Construction of coxsackievirus B5 viruses with luciferase reporters and their applications in vitro and in vivo

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ABSTRACT

Coxsackievirus belongs to the Picornaviridae family and is one of the major pathogens that cause hand, foot and mouth disease (HFMD) in infants and children with potential serious complications and even deaths. The pathogenesis of this virus is not fully elucidated and no vaccine or antiviral drug has been approved. In this study, a full-length infectious cDNA clone of coxsackievirus B5 virus was assembled and the recombinant virus displayed similar growth kinetics and ability to cause cytopathic effects as the parental virus. Luciferase reporter virus was then incorporated to generate both full-length and subgenomic replicon (SGR) reporter viruses. The full-length reporter virus is suitable for high-throughput antiviral screening, while the SGR is a useful tool to study virus-host interactions. More importantly, the full-length reporter virus has also been shown to infect the suckling mouse model and the reporter gene could be detected using an in vivo imaging system, thus providing a powerful tool to track viruses in vivo. In summary, we have generated coxsackievirus B5 reporter viruses and provided unique tools for studying virus-host interactions in vitro and in vivo as well as for high-throughput screenings (HTS) to identify novel antivirals.

1. Introduction

Enteroviruses are a group of non-enveloped viruses belonging to the Picornaviridae family. Enterovirus infections can lead to hand-foot-and-mouth disease (HFMD), which is usually mild and self-limiting, although some strains could result in serious and life-threatening complications, such as encephalitis, myocarditis, meningitis, and poliomyelitis (Chan et al., 2003; Baggen et al., 2018). Enteroviruses have been divided into four groups, polioviruses, coxsackie A viruses (CVA), coxsackie B viruses (CVB), and echoviruses according to their pathogenesis and hepatitis, while CVA mainly falls into Enterovirus A or C and they mostly cause HFMD (Nikonov et al., 2017; Mehta et al., 2018), arguing their different pathogenicity in clinics.

Coxsackievirus is a (+) ssRNA virus with a genome approximately 7400 nucleotides long. The genome consists of a major open reading frame (ORF), flanked by the 5’- and 3’-untranslated regions (UTRs). Recently, a second ORF (uORF) has been identified in members of Enterovirus A, B and C. This ORF is upstream of the major ORF and it encodes a small protein that could enhance viral infection by facilitating viral release (Guo et al., 2019; Lulla et al., 2019). The 5’ end of the genome is linked to a small protein-VPg, while the 3’ end terminates with a poly(A) tract (Tuthill et al., 2010; Tariq and Kyriakopoulos, 2022). The major ORF encodes a large polyprotein precursor, which is further processed by viral proteases into four structural capsid proteins (VP1, VP2, VP3, and VP4), and seven non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D) that are responsible for viral replication and virus-host interactions (Sean and Semler, 2008; Tuthill et al., 2010). Recent studies have shown that sequences encoding the nonstructural proteins are
sufficient to drive enterovirus viral RNA replication in vivo (Kaplan and Raciello, 1988; Chen et al., 2012; Wang et al., 2020), although part of the capsid-coding region has been reported to be required for viral RNA replication of rhinovirus 14 (Mcknight and Lemon, 1996), a close relative strain of enterovirus in the family of Picornaviridae.

To better understand the pathogenesis of enteroviruses, novel tools are required to study virus-host interactions. In addition, sensitive and straightforward antiviral screening platforms will facilitate antiviral development. In this study, we first assembled a full-length infectious clone of CVB5 driven by T7 promoter based on an isolated virus and then established a full-length, infectious virus that stably bears a NanoLuc luciferase, which could be used for high-throughput antiviral screening. In addition, a subgenomic replicon with part of the capsid-encoding region replaced with Renilla luciferase reporter gene was also constructed and demonstrated to be a powerful tool to study virus-host interactions. Finally, the full-length reporter virus could infect suckling mice and NanoLuc luciferase could be visualized with imaging system, which will be useful to study viral tropism in vivo.

