Antiviral Research 97 (2013) 198–205

Contents lists available at SciVerse ScienceDirect

Antiviral Research

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

Development and characterization of a stable eGFP enterovirus 71 for antiviral screening

Baodi Shang [a], Chenglin Deng [a], Hanqing Ye [a], Wenbo Xu [b], Zhiming Yuan [a], Pei-Yong Shi [c,*], Bo Zhang [a,*]

[a] Center for Emerging Infectious Diseases, Wuhan Institute of Virology, Chinese Academy of Science, China
[b] National Lab for Poliomylitis, Institute of Viral Disease Control and Prevention, China Center for Disease Control and Prevention, China
[c] Wadsworth Center, New York State Department of Health, Albany, NY, USA

ARTICLE INFO

Article history:
Received 24 October 2012
Revised 6 December 2012
Accepted 15 December 2012
Available online 22 December 2012

Keywords:
Enterovirus
Reporter virus
High-throughput assay
Antiviral drug discovery

ABSTRACT

Enterovirus 71 (EV71) is one of the major causative agents for hand, foot, and mouth disease. There is currently no clinically approved vaccine or antiviral treatment for EV71 infection. To facilitate antiviral drug discovery, we developed an infectious cDNA clone of an epidemic strain of EV71 and a stable eGFP reporter EV71. The reporter virus was generated by engineering the eGFP gene between the 5′ untranslated region and VP4 gene of the EV71 genome. Vero cells transfected with the cDNA clone-derived RNA generated high titers (>10^6 PFU/ml) of the eGFP reporter virus. The reporter virus was infectious to Vero cells, producing robust eGFP fluorescence signals. Compared with the wild type virus, the reporter virus replicated slower in cell culture. To examine the stability of the reporter virus, we continuously passaged the virus on Vero cells for five rounds. The passaged viruses maintained the eGFP gene, demonstrating the stability of the reporter virus. Using a known EV71 inhibitor, we demonstrate that the reporter virus could be used for antiviral screening. The infectious cDNA clones of the wild type virus and the eGFP reporter viruses will be useful for antiviral research as well as for studying viral replication and pathogenesis of EV71.

1. Introduction

Enterovirus 71 (EV71), a member of *enterovirus* genus within *picornaviridae* family, is one of the major causative agents for hand, foot, and mouth disease (HFMD) associated with neurological disease in children (Nishimura et al., 2009). It was first identified from a child with neurologic symptoms in California in 1969 (Schmidt et al., 1974). During the past 20 years, EV71 epidemics have frequently occurred in Asian countries including China, Japan, Malaysia, and Singapore (Chua and Kasri, 2011; Shih et al., 2011; Tan et al., 2011). In China alone, a total of 488,955 HFMD cases and 126 deaths were reported in 2008; most of these human cases were caused by EV71 infection (Tan et al., 2011). There is currently no effective therapy against EV71 virus although a few candidates are under clinical development (Thibaut et al., 2012). Development of antiviral agents and vaccine represents an urgent unmet medical need to control EV71 infections.

EV71 is a small, non-enveloped virus containing a single plus-strand RNA of approximately 7000 nucleotides in length. The genomic RNA contains a single long open reading frame (ORF) that is flanked by 5′ untranslated region (UTR) and 3′UTR with a poly (A) tail. The 5′UTR consists of two functional domains, the cloverleaf and internal ribosomal-entry site (IRES). VPg (3B) is covalently linked to the 5′ end of the viral genome and involved in the initiation of viral RNA replication (Paul et al., 2000). Viral genomic RNA is translated into a single polyprotein. The polyprotein is processed into three primary precursor molecules: one structural region (P1) and two non-structural regions (P2 and P3) (De Palma et al., 2009). P1 region is further cleaved into VP0, VP3, VP1; VP0 is finally cleaved into VP2 and VP4 during virion formation. P2 and P3 are proteolytically cleaved into various precursors and mature proteins such as $2A^{pro}$, 2BC, 2B, 2C, 3AB, 3A, 3B (VPg), $3CD^{pro}$, $3C^{pro}$, and $3D^{pol}$; these proteins are important for viral replication (Thibaut et al., 2012; Wu et al., 2010).

The current cell-based antiviral assay for EV71 relies on viral infection of susceptible cells (Lee et al., 2008; Shum et al., 2010; Wu et al., 2010). The antiviral activity is monitored through plaque assay, measurement of cytopathic effects, or quantification of viral RNA by RT–PCR. These methods are time-consuming and labor-

Abbreviations: EV71, enterovirus 71; HTS, high-throughput screen; hpi, hour post-infection; pt, post-transfection; pi, post-infection; eGFP, enhanced green fluorescent protein; GuHCl, guanidine hydrochloride.

* Corresponding authors. Addresses: Novartis Institute for Tropical Diseases, 10 Biopolis Road, #05-01 Chromos, Singapore 138670, Singapore (P.-Y. Shi). Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China. Tel.: +86 27 87197822 (B. Zhang).

E-mail addresses: pei_yong.shi@novartis.com (P.-Y. Shi), zhangbo@wh.iov.cn (B. Zhang).

0166-3542/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.antiviral.2012.12.010

intensive, making it impractical for high throughput screening of large compound libraries. The development of alternative cell-based methods for rapid and reliable determination of enterovirus infection remains to be developed (Tsai et al., 2009). Various reporter enteroviruses have been constructed to study the molecular mechanism of viral replication and morphogenesis (Liu et al., 2010; Nishimura et al., 2009; Song et al., 2012; Teterina et al., 2010; Teterina et al., 2011a,b). Ehrenfeld and colleagues inserted red fluorescent protein (DsRed) after residue 144 of 2A protein of poliovirus and three different epitope tags (FLAG, hemagglutinin [HA], and c-myc) into the N-terminal region of protein 3A to study their subcellular localizations (Teterina et al., 2010, 2011b). Using luciferase poliovirus and Coxsackie A virus, Jiang and coworkers demonstrated the interaction between VP3 and 2C during viral encapsidation (Liu et al., 2010). These studies have demonstrated the feasibility to generate a reporter virus for EV71 that could potentially be used for screening of large compound libraries. In this study, we developed and characterized a stable eGFP reporter EV71. Using a guanidine hydrochloride (GuHCl) as a known inhibitor, we showed that the reporter EV71 could be used for antiviral screening.

2. Materials and methods

2.1. Cell lines, viruses, antibodies

Vero (African green monkey kidney) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 10% FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin. The parental EV71 strain HeN09 was isolated from patient collected in October 2009 from MinQuan, HeNan province, China. A single viral stock was made from two round passage in Vero cells without plaque purification, stored as aliquots at −80 °C, and designated as parental EV71. This stock was used as parental virus in all assays. The rabbit polycolonal antibody against EV71 VP1 is a gift from Hualin Wang's group in Wuhan Institute of Virology.

