ISSN 2706-6819 Vol.4, Issue 2: 35-41, DOI: 10.25236/IJFM.2022.040207

EV71 was Detected by Real-Time Fluorescence Quantitative RT-PCR Combined with Hand-Foot-Mouth Disease Rhesus Monkey Model

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Abstract: *In this study, a real-time fluorescence quantitative RT-PCR method was developed for the detection of enterovirus 71 (EV71) and the detection of EV71 viral load during the establishment of hand-foot-mouth disease (HFMD) model in rhesus monkeys. The model of hand-foot-mouth macaque was established by injecting newborn macaques infected with the virus venom, and the dynamic changes of virus content in blood samples from 0 to 14 days after infection were monitored by real-time quantitative RT-PCR. The linear range of the standard curve was between 10^3 and 10^8 copies/μL, and the PCR amplification rate was 99.37%. The results showed that the established method could detect a minimum of 10^2 copies of viral RNA, 100 times higher than the conventional RT-PCR method. It has the characteristics of strong specificity, high sensitivity and good stability. The results of blood sample analysis showed that the three groups of rhesus monkey models were successfully infected with EV71, and the copy number of virus proliferation was correlated with time and challenge dose. The results showed that real-time quantitative PCR could be used for quantitative detection of EV71 viral load.*

Keywords: *EV71, Real-Time Fluorescence Quantitative RT-PCR, Hand-Foot-Mouth Macaque Model, Viral Load*

1. Introduction

In recent years, the prevalence of EV71 in the Asia-Pacific region is on the rise. However, there is no effective vaccine and treatment for EV71 in clinical practice [1], and there is a lack of basic research on the pathogenic mechanism of EV71 infection and hand-foot-mouth disease. At present, neurotoxic animal models of EV71 are mainly established in rodents and non-primates, including lactating rat model and rhesus monkey model [2]. EV71 infection in animals is not only species dependent, but also age dependent. EV71 can only infect ICR mice aged from 1 to 7 days [3], which has certain limitations in establishing an infection model of EV71 in Suckling mice. EV71 injection through spinal canal or intravenous injection can paralyse rhesus monkeys and cause poliomyelitis in nervous system similar to human clinical symptoms [4]. It has been reported that newborn macaques can be infected with EV71 virus through respiratory tract and show herpetic lesions [5]. Therefore, rhesus macaques can be the best and ideal animal model for EV71 infection research mechanism, vaccine research and drug screening [6].

Current detection methods for EV71 include virus isolation and culture, serological detection, immunohistochemistry and RT-PCR detection [7], of which virus isolation and culture is the "gold standard" for EV71 detection [8]. However, the first three detection methods are complicated, long test period, low sensitivity and poor specificity, while RT-PCR is fast and efficient. But the content of virus nucleic acid cannot be determined, and easy contamination causes false positive. Therefore, the establishment of a rapid and effective detection method is of great importance to control the transmission of EV71 and the development of vaccines and drugs. This paper mainly studied the fluorescence quantitative real-time PCR detection method combined with the macaque model of HAND, foot and mouth disease. With its advantages of high sensitivity, rapid detection and high specificity, this method has been widely used in the analysis of gene expression level and quantitative and qualitative detection of pathogens [9].

Quantitative real-time PCR is a method that uses the fluorescent groups added to the PCR reaction system to bind specifically to PCR product, releases the fluorescence signal to monitor the whole PCR process in Real time, and then performs quantitative analysis of the unknown template through the

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standard curve. Compared with other detection methods, Quantitative Real-time PCR method has higher sensitivity than serological detection method. Quantitative Real-time PCR is designed for the specific sequence of the virus, with higher specificity; And the quantitative detection of viral load can truly reflect the copy number and replication of pathogens in cells. The PCR sensitivity was combined with the characteristics of fluorescent dyes to shorten the reaction time and simplify the operation. The completely closed tube operation does not require electrophoresis detection of PCR products, which can avoid false positive and environmental pollution caused by cross-contamination between samples [10]. Compared with conventional RT-PCR, it has advantages in sensitivity, specificity and speed.

