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ORIGINAL ARTICLE

Rescue and characterization of a recombinant HY12 bovine enterovirus carrying a foreign HA epitope in the 3A nonstructural protein

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Abstract
Full-length infectious cDNA clones for recombinant HY12 bovine enteroviruses designated as rHY12-3A-2-HA, rHY12-3A-3-HA, and rHY12-3A-9-HA were constructed by the insertion of an epitope from influenza virus hemagglutinin (HA) at the N-terminus of the HY12-encoded 3A protein at amino acid positions 2, 3, and 9. The recombinant HY12 viruses expressing the HA epitope were rescued and characterized using immunoperoxidase monolayer assay, western blotting, and electron microscopy. The three rescued recombinant marker viruses showed similar characteristics, such as $TCID_{50}$ titer, plaque size, and growth properties, to those of parental rHY12 virus. Comparative analysis of the nucleotide sequences demonstrated the three recombinant marker viruses remained stable for 15 passages with no genetic changes. The recombinant viruses remained viable in various permissive cell lines, including BHK-21, Vero, and PK15 cells, suggesting that the insertion of the HA epitope tag had no effect on virus infectivity. Mice infected with the recombinant marker viruses and the parental virus produced anti-HY12-virus antibodies, while the recombinant marker viruses also produced anti-HA-epitope-tag antibodies. Taken together, these results demonstrate that HY12 viruses containing genetic markers may be useful tools for future investigations of the mechanisms of viral pathogenesis and virus replication, as well as for vaccine development.

Introduction

The genus *Enterovirus* in the family *Picornaviridae* consists of 12 species of enteroviruses (*Enterovirus A, B, C, D, E, F, G, H, I, J, K*, and *L*) and three species of rhinoviruses (*Rhinovirus A, B*, and *C*) [34]. Enteroviruses contribute to digestive and respiratory diseases in humans and animals. Members of the enterovirus species *A-D* are primarily responsible for human diseases such as poliomyelitis, coxsackievirus syndrome, and hand, foot, and mouth disease. Members of enterovirus species *E* and *F*, including bovine enterovirus (BEV), cause infections (BEV) infection, whose clinical signs are characterized by digestive, respiratory, and reproductive disorders [1, 4, 14, 22, 36]. However, some BEV infections cause no clinical signs, and the strains causing these infections are generally considered avirulent and can potentially be used as viral vectors [17].

Enteroviruses are single-stranded, positive-sense, non-enveloped RNA viruses with icosahedral symmetry and a diameter of 27-30 nm. The full-length viral genome is approximately 7,400 nucleotides in length and contains a single open reading frame (ORF) that encodes a large polyprotein. The polyprotein is cleaved into four structural proteins and seven non-structural proteins. The N-terminus of the polyprotein (P1) is processed to form the viral capsid proteins VP1, VP2, VP3, and VP4, which are responsible for viral entry, encapsidation, and assembly of infectious particles [12]. The remainder of the polyprotein (P2 and P3) generates seven non-structural proteins, 2A, 2B, 2C, 3A, 3B,

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3C, and 3D, which are involved in viral translation and replication, disruption and/or reorganization of numerous cellular processes, and activities that restrict or combat viral replication [6, 8, 13, 20, 21, 23, 28, 31]. The proteins located in the P3 region are most directly involved in RNA synthesis, including two relatively stable precursor proteins 3CD and 3AB, which specifically interact with a cloverleaf-like structure at the 5 end of the viral RNA and participate in RNA genome replication [12]. The precursors are slowly processed to generate the mature polypeptides 3A, 3B (VPg), $3C^{pro}$, and $3D^{pol}$ [24]. The 3A protein is a small protein of 87 amino acids (aa). Changes in the 3A protein of enteroviruses [15] and rhinoviruses [11], such as point mutations or deletions, have been associated with altered host cells adaptation or virulence. A highly conserved hydrophobic domain of 22 residues in the C-terminal domain of the 3A protein is believed to be responsible for association of viral proteins with host membranes and is important for enterovirus replication. However, the biological significance of N-terminal domain of the protein and the biological characteristics of the BEV-encoded 3A protein remain largely unknown [3, 6, 32, 33]. In 2011, Teterina et al. inserted three different epitope tags (FLAG, HA, and c-myc) into the N-terminal region of protein 3A in a poliovirus infectious clone and found the resulting viruses exhibited growth properties and characteristics similar to those of the parental virus [31]. In 2013, Chu et al. reported the construction of a recombinant infectious BEV clone by insertion of a foot-and-mouth disease virus (FMDV) VP1 epitope (amino acid residues 141–160) at the VP1/2A junction of BEV, which had no effect on growth kinetics or plaque morphology when compared to the parental virus [2]. Teterina et al. [29] generated a full-length poliovirus cDNA clone with a red fluorescent protein (DsRed) inserted at a position following amino acid residue 144 in protein 2A, from which an infectious particle was rescued, and the progeny virus was stable for three to four passages. Moreover, Shang et al. (2013) generated a cDNA infectious clone by inserting the eGFP gene between the 5'-untranslated region and the VP4 gene of the EV71 genome. A high titer ($> 10^6$ PFU/ml) of the reporter viruses was rescued in Vero cells transfected with cDNA-derived RNA. The resulting reporter viruses remained stable for at least five passages in Vero cells, thus demonstrating the stability of the recombinant virus [26]. Although much progress has been made in construction of recombinant and chimeric human enteroviruses such as poliovirus and EV-71 virus, techniques for making recombinant BEV remain largely unexplored. Previously, we demonstrated that enterovirus has a unique secondary structure in its 5'-untranslated region and can be grown to a $TCID_{50}$ titer of 10^{9-10}/ml [35], suggesting its potential for application as a live viral vector to express exogenous tags. In an earlier study, we identified an insertion site for an influenza virus hemagglutinin (HA) epitope in viral structural protein VP1 [19]. Here, we further explore the potential of HY12 virus as a viral vector to express an exogenous tag by inserting an HA epitope at the N-terminal end of viral nonstructural protein 3A. Our findings demonstrate that the insertion of the HA epitope at the N-terminus of the HY12-encoded 3A protein does not affect HY12 replication or biological characteristics of the three rescued recombinant marker viruses, thus providing an experimental basis for the development of genetically tagged BEV recombinants, which may be used for future investigation of viral pathogenesis and virus replication mechanisms.