2.2. Plasmid construction

Fig. 1 shows the overall scheme of the cloning strategy for the full-length EV71 cDNA clone. The viral RNA was extracted from the parental virus with QIAamp Viral RNA Mini Kit (Qiagen), and reverse transcribed using oligo(dT) primers and M-MLV Reverse Transcriptase (Invitrogen) to produce the first strand cDNA of the complete viral genome. By using this first strand cDNA as a template, four cDNA fragments covering the complete genome were amplified with high fidelity Taq polymearase (NEB) and four pairs of primers, and cloned into pACYC177 at XbaI and HindIII sites, yielding four constructs called EV71-A, EV71-B, EV71-C, and EV71-D. The primer sequences were listed in Table 1. Construct EV71-A contained the sequence from the 5′ end to the nucleotide position 1,489 with a T7 promoter before the 5′ end of the genome. Construct EV71-B contained the sequence from nucleotide position 1341–3821. Construct EV71-C contained the sequence from nucleotide position 3641–6420, and construct EV71-D contained the sequence from nucleotide position 5878 to the 3′ end with a poly(A)$_{29}$ tail. Next, the subclone EV71-AB was constructed by pasting the fragment between BsiWI (nucleotide position 1438) and HindIII from EV71-B into EV71-A. The subclone EV71-ABC was constructed by pasting the fragment between AatII (nucleotide position 3739) and HindIII from EV71-C into EV71-AB. Finally, fragment between SpeI (nucleotide position 6276) and HindIII from EV71-D was inserted into EV71-ABC, resulting in the full-length cDNA clone pACYC-EV71-FL.

A two-step cloning strategy was used to construct EV71 reporter virus cDNA. First, a standard overlap PCR was used to create a cassette containing "XbaI-T7pro-5′UTR-SphI-MluI-VP42" by using pACYC-EV71-FL as a template. This PCR product was inserted into pACYC-EV71-FL at sites XbaI and BsiWI to produce the plasmid pACYC-SphI-MluI-EV71-FL. Second, the reporter gene with the unique 5′-SphI and 3′-MluI site was inserted at the corresponding sites into pACYC-SphI-MluI-EV71-FL to produce EV71 reporter virus cDNA clone.

Plasmid pACYC177 was used as the cloning vector throughout the experiments. Bacterial strain HB101 (Promega) was used as the host for propagation of the cDNA clones. The virus-specific sequence of each intermediate cloning product was validated by sequencing before it was used in the subsequent cloning step. All restriction endonucleases were purchased from New England Biolabs.

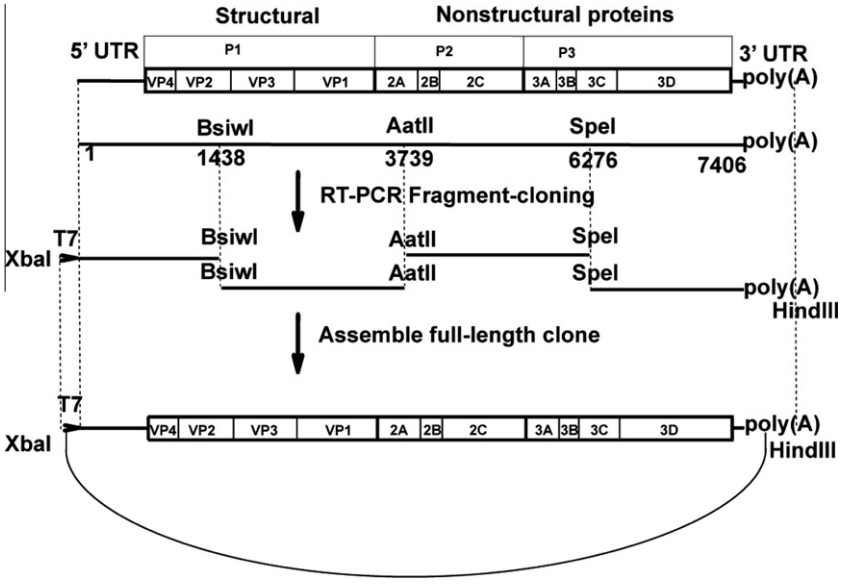

Fig. 1. Construction of the full-length cDNA clone of EV71. Genome organization and unique restriction sites as well as their nucleotide numbers are shown at the top. Four cDNA fragments represented by thick lines were synthesized from genomic RNA through RT–PCR to cover the complete EV71 genome. Individual fragments were assembled to form the full-length cDNA clone of EV71 (pACYC-EV71-FL) as described in Section 2. The complete EV71 cDNA is positioned under the control of T7 promoter elements for *in vitro* transcription. The numbers are the nucleotide positions based on the sequence from GenBank accession No. JN256064.

Table 1
Primer sequences.

Primer	Sequence
EV71-XbaI-T7-5UTR-b	CTAGTCTAGATAATACGACTCACTATAGGTTAAAACAGCCTGTGGGTTGCACCCACT
EV71-HidIII-1489-R	CTAGAAGCTTCCTCAAATTAATCCACTGGT
EV71-XbaI-1341F	CTAGTCTAGA GTCATTGGGACAGTAGCAGGC
EV71-HindIII-3821R	CTAGAAGCTTCTGCGTCAGTGAAGCCTGTT
EV71-XbaI-3641F	CTAGTCTAGACTCAGAACCTGGTGATTGC
EV71-HindIII-6420R	CTAGAAGCTTAATTTTATCAATCGAGCGCAG
EV71-XbaI-5878F	CTAGTCTAGAGCAATGGCAGACAAGGTTTT
EV71-HidIII-poly(T)	CTAGAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTT
EV71-746-R	ACGCGTACATACATACATGCATGCTGAGCCCATGTTTAGCTGCGTTAAGGGTC
EV71-747-F	ATGTATGTATGTACGCGTGCCATTACTACCCTTGGTTCGCAAGTGTCCACACAGC
DsRed-F	ACATGCATGCATGGACAACACCGAGGACGTCATC
Rluc-F	ACATGCATGCATGGCTTCCAAGGTGTACGACC
Rluc-R	CGACGCGTCTGCTCGTTCTTCAGCACGCGCTC
SphI-eGFP-F	ACATGCATGCATGGTGAGCA AGGGCGAGGA
MluI-eGFP-R	CGACGCGTCTTGTACAGCTCGTCCATG
EV71-HidIII-1489-R	CTAGAAGCTTCCTCAAATTAATCCACTGGT
EV71-XbaI-1341F	CTAGTCTAGA GTCATTGGGACAGTAGCAGGC
EV71-HindIII-3821R	CTAGAAGCTTCTGCGTCAGTGAAGCCTGTT
EV71-XbaI-3641F	CTAGTCTAGACTCAGAACCTGGTGATTGC
EV71-HindIII-6420R	CTAGAAGCTTAATTTTATCAATCGAGCGCAG
EV71-XbaI-5878F	CTAGTCTAGAGCAATGGCAGACAAGGTTTT

2.3. RNA transcription and transfection

Genome-length and reporting viral RNAs were *in vitro* transcribed from the HindIII-linearized cDNA plasmids using MEGAscript® T7 Kit (Ambion) according to the manufacturer's protocols. The RNAs were electroporated into Vero cells as previously described (Zhang et al., 2010). After transfection, supernatants were collected at different time points. The culture medium containing viruses were aliquotated and stored at −80 °C.