2. Materials and methods

2.1. Antibodies and reagents

Antibodies used in this study include: β-actin (AC006, Abclonal, Woburn, MA); VP3 (MA5-18206, ThermoFisher Scientific); DAF (A11283, Abclonal, Woburn, MA); PI4KB (13247-1-AP, Proteintech, Wuhan, China); dsRNA(J2) (J2-2007, English and Scientific Consulting, Hangzhou); GAPDH (60004-1-Ig, Proteintech, Wuhan, China).

GuHCl and crystal violet were purchased from Sigma-Aldrich; PI4KIIIα-IN-9 and furimazine were purchased from MCE. DAPI, Alexa Fluor 488 conjugated secondary antibody for microscopy experiments were purchased from ThermoFisher Scientific.

2.2. Cell lines, virus

Human rhabdomyosarcoma (RD) cells and 293 T cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 1 μg/mL streptomycin at 37 °C in the presence of 5% CO2. The parental CVB5 virus (GenBank accession: QO919261) was isolated from patients at Xi’an municipal CDC and was propagated in RD cells.

2.3. Plasmids construction

To construct the full-length CVB5 infectious cDNA, viral RNA was first extracted from CVB5-infected RD cells, and reverse transcribed into cDNA using random primer. The whole CVB5 cDNA genome was then divided into three segments with overlapping primers. The three fragments and the vector pRBCU were assembled into pRBCU-CVB5 with 2× MultiF Seamless Assembly Mix (Abclonal, Woburn, MA). The T7 promoter sequence (TAATACGAACTCACTATAGG) was inserted upstream of the 5’UTR for in vitro transcription purpose. The primers used are listed in the table below.

To make the NanoLuc-CVB5, NanoLuc as well as the 3C self-cleavage site encoding sequences (GCTTTGTTTCAAGGA) were inserted in front of the start codon of VP4 gene with 2× MultiF Seamless Assembly Mix (Abclonal, Woburn, MA). The primers used are listed in Table S1.

To make CVB5 subgenomic replicon (pSGR-Rluc-CVB5), a Renilla luciferase (RLuc)-Neo fusion gene was first amplified from the pSGR-Rluc-JFH1 (Wang and Tai, 2017). Together with the 3C self-cleave site encoding sequences, they were inserted into pRBCU-CVB5 to replace VP4, VP2, and the first 167aa of VP3 with 2× MultiF Seamless Assembly Mix (Abclonal, Woburn, MA). The primers used are listed in Table S1.

The polymerase-dead subgenomic replicon (pSGR-Rluc-CVB5/GND) was made by mutating the 3D polymerase catalytic sites GDD to GND with 2× MultiF Seamless Assembly Mix (Abclonal, Woburn, MA). The primers used are also listed in Table S1.

All constructs were confirmed by sequencing and detailed sequences for these constructs are available upon request.

2.4. In vitro transcription and RNA transfection

In vitro transcription was carried out as previously described (Wang and Tai, 2017; Yang et al., 2021). Briefly, all the constructs were first linearized with HindIII (NEB, Ipswich, MA, USA) and then transcribed into RNA using HiScribe™ T7 High Yield RNA Synthesis Kit (NEB, Ipswich, MA, USA). Viral RNA was then purified using the RNA Isolation Kit (FOREGENE, Chengdu, China). The purified RNA (0.5 μg) was transfected into 1.2 × 10⁶ RD cells with TransIT-mRNA transfection reagent (Mirus Bio, Madison, WI) according to manufacturer’s instruction.

2.5. Plaque assay

Plaque assays were performed as previously described (Yang et al., 2021). Briefly, viruses were first adjusted to the same titration (MOI = 0.2). Ten-fold series dilutions of viruses were then inoculated to RD cells in 6-well plates. Viruses were incubated at 37 °C for 1 h before they were removed, and 2 mL of DMEM containing 2% FBS and 1% low-melting agarose was added to each well. Cells were incubated for another three days before stained with 1% crystal violet containing 10% formalin solution.

