Journal of Virological Methods 189 (2013) 101–104

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

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

Short communication

Construction of a bovine enterovirus-based vector expressing a foot-and-mouth disease virus epitope

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Article history:
Received 14 March 2012
Received in revised form 15 January 2013
Accepted 28 January 2013
Available online 4 February 2013

Keywords:
Bovine enterovirus
Viral vector
Foot-and-mouth disease
Epitope

A B S T R A C T

A recombinant infectious bovine enterovirus (BEV) vector was constructed to express a foot-and-mouth disease virus (FMDV) capsid protein (VP1) epitope. Sequences encoding the VP1 epitope (amino acid residues 141–160) of FMDV (vaccine strain O1/Manisa/Turkey/69) were inserted into pBLUBEV at the VP1/2A junction. The growth characteristics of the parental virus and viruses derived from recombinant plasmids (pBLUBEV, pBLUBEV-Manisa-epi) were determined by plaque assay and one-step growth curve analysis. There were no significant differences in the growth kinetics and plaque morphologies between transfectant viruses and their parental virus. The expressed VP1 epitope was detected successfully by using indirect immunofluorescence assay with a polyclonal antibody against the FMDV VP1 epitope from Madin Darby bovine kidney (MDBK) cells infected with BEV-Manisa-epi transfectant virus. This study demonstrated a novel alternative live viral vector that may be utilized as a candidate vaccine vector for veterinary applications.

Bovine enterovirus (BEV) is a picornavirus of the genus *Enterovirus*. BEVs were originally classified into two serotypes, BEV-1 and BEV-2 (Knowles and Barnett, 1985), but recently it has been proposed that BEVs could be divided into two distinct species as BEV-A and BEV-B (Zell et al., 2006). The pathogenesis of BEV is unclear, but it appears to produce no or only mild clinical signs (Kalter, 1960). Consequently, BEV has not been well studied until recently, when it was proposed that its mild pathogenesis could make it useful as a vaccine vector to immunize against bovine diseases (Hoey, 1984; Zhang and Burgess, 1990). BEV has several characteristics that make it attractive as a good candidate for a vaccine vector. Initially, resistance of BEV in the treatment at pH 3 and protection from inactivation at 56 °C were used to distinguish enteroviruses from other picornaviruses (Afshar et al., 1964; Durham and Burgess, 1979; Spradbrow, 1964).

Viral vectors are a tool commonly used by scientists to deliver genetic material into cells. Currently, a wide range of virus families is under intensive development as vaccine vectors for either human or veterinary use, including some virus families that are replication competent, but also many that are specifically attenuated (Draper and Heeney, 2009). One of the most important indexes for the evaluation of a viral vector is its capacity to accept exogenous sequences. Therefore, it is essential to know the maximum size limit of the inserted sequence that a virus can stably accept without disrupting the virion or decreasing gene expression. Viruses whose genome can accept larger sequences may be more suitable for modification, as they may be engineered to contain the sequence of more than one antigen.

Foot-and-mouth disease (FMD) virus (FMDV) is a highly contagious picornavirus (genus *Aphthovirus*) that causes a widespread and economically damaging disease of cattle and other cloven-hoofed animals. It has been reported that residues 141–160 and 200–213 of VP1 are the major immunogenic regions that provide protective immunity in experimental animals (Bittle et al., 1982). Several candidate vaccines have been developed containing either chemically synthesized peptides or biosynthetic proteins fused with the VP1 epitope (Kit et al., 1991; Wong et al., 2000). Although promising, the candidate FMD vaccines developed to date have not been made commercially available, either because the vaccines were insufficiently immunogenic or because the yields were poor for commercial purposes.

Potential insertion sites within the BEV genome were determined on the basis of alignments of sequences and extrapolation of strain VG527 structural motifs (Smyth et al., 1995). The major immunogenic sites of BEV and FMDV were located in the VP1 region (Smyth et al., 1990, 1992). Consequently, this position would be an excellent target for the insertion or replacement of a foreign gene. Unlike FMDV, the BEV proteinase 2A cleaves its own N-terminus.

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http://dx.doi.org/10.1016/j.jviromet.2013.01.010

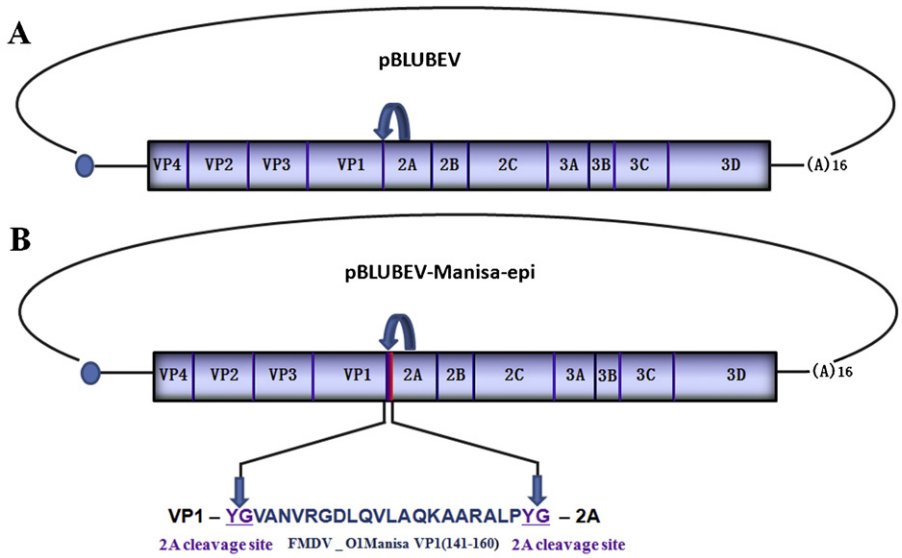

Fig. 1. (A) Schematic representation of bovine enterovirus (BEV) full infectious clone. (B) Strategy for the insertion of O1/Manisa/Turkey/69 VP1 epitope (141–160) into the VP1/2A site of the BEV viral vector. The 2A cleavage site (Y/G) is underlined.