Therefore, in this study, the recombinant plasmid standard was constructed by cloning the outer membrane protein VP1 of EV71, and fluorescence quantitative primers were designed in the VP1 fragment to construct the standard curve. The specificity, sensitivity and repeatability of the method were evaluated, and a real-time fluorescence quantitative RT-PCR method with high specificity and sensitivity was established. It was applied to detect EV71 viral load during the establishment of hand-foot-mouth disease model in rhesus monkeys.

2. Materials, Reagents and Instruments

2.1. Strains, Bacterial Strain and Experimental Animals

Enterovirus EV71 (Laboratory of Animal Disease Model, Sichuan Agricultural University), Coxsackie virus CA16 (Laboratory of Animal Disease Model, Sichuan Agricultural University); E.coli DH5α strain (Tiagen Biochemical Technology Company); Newborn juvenile macaques were used in the experiment (National experimental Macaque provenance Base).

2.2. The Main Reagent

TaKaRa MiniBEST Universal RNA Extraction Kit (Bao Biology (Dalian) Co., Ltd); TaKaRa Taq™ HS Perfect Mix (Bao Biology (Dalian) Co., Ltd); PrimeScript™ RT Master Mix (Perfect Real Time) (Bao Biology (Dalian) Co., Ltd); TIANgel Midi Purification Kit Generic Agarose Gel DNA Recovery Kit (Tiangen Biochemical Technology Co), pGM-Simple-T Fast Cloning Kit (Tiangen Biochemical Technology Co); SanPrep Column Plasmid DNA Small-amount Extraction Kit (Shanghai Biotechnology Biotechnology Company).

Primer synthesis was completed by Baosheng Biology (Dalian) Co., Ltd.

2.3. Key Instrument

Gel imaging system, MyCycler PCR instrument, PowerPac Universal electrophoresis instrument, Bio-RAD fluorescence quantitative PCR instrument (Bio-RAD); High-speed low temperature Centrifuge 5408, water bath shaker, pipette and constant temperature incubator (Eppendorf company); Electric thermostatic water bath DK-8D (Shanghai Jinghong Experimental Equipment Co., LTD.) etc.

3. Empirical Method

3.1. Establishment of a Quantitative Real-Time RT-PCR

3.1.1. Design of the Quantitative Fluorescence Primers

According to the nucleic acid sequences of all EV71 strains and VP1 region of each strain published in GenBank, DNAStar software was used to compare the sequences of different strains. According to the design principle of fluorescence quantitative PCR primers [11], Three pairs of fluorescent quantitative primers, EV71-F_1/R_1, EV71-F_2/R_2 and EV71-F_3/R_3, were designed with Primer5.0 software in the conserved region of VP1. The primer sequences were shown in Table 1.

PCR products amplified by three pairs of EV71 fluorescent quantitative primers were analyzed by 2% agarose gel electrophoresis as shown in Figure 1, indicating that all three pairs of primers could amplify the target band, but EV71-F_2/R_2 amplification products had non-specific primer dimers. The PCR products amplified by EV71-F_3/R_3 were less; The EV71-F_1/R_1 target band was bright and dimer-free, showing good specificity.

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Table 1: Primer design for RT-PCR.