2.6. Viral RNA quantification

RNA from virus infected cells was isolated with RNA Isolation Kit (FOREGENE, Chengdu, China) and reverse-transcribed with ABScript III RT Master Mix from Abclonal. Viral RNA was then quantified with RealStar Power SYBR Mix (GenStar, Beijing, China). The following primers were used: EV-F, 5’- GTGTGAAAGGTCTATGGACG-3’; EV-R, 5’- ATTGTACCATAAGCAGCCA-3’; NL-F, 5’- GATTGCTCTGAGCGGTGAAA-3’; NL-R, 5’- CATACGGCGCTCAGAATAG-3’. For NanoLuc gene copy number calculation, a standard curve was established with serially diluted, linearized NanoLuc-CVB5 construct.

2.7. Immunoblotting

Cells were treated and harvested as indicated and lysed with 1× LDS sample buffer (Thermo Scientific, Waltham, MA, USA); Mice tissues were homogenized with DRAGONLAB Homogenizer on ice and centrifuged at 12,000 ×g for 10 min at 4 °C to remove debris, and supernatants were mixed with LDS sample buffer before they were subjected to 10% SDS-PAGE gel and transferred onto PVDF membranes (Millipore, Burlington, VT, USA). The membranes were then incubated with specific primary antibodies and corresponding secondary antibodies. Images were taken with FUSION SOLO3.

2.8. Immunofluorescence

Immunofluorescence staining was carried out as previously described (Wang et al., 2014). Briefly, cells seeded on poly-o-lysine treated coverslips were infected with indicated virus at MOI of 1.0. After 12 h (hrs), cells were fixed with cold methanol for 15 min and probed with Anti-dsDNA(J2) antibody diluted at 1:500 for 1 h at 25 °C, followed by Alexa Fluor 488 goat anti-mouse IgG staining (Thermo Scientific, Waltham, MA, USA). Cells were then counterstained with DAPI before mounted on slides. Images were taken with Olympus Fluoview FV3000 confocal microscope.
2.9. Luciferase assay

RD cells, DAF knock-out (KO) or PI4KB knock-down (KD) cells were infected with NanoLuc-CVB5 reporter virus and treated as indicated before they were lysed and tested with the Nano-Glo luciferase Assay System (Promega Corporation) according to the manufacturer’s instruction. For SGR luciferase measurement, cells were first transfected with pSGR-Null-CVB5 RNA and Renilla luciferase was assayed with Renilla Luciferase Assay System (Promega Corporation) according to the manufacturer’s instruction. Luminescence was acquired with BioTek Neo2 multifunctional microplate reader.

For Z-factor determination, 10,000 RD cells per well were seeded in 384-well plate and infected with NanoLuc-CVB5 (MOI = 10) for 2 h before half plate of cells were treated with 100 nmol/L PI4KIIIβ-IN-9. About 12 h later, NanoLuc luciferase activity was measured as described above. Z-factor was calculated as previously described (Zhang et al., 1999).

2.10. Mouse infection experiment

The mice at an age of three days were intraperitoneally (i.p.) inoculated with parental CVB5 or NanoLuc-CVB5 at 2 × 10^3 TCID50s/mouse. All mice were monitored daily to record their survival rate or were sacrificed to dissect organs. Viral RNAs from 20 mg tissue were extracted with Tissue RNA Isolation Kit (FOREGENE, Chengdu, China) after homogenization and quantified with qPCR using primers as described above.

For in vivo imaging, mice were first infected with virus as described above, and one or two days later, mice were intraperitoneally injected with furimazine (2 g/mouse). Four or eight minutes after injection, images were taken with VISQUE™ InVivo Smart-LF.

2.11. Statistical analyses

Unless otherwise indicated, all values represent means ± standard deviations and represent the results of a minimum of three independent experiments. The two-tailed Student’s t-test was used to compare the means of control and experimental groups. ns, not significant; ****, P < 0.0001.