Materials and methods

Cells, viruses and antibody

Madin-Darby bovine kidney (MDBK) cells, baby hamster kidney cells (BHK-21), African green monkey kidney cells (Vero) and porcine kidney cells (PK15) were cultured in Dulbecco's modified Eagle's Medium (DMEM, Life Technologies, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. HY12 enterovirus (EV-E) was isolated and kept in our laboratory [36]. Mouse anti-HY12-VP2 monoclonal antibody (mAb) 3H4 was prepared in our laboratory [19]. Mouse anti-HA mAb, rabbit anti-HA polyclonal antibody (PcAb), horseradish peroxidase (HRP)-conjugated rabbit anti-mouse secondary antibody, and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). Alexa Fluor™ 633–conjugated goat anti-rabbit secondary IgG antibody and mouse anti-β mAb were purchased from Invitrogen (Carlsbad, CA, USA). DyLight™ 680-labeled goat anti-mouse IgG (H+L) was purchased from KPL (Gaithersburg, MD, USA).

RNA extraction and cDNA synthesis

Viral RNA was extracted from MDBK cells infected either with wild-type (wt) HY12 virus or recombinant marker viruses (rHY12-3A-2-HA, rHY12-3A-3-HA and rHY12-3A-9-HA) and rescued parental HY12 (rHY12) using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). The resulting RNAs were digested with DNase to remove potential DNA contamination. cDNA synthesis was performed using oligo(dT) primers and ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) according to the manufacturer's protocol.

Construction of infectious cDNA clones of the recombinant marker viruses

Full-length HA-epitope-tagged HY12 infectious cDNA clones were generated by insertion of the oligonucleotide sequence of the HA epitope in frame in the HY12-encoded 3A sequence at amino acid (aa) positions 2, 3, and 9 following the strategy shown in Fig. 1. The synthesized cDNA was used as a template for subsequent PCR amplification. Two fragments, fragment A and fragment B, encompassing nucleotides 1–4673 and 4562–7415, respectively, were amplified using a KOD-plus Kit (Toyobo), using two primer pairs (A1-F/R, B1-F/R) that were designed based on the genomic sequence of HY12 virus (GenBank accession no. KF748290) (Table 1), and cloned into the pBluescript II SK(+) vector to generate the full-length cDNA clone (pBlu-HY12) (Fig. 1B and C). The HY12-3A gene was amplified using primers B1-F/R6138 (Table 1) and ligated into the pMD18-T vector to generate pMD18-3A, which was used as a template for mutagenic PCR. The HA epitope sequence was introduced into pMD18-3A to generate three marker plasmids using primer pairs F1-HA/R1-HA, F2-HA/R2-HA, and F3-HA/R3-HA (Table 1). The recombinant marker plasmids pBlu-HY12-3A-2-HA, pBlu-HY12-3A-3-HA, and pBlu-HY12-3A-9-HA were generated by replacing the fragment spanning the nucleotide sequence from base pairs 4562 to 6138 in pBlu-HY12 with the corresponding PCR-amplified fragments from the above marker plasmids, which contained the HA epitope sequence (Fig. 1D and E). All recombinant plasmids were confirmed by sequencing.