Primers	Primer sequence	Position	Product Size
EV71-F_1	5′-CAAGGTTCCAGCACTCCAAGC-3′	2543-2563	150
EV71-R_1	5′-TCTCCAACTAATCCCGCCCTA-3′	2672-2692	
EV71-F_2	5′-AGTTGTGCAAGGATGCTAGT-3′	2371-2390	230
EV71-R_2	5′-CTCGTCACTAGCATTTGATG-3′	2581-2600	
EV71-F_3	5′-GCCCCTGAATGCGGCTAAT-3′	2732-2750	148
EV71-R_3	5′-ATTGTCACCATAAGCAGCCA-3′	2860-2879	

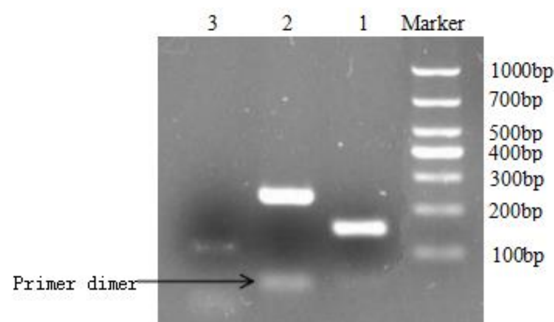

Figure 1: Selection of the best primers.

1~3: Amplifications of EV71-F1/R1, EV71-F2/R2, EV71-F3/R3 in turn

3.1.2. Identification of Quantitative Fluorescence Primers and Reaction Conditions

According to TaKaRa PrimeScript™ RT Master Mix (Perfect Real Time) kit recommendation system and conditions, the diluted plasmid was used as template for common PCR amplification with 3 pairs of fluorescent quantitative primers of EV71. According to the characteristics of the amplification bands and the presence of dimers, the primer concentration and annealing temperature were adjusted after confirming the availability of the designed primer and reaction system.

3.1.3. Optimization of the Annealing Temperature of the Fluorescence-Based Quantitative PCR Reaction

After selecting the best quantitative fluorescence primers, to improve the reaction specificity, an annealing temperature gradient (55 to 60℃) was set for a two-step ordinary PCR amplification.

PCR reaction condition:

Predenaturation(1 Cycle):	95 ℃	30 s
PCR reaction(40 Cycle):	95 ℃	5 s
	58 ℃	30 s

3.2. Establishment of the Standard Curve

The quantitative standard was diluted at 10 times ratio, and the concentration of the standard was 10^9-10^2 copies/μL with 8 gradients, respectively, as the template. The optimized fluorescence PCR system and procedure were used for detection, and blank control was set at the same time. Three parallel replicates were conducted for each standard and blank control, and the standard curve was obtained by real-time fluorescence PCR. The linear range of detection was determined by the shape of fluorescence curve of each concentration standard and the correlation of fluorescence curve of each concentration.

3.3. The Dynamics of Virus Content in the HFMD Macaque Model Was Monitored

In order to further study the pathogenesis of EV71 and improve the establishment of rhesus monkey model, the rhesus monkey model of respiratory tract infection EV71 was replicated, and three newborn monkeys, NO.13402, NO.0106 and NO.3384, were assigned to low, medium and high dose groups. EV71 venom of $1\times10^{6.5}$ CCID50, $4.29\times10^{6.5}$ CCID50 and $1.86\times10^{7.5}$ CCID50 were injected via trachea respectively. 250 μL of peripheral blood samples from the day before and day 1-14 after challenge were collected. The blood samples from the day before challenge were used as negative control, and 50 μL of whole blood RNA was extracted with Trizol. The established EV71 fluorescent quantitative RT-PCR method was used to quantitatively detect the viral load in blood.

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4. Results and Discussion

4.1. Experimental Result

Dynamic monitoring of EV71 virus infection in foot-mouth macaque model

After EV71 challenge, all 3 rhesus monkeys showed typical herpetic lesions in oral mucosa, lips and palm (Fig 2). According to the measured kinetic curves (Fig 3), on the second day after challenge, the virus content in the blood of the three experimental animals increased significantly, showing an obvious trend of growth, and then gradually decreased after reaching the peak value, and then fluctuated up and down within a certain range. On day 6 of challenge, the virus load of EV71 in the blood of juvenile monkey NO.13402 was the highest, which was 7.91×10^4 copies/µL, and the virus load remained relatively high. The viral loads of the two monkeys, NO.3384 and NO.0106, showed similar trends, with the maximum viral loads in the blood reaching 1.21×10^4 copies/µL and 4.86×10^3 copies/µL, respectively, on the second day after infection.