3. Results

3.1. Construction and characterization of infectious full-length cDNA clone of coxsackievirus B5

To generate a full-length infectious clone of CVB5, the genome of CVB5 was subdivided into three fragments and assembled with the pRBCU backbone. A T7 promoter was inserted upstream of 5’ transcription purpose (Fig. 1A). Purified viral RNA was then transfected into RD cells and cytopathic effect (CPE) was observed 48 h post-transfection (Supplementary Fig. S1), suggesting new virions were produced and released into the supernatant. When the supernatant was further inoculated to RD cells, CPE was getting stronger. Viral supernatants were further passaged for another four rounds and viral stocks were prepared for further characterization.

Compared to parental CVB5 (pCVB5), the recombinant virus (rCVB5) showed comparable ability to cause CPE, which was observed in less than 24 h post-infection (h.p.i) (Fig. 1B). When cell lysis were immunoblotted, both pCVB5 and rCVB5 showed VP3 expression around 12 h.p.i (Fig. 1C), confirming that the CPE observed was caused by CVB5 infection. More importantly, when cells were immunostained with double-stranded RNA (dsRNA) antibody, both pCVB5 and rCVB5 infected cells showed puncta staining, indicating the formation of the intermediates of viral genome replication, while no puncta were observed in uninfected cells (Fig. 1D). These results all suggest the successful recovery of a full-length infectious clone of CVB5.

We next further compared the viral titer and replication kinetics of rCVB5 to those of pCVB5. Plaque formation assay showed that both viruses reached viral titer above 10^11 PFU/mL (6.8 × 10^11 for pCVB5 and 5.2 × 10^11 PFU/mL for rCVB5) (Fig. 1E). Replication kinetics measuring the RNA copy number of viruses at different time points showed that the recombinant virus replicated as efficiently as the parental virus (Fig. 1F). Overall, these data demonstrated that the recombinant virus had similar ability of replication, release and to cause CPE to those of the parental virus.

3.2. Generation of CVB5 infectious clone with NanoLuc luciferase reporter gene

Most viral genomes are small and compact and do not allow large fragment insertion. Therefore, to generate a full-length, infectious reporter CVB5 virus, we chose the relatively small NanoLuc luciferase as the reporter gene. The NanoLuc luciferase reporter gene as well as the 3Cpro self-cleavage site (ALFQG) were inserted between CVB5 5’UTR and the start codon of VP4 (Fig. 2A). Cleavage of viral polyprotein by 3Cpro will release the NanoLuc luciferase as a reporter to indicate viral replication.

Transfection of in vitro transcribed RNA into RD cells led to luciferase expression at 6 h post-transfection and luciferase kept increasing for about 24 h, while rCVB5 without luciferase only showed background reading (Fig. 2B), suggesting the successful expression of the luciferase reporter gene. To ensure that the reporter gene could be successfully encapsided into virions and was not lost during passing, viral supernatants were kept inoculating into RD cells for another 15 passages and luciferase activity as well as NanoLuc gene from selected passages were monitored. Fig. 2C showed that luciferase activity kept increasing until reaching a plateau around P13, suggesting the reporter virus could successfully fulfill its life cycle and indicating that the virus might accumulate adaptive mutations for efficient replication. Similarly, quantitative RT-PCR of virus infected cells showed that the gene copy number of NanoLuc gene was increasing during passage (Fig. 2D), indicating the reporter gene was stably maintained during the passage. In addition, immunostaining also confirmed that the dsRNA was easily detected in NanoLuc-CVB5 infected cells (Fig. 2E) and viral structural protein VP3 was detected by immunoblotting 12 h.p.i (Fig. 2F), similar to parental or recombinant non-tagged virus.