Transfection and rescue of the recombinant marker viruses

Transfection and rescue of the recombinant marker viruses were performed according to the manufacturer's instructions (Invitrogen). Briefly, the linearized pBlu-HY12-3A-2-HA, pBlu-HY12-3A-3-HA, and pBlu-HY12-3A-9-HA plasmids were co-transfected with the plasmid expressing T7 RNA polymerase (pCI-T7), using Lipofectamine^{TM} 3000 (Invitrogen, Carlsbad, CA, USA). MDBK cells were transfected with the linearized parental rHY12 plasmid and empty plasmid as positive and negative controls, respectively. After incubation for 5 h at 37°C, the medium was replaced with DMEM containing 5% FBS until a cytopathic effect (CPE) was visible. The rescued viruses, designated rHY12-3A-2-HA, rHY12-3A-3-HA, rHY12-3A-9-HA, and rHY12, were harvested after three passages in MDBK cells and were stored at −80°C for subsequent experiments.

Preliminary identification of the recombinant HY12 marker viruses

The rescued recombinant HY12 marker viruses (third passage) were initially identified by PCR amplification using the primer pairs 3A-JD-F and 3A-JD-R (Table 1). The 150-bp amplified fragments were confirmed by sequencing. Sequence analysis was performed using DNAMAN7 software (LynnonBiosoft, USA).

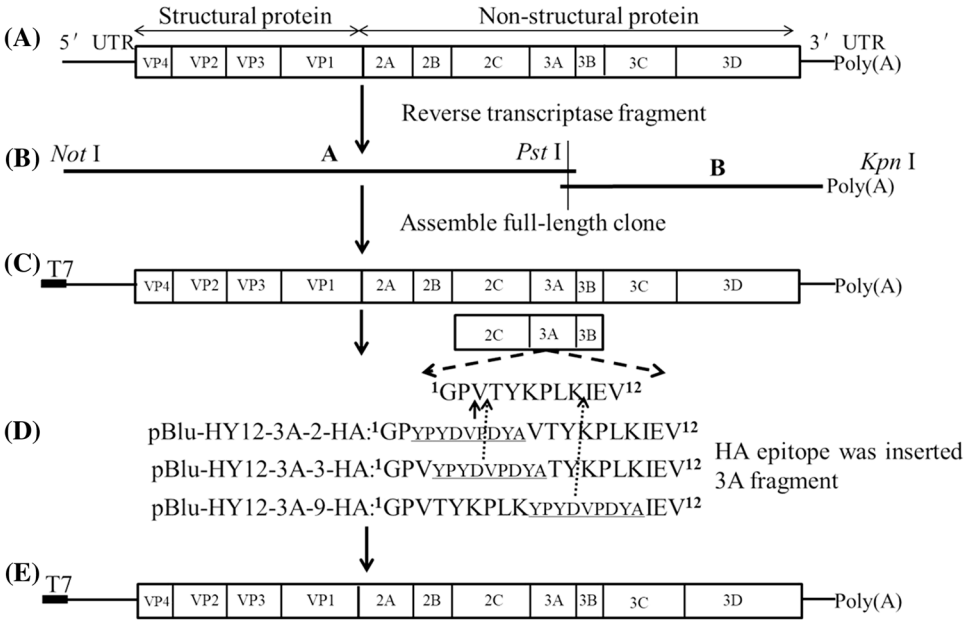

Fig. 1 Schematic diagram for the construction of full-length infectious cDNA clones of the three recombinant marker viruses. (A) Structure of the viral genome and its encoded proteins as well as the 5' and 3' untranslated regions (UTRs). (B, C) Construction of the cDNA clone of the full-length genome, using three unique restriction sites. The T7 promoter sequence is indicated by a solid black rectangle before the 5'-UTR. (D, E) Insertion of the HA epitope at amino acid positions 2-3, 3-4, and 9-10 at the N-terminus of the 3A protein to construct infectious recombinant cDNA clones. The insertion sites are marked by ↑↑. The amino acid sequence of the HA epitope is underlined

Table 1 Primers used for the construction and identification of the recombinant marker viruses

Primer	Sequence[a] (5'→3')
A1-F	S: ATAAGAATGCGGCCGCTTAAAACAGCCTGGGGGTTGTAC
A1-R	AS: GTGGAGACCATTTGACAAAACAGGG
B1-F	S: CTGATCCAGATCATTTTGACGGCTA
B1-R	AS: CGGGGTACCTTTTTTTTTTTTTTTTTACACCCCATCCGGTGGGTGTA GGGTGTA
R6138	AS: GAACAGGGCTTCTTCAAAGTTAGTT
F1-HA	S: GCTCTTTTTCAAGGCCCCTACCCATACGATGTTCAGATTACGCTCCCGT AACCTACAAACCC
R1-HA	AS: GGGTTTGTAGGTTACGGGAGCGTAATCTGGAACATCGTATGGGTAGG GGCCTGAAAAAGAGC
F1-HA	S: CTTTTTCAAGGCCCCGTATACCCATACGATGTTCCAGATTACGCTGTA ACCTACAAACCCTTG
R2-HA	AS: CAAGGGTTTGTAGGTTACAGCGTAATCTGGAACATCGTATGGGTAT ACGGGGCCTTGAAAAAG
F3-HA	S: ACCTACAAACCCTTGAAGTACCCATACGATGTTCCAGATTACGCTAAG ATTGAAGTCATTGAG
R3-HA	AS: CTCAATGACTTCAATCTTAGCGTAATCTGGAACATCGTATGGGTACTT CAAGGGTTTGTAGGT
3A-JD-F	S: TGGTAACGTTCTCGAGGCTCTTTTTCAAG
3A-JD-R	AS: TGCCTGTAGTAGATCGCTGATGGCTGAGGG

