Molecular and Cellular Probes xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Molecular and Cellular Probes

journal homepage: www.elsevier.com/locate/ymcpr

Development of a SYBR Green I real-time PCR for detection and quantitation of *orthopoxvirus* by using Ectromelia virus

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ARTICLE INFO

Keywords:
Ectromelia virus
Real-time PCR
SYBR Green I
Detection
Quantitation

ABSTRACT

Ectromelia virus (ECTV) is the causative agent of mousepox, which has devastating effects in laboratory-mouse colonies and causes economic loss in biomedical research. More importantly, ECTV has been extensively used as an excellent model for studies of the pathogenesis and immunobiology of human smallpox. A rapid and sensitive SYBR Green I-based real-time PCR assay was developed and used for the detection and quantitation of orthopoxvirus by using ECTV in this study. Primers targeted to the highly conserved region of major core protein P4b gene of orthopoxvirus were designed and the standard plasmid was constructed. This assay was able to detect a minimum of 10 copies of standard DNA and 5 $TCID_{50}$ units of ECTV. In addition, no cross-reactions were observed with two DNA viruses, such as herpes simplex virus and swine pseudorabies virus, and one RNA virus, vesicular stomatitis virus. Furthermore, intra- and inter-assay variability data showed that this method had a highly reproducibility and reliability. Moreover, the current assay was faster and had a higher sensitivity for detection of ECTV genomic DNA in cell cultured and clinical test samples. Therefore, the high sensitivity and reproducibility of this SYBR Green real-time PCR approach is a more effective method than the conventional PCR for ECTV diagnosis and quantitation.

1. Introduction

Poxviruses are viruses with an ancient origin and infect a wide variety of organisms, including invertebrates, reptiles, birds, and mammals [1–3]. Poxviruses infecting mammals are further divided into a number of subgroups, including the well-studied *orthopoxvirus* genus [3]. Ectromelia virus (ECTV) is an excellent model for orthopoxvirus study, which has a restricted host range and causes mousepox with a high mortality rate [4–6]. The first strain of ECTV was isolated in 1930 in a laboratory-mouse colony. The infection showed high mortality and amputations in mice recovered from infection [7,8]. Since then, other strains have been successively isolated from outbreaks in Moscow, Japan, the United States, and China [9–12]. These outbreaks had caused deaths in tens of thousands of laboratory mice and as a result in loss of millions of dollars in biomedical research funds [13].

Animal husbandry and diseases surveillances development and improvements have reduced the incidence of ECTV but infections still occur in sporadic forms [8,14–16]. Although ECTV can infect all laboratory mouse strains, the disease picture varies between fatal, mild or un-apparent signs in different mouse breeds [17,18]. Since the clinical signs of mousepox are not evident of resistance in different mouse strains, ECTV infections could be present in colonies of mice for long periods before their detection in animal house facilities [7,8]. Control of the spread of mousepox has involved the entire execution of the colonies [8]. Therefore, it is necessary to apply regular tests for the microorganism's status of laboratory mice to achieve statistically significant results.

Many diagnostic techniques, including virus isolation, electron microscopy, polymerase chain reaction (PCR), animal inoculation, serological tests, and quantum dot-fluorescence hybridization, had been developed for the detection of ECTV in mice and their secretions [8,19,20]. However, each one of these methods have drawbacks. The virus isolation in cell culture is technically difficult and time consuming, and electron microscopy is inconvenient and not applicable. Serological testing lacks sensitivity and specificity, and is not suitable for confirming the presence of ECTV in culture media and suspected tissues in mouse. Thus, the development of a rapid, sensitive, and effective method for the detection and quantification of ECTV in high-throughput clinical samples or in cell-cultured samples would offer advantages over current methods.

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https://doi.org/10.1016/j.mcp.2017.12.001
Received 3 August 2017; Received in revised form 2 December 2017; Accepted 4 December 2017

In the present study, we established a SYBR Green real-time PCR detection method for the molecular diagnosis of ECTV, and also for other orthopoxvirus members. The real-time PCR method was further applied to evaluate cell cultured and tissue samples.

2. Materials and methods

2.1. Ethics statement

All animal experiments were handled in accordance with the Good Animal Practice Requirements of the Animal Ethics Procedures and Guidelines of the People's Republic of China. The study was reviewed and approved by the Animal Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Science.

2.2. Virus, cells, and animals

The wild-type strain of ECTV was originally isolated from a naturally infected laboratory mouse and propagated in Vero cells (data unpublished). Plaque-purified ECTV was serially passaged and virus titer was measured by the 50% tissue culture infective dose ($TCID_{50}$) assay. The Ankara strain of modified vaccinia virus (MVA, VR-1508) was purchased from American Type Culture Collection (ATCC) and passaged on Vero cells. Herpes simplex virus (HSV), swine pseudorabies virus (PRV), and vesicular stomatitis virus (VSV) were cultured by our laboratory. Vero cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, USA) supplemented with antibiotics (penicillin 100 IU/mL, streptomycin 100 μg/mL) and 10% fetal bovine serum (FBS; Biological Industries, USA), and incubated at 37 ˚C in the presence of 5% CO_2.

Eight-week-old, specific-pathogen-free, male C57BL/6 mice were obtained from the experimental animal center of Lanzhou University. The mice were housed in individual ventilation cages of a biosafety level 3 room at the Lanzhou Veterinary Research Institute of the Chinese Academy of Agricultural Sciences.

