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A replication-competent foot-and-mouth disease virus expressing a luciferase reporter

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ABSTRACT

Bioluminescence is a powerful tool in the study of viral infection both *in vivo* and *in vitro*. Foot-and-mouth disease virus (FMDV) has a small RNA genome with a limited tolerance to foreign RNA entities. There has been no success in making a reporter FMDV expressing a luciferase in infected cell culture supernatants. We report here for the first time a replication-competent FMDV encoding Nanoluciferase, named as Nano-FMDV. Nano-FMDV is genetically stable during serial passages in cells and exhibits growth kinetics and plaque morphology similar to its parental virus. There are applications for the use of Nano-FMDV such as real-time monitoring of FMDV replication *in vitro* and *in vivo*.

1. Introduction

Foot-and-mouth disease virus (FMDV) causes foot-and-mouth disease (FMD) in cloven-hoofed farm animals (Grubman and Baxt, 2004). Viral particles have a positive-sense RNA genome of approximately 8500 nt consisting of a 5′ untranslated region (UTR), a single open reading frame (ORF), and a 3′ UTR with a polyadenylic acid [poly(A)] tail. The ORF encodes a polyprotein that is processed by a viral encoded protease into at least 13 proteins; four structural (VP1, VP2, VP3 and VP4) and nine non-structural proteins (leader proteinase (Lpro), 2A, 2B, 3A, 3B1, 3B2, 3B3, 3Cpro and 3Dpol). FMD outbreaks have devastating economic and social impacts (Knight-Jones and Rushton, 2013), however many aspects of the FMDV life cycle, cell tropism and pathogenesis are still not well characterised. There is a need to develop new tools such as a reporter virus that can be used to trace infection *in vitro* and *in vivo* in a real-time manner (Robinson et al., 2016).

FMDV has a diameter of 25 nm (Wild et al., 1969) and a small, highly structured RNA genome, which offers limited packaging capacity and choices of insertion sites for foreign reporter genes. Our early attempts to generate recombinant FMDV expressing green fluorescent protein (GFP) of *Aequorea victoria* or the *Renilla* luciferase protein (RL) of *Renilla reniformis*, which are about 720 nt and 930 nt respectively, were unsuccessful. A panel of truncated GFP gene fragments were subsequently used to show a packaging limit of between 417 nt and 504 nt for the targeted insertion site located between VP1 and 2A in the FMDV genome. Based upon this finding we successfully constructed a viable reporter FMDV expressing a fluorescence protein, termed iLOV (Seago et al., 2013; Chapman et al., 2008). Although iLOV-FMDV has particular application for monitoring FMDV infection in real-time with microscopy, emitted levels of fluorescence are relatively faint and an alternative FMDV reporter is required to develop quantitative assays (Seago et al., 2013).

Recently Promega has developed a new luminescent reporter, namely Nanoluciferase, NanoLuc® (NLuc), which is smaller and brighter than other luciferases available (Hall et al., 2012a). This prompted us to construct a recombinant FMDV expressing NLuc so that we can make use of the powerful bioluminescence technique in various fields of FMDV study. We report here that we have rescued an infectious FMDV expressing NLuc and investigated its application to monitor infection *in vitro*.

2. Material and methods

2.1. Cell lines

Cells were maintained at 37 °C in 5% CO_2. IB-RS2 cells were cultivated in Glasgow's modified Eagle's medium (GMEM, Sigma) with 10% foetal calf serum (FCS), BHK-21 cells in GMEM with 10% FCS and 5% tryptose phosphate broth, and ZZ-R 127 cells in Dulbecco's modified Eagle's medium/Ham's F12 (Sigma) with 10% FCS. All media

Abbreviations: FMDV, foot and-mouth disease virus; qRT-PCR, quantitative reverse transcription-PCR; RLU, relative light unit; hpi, hours post infection; pfu, plaque forming unit; MOI, multiplicity of infection; GFP, green fluorescent protein

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were supplemented with 100 SI units/ml penicillin and 100 μg/ml streptomycin (all from Sigma).