[a]Nucleotides corresponding to the HA epitope tag are underlined

S, sense; AS, antisense

Detection of the recombinant marker viruses by IPMA

Rescued recombinant marker viruses were detected using a modified immunoperoxidase monolayer assay (IPMA) as described previously [18]. Briefly, MDBK cells were infected with the tenth passage of rHY12-3A-2-HA, rHY12-3A-3-HA, and rHY12-3A-9-HA or parental rHY12 virus at a multiplicity of infection (MOI) of 10. Eight hours after infection, the cells were washed three times with phosphate-buffered saline (0.01 M PBS, pH 7.4), fixed with ice-cold methanol for 20 min at −20°C, and incubated with mouse anti-HA mAb (1:1000, Sigma) or anti-HY12-VP2 mAb 3H4 (1:300) for 1 h at 37°C before they were probed with HRP-conjugated rabbit anti-mouse IgG antibody (1:500, Sigma) for 40 min at 37°C. The cells were then stained with 3-amino-9-ethylcarbazole (Amresco, Olympia, WA, USA) substrate and visualized under a light microscope (Nikon, Tokyo, Japan). Mock-infected cells were used as a negative control.

Detection of the recombinant marker viruses by western blotting

Western blotting was performed to detect the rescued recombinant marker viruses according to a standard procedure. Briefly, MDBK cells were infected with the tenth passage of the recombinant marker viruses. After 8 h of infection, an equivalent amount of protein was loaded and separated by 12% SDS-PAGE. The proteins were then transferred to nitrocellulose filter membranes, and the blots were blocked with 5% skim milk in PBS for 30 min at room temperature, probed with mouse anti-HA mAb (1:5000, Sigma), anti-HY12-VP2 mAb (1:300) or anti-β-actin (1:5000, Invitrogen) for 1 h at room temperature, and then incubated with goat anti-mouse IgG (H+L) (1:10,000, KPL) for 1 h. The blot was then washed three times with PBS containing 0.05% Tween 20 (PBS-T), and the signal was captured using an Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE, USA).

Electron microscopy

The rescued recombinant marker viruses were examined by electron microscopy using a routine procedure. The cells infected with the rescued recombinant marker viruses or parental virus were frozen and thawed three times and then centrifuged at 8000 × *g* for 30 min. The collected supernatants were added to a 15% sucrose (Amresco) solution and centrifuged at 36,000 × *g* for 3 h. The resulting pellet was dissolved in PBS containing 12% CsCl (Amresco) and centrifuged at 40,000 × *g* for 10 h. The liquid layer containing viruses was extracted using plat syringe (100 mm) and stained with 3% phosphotungstic acid, pH 7.2. The viral particles were examined and imaged using an electron microscope (Hitachi H-7650, Hitachi, Tokyo, Japan).

Virus propagation dynamics and plague morphology observations

The propagation dynamics of the rHY12-3A-2-HA, rHY12-3A-3-HA, and rHY12-3A-9-HA marker viruses were examined in MDBK cells. Briefly, MDBK cells in 96-well plates were infected at an MOI of 1 with the tenth passage of the recombinant marker viruses or parental rHY12 virus and were incubated at 37°C in 5% CO_2. After adsorption for 1 h, the cells were washed with PBS, and DMEM containing 2% FBS was added to the wells. Infected cells were harvested at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 h postinfection and then frozen and thawed three times, after which the virus titers were determined using the $TCID_{50}$ assay. Each sample was assayed in triplicate.

Plaque assays were performed in a six-well plate. Briefly, confluent MDBK cells were incubated with serial tenfold dilutions of rHY12-3A-2-HA, rHY12-3A-3-HA, and rHY12-3A-9-HA or parental rHY12 viral stocks for 1 h at 37°C and were covered with 1.25% low-melting-point agarose for a further 24 to 48 h at 37°C. Cells were then fixed with 0.4% paraformaldehyde and stained with crystal violet prior to the removal of the overlay agarose. The assay was repeated at least three times for each rescued recombinant HY12 virus.