2.4. Plaque assay

A series of 1:10 dilutions were made by mixing 15 μl of virus sample with 135 μl of DMEM. One hundred microliters of each dilution were seeded to individual wells of 12-well plates containing confluent Vero cells (2×10^5 cells per well, plated one day in advance). The plates were incubated at 37 °C with 5% CO_2 for 1 h before the layer of 2% methyl cellulose was added. After 4 days of incubation at 37 °C with 5% CO_2, the cells were fixed in 3.7% formaldehyde and then stained with 1% crystal violet. Plaque morphology and numbers were recorded after washing the plates with tap water.

2.5. Immunofluorescence assay (IFA)

Vero cells transfected with the EV71 genome-length RNA were seeded on Chamber Slide (Nalge Nunc). At 24, 36, and 48 h post-transfection (hpt), the cells were fixed by cold (−20 °C) 5% acetone in methanol for 20 min at room temperature. The fixed cells were washed with PBS and incubated with EV71 VP1 rabbit polyclonal antibody (1:300 dilutions with PBS) for 1 h. After washing with PBS for three times, the cells were incubated with goat anti-rabbit IgG conjugated with FITC at room temperature for another hour. After three times of PBS wash, the slide was mounted with 90% glycerol and examined under a fluorescent microscope. Cell images were taken at 200× magnification.

2.6. Viral growth kinetics

Vero cells were seeded in a 6-well plate (2×10^5 cells per well). At 24 h post seeding, the cells were infected with virus at an indicated MOI. Culture medium was collected at the indicated time points and was used to quantify viral titers by plaque assay on Vero cells as described above.

2.7. Cytotoxicity assay

A cell proliferation-based MTT kit (a tetrazoliumcolorimetric assay; American Type Culture Collection) was used to estimate the cytotoxicity of guanidine hydrochloride (GuHCl). Briefly, Vero cells (1×10^4 cells in 100 μl DMEM plus 10%FBS) were seeded per well of a 96-well plate. After 6 h incubation, different concentrations of GuHCl (dissolved in water) were added to the cells. After 48 h incubation, 10 μl MTT reagent [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] was added and incubated for 3.5 h, followed by the addition of 100 μl detergent reagents. The plates were then swirled gently and left in darkness at room temperature for 4 h. The absorbance of the plate was measured through a Multimode Microplate Reader (Varioskan Flash, Thermo Fisher, Finland) with a 550-nm filter. The cytotoxicity of the GuHCl was estimated by comparing the absorbance from the compound-treated cells versus the absorbance from the mock-treated cells.

2.8. Inhibition assay of eGFP-EV71 by GuHCl

Vero cells were seeded in black 96-well plates at a density of 1×10^4 cells per well. After 24 h, the cells were infected with eGFP-EV71 virus at an MOI of 0.5 and incubated with various concentrations of GuHCl (0–1 mM). Incubations were done in triplicate and 6 control wells were also included. The eGFP fluorescence intensity was measured with Multimode Microplate Reader (Varioskan Flash, Thermo Fisher, Finland) at 488 nm excitation (5 nm bandwidth) and 507 nm emission wavelengths, respectively, and the measurement time was set at 100 ms.

3. Results

3.1. Construction and characterization of the full-length EV71 cDNA clone

As shown in Fig. 1 and described in Material and Method, the full-length EV71 cDNA clone, pACYC-EV71-FL, was assembled by four fragments, and contained a T7 promoter at the 5′ end for *in vitro* transcription. Followed by linearization with HindIII, the

full-length cDNA was *in vitro* transcribed that has a poly (A) tail at 3′ end of the EV71 genomic RNA and a 5′ non-viral G nucleotide derived from the T7 promoter.

IFA were used to detect the expression of viral VP1 protein in Vero cells transfected with the *in vitro* genome-length RNA (Fig. 2A). The number of cells expressing viral VP1 protein was increasing from 24 to 48 hpt; more than 70% cells were IFA-positive at 48 hpt. The staining intensity varied among the IFA-positive cell population. High intensity of positive cells were rounded and lost normal cell morphology which indicates viral replication-mediated cytotoxicity; the cells with earlier stage of viral infection still maintained normal morphology and showed comparably lower fluorescence intensities. Apparent cytopathogenic effect (CPE) appeared at 48 hpt (Fig. 2B). The culture medium from the transfected cells was collected at different time points, and virus titers were determined on Vero cells by plaque assays. High viral titers (around 10^6 PFU/ml) were consistently obtained (Fig. 2C). The recombinant virus showed plaque morphology identical to the parental virus (Fig. 2D). To further confirm the release of virions, RNAs extracted from the culture medium was reversely transcribed followed by PCR using primers specific to viral genes. The recombinant virus produced the same size of virus-specific RT–PCR product as the parental virus (Fig. 2E). An equivalent viral growth curves at MOI of 1 were observed between these two viruses (Fig. 2F). Overall, these data demonstrate that the parental and recombinant viruses are indistinguishable in replication and spread in Vero cells.

3.2. Construction and characterization of the EV71 reporter virus

As described in Section 2, the cDNA clone of EV71 reporter virus was constructed by inserting the reporter gene between the 5′UTR and the N-terminus of VP4 gene. In order to release the reporter gene from VP4, an EV71 2A protease cleavage site (ITTLG) was introduced between the reporter gene and VP4 (Fig. 3A). eGFP or DsRed gene was engineered into the EV71-FL cDNA clone at SphI and MluI sites to produce eGFP-EV71 or DeRed-EV71 reporter virus cDNA clone. The primers used for reporter virus cDNA clone are listed in Table 1.

