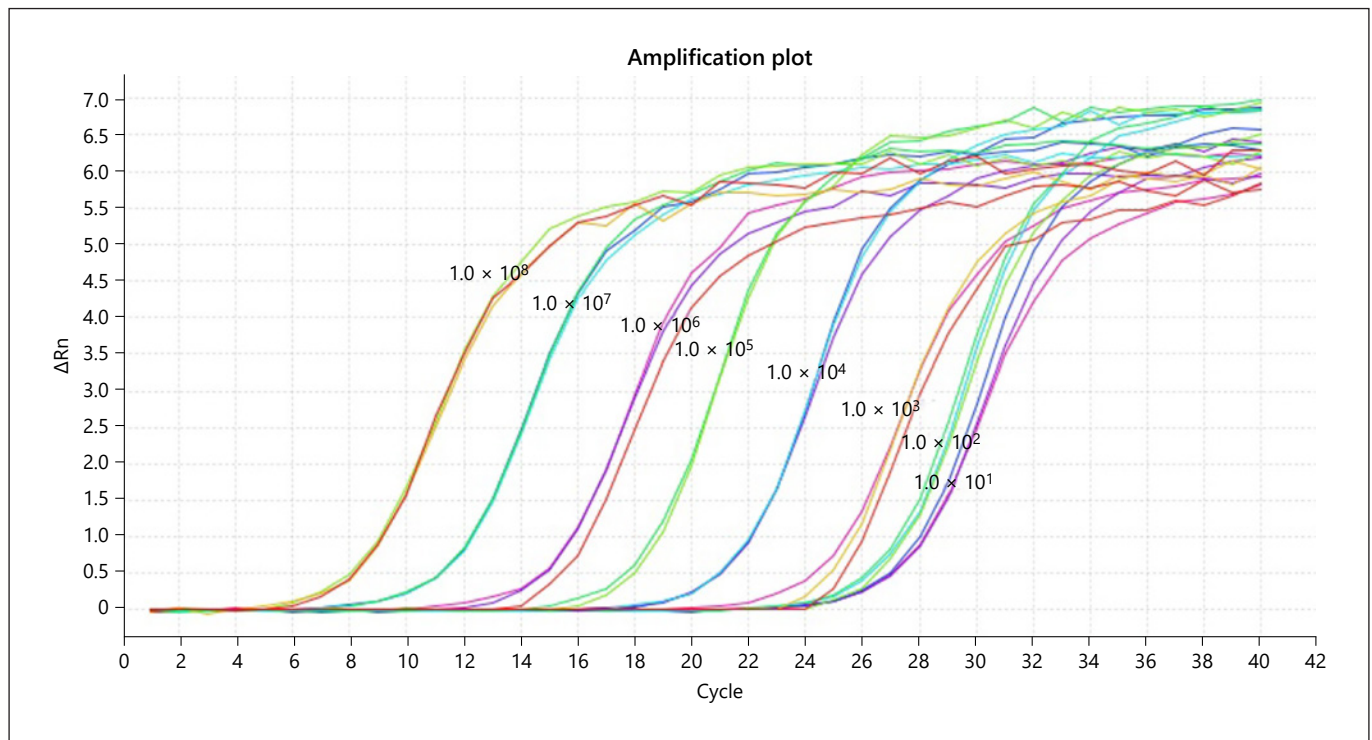
Viral total RNA of JEV vaccine strain SA 14-14-2 were extracted using Invitrogen™ TRIzol® Reagent (Life Technologies Corporation, Carlsbad, CA, USA) under the user guide. Viral RNA was reverse transcribed into cDNA. RT was carried out with PrimeScript™1st Strand cDNA Synthesis Kit (TaKaRa, Beijing, China) subsequently. The resulting cDNA was used as a template in the following PCR reactions.