Determination of the stability of the recombinant marker viruses

The stability of the rescued recombinant marker viruses, rHY12-3A-2-HA, rHY12-3A-3-HA, and rHY12-3A-9-HA was determined by plaque assay, PCR, and sequencing. After plaque assay, 50 colonies for 5^{th}, 10^{th} and 15^{th} passages of the three recombinant marker viruses and parental HY12 virus were selected to infect MDBK cells. RNAs were extracted and treated with DNase to remove potential plasmid DNA contamination. cDNA was synthesized and used as template for PCR amplification using the primers 3A-JD-F and 3A-JD-R (Table 1). The resulting PCR-amplified fragments were sequenced to confirm the corresponding HA epitope in the rescued viruses.

Confocal microscopy

MDBK cells infected with the tenth passage of the three recombinant marker viruses or parental rHY12 virus at an MOI of 0.1 were fixed separately at 2, 8, and 16 h postinfection with 4% paraformaldehyde in PBS for 30 min and permeabilized with 0.2% Triton X-100 in PBS for 10 min, followed by incubation with both rabbit anti-HA PcAb (1: 70) (Sigma) and mouse anti-VP2 mAb (1:300) for 1 h at 37°C [19]. The cells were washed three times with PBS and incubated with a goat anti-rabbit antibody conjugated with Alexa Fluor™ 633 (1:1000) (Invitrogen) or an FITC-conjugated goat anti-mouse antibody (1:1000) (Sigma) at 37°C for 1 h. Cell nuclei were stained with Hoechst 33258 (Sigma) for 10 min at room temperature. Fluorescence signals were visualized using a LeicaSP2 confocal system (Leica Microsystems, Wetzlar, Germany).

Infectivity of the recombinant marker viruses in different cell lines

Different cell lines (MDBK, BHK-21, Vero and PK15 cells) were infected with the fifth passage of the recombinant marker viruses or parental rHY12 virus using a tenfold serial dilution (10^8 to 10^0 $TCID_{50}$). After incubation for 1 h, the inoculum was removed, and the cells were washed with PBS to remove the unabsorbed virus. The cells were then cultured with DMEM containing 2% FBS at 37°C. Infected cells were fixed at 30 h postinfection using ice-cold methanol, and viral titers were subsequently determined by IPMA assay. Infection of each cell line was repeated independently three times.

Immunogenicity of the rescued recombinant marker viruses

Twenty 4-week-old ICR mice were purchased from the Laboratory Animal Center of Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Harbin, China). The mice were randomly divided into four test groups (n = 4) (groups A-C) and a parental virus group D. They were intramuscularly inoculated with 0.2 ml containing 10^8 $TCID_{50}$ of the parental rHY12 virus or one of the three recombinant marker viruses, which were emulsified with ISA 15 (Seppic, Castres, France) adjuvant [7]. Serum samples were collected and prepared from the mice in each group by tail bleeding at 7-day intervals until 42 days after immunization. All serum samples were kept at −20°C until further use.

Detection of seroconversion in mice infected with recombinant marker viruses or parental rHY12 virus

HY12-VP2- and HA-epitope-tag-based ELISA was performed to detect anti-HY12 and anti-HA antibodies in the immunized mice. HY12-VP2 protein was purified using a nickel column (Genscript, Nanjing, China), and the 27-mer HA epitope peptide (three HA epitopes) was biosynthesized by GL Biochem (Shanghai, China). Briefly, 96-well ELISA plates were coated with purified VP2 protein and 27-mer HA epitope peptide at a concentration of 8 μg/ml and incubated at 4°C overnight. The plates were washed three times with PBS-T and blocked with 1% BSA in PBS-T, followed by the addition of serum samples diluted 1/50 (v/v) in 1%

BSA-PBS-T. After incubation for 1 h at 37°C, 100 µl of HRP-conjugated rabbit anti-mouse IgG (H+L) (1:5000, Sigma) was added to each well, and the plate was incubated for a further 1 h at 37°C. Next, 2, 2'-azino-bis (3-ethylbenzothiazole-6-sulfonic acid) (Amresco) substrate was added for color development, and the reaction was stopped by the addition of 50 µl of 1% NaF solution per well. Mouse pre-immune sera were used as negative controls. The OD_{405} nm cutoff value for each ELISA was determined using a microplate reader (Biotek, Winooski, VT, USA).

Statistical analysis

All data are presented as the average of three independent experiments with the standard deviation (SD). Data was analyzed statistically using two-way ANOVA in the GraphPad Prism 7.0 software (GraphPad Software, CA, USA).

Results

Introduction of the HA epitope tag into the N-terminus of the 3A protein

To generate the recombinant marker infectious HY12 cDNA clones, the strategy described in the methods section was used (Fig. 1). After the infectious HY12 clones were generated and confirmed by sequencing, the PCR-amplified 3A coding sequence was cloned into pMD18 to generate the pMD18-3A plasmid. The HA epitope was then introduced at different positions in the 3A coding sequence using a site-directed mutagenesis kit (Toyobo, Osaka, Japan). The HA epitope-tagged 3A coding sequence was amplified by PCR and used to replace the 3A coding sequence in the pBlu-HY12 plasmid to generate the recombinant marker infectious HY12 clones pBlu-HY12-3A-2-HA, pBlu-HY12-3A-3-HA, and pBlu-HY12-3A-9-HA. Sequencing results indicated that all three recombinant infectious HY12 clones contained the HA epitope sequence in the correct position.