To test whether the reporter full-length viral RNA is replicative, equal amounts of EV71-FL, eGFP-EV71 and DsRed-EV71 genome-length RNA transcripts were transfected into Vero cell as described in Section 2. Both eGFP and DsRed expressions (Fig. 3B) were monitored under a fluorescent microscope. The eGFP or DsRed positive cells were observed as early as 12 hpt. In addition, there were more eGFP or DsRed positive cells at 24 and 48 hpt which indicated viral replication and viral spread. More CPE were observed in eGFP-

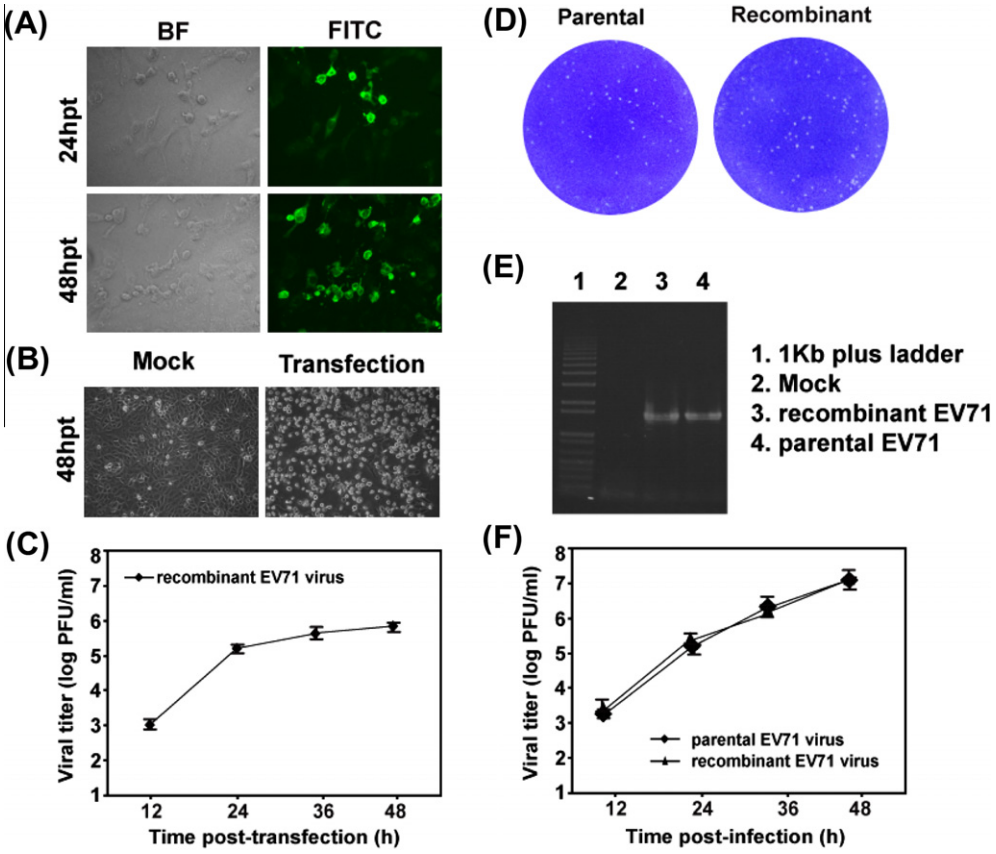

Fig. 2. Characterization of full-length EV71 recombinant virus. (A) IFA of viral protein expression in cells transfected with full-length EV71 RNA transcript. Vero cells transfected with full-length EV71 RNA transcript were analyzed by IFA at the indicated times post transfection. The left and right panels represent the same field of view for each time point. The left panels were visualized with differential interference contrast, and the right panels were visualized with a fluorescein isothiocyanate (FITC) filter set. (B) Cytopathogenic effect of EV71 RNA transcript transfected Vero cells. Mock transfected (left) and EV71 RNA transcript transfected (right) Vero cells were observed 48 hpt. (C) Viral titer which is from EV71 RNA transcript transfected Vero cells at different times of post-transfection. Viral titers were calculated as the number of plaque-forming units (PFU)/μL by plaque assays. Plaque assays were performed using a standard protocol mentioned in Section 2. (D) Plaque morphology of parental and clone-derived EV71 on Vero cells. (E) RT–PCR detection of recombinant viral RNA. Expected band were observed for parental and recombinant virus. As a negative control, no RT–PCR product was detected from the extracted supernatant of mock transfected Vero cells. (F) Comparison of the growth kinetics of recombinant and parental EV71. The growth of recombinant and parental viruses was compared at MOI = 1 on Vero cells. Recombinant virus and parental virus were designated by diamond and triangle, respectively. Three independent experiments were performed in duplicate, and the representative data were presented.