### qRT-PCR Standard Plasmid Template Preparation

PCR assay was carried out in a 20-μL volume containing 2 μL of cDNA template, 2 μL primers (each 0.2 μM), 10 μL Premix Taq™ (TaKaRa, Beijing, China), and 6 μL RNase-free water. Amplification began with a 7-min incubation at 95°C, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 30 s, extension at 72°C for 30 s, and 1 cycle of final extension at 72°C for 10 min. After examination by 1% (w/v) agarose gel electrophoresis, the amplified fragments were digested with *Bam*HI and *Hind*III and inserted into pMD™19-T-Vector (TaKaRa, Beijing, China). The constructed plasmids were confirmed by PCR and DNA sequencing (Sangon Biotech, China). The extracted plasmids were determined by OD<sub>260</sub> optical absorption value in NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and diluted at 1.0 × 10<sup>10</sup> copies/μL ultimately.

### Fluorescent Quantitative PCR Assay

The final volume of TaqMan Probe-based fluorescent quantitative PCR is 20 μL, which contains 10 μL 2× FastStart Universal



**Fig. 1.** The sensitivity of qRT-PCR. qRT-PCR amplification plot of 10-fold serial dilutions of standard positive plasmids DNA (from  $1.0 \times 10^8$  copies/ $\mu\text{L}$  to  $1.0 \times 10^1$  copies/ $\mu\text{L}$ ). JEV, Japanese encephalitis virus.

Probe Master (Roche), 10  $\mu\text{M}$  upstream and downstream each primer 0.5  $\mu\text{L}$ , 10  $\mu\text{M}$  probe 1  $\mu\text{L}$ , templates 1  $\mu\text{L}$ , and RNase-free water 6  $\mu\text{L}$ . PCR cycling parameters were 94°C for 5 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

#### *Standard Curve, Sensitivity, Specificity, and Reproducibility of the Assay*

To evaluate the sensitivity, 10-folds serially diluted standard plasmid DNA (from  $1.0 \times 10^8$  copies/ $\mu\text{L}$  to  $1.0 \times 10^1$  copies/ $\mu\text{L}$ ) were used to perform the PCR. The results were compared with conventional RT-PCR. Meanwhile, to generate a standard curve of the assay, 10-folds serially diluted standard plasmid DNA (from  $1.0 \times 10^8$  copies/ $\mu\text{L}$  to  $1.0 \times 10^4$  copies/ $\mu\text{L}$ ) were used. The standard curve of JEV detection is established by taking the logarithm of the number of starting templates as the X-axis and the Ct value as the Y-axis as the regression curve. The specificity was determined using RNA/DNA extracted from PPV, PCV2, CSFV, PRV, PRRSV, and TGEV, which were used as templates in parallel to evaluate the specificity of the assay.

For verifying the reproducibility, 3 different dilutions of the standard plasmid DNA ( $1.0 \times 10^7$  copies/ $\mu\text{L}$ ,  $1.0 \times 10^6$  copies/ $\mu\text{L}$ , and  $1.0 \times 10^5$  copies/ $\mu\text{L}$ ) were tested for 10 replicates and statistically analyzed.

#### *Comparison of the Dynamic Proliferation of JEV in BHK-21 Cells*

##### *Cell Culture and Inoculation*

BHK-21 cells (inoculation concentration of  $5.0 \times 10^5$  cells/mL) were seeded in DMEM (Gibco, UK) supplemented with 8% fetal

bovine serum (FBS, Hyclone, Logan, UT, USA) and cultivated for 24 h at 37°C in a 5%  $\text{CO}_2$  incubator routinely. And then, the cells were infected with 0.1 mL JEV at a titer of  $10^2$  TCID<sub>50</sub> in T-25 flasks for 1 h at 37°C.

#### *Virus Collection*

After incubation, the cells were washed 3 times with PBS (pH 7.2) to remove unbound virus particles and then maintained in 5 mL mediums DMEM containing 2% FBS. At 0, 12, 24, 36, 48, 60, 72, 96, and 120 h post infection (hpi), 500  $\mu\text{L}$  culture supernate and cell suspension were collected from every 3 samples after frozen and thawed 3 times.