Rescue of the recombinant marker viruses

To rescue the recombinant marker viruses, pBlu-HY12-3A-2-HA, pBlu-HY12-3A-3-HA, and pBlu-HY12-3A-9-HA plasmids were linearized by digestion with *Kpn*I and used to cotransfect MDBK cells with pCI-T7 plasmid expressing T7 RNA polymerase. As shown in Fig. 2A, a cytopathic effect (CPE) was observed at 30-36 h post-transfection in cells transfected with pHY12-3A-2-HA, pHY12-3A-3-HA, and pHY12-3A-9-HA plasmids, but not in cells transfected with the empty vector control. The CPE observed in cells transfected with HA-epitope-tagged plasmids was similar to that observed in cells transfected with parental rHY12 plasmid, suggesting that transfection with the plasmids resulted in the production of cytopathic virus. To confirm that the three rescued marker viruses contained the HA epitope, PCR was used to amplify the sequence spanning the position of the HA epitope insertion, and the presence of the HA epitope sequences was confirmed (data not shown).

Expression of the HA epitope in cells infected with the three recombinant marker viruses

To detect the expression of HA epitope in cells infected by the rescued recombinant marker viruses, IPMA was performed using an anti-HY12-VP2 mAb and an anti-HA mAb. As shown in Fig. 2B, cells infected with one of the three recombinant marker viruses or the parental rHY12 virus all reacted with the anti-VP2 mAb, while only cells infected the marker viruses reacted with the anti-HA mAb. Mock-infected MDBK cells showed no reaction to either anti-HY12-VP2 or anti-HA mAb antibodies. These results verified that the inserted HA epitope was expressed in cells infected with the three recombinant marker viruses.

To confirm the IPMA results, we performed western blotting to detect VP2 and HA epitope expression in lysates of MDBK cells infected with each of the three recombinant marker viruses and parental rHY12 virus as well as mock-infected cells. As shown in Fig. 3, two bands were detected in the samples from the parental rHY12 virus and all three marker viruses using anti-VP2 mAb (Fig. 3A), while the mock-infected cells did not produce a band. The upper band is the unprocessed precursor protein VP0 (VP4 and VP2), and the lower band is the processed VP2 protein [6]. Two bands were also obtained when cells infected with the three marker viruses were analyzed using an anti-HA mAb, and these were not observed when cells infected with the parental rHY12 virus or mock-infected cells were analyzed (Fig. 3B). These two bands corresponded to the precursor 3AB (upper) and processed 3A (lower) proteins. These results confirmed the expression of the HA epitope in cells infected with the recombinant marker viruses.

Similar characteristics of recombinant marker viruses and the parental rHY12 virus

To determine whether the introduction of the HA epitope affects the morphology of the rescued recombinant viruses, the purified rescued recombinant marker viruses and parental rHY12 virus were stained and examined by electron microscopy. As shown in Fig. 4A, the recombinant HY12 viral particles, like the parental rHY12 virus, were approximately 30 nm in diameter with a uniform shape, and no foreign viral contamination was detected.

To investigate the effect of the HA epitope tag insertion on the growth of the recombinant marker viruses,