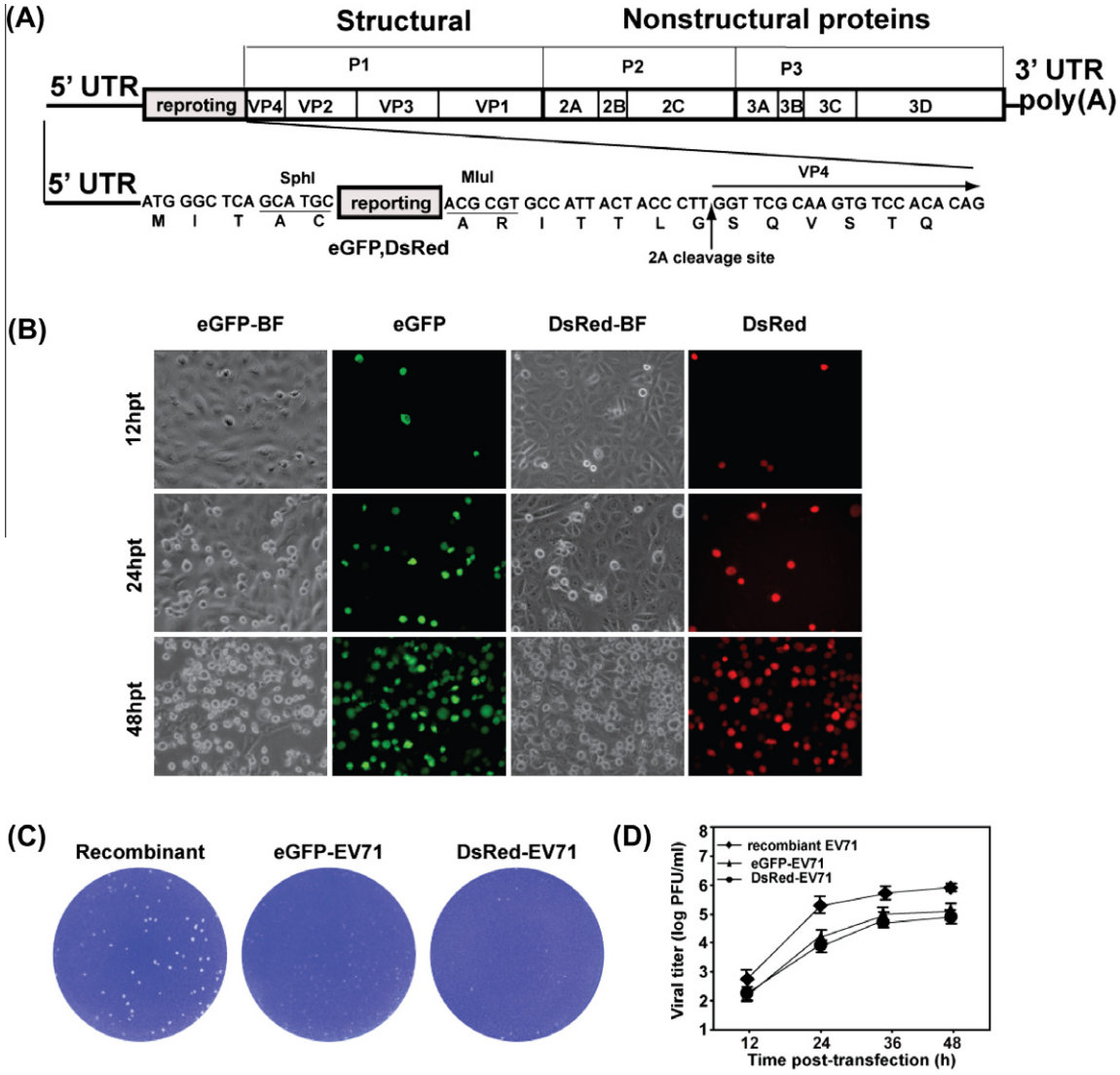

Fig. 3. Characterization of EV71 reporter virus. (A) Schematic of the construction of the EV71 reporter virus. An infectious cDNA clone of pACYC-EV71-FL was used as backbone for construction of EV71 reporter virus. Reporter genes were inserted at the engineered SphI and MluI sites. 2A protease cleavage site (ITTLG) was indicated by arrow. (B) eGFP and DsRed expressions in cells transfected with full-length EV71 reporter RNA transcripts. Vero cells transfected with full-length EV71 reporter RNA transcript were analyzed by fluorescent microscopy at the indicated times post-transfection. (C) Plaque morphology of recombinant EV71 virus, eGFP-EV71 virus and DsRed-EV71 virus on Vero cells. (D) Comparison of the growth kinetics of recombinant and parental EV71. The growth of recombinant and reporter viruses were compared at MOI = 0.1 on Vero. Recombinant EV71 virus, eGFP-EV71 virus and DsRed-EV71 virus were designated by diamond, triangle and circle, respectively. Three independent experiments were performed in duplicate, and the representative data were presented.

EV71 or DsRed-EV71 genome-length RNA transfected Vero cells when cells were cultured for longer time post transfection (Fig. 3B). Plaque morphology and viral titer were also compared among wild type EV71-FL, eGFP-EV71 and DsRed-EV71. As measured by plaque assay, both eGFP-EV71 and DsRed-EV71 displayed smaller plaque size than EV71-FL (Fig. 3C), and the viral titer of EV71 reporter virus was about 10-fold lower than that of EV71-FL (Fig. 3D). Overall, the results show that the eGFP-EV71 and DsRed-EV71 genome-length RNA are replication-competent and infectious, although the viral titer is lower than wild type EV71-FL virus.

3.3. Stability of the reporter virus in cell culture

The eGFP and DsRed EV71 reporter viruses were blind passaged on Vero cells respectively for five rounds to test their stability. The viruses which were derived from eGFP or DsRed EV71 RNA-transfected Vero cells were defined as P0 and used for passaging. At 60 hour post-infection (hpi), 10 μl viral supernatant was transferred to a new T-25 flask containing naïve Vero cells in 5 ml of culture medium. The viruses from each passage were defined as P1 to P5, respectively. As shown in Fig. 4A, strong fluorescence signals were detected in cells infected with P5 viruses, indicating the reporter gene was stably maintained during passaging. To further confirm the stability of these reporter viruses, viral RNA were extracted from P1, P3 and P5 passage, and RT–PCR was performed to amplify the region from 5′UTR to VP2 using primer EV71-XbaI-T7-5UTR-b and EV71-HindIII-1489-R (Table 1). An expected 1.5 and 2.0 Kb band was detected from wild type and reporter viruses RT–PCR product, respectively (Fig. 4B). Plaque morphology was also compared between P0 and P5 passage viruses. As shown in Fig. 4C, the plaque sizes were similar between these two passage viruses. These results indicate that EV71 reporter viruses are stable during passaging.