In contrast to a previously reported NanoLuc-tagged EV71 virus, which showed little CPE in infected RD cells (Yang et al., 2021), this NanoLuc tagged CVB5 caused strong CPE in RD cells even at 24 h.p.i, comparable to that of pCVB5 or rCVB5 (Fig. 2G). Plaque assay demonstrated that the virus titer of this reporter virus is 2.5 × 10^10 PFU/mL (Fig. 2H), suggesting the reporter virus could still reach very high infection efficiency and the incorporation of this NanoLuc reporter had little effect on virus titers. In line with these observations, the viral replication kinetic study showed that the replication ability of this NanoLuc-CVB5 virus was only slightly reduced compared to recombinant virus (Fig. 2I).

3.3. Adapting NanoLuc-CVB5 to high-throughput screening

NanoLuc luciferase shows much brighter signal than firefly or Renilla luciferase and could be detected in a homogeneous assay, thus is ideal for high-throughput screening (HTS) (Hall et al., 2012). To adapt this NanoLuc-CVB5 virus for HTS purpose, we first tested whether this reporter virus was suitable for antiviral testing. NanoLuc-CVB5 infected cells were treated with different concentrations of guanidine hydrochloride (GuHCl), a known inhibitor of enterovirus 2C protein (Pfister and Wimmer, 1999), or PI4KIIIβ-IN-9, an inhibitor of an essential host factor (phosphatidylinositol 4-kinase β), PI4KB) for enterovirus infection (Hsu et al., 2010). While both drugs showed little cytotoxicity, they both inhibit viral replication in a dose-dependent manner (Fig. 3A and B). The calculated IC50 for these two inhibitors are 40 μmol/L and 2 nmol/L, respectively, which are consistent with previously reported
values (Pfister and Wimmer, 1999; Rutaganira et al., 2016), suggesting this model is suitable for different types of anti-enterovirus inhibitor screening.

We then proceeded to optimize this assay in 384-well plate for HTS purpose. After determining the optimum cell density and virus titer of infection, we measured the Z-factor of this assay. The Z-factor is a widely used parameter in the evaluation and validation of HTS experiment (Zhang et al., 1999). In general, a Z-factor > 0.5 is considered as a robust assay. We measured the luciferase activity of NanoLuc-CVB5 infected cells in a 384-well plate, in which 192 wells were treated with PI4KIII-β-IN-9 and 192 wells were treated with DMSO. Fig. 3C showed that the dynamic range of this assay is high, and the wide separation of these two data sets indicated that this assay was robust. In two independent experiments, we obtained Z-factors of 0.62 and 0.65, suggesting this assay is suitable for HTS in 384-well plate, and with proper optimization, it is possible that acceptable Z-factor could be achieved in 1536-well format.

3.4. Construction and characterization of the subgenomic CVB5 replicon

In addition to the full-length reporter virus, a subgenomic replicon (SGR) with reporter gene is also widely used in virology studies. In general, the structural genes of viral genome are replaced with reporter gene in the subgenomic replicon system, and therefore, the viral RNA could not package into mature virions. To generate a subgenomic replicon for CVB5, we replaced the structural gene of VP4, VP2 and part of VP3 with Renilla luciferase encoding gene followed by G418 selection marker and the 3Cpro cleavage site (Fig. 4A). Part of VP3 and VP1 genes were kept as a previous report has shown that this region is required for efficient replication of rhinovirus 14, another member of Enterovirus genus (Mcknight and Lemon, 1996). When transcribed RNA was transfected into RD cells, luciferase was monitored over different time points and Fig. 4B showed that luciferase kept increasing until 12 h post-transfection, while the polymerase-dead replicon RNA bearing the
GND mutation in 3D polymerase only showed background signals, suggesting the successful replication of SGR in RD cells. We also tried to obtain RD cells stably harboring the SGR with G418 selection, but this was unsuccessful after several trials, suggesting the replication of this SGR is not strong in RD cells or adaptive mutations are required for stable SGR replication.