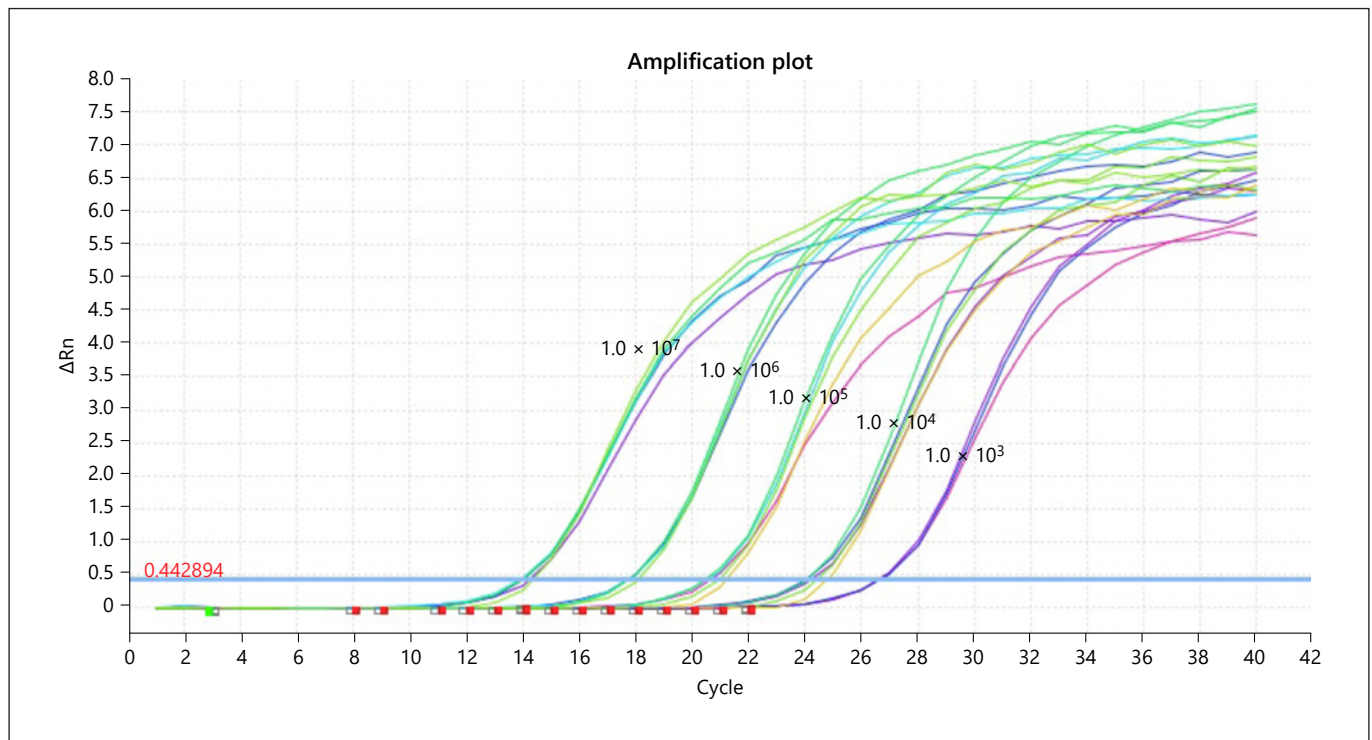
#### *Determination of Virus Titer*

qRT-PCR assay and TCID<sub>50</sub> determination were performed on samples taken at different time points. According to the average determination results, the proliferation curve of the virus was drawn, and comparative analysis was performed to calculate the correlation coefficient.

## **Results**

#### *Identification of Standard Positive Plasmids*

PCR products were analyzed by 1% agarose gel electrophoresis, and a specific fragment of approximately



**Fig. 2.** Reproducibility of qRT-PCR. Amplification plot of plasmid DNA (from  $1.0 \times 10^7$  copies/ $\mu\text{L}$  to  $1.0 \times 10^3$  copies/ $\mu\text{L}$ ).