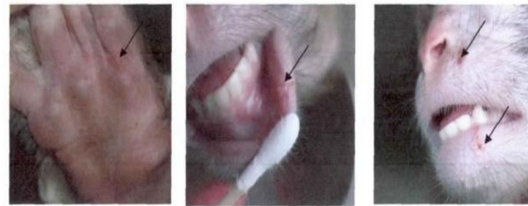

Figure 2: Herpetic lesions on forefoot mouth and lips after inoculation.

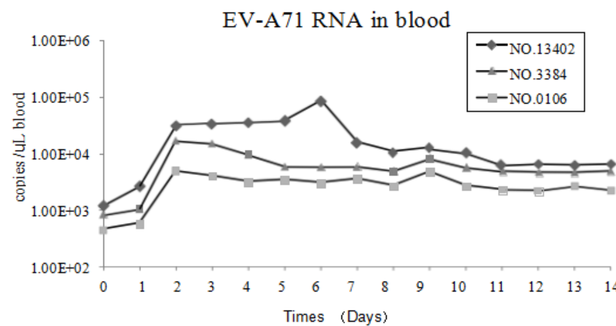

Figure 3: EV71 RNA detection in infected blood of infant macaque.

4.2. Experimental Optimization

4.2.1. Standard Curves for Quantitative RT-PCR

The standard curve was obtained by fluorescence quantitative PCR using double dilution recombinant plasmid as template. According to the dissolution curve analysis, 10^8~10^3 copies were selected as the standard curve range.

Y is the number of cycles (Cq value) corresponding to the inflection point of the amplification curve from the fluorescence threshold level to the exponential growth stage, and X is the log value of the initial copy content of the corresponding specimen. The intercept Slope of the standard curve is -3.337, and the PCR efficiency is 99.37%. The results show that EV71 fluorescence quantitative standard curve has a good correlation and a wide linear range.

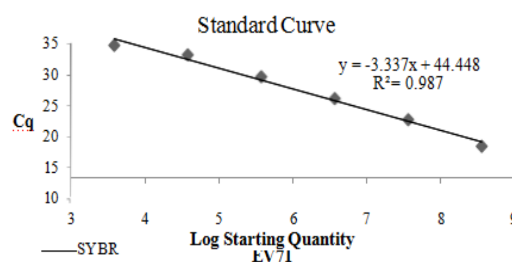

Figure 4: Standard curve of the real-time RT-PCR of EV71 detection.

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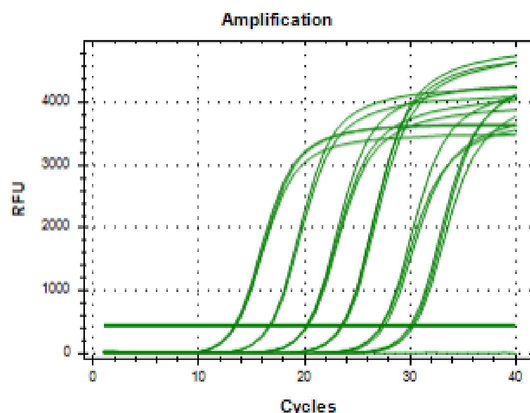

Figure 5: Amplification curve of standard curve.

The curves from left to right in turn are plasmid standard 3.79×10^8, 3.79×10^7, 3.79×10^6, 3.79×10^5, 3.79×10^4, 3.79×10^3 copies/μL, straight lines are black control.