SGRs are especially useful to elucidate the role of host factors during viral replication. For this purpose, we generated decay-accelerating factor knock-out (DAF KO) cells with CRISPR/Cas9 or PI4KB knock-down (PI4KB KD) cells with shRNAs. Immunoblotting confirmed that DAF or PI4KB protein expression was inhibited as expected (Fig. 4C) and these cells were unable to support CVB5 infection as demonstrated by viral VP3 expression (Fig. 4C), full-length NanoLuc-CVB5 infection (Fig. 4D) or cell viability assay (Fig. 4E). DAF is an attachment factor for certain coxsackievirus B isolates (Bergelson et al., 1995; Shieh and Bergelson, 2002), while PI4KB is an essential host factor supporting enterovirus replication (Hsu et al., 2010). When these cells were transfected with SGR RNAs, we found that PI4KB KD cells do not support CVB5 replication, while DAF

Fig. 2. Generation and characterization of NanoLuc-CVB5. A Diagram showing the gene structure of the NanoLuc-CVB5. NanoLuc luciferase reporter gene as well as 3C cleavage site was inserted immediately upstream of VP4. B RD cells (1.2 × 10⁵ cells) transfected with 0.5 μg viral RNAs from rCVB5 or NanoLuc-CVB5 were analyzed for luminescence at indicated time points. C RD cells (1.2 × 10⁵ cells) were infected with different passages of reporter virus (MOI = 0.1). Luciferase activity was determined at 24 h.p.i. D qRT-PCR analysis of NanoLuc gene copy numbers from RD cells infected with different passages of reporter virus. E Uninfected or RD cells (1.2 × 10⁵ cells) infected with indicated virus (MOI = 1) were immunostained with anti-dsRNA(J2) antibody at 12 h.p.i. Nuclei were counterstained by DAPI. Scale bar, 10 μm. F RD cells infected with NanoLuc-CVB5 were immunoblotted at indicated time points with anti-VP3 antibody. β-actin was used as a loading control. G RD cells (1.2 × 10⁵ cells) infected with indicated rCVB5 or NanoLuc-CVB5 (MOI = 0.1) were observed for CPE at indicated time points. Scale bar, 100 μm. H Plaque assay of RD cells infected with serial dilutions of NanoLuc-CVB5, RD cells (1.2 × 10⁵ cells) were infected with ten-fold diluted NanoLuc-CVB5 with starting MOI of 0.2. The plaque assay was performed at day 3 post infection. I q-RT-PCR analysis of viral RNA. RD cells (1.2 × 10⁶ cells) infected with rCVB5 or NanoLuc-CVB5 (MOI = 0.1). The viral RNA was quantitated by q-RT-PCR analysis at various time points. Data presented as mean ± SD from three repeat experiments.
KO cells could still support viral replication (Fig. 4F), arguing that PI4KB, but not DAF plays essential role during viral replication. These data indicated that this SGR is useful to validate the role of host factors in viral replication.

3.5. Application of NanoLuc-CVB5 for in vivo imaging

Luciferase, especially firefly luciferase has been widely used for in vivo imaging. However, the ATP and Mg$^{2+}$ requirement of firefly luciferase limit its usage in vivo under certain circumstances (Close et al., 2011). NanoLuc is about 1000× brighter than firefly luciferase and the ATP-independence nature allows it for in vivo monitoring of both intracellular and extracellular events (Stacer et al., 2013). To analyze real-time infection of CVB5 in vivo, we first infected 3-day old mice with either parental CVB5 or NanoLuc-CVB5 at $2 \times 10^{10}$ TCID$_{50}$ per mouse, and determined the survival rate after virus challenge. We found both viruses exhibited similar fatality rate and all mice died within 6 days post-infection (Fig. 5A), suggesting NanoLuc-CVB5 had similar infection efficiency to parental virus in vivo. We next determined the viral loads in different organs, and we found that in this suckling mice infection model, parental CVB5 could replicate in muscle, lung, liver, and intestine at relative high level, while heart and brain showed low virus load (Fig. 5B–D). Similarly, NanoLuc-CVB5 replicates well in muscle, liver, intestine and lung, compared to heart and brain (Fig. 5B–D), suggesting a similar tissue tropism between parental CVB5 and NanoLuc-CVB5. However, the NanoLuc-CVB5 did show slightly lower virus titers compared to parental CVB5, indicative of virus attenuation in vivo due to the introduction of the luciferase reporter.