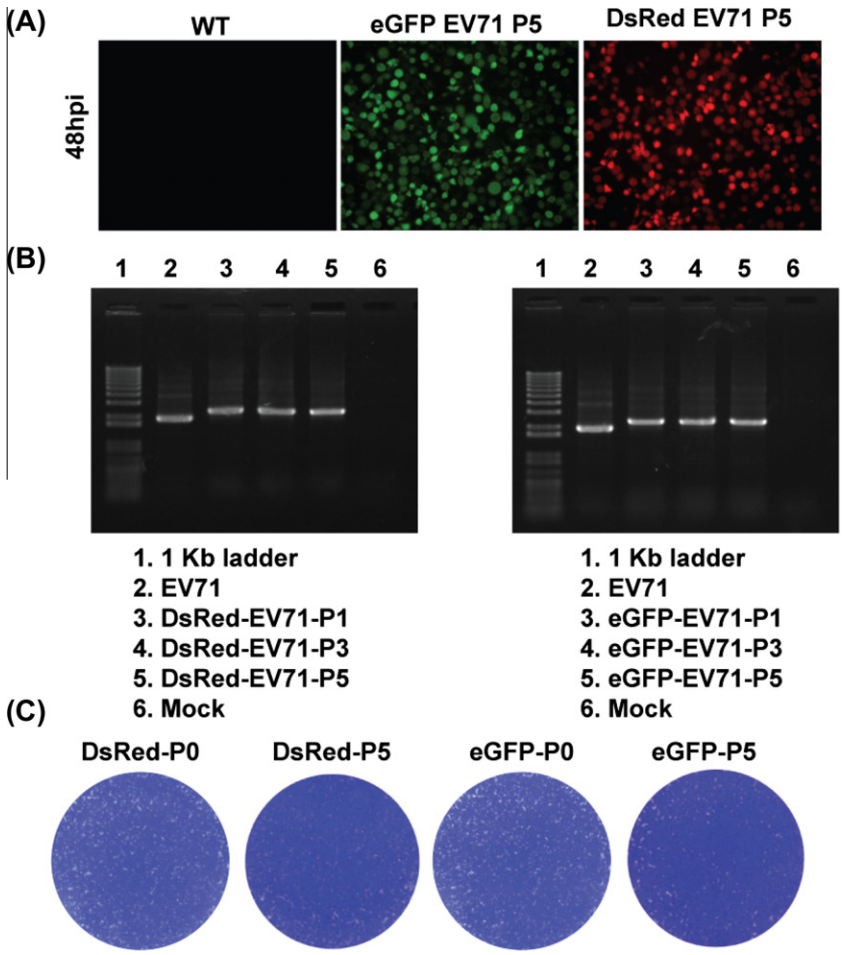

Fig. 4. Stability of EV71 reporter virus in Vero cells. (A) Detection of eGFP and DsRed expression on Vero cells infected with passaged reporter virus. eGFP and DsRed positive cells were observed under a fluorescent microscope when Vero cells were infected with P5 viruses 48 hpi, which indicates the reporter gene still maintained in the reporter viruses. (B) Detection of the reporter gene during virus passage. Viral RNA was extracted from culture supernatants of P1, P3 and P5 passage, respectively. RT–PCR was performed with primer pair locating 5′UTR and VP4 region. The resulting RT–PCR products were resolved by 1% agarose gel electrophoresis. (C) Plaque morphologies of eGFP-EV71 and DsRed-EV71 from P0 and P5. Virus from culture supernatants was subjected to plaque assay on Vero cells.

3.4. Optimization and validation of an eGFP-EV71 reporter virus based antiviral screening assay

To develop reporter virus-based antiviral screening assay, we first identified the cell number and MOI value for viral infection based on eGFP-EV71 reporter virus. The cells should be almost 100% confluent at the end of the assay. Ten thousand Vero cells per well in 96-well plate were seeded 24 h before viral infection, followed by infection with eGFP-EV71 at varying MOI. At 24 hpi, the plate was read for the intensity of eGFP fluorescence. The fluorescence value was calculated by eGFP fluorescence signal level in infected cells subtracting the background signal in naïve cells. As shown in Fig. 5A, there was a good correlation between the intensity of eGFP fluorescence and the value of MOI. Vero cells infected with eGFP-EV71 at MOI of 0.5 yielded the signals high enough for fluorimeter measurement. Therefore, we chose 0.5 MOI in the following assays.

Since chemical compounds are dissolved in DMSO, the effect of DMSO concentration on eGFP-EV71 infection in 96-well plate was examined. Increasing amount of DMSO had no obvious effect on the signals of eGFP fluorescence; up to 1% DMSO was tolerated which is a concentration of DMSO used for antiviral screening. We also tested whether phenol red, FBS in media could interfere with fluorescence measurements and found that they did not have dramatic effect on measurement (data not shown). Therefore, we chose 1% DMSO as our final assay concentration.

To validate whether this reporter virus could be used as an antiviral screening assay, we used GuHCl to validate our antiviral assay. GuHCl is an inhibitor of the replication of enterovirus without adversely affecting host cell metabolism; protein 2C of enterovirus is the target of GuHCl (Baltera and Tershak, 1989; Pfister and Wimmer, 1999). The reported 50% effective concentration (EC50) of this drug against different strains of polioviruses in Hela cells varied between 320 and 720 μM (Shimizu et al., 2000). We examined the inhibitory effects of GuHCl at different concentrations on eGFP-EV71 infection by fluorescence measurement using both fluorescent microscopy and microplate fluorimeter. As shown in Fig. 5B, the number of eGFP-positive cells decreased with the increase of GuHCl concentration. In consistent with it, the data from microplate fluorimeter also showed the expression of eGFP was inversely proportional to the concentration of GuHCl, depicted as the curve of eGFP fluorescence signal versus GuHCl concentration (Fig. 5C). The EC50 was estimated to be 150 μM in eGFP-EV71-based assay. As a control, the inhibitory effect of GuHCl on full-length recombinant EV71 (EV71-FL) infection was also determined by viral titer reduction assay, and the resulting EC50 was 134 μM (Fig. 5D). This discrepancy among EC50 values from different assay system is not surprising due to intrinsic differences among the

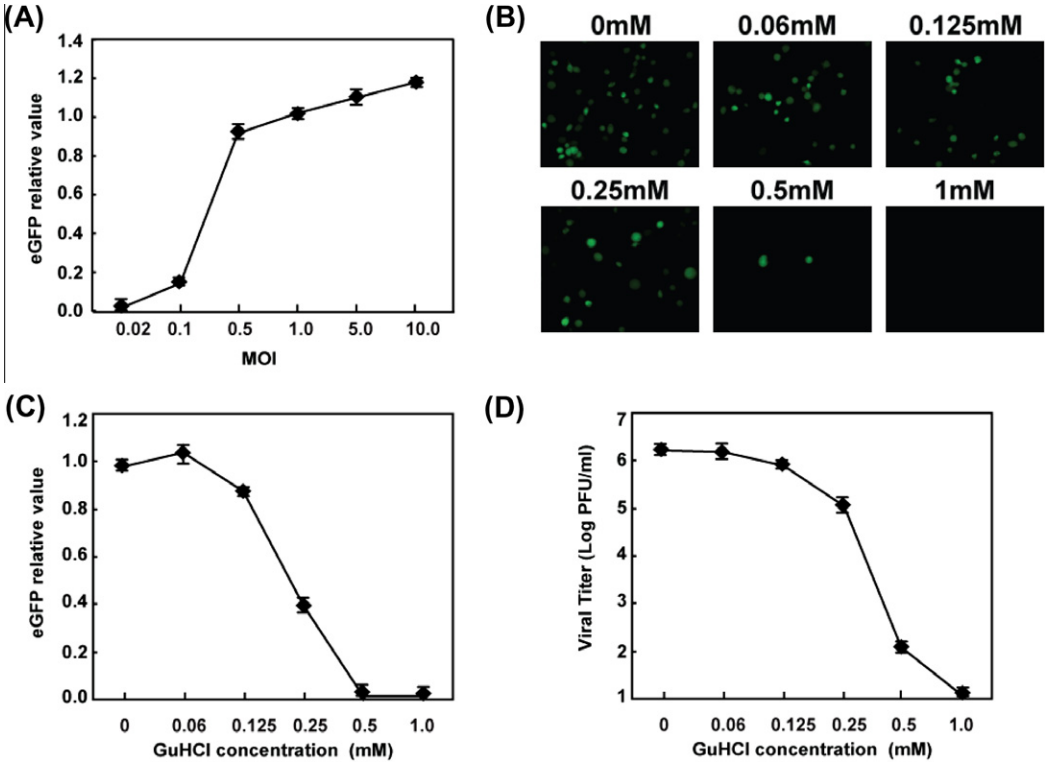

Fig. 5. Antiviral activity of GuHCl on recombinant WT-EV71 and eGFP-EV71 reporter virus. (A) Correlation between eGFP signal and different MOI infection. Ten thousand Vero cells per well in 96-well plate were infected with eGFP-EV71 at the indicated MOI and the infected cells were assayed for eGFP fluorescence intensity at 24 hpi. (B) Detection of eGFP expression by fluorescent microscopy at different concentration of GuHCl. (C) Antiviral activity of GuHCl by using eGFP-EV71 on a 96-well plate. Vero cells were seeded in black 96-well plates at a density of ten thousand cells per well. After 24 h, the cells were infected with eGFP-EV71 virus at MOI of 0.5 and incubated with various concentrations of GuHCl (0–1 mM). The infected cells were assayed for eGFP fluorescence intensity at 24 hpi. Three independent experiments were performed in duplicate, and the representative data were presented. (D) Viral titer reduction assay of EV71-FL at different concentration of GuHCl.