2.2. Construction of infectious copy plasmids

New recombinant viruses were generated from a previously described (Seago et al., 2012) infectious copy plasmid, herein termed AvrII-O1K/OUKG. In brief, AvrII-O1K/OUKG encodes the VP2, VP3, VP1 and the 2A proteins of FMDV O UKG/35/2001 and the Lpro, VP4, 2B, 2C, 3A, 3B, 3C and 3D proteins of FMDV O1K. The AvrII-O1K/OUKG genome contains a unique Avr II site between VP1 and 2A for insertion of exogenous cDNAs that encode reporter proteins (Botner et al., 2011). In this study, one targeted amino acid mutation (VP3 56 His to Arg) was introduced into the AvrII-O1K/OUKG infectious clone using the QuikChange Lightning Mutagenesis kit (Agilent Technologies), producing a new parental infectious copy plasmid termed AvrII-O1K/OUKG-$_{HS+}$. This targeted mutation enabled the use of heparan sulphate for viral entry and thus virus infection of BHK-21 cells (Fry et al., 2005; Jackson et al., 1996). The Avr II sites of both AvrII-O1K/OUKG and AvrII-O1K/OUKG-$_{HS+}$ were then used for insertion of DNA encoding the NanoLuc open reading frame, which was PCR amplified from plasmid pNL1.1 (Promega) using the following primer pair: forward/5′-*AGG*CTCTAGAATGGTCTTCACACTCGAAGA-3′ and reverse/5′-*ATA*CCTAGGCGCCAGAATGCGTTCGCAC-3′ (clamp bases in italics). Xba I (TCTAGA) and Avr II (CCTAGG) sites (underlined in primers) were engineered to the 5′ and 3′ ends of the amplicon to facilitate cloning and screening. The resultant infectious copy plasmids were termed Nano-FMDV and Nano-FMDV-$_{HS+}$, respectively. Construction of infectious copy plasmids and all virus work was performed at a SAPO4 level of biocontainment.

2.3. In vitro RNA transcription and transfection

RNA was transcribed from infectious copy plasmids using the MEGAscript T7 kit (Ambion). After RNA synthesis was complete, the *in vitro* transcription reactions were treated with 1 μl of RNase-free DNase (Ambion) at 37 °C for 15 min to degrade the DNA templates and the RNA was purified using the MEGAclear kit (Ambion). The TransIT-mRNA Kit (Mirus Bio Corporation) was used to deliver infectious RNA into BHK-21 cells. After 24–48 h, the cells were freeze-thawed to release the initial virus stock, designated as passage 0 (P0). ZZ-R 127 cells (Brehm et al., 2009) were used for the subsequent passages to optimise the rescue of recombinant virus and maximise virus yields.

2.4. Luciferase assay

The Nano-Glo® Luciferase Assay System (Promega) was used according to the manufacturer's instructions. Briefly, 10 μl of infected supernatant was mixed with an equal volume of assay buffer containing the Nano-Glo substrate in a white 96-well plate (Nunc, Thermo Scientific) and incubated at room temperature for 10 min. The plate was then read in a Synergy 2 plate reader (BioTek) and light signal determined as Log_{10} relative light units (RLU)/10 μl.

2.5. Plaque assay

IB-RS2, BHK-21 or ZZ-R 127 cells were plated in 6 or 24-well plates to produce confluent cell monolayers the following day. Cells were infected by incubation with ten fold dilutions (made in 1 X PBS) of P3 virus stocks for 1 h at 37° C and then overlaid with medium containing 0.6% UltraPure LMP Agarose (Invitrogen). Cells were fixed and stained after 24 h with a solution containing 4% formaldehyde and crystal violet or methylene blue before removal of the overlay. Viral titres were determined as Log_{10} plaque forming units (PFU)/ml.