In order to visualize CVB infection in living animals, we infected mice with NanoLuc-CVB5 and monitored the NanoLuc luciferase signal over time. No signal was detected when mice were inoculated with PBS (Fig. 5E, upper left panels) or parental CVB5 (Fig. 5E, upper right panels), suggesting a very low background of NanoLuc luciferase for in vivo imaging. At 24 h.p.i, strong luciferase signals were observed in the abdominal area (ventral side) and thigh area (dorsal side) (Fig. 5E, lower panels). Luciferase signals with similar distribution pattern were also observed at 48 h.p.i, but not as strong as 24 h.p.i (Fig. 5E, lower panels). These distributions are in general, in agreement with the results demonstrated by TCID$_{50}$ or by qPCR, suggesting this model could be used for observations of viral load and dissemination in infected animals.

4. Discussion

Enterovirus are ubiquitous worldwide and are transmitted mainly through the fecal–oral and respiratory routes following a seasonal pattern (Nekoua et al., 2022). With the control of poliovirus in much of the world, nonpolio enteroviruses, such as coxsackievirus have drawn much attention. Both coxsackievirus A and coxsackievirus B could cause nonspecific febrile illness, rashes, upper respiratory tract disease and aseptic meningitis. However, CVA tends to infect the heart, pleura, pancreas, and liver (Abuelreish and Rathore, 2008). No drug or vaccine has been approved for coxsackievirus infection and therefore, much effort is still needed to elucidate the pathogenesis of coxsackievirus infection and for antiviral development. Here in this study, we established CVB5 reporter viruses with luciferase reporter and thus providing useful tools to study virus-host interactions and for antiviral screening.

Reverse genetics provides a powerful tool to study RNA viruses and it has led to important advances in our understanding of viral gene function and interaction with host cells (Bridgen, 2012). Here we first generate an infectious clone of CVB5 with reverse genetics and showed that this recombinant virus has similar viral infection parameters to
those of parental viruses, indicating this technique could be successfully applied to this virus. We then proceeded to generate a full-length, infectious CVB5 with luciferase reporter gene and showed that the luciferase reporter gene was stably maintained in the viral genome and the infectious CVB5 with luciferase reporter gene and showed that the luciferase activity was determined at 24 h.p.i. with immunoblotting. Right panel: RD cells were stably transduced with control or PI4KB shRNA and infected with CVB5 and infected with NanoLuc-CVB5 (MOI = 0.1). The luciferase activity was determined at 24 h.p.i. E RD cells (1.2 × 10^5) stably transduced with DAF sgRNA or PI4KB shRNA were either uninfected or infected with NanoLuc-CVB5 (MOI = 0.1) and cell viability was determined at 24 h.p.i with Cell Titer Glo. F RD cells (1.2 × 10^5) were stably transduced with DAF sgRNA (left panel) or PI4KB shRNA (right panel) lentivirus and transfected with 0.5 μg CVB5-SGR RNA. The luciferase activity was determined at 12 h post-transfection. Data presented as mean ± SD from three repeat experiments. Statistical significance was determined by Student’s t-test. ns, not significant; ****, P < 0.0001.