systems (Puig-Basagoiti et al., 2005). These results provided an evidence that eGFP-EV71 reporter virus could be used in a high-throughput assay for screening inhibitors of EV71.

4. Discussion

The prevention and treatment of enterovirus 71 infection are presently public health priorities. This study addressed the development and characterization of an infectious cDNA clone of EV71 and a novel reporter EV71 virus system for antiviral study. The recombinant virus showed biological properties indistinguishable from the parental virus, including plaque morphology and growth kinetics (Fig. 2). These results indicated that an efficient reverse genetic system has been established for EV71 virus. For eGFP and DsRed reporter virus, the fluorescent gene was inserted between the 5′UTR and the N-terminus of VP4 gene with the addition of an EV71 2A protease cleavage site (ITTLG) at its C-terminus (Fig. 3A). Upon the cleavage by 2A protein, the fluorescent protein is released and visible under a fluorescent microscope. Fig. 3B–D showed that the levels of eGFP expression in the eGFP-EV71 reporter virus-infected cells correlate with viral RNA replication. The reverse genetic systems developed in this study will be useful for making recombinant viruses required for mode-of-action analysis of future inhibitors, as well as for studying viral replication and pathogenesis. In addition, the reporter viruses could be readily used for high throughput antiviral screening.

A number of high-throughput assays have been reported to identify the inhibitor of enterovirus infection. A 6-amino-acidpeptide, LVLQTM, was identified as a decoy substrate of the human rhinovirus (HRV) 2A cysteine protease ($2A^{pro}$) through a high-throughput yeast two-hybrid screening assay. It was further demonstrated to be able to inhibit viral replication in both A549 cells and *in vivo* mouse model by blocking the viral 2A protease activity (Falah et al., 2012). Target-based enzymatic high-throughput assays have also been developed for the potential inhibitors of the polymerase $3D^{pol}$ and protease $3C^{pro}$ (Campagnola et al., 2011; Tsai et al., 2009). The main advantage of the target-based assay is that the targets of the identified inhibitors are known. Several cytopathic effects (CPE)-based antiviral assays were adapted to high-throughput formats to identify antiviral compounds for coxsackie B virus and HRV (Gong et al., 2008; Phillips et al., 2011; Schmidtke et al., 2001). The antiviral assay described here is based on eGFP-EV71 reporter virus infection of Vero cells. Our results showed that the decrease in eGFP signal could be used as a surrogate readout to measure the antiviral activity of potential inhibitors. Since the assay is based on reporter virus infection and replication, it allows for identification of inhibitors that target any steps of the viral life cycle, including viral entry, translation, RNA synthesis, RNA encapsidation, and virion maturation. The mode-of-action of viral inhibitors can further be identified through time-of-addition studies with compound or individual biochemical assays such as protease and RNA-dependent RNA polymease (RdRp) assays. Alternatively, the antiviral target could potentially be identified through selection of drug resistant virus (Lo et al., 2003). Another advantage of the cell-based assay is that the assay requires the compound to penetrate into cells in order to exhibit antiviral activity. Therefore, compounds identified from the cell-based assay should have a high success rate in subsequent animal experiments (Lo et al., 2003).

The fluorescent protein-based reporter viruses could also be used for high content image-based assay (Shum et al., 2010). The

high-content image-based assay can evaluate antiviral activity and cytotoxicity of compounds simultaneously in living cells. A high-content assay to identify inhibitors of dengue virus infection has also been established; the assay was validated to screen a chemical library of known drugs and bioactive (Shum et al., 2010). A series of fluorescent protein-based reporter vaccinia viruses was developed for rapid, high content analysis of viral function at all stages of gene expression in plate reader assays (Dower et al., 2011). Our eGFP-EV71 reporter virus could also be developed for a high-content image antiviral assay. Experiments are ongoing in our laboratory to establish such high-content image assay.

It is worth noting that although the eGFP reporter EV71 was stable, both *renilla* luciferase (Rluc) and *gaussia* luciferase (Gluc) reporter EV71 using the same strategy were unstable and not viable (data not shown). The Rluc reporter gene was completely lost during passaging on Vero cells. The possibility is due to the size selection of viral genome during RNA synthesis and encapsidation, deletion of foreign gene commenced very quickly during viral passage (Song et al., 2012). The possible explanation for Gluc reporter virus is that certain RNA elements within the Gluc gene or Gluc protein may inferfere with viral replication (Song et al., 2012).

In summary, we have established the reverse genetic systems using an epidemic strain of EV71. The eGFP reporter EV71 assay developed in this study should be useful for screening novel anti-EV71 agents. The reporter virus will also be useful for studying EV71 replication and pathogenesis.

Acknowledgements

This work was supported the National Basic Research Program of China (Grant Nos. 2011CB5047 and 2012CB518904), the National Natural Science Foundation of China (Grant No. 31170158) and the '100 Talents Project' of Chinese Academy of Sciences, China (Grant No. Y002171YC1).

References

Baltera Jr., R.F., Tershak, D.R., 1989. Guanidine-resistant mutants of poliovirus have distinct mutations in peptide 2C. J. Virol. 63, 4441–4444.

Campagnola, G., Gong, P., Peersen, O.B., 2011. High-throughput screening identification of poliovirus RNA-dependent RNA polymerase inhibitors. Antiviral Res. 91, 241–251.

Chua, K.B., Kasri, A.R., 2011. Hand foot and mouth disease due to enterovirus 71 in Malaysia. Virol. Sin. 26, 221–228.

De Palma, A.M., Thibaut, H.J., van der Linden, L., Lanke, K., Heggermont, W., Ireland, S., Andrews, R., Arimilli, M., Al-Tel, T.H., De Clercq, E., van Kuppeveld, F., Neyts, J., 2009. Mutations in the nonstructural protein 3A confer resistance to the novel enterovirus replication inhibitor TTP-8307. Antimicrob. Agents Chemother. 53, 1850–1857.