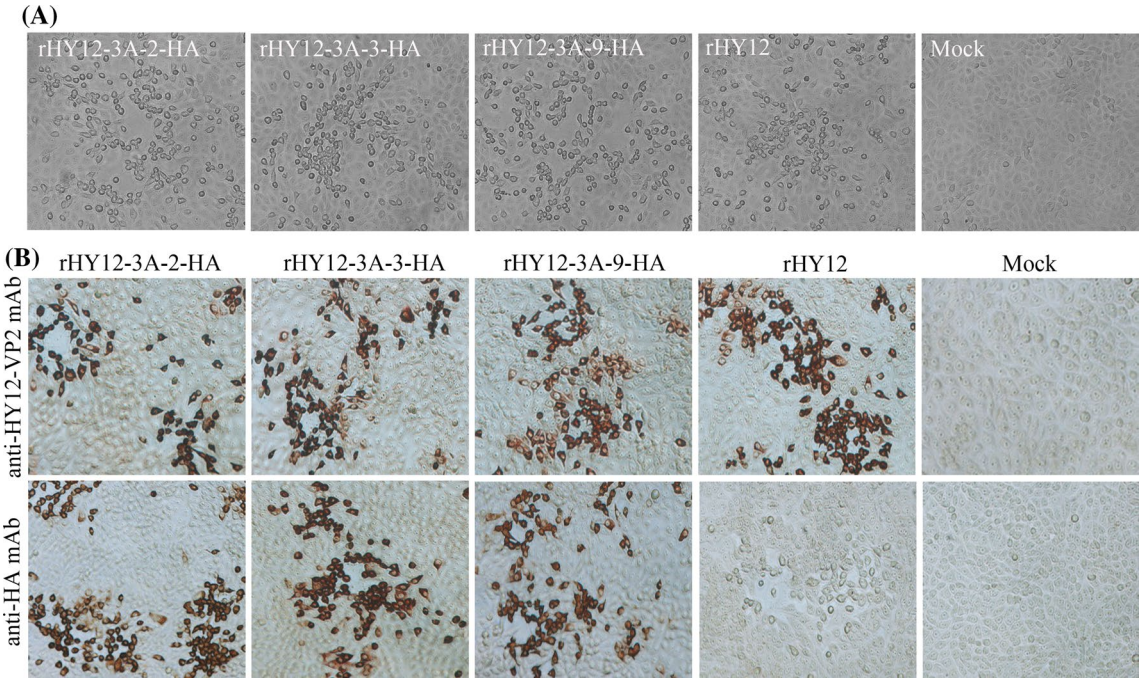

Fig. 2 Rescue and identification of the recombinant HY12 marker virus. (A) CPE after infection with rescued recombinant marker viruses. CPE was observed in MDBK cells transfected with linearized pBlu-HY12-3A-2-HA, pBlu-HY12-3A-3-HA, or pBlu-HY12-3A-9-HA plasmids. The pBlu-HY12 plasmid and empty pBlu-SK(+) vector were used as a positive and negative control, respectively. (B) Detection of HA epitope expression in cells infected with the recombinant viruses by IPMA. MDBK cells were infected with rHY12-3A-2-HA, rHY12-3A-3-HA, rHY12-3A-9-HA or the parental rHY12 virus (MOI = 10). After 8 h of infection, the cells were fixed and incubated with an anti-HY12-VP2 mAb and an anti-HA mAb, followed by incubation with secondary antibody. Uninfected MDBK cells were used as a mock control

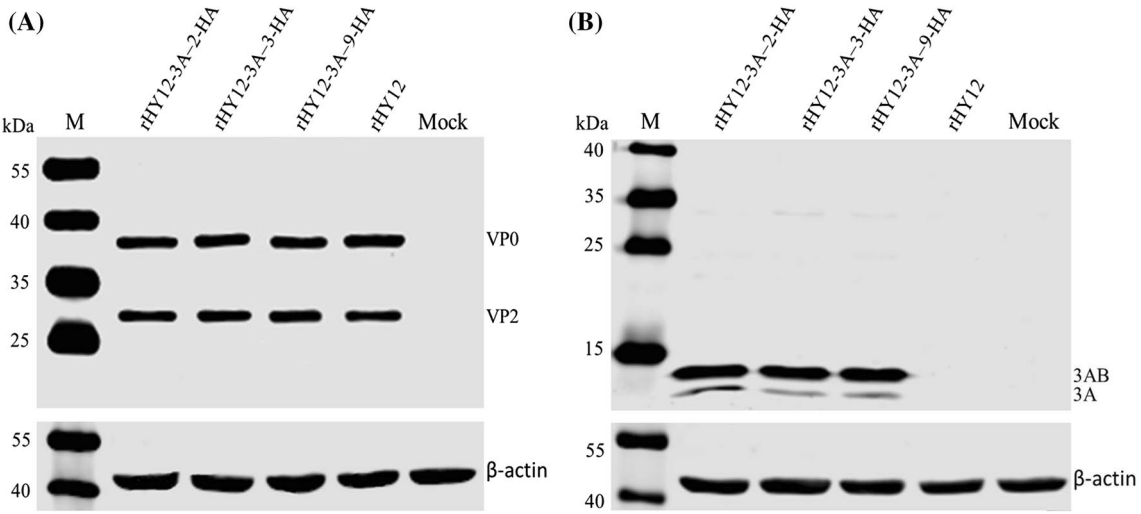

Fig. 3 Identification of the recombinant marker viruses by Western blot. MDBK cells infected with the parental rHY12 virus and rHY12-3A-2-HA, rHY12-3A-3-HA, or rHY12-3A-9-HA viruses (MOI = 10) were used for Western blot assay. (A, B) Reactivity of the parental rHY12 virus and the three recombinant marker viruses to (A) HY12-VP2 mAb and (B) anti-HA mAb. The β-actin protein was used as a loading control. Protein size markers are shown on the left; molecular weights of the proteins are indicated on the right (VP0, 37 kDa; VP2; 27.3 kDa; 3AB, 12.7 kDa; 3A, 2.5 kDa)