Whether this longer uORF could still express itself and how it affects enterovirus infection needs further investigation. However, the incorporation of this short NanoLuc-encoding gene and the high virus titer of this reporter virus still showed great advantage compared to previously reported coxsackievirus or EV71 virus with green fluorescent protein (GFP) (Shang et al., 2013; Deng et al., 2015) or luciferase reporters (Yu et al., 2015; Yang et al., 2021; Yu et al., 2023), which all showed apparent replication inhibition due to the introduction of exogenous reporter genes. Another advantage of this luciferase reporter virus compared to GFP-tagged virus is that luciferase is more suitable for drug screening, especially for HTS assays. We here showed that this NanoLuc-CVB5 could be used for antiviral testing and have optimized this virus for HTS purposes.
In addition, luciferase reporter virus offers an indispensable method for the noninvasive visualization of viral infections in living animals (Song et al., 2022). Here we used this NanoLuc-CVB5 to infect suckling mice and demonstrated that CVB5 could infect muscle, intestine, and liver at high virus titers and brain to a lesser extent, this pattern of infection was in general consistent with previous reports (Myers et al., 2009; Nekoua et al., 2022) and thus proved this is a useful tool to study CVB5 infection in vivo. Of note, the most widely used luciferase for in vivo imaging is firefly luciferase, which produces yellow light (around 560 nm) that can penetrate through several centimeters of tissue and thus suitable for internal organ visualization (Sadikot and Blackwell, 2005; England et al., 2016). On the other hand, NanoLuc has a blue-shifted emission spectrum (around 460 nm), which has limited ability of tissue penetration and therefore is most widely used for shallow tissues in vivo imaging (England et al., 2016; Liu et al., 2021). However, the high brightness, small size and high stability have gained NanoLuc some advantage over firefly or Renilla luciferase (Liu et al., 2021). With directed evolution towards red-shifted emission and novel substrates (Yeh et al., 2017; Su et al., 2020), improved in vivo visualization of enterovirus infection is anticipated.

Finally, we also generated a subgenomic replicon of CVB5 with Renilla luciferase reporter. As part of the structural gene was deleted and the SGR is in vitro transcribed and transfected into host cells and therefore it could bypass the viral entry and viral assembly/release steps, rendering this system especially useful to study viral protein translation and replication (Hannemann, 2020). We employed this system to test the role of DAF and PI4KB during CVB5 infection and demonstrated that this is a useful tool to elucidate the roles of host factors during viral replication.

Fig. 5. In vivo imaging of NanoLuc-CVB5 infected mice. A Survival rate of CVB5 infected mice. 3-Day old mice (n = 7) were inoculated with 2 × 10^10 TCID50 pCVB5 or NanoLuc-CVB5 and monitored for 7 days. B Viral titers from organs of pCVB5 or NanoLuc-CVB5 infected mice were quantified and expressed as TCID50. C Viral RNAs from organs of CVB5 or NanoLuc-CVB5 infected mice were quantified by qRT-PCR and expressed as viral copy number per mg tissue. D Indicated tissues from NanoLuc-CVB5 infected mice were homogenized and subjected to immunoblotting. VP3 expression for CVB5 infection was determined and GAPDH was detected as loading control. E Bioluminescence measurement of mice infected with parental CVB5 (upper right panels) or NanoLuc-CVB5 (lower panels) using furimazine substrate. PBS injected mice was shown as control (upper left panels). Mice infected with virus for 24 and 48 h were injected with furimazine. Four or eight minutes after injection, images were taken with VISQUE™ InVivo Smart-LF. Data presented as mean ± SD from three repeat experiments.
5. Conclusions

In summary, an infectious clone of CVB5 as well as a full-length, infectious virus and subgenomic replicon with luciferase reporter have been established with reverse genetics. Validation of these viral genomes showed that constructions are very successful and inclusion of the luciferase reporter has little effect on viral viability and fitness. These results provide useful tools to study viral-host interactions both in vitro and in vivo and are also useful for antiviral screening.

Data availability

All data generated or analyzed during this study are included in this published article.

Ethics statement

All animal experiments were conducted under protocols approved by the Committee on the Ethics of Animal Experiments of Xi’an Jiaotong University. Specific-pathogen-free ICR mice (Xi’an Jiaotong University animal facility, China) were used to develop an animal model. All institutional guidelines for animal care and use were strictly followed.

Author contributions


Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2023.05.010.

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