1,500 bp was amplified, which was consistent with the expected results (online suppl. Fig. 1a; for all online suppl. material, see [www.karger.com/doi/10.1159/000510585](http://www.karger.com/doi/10.1159/000510585)). The constructed plasmid DNA was digested with *Bam*HI and *Hind*III, and the band of interest of about 1,500 bp and the vector of plasmid 2,700 bp were excised (online suppl. Fig. 1b). Simultaneous sequencing of the recombinant plasmids revealed that the JEV E genetic fragment had been cloned into the T-vector.

#### Standard Curve

The qRT-PCR amplification was performed on a 10-fold serial dilution of standard positive plasmids DNA ( $1.0 \times 10^8 \sim 1.0 \times 10^1$  copies/ $\mu\text{L}$ ) as a template. The amplification showed a good linear relationship, the standard curve equation is  $C_t = -3.336 \log(\text{copy nr}) + 44.18$ , the correlation coefficient is 0.997, and the amplification efficiency is 99.3% (online suppl. Fig. 2).

#### Sensitivity, Specificity, and Reproducibility

The analytical sensitivity of the assay was assessed through serial dilutions of standards of plasmids DNA (from  $1.0 \times 10^8$  copies/ $\mu\text{L}$  to  $1.0 \times 10^1$  copies/ $\mu\text{L}$ ). There

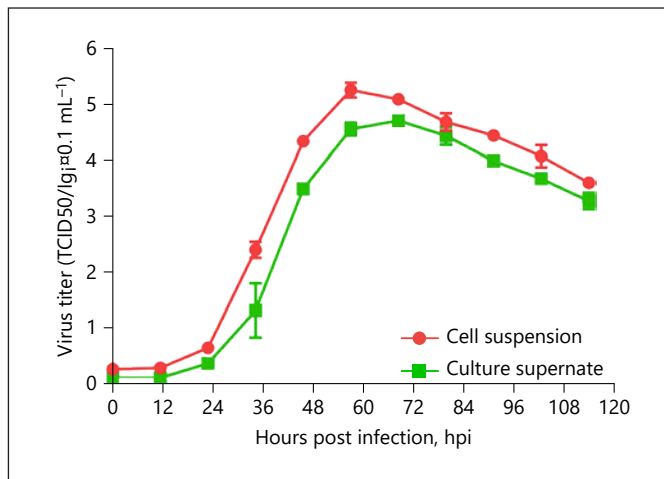
is still a fluorescence curve of  $1.0 \times 10^1$  copies/ $\mu\text{L}$ , indicating that the detection sensitivity of the method can reach  $1.0 \times 10^1$  copies/ $\mu\text{L}$ . The results of the sensitivity test are shown in Figure 1. Under the same reaction system and conditions for fluorescent quantitative PCR, only JEV-positive standards showed positive signals, and other virus samples controls were negative.

In this experiment, 5 concentrations ( $1.0 \times 10^7$ ,  $1.0 \times 10^6$ ,  $1.0 \times 10^5$ ,  $1.0 \times 10^4$  and  $1.0 \times 10^3$  copies/ $\mu\text{L}$ ,  $n = 3$ ) of plasmid DNA were selected as standard samples for repeated determination (Fig. 2), and the results were obtained by statistical analysis. As can be seen from Table 2, the final actual measured values of the standards at initial concentrations of  $1.0 \times 10^7$ ,  $1.0 \times 10^6$ ,  $1.0 \times 10^5$ ,  $1.0 \times 10^4$ , and  $1.0 \times 10^3$  copies/ $\mu\text{L}$  were  $0.988 \times 10^7$ ,  $0.933 \times 10^6$ ,  $0.975 \times 10^5$ ,  $0.930 \times 10^4$ , and  $0.898 \times 10^3$  copies/ $\mu\text{L}$ , respectively. The coefficients are 8.40, 5.79, 2.77, 3.33, and 3.34%, respectively. The determination result indicated that the method has good accuracy and reproducibility.