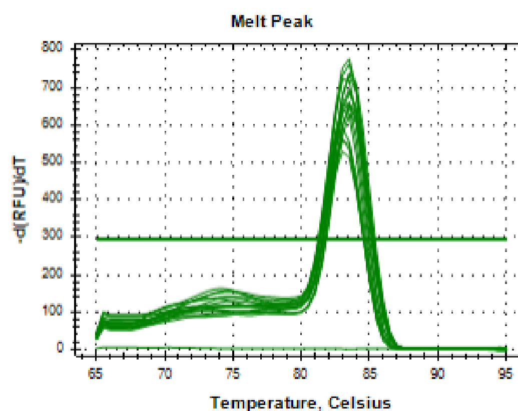

Figure 6: Melt curve of standard curve.

4.2.2. Methodological Evaluation

(1) Sensitivity

Standard substance was diluted 10x gradient to 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 copies respectively and detected by conventional RT-PCR and established fluorescence quantitative RT-PCR. As shown in fig 7, with the decrease of plasmid copy number, the specific products of ordinary RT-PCR gradually decreased. When the plasmid copy number decreased to 10^3 copies, no specific amplification bands were found, the lowest detectable level was 10^4 copies. The sensitivity of the established fluorescence quantitative RT-PCR method was tested with Cq value >35 cycles as the lower limit, as shown in fig 8. It can be seen from the amplification curve that the established fluorescence quantitative RT-PCR detection method can detect at least 10^2 copy number levels. Therefore, the sensitivity of the established fluorescence quantitative RT-PCR detection method is 100 times higher than that of ordinary RT-PCR detection method.

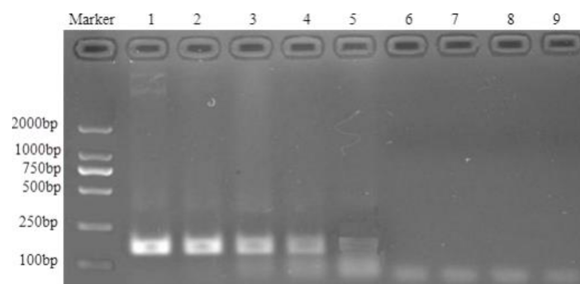

Figure 7: Sensitivity of regular RT-PCR of EV71 detection.

1~9: In turns are plasmid standard 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 copies and black control

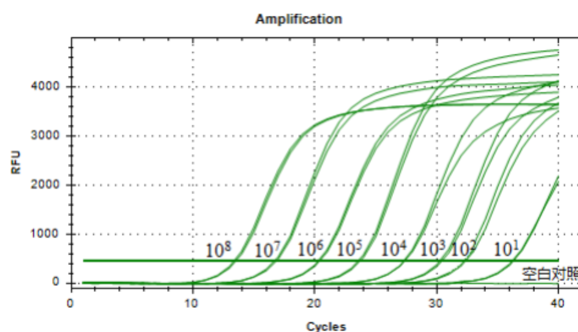

Figure 8: Sensitivity of real-time RT-PCR of EV71 detection.

(2) Specific

The RNA extracts of EV71 and COxsackie CA16 virus of the same genus of enterovirus were used as templates, and the established fluorescence quantitative PCR method was used for detection. The detection results showed that only EV71 virus was positive (Fig 9), indicating that the detection system had good specificity.

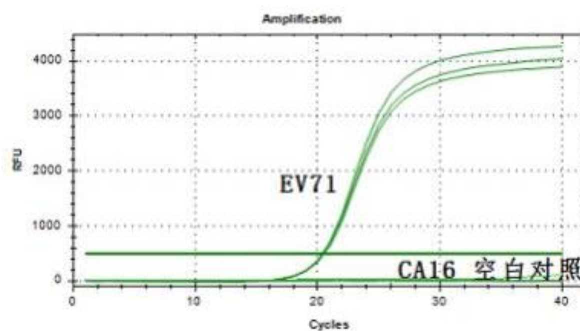

Figure 9: Specificity of real-time RT-PCR of EV71 detection.