2.3. Viral infection and sample collection

Vero cell monolayers were digested with 0.25% trypsin (Gibco, USA). The dislodged cells were suspended (5×10^5 cells) in 2 mL DMEM with 10% FBS and aliquots were seeded in each well of a 6-well plate and incubated at 37 ˚C and 5% CO_2 until they reached approximately 90% confluence. The cells were infected with ECTV (multiplicity of infection = 1) for 2 h. The medium was replaced with fresh medium containing DMEM with 2% FBS. The supernatant was then harvested at 24, 48, 72 and 96 h post-infection for virus titration and viral DNA extraction.

Twelve C57BL/6 mice were randomly assigned to the three study groups. The mice were infected in the footpad of both hind legs with 4.3×10^3 $TCID_{50}$ virus in a total volume of 100 μL phosphate-buffered saline (PBS) with injection of 50 μL per leg. The mice were sacrificed at 3, 5 and 7 days post-infection (dpi) for blood, liver, and spleen samples collection and viral DNA extraction.

2.4. Primers pairs design

The A4L gene of VACV was conserved as documented in a previous study. Alignment was performed with the homologous P4b genes of ECTV (NC_004105, KJ563295 and JQ410350), VACV (AM501482), CPXV-BR (AF482758) from GenBank, which are highly conserved and were selected for primers design (Fig. S1). The primers (5′-AGAAGAT ATCAGACGATCCACAATC-3′ and 5′- GTAGAACGACGCCAGAATAAGA ATA-3′) were used for standard plasmid construction and real-time amplification of ECTV DNA. To prove the specificity of the primers, a Taqman probe was labelled with FAM (6-carboxyfluorescein) (5′-FAM-TTGGTCTCCGAGTTTATAACAGACTGCGGAC-BHQ-3′) and synthesized by Invitrogen (Beijing, China).

2.5. Preparation of standard plasmid DNA

The genomic DNA of ECTV was extracted from ECTV-infected Vero cells using a MiniBEST viral RNA/DNA extraction kit (TaKaRa) according to the manufacturer's instructions. The obtained DNA was used as a template to amplify the fragment of the P4b gene (Gene ID: 951664), and then was cloned into the pJET1.2 vector (Thermo Fisher Scientific, USA). The recombinant plasmid was transformed into competent *Escherichia coli* DH5α cells, extracted with a Mini Kit I (Omega Biotek, USA), and verified by sequencing. The plasmid DNA concentrations were measured using a NanoDrop 2000c system (Thermo Fisher Scientific) and used to calculate the number of copies of the plasmid (copies/μL). Aliquots of DNA were prepared in ten-fold serial dilutions from 1×10^7 to 1×10^1 copies/μL and stored at −20 ˚C until used for establishment of the standard curve.

2.6. SYBR Green real-time quantitative PCR assay

Real-time PCR was performed using SYBR Premix Ex Taq II (TaKaRa). Each PCR was performed with a final volume of 25 μL containing 12.5 μL of 2 × SYBR Green qPCR Master Mix, 0.4 μM of each primer, 8.5 μL of nuclease-free water, and 2 μL of DNA or standard plasmid DNA. PCR amplification was performed using the CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) and run in triplicate with the following conditions: one cycle of 95 ˚C for 30 s, followed by 40 cycles of 95 ˚C for 5 s, 63 ˚C for 30 s. Melting curve analysis was performed at 95 ˚C for 10 s, 65 ˚C for 5 s, then followed by a slow increase from 65 ˚C to 95 ˚C with a speed of 0.5 ˚C per second.

As for the TaqMan real-time PCR, a 20 μL total volume was consisted of 10 μL probe mix, 0.4 μM of each primer, 0.4 μM of TaqMan probe, 6.8 μL nuclease-free water and 2 μL cDNA. PCR amplification was run in triplicate under the following conditions: one cycle of 95 ˚C for 3 min, followed by 40 cycles of 95 ˚C for 15 s, 67 ˚C for 30 s.

2.7. Sensitivity, specificity, and reproducibility analyses

To determine the specificity of the assay, DNA extracted from MAV, PRV and HSV were tested in triplicate. ECTV-positive samples and an ultrapure water negative control were included in each run. To check the reproducibility of the real-time PCR assay, standard plasmid DNA was serially diluted ten-fold with sterile water to concentrations of 1×10^7 to 1×10^1 copies/μL. The intra-assay test was conducted in triplicate within the same run. The inter-assay test was performed independently as three different runs on different days. The mean, standard deviation (SD), and coefficient of variation (CV) for the intra- and inter-assay tests were calculated separately for each standard DNA dilution based on their cycle-threshold (Ct) values using Microsoft Excel software.

2.8. Detection of ECTV from virus-infected cells and mice

To confirm the applicability of the real-time PCR in sample diagnostics, DNA was extracted from the supernatant of virus-infected cells and organs of virus-infected mice using MiniBEST viral RNA/DNA extraction kit (TaKaRa) according to the manufacturer's instructions. The extracted DNA was then used for real-time PCR.

3. Results

3.1. Standard curve for SYBR Green I real-time PCR

Serial dilutions from 10^7 to 10^1 copies/μL of the recombinant plasmid were used to produce standard curves for the real-time PCR. Under the optimized conditions, each real-time PCR reaction used 2 μL