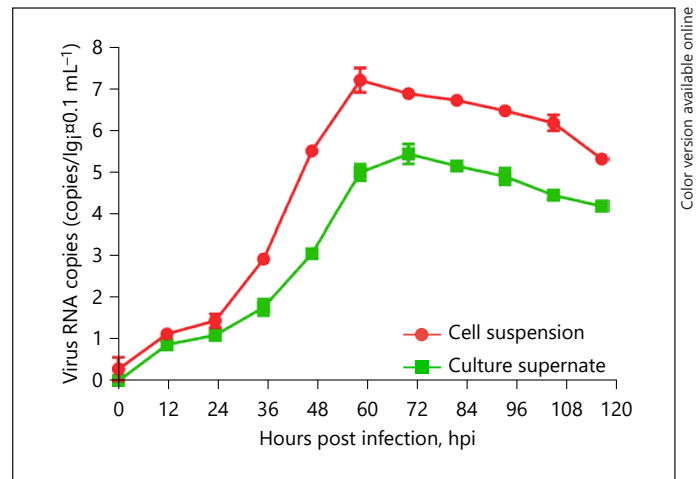
#### Proliferation Profile of JEV on BHK-21 Cells

BHK-21 cells were infected with 0.1 mL JEV at a titer of  $10^2$  TCID<sub>50</sub> and normal cells were used as control. The





**Fig. 3.** One-step proliferation curve of JEV on BHK-21 cells determined by TCID<sub>50</sub> assay. JEV, Japanese encephalitis virus; hpi, hours post infection.



**Fig. 4.** One-step proliferation curve of JEV in BHK-21 cells determined by qRT-PCR. JEV, Japanese encephalitis virus; hpi, hours post infection.

**Table 2.** Reproducibility and recovery of the measurement of replicate standards

Experiment	Quantity, copies/ $\mu$ L				
	$1.0 \times 10^7$	$1.0 \times 10^6$	$1.0 \times 10^5$	$1.0 \times 10^4$	$1.0 \times 10^3$
1	0.964	0.912	0.977	0.966	0.907
2	0.988	0.893	1.000	0.916	0.879
3	1.0137	0.995	0.947	0.909	0.922
Arithmetic mean	0.988	0.93	0.975	0.930	0.898
Standard deviation	0.083	0.054	0.027	0.031	0.030
Coefficient of variation, $n$ (%)	8.40	5.79	2.77	3.33	3.34

$$SD = \sqrt{\left[ (d_1 - x)^2 + (d_2 - x)^2 + \dots + (d_n - x)^2 \right] / (n - 1)};$$

CV =  $S/X \times 100\%$ .  $d_n$  is the measured value and  $x$  is the arithmetic mean value. CV, coefficient of variation; SD, standard deviation.

morphological changes of the cells at 0, 12, 24, 48, 60, 72, 84, 96, and 120 h after challenged were observed, and the cytopathic images were collected. Cytopathic effect (CPE) began to appear at 48 hpi, but it was not obvious. A small number of tiny plaques were scattered on monolayer cells; at 60 h, the CPE was obvious, the cells shrunk, some rounded and gathered, and the cell gap increased. Cells mostly became round and began to fall off at 120 h. Large amounts of cells shed and disintegrated in large areas moreover.

qRT-PCR assay and TCID<sub>50</sub> determination were performed on samples taken at different times after virus inoculation, and the virus proliferation curve was drawn (Fig. 3, 4). As shown in Figure 3, in both cell suspension

and culture supernatant, the virus proliferated slowly within 24 hpi. The virus proliferation rate increased rapidly subsequently within 24 hpi~60 hpi, with the viral load increasing logarithmically. The viral titer reached the highest level of  $10^{5.44}$  TCID<sub>50</sub>/0.1 mL in cell suspension at 60 hpi and  $10^{4.86}$  TCID<sub>50</sub>/0.1 mL in culture supernate at 72 hpi, respectively. The peak of viral titer is 12 h ahead in cell suspension than in culture supernate. After that, the content of JEV showed a decreasing trend up to 120 h, but the decline rate was slow. The dynamic curves of virus titer of JEV on BHK-21 cells were very similar in both cell suspension and culture supernate.