2.6. Virus growth curve

ZZ-R 127 cells grown in 6-well plates were infected with the respective FMDV stocks (P3) at a multiplicity of infection (MOI) of 0.001 by incubation for 1 h at 37° C. Inoculum was then discarded and the monolayers were washed with PBS and 2-morpholinoethanesulfonic acid (MES) buffered saline (25 mM MES [pH 5.5], 145 mM NaCl) to remove non-adsorbed virus and Nanoluciferase carried over from Nano-FMDV-$_{HS+}$ stock. The cells were maintained in 3 ml of medium containing 1% FBS and 100 μl samples of supernatant were taken at 2, 4, 6, 8, 16, and 24 h post infection (hpi) for analysis. The growth kinetics for the Nano-FMDV-$_{HS+}$ was determined by both plaque assay and luciferase assay. Three experimental repeats were performed.

2.7. Inhibition of FMDV replication by guanidine hydrochloride

Guanidine hydrochloride (Sigma) treated ZZ-R 127 cells grown in 24-well plates were infected with AvrII-O1K/OUKG-$_{HS+}$ parental virus or Nano-FMDV-$_{HS+}$ at a MOI of 2 by incubation for 1 h at 37° C. Following infection, cells were washed with PBS and MES buffer to remove residual free viral particles and background luciferase, then incubated at 37° C for different periods of time in fresh medium supplemented with 1% FCS and guanidine hydrochloride at a final concentration of 0, 0.004, 0.04, 0.4 or 4 mM. At 7 and 24 hpi, supernatant samples were analysed by luciferase assay and real-time RT-PCR to measure viral yields. Experiments were performed in triplicate.

2.8. Virus neutralization test

Micro-neutralisation tests were performed according to the protocol recommended by the World Organisation for Animal Health (Office International des Epizooties (OIE)) (OIE, 2012; Golding et al., 1976). Both AvrII-O1K/OUKG-$_{HS+}$ parental virus and Nano-FMDV-$_{HS+}$ were tested in parallel on IB-RS2 cells against a panel of five sera, comprised of post-vaccination bovine anti-FMDV O1Manisa serum (positive control (Serum 1)), guinea pig anti-FMDV O1Manisa serum (Serum 2), guinea pig anti-FMDV O/Tur5/09 virus like particles (VLP) serum (Serum 3), mouse anti-FMDV O/Tur5/09 serum (Serum 4) and mouse anti-FMDV SAT2 serum (negative control (Serum 5)). Sera were inactivated at 56 °C for 30 min before use. Neat serum stocks were initially diluted 1:8 and then in two-fold dilutions for the tests (1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048). For each test a 100 TCID50 of virus was used in a total volume of 50 μl. Neutralizing antibody titres, calculated by the Spearmann-Karber method (Kärber, 1931), were expressed as the last dilution of serum that neutralizes 50% of the virus. In addition, the luminescent signal for each sample was determined to investigate if bioilluminesence correlated with the neutralising titres of tested sera.

2.9. Viral RNA extraction, conventional and real-time RT-PCR

Viral RNA was extracted using the MagVet™ Universal Isolation Kit (Thermo Fisher Scientific) on a KingFisher™ Flex Robot (Life Technologies). Following RNA extraction, cDNA was generated by using the TaqMan RT reagents Kit (Thermo Fisher Scientific) and hexamer random primers. Platinum HiFi Taq DNA Polymerase (Invitrogen) and the following primer pair were used to amplify the region of viral genome containing the inserted Nluc gene and to provide template for sequencing: forward/5′-CCACTCGGGTGACTGAACTGC-3′ and reverse/5′-TGTCCTCGAGTGATGCCATG-3′.

One-step Callahan 3D quantitative real-time RT-PCR was performed according to the standard protocol of the World Reference Laboratory for FMDV (King et al., 2006).

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