4.2.3. The Dynamics of Virus Content in the HFMD Macaque Model Was Monitored

Fluorescence quantitative PCR detection of high efficiency, short time consuming, and through the fluorescence signal can be quantitative calculation template copy number, in the process of macaques, foot and mouth disease model can accurate real-time quantitative monitoring poison attack within 2 weeks after the virus in the blood loads, virus present in rhesus monkeys appeared along with the change of time copy number of dynamic changes, and associated with tapping toxic dose. This study can provide important reference value for subsequent evaluation of EV71 macaque model, vaccine development and antiviral drug screening.

5. Conclusions

EV71 virus is considered to be the most important neurotropic enterovirus after poliovirus has been extinguished. The method established in this paper can detect 10^2 copies of viral RNA at minimum, which is 100 times higher than the ordinary rt-pcr method. The linear range of the standard curve is between 10^3 and 10^8copies/μL, and the PCR amplification rate is 99.37%. The detection results of other enterovirus are negative, showing strong specificity and high sensitivity. Good stability; The results of blood sample analysis showed that the three groups of rhesus monkey models were successfully infected with EV71, and the copy number of virus proliferation was correlated with time and challenge dose. The results showed that the fluorescence quantitative PCR technique could be used for quantitative detection of EV71 viral load. With the further research, it is believed that more efficient and rapid detection methods will be established, laying a solid foundation for the detection of EV71.Consider that the digital PCR can have a higher sensitivity than the qRT-PCR.

References

[1] Liu Wenjuan, Zhang Qiong, Cao Xia, Progress in the mechanism and treatment of CNS damage

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caused by enterovirus EV71 infection [J]. Journal of Chengdu Medical College, 2016, 11(2): 257-262.
[2] Cui Xiaoqing, Wen Hongling, Wang Zhiyu, Progress in the study of animal models of human enterovirus type 71 [J]. Chinese Journal of Virus Diseases, 2013(5): 392-395.
[3] Liu Juan, Li Kang, Yang Jing, et al., Establishment of an ICR milk-mouse animal model of enterovirus type 71 (EV71) [J]. Military Medicine, 2010, 35(11): 862-866.
[4] Chumakov M, Voroshilova M, Shindarov L, et. al., Enterovirus 71 isolated from cases of epidemic poliomyelitis-like disease in Bulgaria [J]. Arch Virol, 1979, 60(3-4): 329-340.
[5] Liu LD, Zhao HL, Zhang YW, et al., Neonatal rhesus monkey is a potential animal model for studying pathogenesis of EV71 infection [J]. Virology, 2011, 412(1): 91-100.
[6] Arita M, Nagata N, Iwata N, et al., An attenuated strain of enterovirus 71 belonging to genotype A showed a broad spectrum of antigenicity with attenuated neurovirulence in cynomolgus monkeys [J]. Journal of Virology, 2007, 81(17): 9386-9395.
[7] Xiao XL, Wu H, Li YJ, et al., Simultaneous detection of enterovirus 70 and coxsackievirus A24 variant by multiplex real-time RT-PCR using an internal control[J]. Journal of Virological Methods, 2009, 159(1): 23-8.
[8] Oberste MS, Maher K, Patterson MA, et al., The complete genome sequence for an American isolate of enterovirus 77 [J]. Arch Virol, 2007, 152(8): 1587-1591.
[9] Yang Fang, Yu Shicheng, Zhang Juying, et al., Analysis of the epidemic characteristics of severe HFMD in mainland China from 2008 to 2010 [J]. Disease surveillance, 2013,28(11): 888-893.
[10] Yu Hejua,. Establishment and evaluation of quantitative RT-PCR detection for enterovirus type 71 [D]. [Master's degree thesis]. Henan province: Zhengzhou University. 2010.
[11] Livak KJ, Flood SJ, Marmaro J, et al., Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization PCR [J]. PCR Methods Appl, 1995, 4: 357-362.