As shown in Figure 4, the amount of JEV presented a tendency of straight climb with the increase of hpi either

in cell suspension or in culture supernate. And the viral load in culture supernate is less than that in cell suspension continuously. The virus RNA copies reached the peak at  $1.0 \times 10^{7.5}$  copies/ $\mu\text{L}$  and  $1.0 \times 10^{5.6}$  copies/ $\mu\text{L}$  in cell suspension and culture supernate, respectively. The peak appeared in cell suspension is 12 h ahead than in culture supernate. The virus RNA copies reduced slowly after the limit value. Combining with Figures 3 and 4, it can be seen that the uptrend and the peak of the proliferation profile of JEV inoculated on BHK-21 cells measured by TCID<sub>50</sub> determination are consistent with which measured by qRT-PCR assay. Meanwhile, the correlation coefficient between the quantitative copy number and the TCID<sub>50</sub> data was  $r = 0.867$  and  $r = 0.888$  in the cell supernatant and cell suspension, respectively.

## Discussion

qRT-PCR could be used for quantitative detection of virus contained in a unit volume. However, it should be noted that the viral load measurement just reflected the amount of viral nucleic acid contained in the test and could not reflect the amount of live virus or intact virus particles. Virus titer is usually expressed as TCID<sub>50</sub>, which could reflect the number of viruses with biological activity or function. The primitive and classical method for the quantitative detection of JEV is to determine its TCID<sub>50</sub>. However, its practical application is subject to many conditions.

The standard curve of qRT-PCR assay explored in this test showed a good linear relationship at  $1.0 \times 10^8 \sim 1.0 \times 10^1$  copies/ $\mu\text{L}$ , with the correlation coefficient 0.997 and the amplification efficiency 99.3%, which provides basic data for precise analysis of virus replication in cells.

The proliferation dynamics of JEV on BHK-21 cells determined by qRT-PCR and TCID<sub>50</sub> assay showed that the number of virions or virus titer of the cell suspension was significantly higher than that of the culture supernatant and reached the peak at first. This is because some cells contracted and fell off and entered into the cell supernatant after 24 hpi. Both of them reached the peak at 60 hpi and then began to fall. However, the TCID<sub>50</sub> value decreased slightly after 60 hpi which may be because the value measured by the qRT-PCR contained 3 types of viral nucleic acids, including live virus (infectious) nucleic acids, inactivated viruses (non-infectious but antigenic and immunogenic) nucleic acids, and nucleic acids of virion that have not yet completed assembly. While the TCID<sub>50</sub> value only determined the titer of live virions.

The proliferation curve determined by the 2 methods has a certain parallel relationship, but the quantitative PCR (4 h) is faster and more sensitive than the TCID<sub>50</sub> assay (3~4 days), and it is more suitable for rapid determination of JEV proliferation titer real timely. The comprehensive analysis showed that the best time for receiving JEV proliferation on BHK-21 cells was at 60 hpi. Nevertheless, the peak of virus proliferation was closely related to various factors, the proliferation curve of different virus strains needed to be studied systematically. However, this study will provide technical support for the preparation of high-titer virus solution or production of JEV.

## Statement of Ethics

Not applicable. There are no human and animal subjects in this study.

## Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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## Author Contributions

Y.H.Z. planned and designed the research. F.L.Z. and J.L. finished the experiments. M.T. and G.X.X. performed the data analysis. J.Q.G. prepared the reagents used in the experiments. F.L.Z. wrote the manuscript. All authors have read and approved the manuscript.

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