Dower, K., Rubins, K.H., Hensley, L.E., Connor, J.H., 2011. Development of Vaccinia reporter viruses for rapid, high content analysis of viral function at all stages of gene expression. Antiviral Res. 91, 72–80.

Falah, N., Violot, S., Decimo, D., Berri, F., Foucault-Grunenwald, M.L., Ohlmann, T., Schuffenecker, I., Morfin, F., Lina, B., Riteau, B., Cortay, J.C., 2012. Ex vivo and in vivo inhibition of human rhinovirus replication by a new pseudosubstrate of viral 2A protease. J. Virol. 86, 691–704.

Gong, E., Ivens, T., Van den Eynde, C., Hallenberger, S., Hertogs, K., 2008. Development of robust antiviral assays for profiling compounds against a panel of positive-strand RNA viruses using ATP/luminescence readout. J. Virol. Methods 151, 121–125.

Lee, J.C., Shih, S.R., Chang, T.Y., Tseng, H.Y., Shih, Y.F., Yen, K.J., Chen, W.C., Shie, J.J., Fang, J.M., Liang, P.H., Chao, Y.S., Hsu, J.T., 2008. A mammalian cell-based reverse two-hybrid system for functional analysis of 3C viral protease of human enterovirus 71. Anal. Biochem. 375, 115–123.

Liu, Y., Wang, C., Mueller, S., Paul, A.V., Wimmer, E., Jiang, P., 2010. Direct interaction between two viral proteins, the nonstructural protein 2C and the capsid protein VP3, is required for enterovirus morphogenesis. PLoS Pathog. 6, e1001066.

Lo, M.K., Tilgner, M., Shi, P.Y., 2003. Potential high-throughput assay for screening inhibitors of West Nile virus replication. J. Virol. 77, 12901–12906.

Nishimura, Y., Shimojima, M., Tano, Y., Miyamura, T., Wakita, T., Shimizu, H., 2009. Human P-selectin glycoprotein ligand-1 is a functional receptor for enterovirus 71. Nat. Med. 15, 794–797.

Paul, A.V., Rieder, E., Kim, D.W., van Boom, J.H., Wimmer, E., 2000. Identification of an RNA hairpin in poliovirus RNA that serves as the primary template in the in vitro uridylylation of VPg. J. Virol. 74, 10359–10370.

Pfister, T., Wimmer, E., 1999. Characterization of the nucleoside triphosphatase activity of poliovirus protein 2C reveals a mechanism by which guanidine inhibits poliovirus replication. J. Biol. Chem. 274, 6992–7001.

Phillips, T., Jenkinson, L., McCrae, C., Thong, B., Unitt, J., 2011. Development of a high-throughput human rhinovirus infectivity cell-based assay for identifying antiviral compounds. J. Virol. Methods 173, 182–188.

Puig-Basagoiti, F., Deas, T.S., Ren, P., Tilgner, M., Ferguson, D.M., Shi, P.Y., 2005. High-throughput assays using a luciferase-expressing replicon, virus-like particles, and full-length virus for West Nile virus drug discovery. Antimicrob. Agents Chemother. 49, 4980–4988.

Schmidt, N.J., Lennette, E.H., Ho, H.H., 1974. An apparently new enterovirus isolated from patients with disease of the central nervous system. J. Infect. Dis. 129, 304–309.

Schmidtke, M., Schnittler, U., Jahn, B., Dahse, H., Stelzner, A., 2001. A rapid assay for evaluation of antiviral activity against coxsackie virus B3, influenza virus A, and herpes simplex virus type 1. J. Virol. Methods 95, 133–143.

Shih, S.R., Stollar, V., Li, M.L., 2011. Host factors in enterovirus 71 replication. J. Virol. 85, 9658–9666.

Shimizu, H., Agoh, M., Agoh, Y., Yoshida, H., Yoshii, K., Yoneyama, T., Hagiwara, A., Miyamura, T., 2000. Mutations in the 2C region of poliovirus responsible for altered sensitivity to benzimidazole derivatives. J. Virol. 74, 4146–4154.

Shum, D., Smith, J.L., Hirsch, A.J., Bhinder, B., Radu, C., Stein, D.A., Nelson, J.A., Fruh, K., Djaballah, H., 2010. High-content assay to identify inhibitors of dengue virus infection. Assay Drug Dev. Technol. 8, 553–570.

Song, Y., Paul, A.V., Wimmer, E., 2012. Evolution of poliovirus defective interfering particles expressing Gaussia luciferase. J. Virol. 86, 1999–2010.

Tan, X., Huang, X., Zhu, S., Chen, H., Yu, Q., Wang, H., Huo, X., Zhou, J., Wu, Y., Yan, D., Zhang, Y., Wang, D., Cui, A., An, H., Xu, W., 2011. The persistent circulation of enterovirus 71 in People's Republic of China: causing emerging nationwide epidemics since 2008. PLoS ONE 6, e25662.

Teterina, N.L., Levenson, E.A., Ehrenfeld, E., 2010. Viable polioviruses that encode 2A proteins with fluorescent protein tags. J. Virol. 84, 1477–1488.

Teterina, N.L., Lauber, C., Jensen, K.S., Levenson, E.A., Gorbalenya, A.E., Ehrenfeld, E., 2011a. Identification of tolerated insertion sites in poliovirus non-structural proteins. Virology 409, 1–11.

Teterina, N.L., Pinto, Y., Weaver, J.D., Jensen, K.S., Ehrenfeld, E., 2011b. Analysis of poliovirus protein 3A interactions with viral and cellular proteins in infected cells. J. Virol. 85, 4284–4296.

Thibaut, H.J., De Palma, A.M., Neyts, J., 2012. Combating enterovirus replication: state-of-the-art on antiviral research. Biochem. Pharmacol. 83, 185–192.

Tsai, M.T., Cheng, Y.H., Liu, Y.N., Liao, N.C., Lu, W.W., Kung, S.H., 2009. Real-time monitoring of human enterovirus (HEV)-infected cells and anti-HEV 3C protease potency by fluorescence resonance energy transfer. Antimicrob. Agents Chemother. 53, 748–755.

Wu, K.X., Ng, M.M., Chu, J.J., 2010. Developments towards antiviral therapies against enterovirus 71. Drug Discov. Today 15, 1041–1051.

Zhang, B., Dong, H., Ye, H., Tilgner, M., Shi, P.Y., 2010. Genetic analysis of West Nile virus containing a complete 3′CSI RNA deletion. Virology 408, 138–145.