In this way, the P1 (capsid proteins) and P2 (replicative proteins) regions of the polyprotein are separated by processing at the 1D/2A site. Therefore, in this study, the 1D/2A site has been chosen as the insertion site for the VP1 epitope (141–160) of the FMDV vaccine strain O1/Manisa/Turkey/69.

Viral RNA was extracted from a viral suspension of BEV type 1 strain LCR4 (ATCC® Number: VR-248™) using the RNeasy kit (QIAGEN, Hilden, Germany). The first-strand cDNA was synthesized with superscript III reverse transcriptase (Invitrogen, Grand Island, NY, USA). The full length of the viral genome was amplified using primers that were flanked with the restriction enzyme site *Sal*I in the forward primer and *Sac* I in the reverse primer (forward: 5′-GCG ATA GTC GAC TTA AAA CAG CCT GGG GGT TGT A-3′; reverse: 5′-GCC GAG CTC TTT TTT TTT TTT TTT TAC ACC CCA TCC GGT GGG TGT-3′). PCR amplification was performed with Phusion® High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Vantaa, Finland) according to the manufacturer's instructions. The PCR product was cloned into a pBluescript II SK (+) vector (Fermentas, Glen Burnie, MD, USA). The redundant sequence between T7 promoter and BEV 5′UTR was deleted using a KOD-Plus-Mutagenesis Kit (TOYOBO, Osaka, Japan). The resulting plasmid was named as pBLUBEV and confirmed by restriction analysis and sequencing (Fig. 1A).

The sequence encoding the VP1 epitope (amino acid residues 141–160) of FMDV (vaccine strain O1/Manisa/Turkey/69) was inserted into pBLUBEV at the VP1/2A junction using the KOD-Plus-Mutagenesis Kit. The cleavage site Y/G was added to both the N′-terminus and the C′-terminus of the FMDV epitope. The resulting plasmid was designated as pBLUBEV-Manisa-epi, and a schematic representation was shown in Fig. 1B.

Plasmids pBLUBEV and pBLUBEV-Manisa-epi were linearized with *Sac*I and purified with a QIAquick PCR purification kit (QIAGEN, Hilden, Germany). One day before DNA transfection, baby hamster kidney cells that stably expressing T7 RNA polymerase (BHK/T7-9 cells) (Ito et al., 2003) were seeded on a 48-well plate. At 80–90% confluency, the cells were transfected with the linearized DNA using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. At 2 days post-transfection, the cells were freeze-thaw treated and blind passaged twice on fresh MDBK monolayer cells. Viral particles were released by three successive cycles of freezing and thawing, and the viral titers were determined. Monolayers of MDBK cells were infected with rescued viruses and parental virus diluted serially in 6-well tissue culture plates for 1 h, respectively. After removing the unabsorbed virus, the cells were overlaid with 2 ml 1.5% SeaPlaque® agarose (Lonza, Rockland, ME, USA) containing 2% FBS in DMEM and cultured at 37 °C in 5% CO_2 for 3 days. The plaques were visualized by staining with a 0.1% crystal violet solution (Fig. 2A). The recovered virus had plaque morphology similar to that of the parental virus. To compare further the growth characteristics of the parental virus and the viruses derived from recombinant plasmids, the growth kinetics of these viruses were examined. There was no significant difference between transfectant viruses and the parental virus (Fig. 2B). Western blot analysis was performed to confirm that the epitope was cleaved successfully from the polyprotein. In brief, viral supernatant was loaded on a 15% polyacrylamide gel, transferred to polyvinylidene difluoride membranes and then blocked with 5% skim milk in Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) containing 0.1% Tween 20 (TBST). The membrane was washed three times with TBST and then incubated with diluted (1:500) homemade mouse antibody against the epitope (amino acid residue 141–160) of FMDV O1/Manisa/Turkey/69 for 2 h at room temperature. Unreacted antibody was washed out with TBST, followed by incubation with goat anti-mouse IgG-peroxidase conjugate (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:10,000 in 5% skim milk–TBST. Peroxidase activity was detected using an enhanced chemiluminescence Western blotting detection system (Amersham-Pharmacia, Freiburg, Germany) (Fig. 2C).

For detection of the inserted epitopes, indirect immunofluorescence assay was routinely performed. Typically, a total of 0.5×10^5 MDBK cells were seeded onto 12 mm diameter coverslips until the cells reached approximately 80% confluence. The culture medium was removed, and the cells were washed three times with phosphate buffered saline (PBS) (pH 7.4). The cells were then infected with 2 MOI of the O1/Manisa/Turkey/69 FMDV strain or cloned viruses. Incubation was stopped at 6 h post-infection, and the cells were fixed with cold acetone for 5 min. The fixed cells were washed with PBS and incubated at room temperature for 1 h with a polyclonal antibody (an immune serum from rabbit, 1:500 dilution) against the FMDV VP1 epitope. Coverslips were washed 3 times with PBS containing 0.1% Triton X-100 (PBST) and then incubated with goat anti-rabbit IgG conjugated with fluorescent isothiocyanate (KPL, Gaithersburg, MD, USA) at room temperature for 2 h (1:200 dilution). The coverslips were washed with PBS, mounted