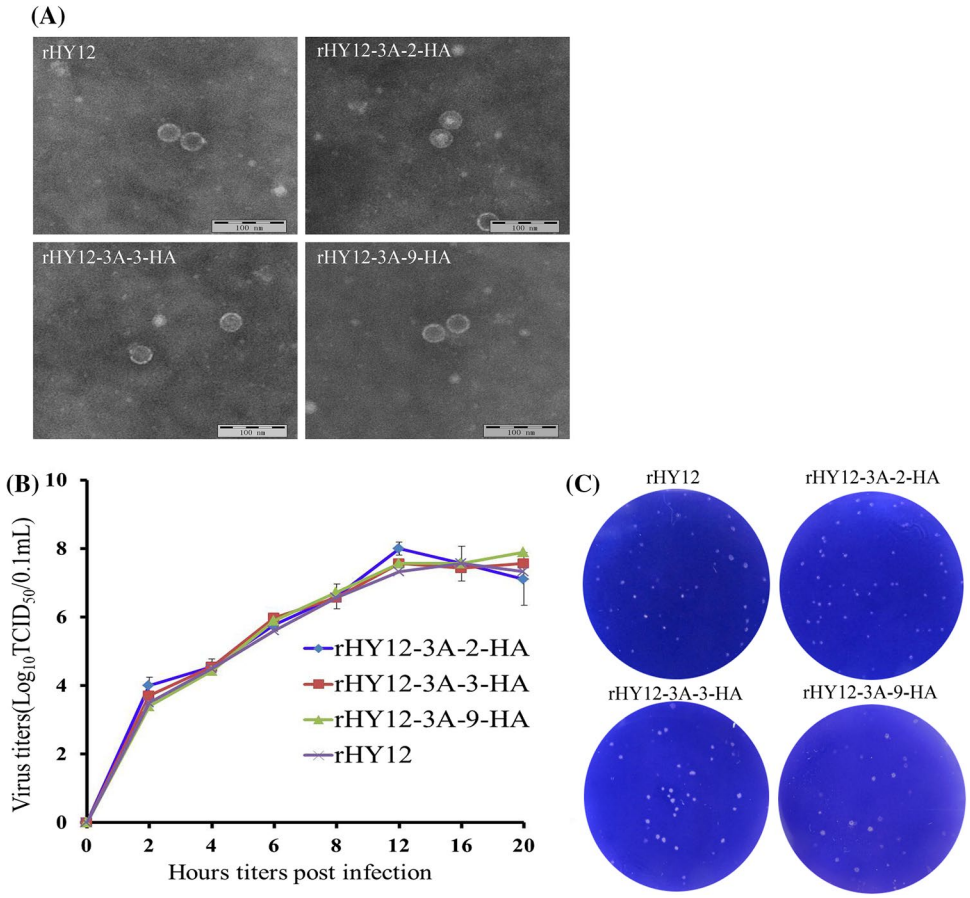

Fig. 4 Morphology and propagation dynamics of the recombinant marker viruses. (A) Observation of the recombinant marker viruses and the parental rHY12 virus by EM. Scale bar = 100 nm. (B) Propagation dynamics of the recombinant marker viruses and parental rHY12 in MDBK cells. MDBK cells were infected with rHY12-3A-2-HA, rHY12-3A-3-HA, or rHY12-3A-9-HA viruses and the parental rHY12, at a MOI of 1. The data from three independent experiments are expressed as $\log_{10}$ $TCID_{50}$/0.1 ml. Error bars represent standard deviations. (C) Representative plaques formed by the recombinant marker viruses and parental rHY12. The plaque assay was performed using low-melting-point agarose (1.25%) overlaid on MDBK cells. Cells were stained with crystal violet at 48 h postinfection

propagation dynamics assays were performed. As illustrated in Fig. 4B, the three recombinant marker viruses exhibited similar growth characteristics and replication efficiency to that of the parental rHY12 virus, indicating that the insertion of the HA epitope had no effect on viral growth characteristics. Moreover, the plaque size and morphology of all three recombinant marker viruses was not significantly different from those of the parental HY12 virus (Fig. 4C).

Together, the above results indicate that the insertion of the HA epitope into the N-terminus of the 3A protein had no significant effect on the biological characteristics of the virus.

Genetic stability of the recombinant marker viruses

To determine the genetic stability of the rescued recombinant viruses *in vitro*, the 5th, 10th, and 15th passages of the three recombinant marker viruses were used to perform plaque assays in MDBK cells with the corresponding passages of the parental rHY12 virus as a control. The plaque clones were then picked to infect cells. RNA extracted from the infected cells was used to amplify the sequence spanning the HA epitope tag. Sequencing of amplicons from 50 plaque-purified colonies from each passage revealed that all of the recombinant marker viruses retained the HA epitope tag during their passage in MDBK cells, even after the 15th passage, while no HA epitope sequence was found in the parental virus. These results demonstrate the stability of the HA epitope tag inserted in the N-terminus of the 3A coding sequences (Table. 2).

Localization of 3A protein

To determine the localization of the HA-tagged 3A protein, MDBK cells infected with three recombinant marker viruses or parental rHY12 virus were detected by confocal microscopy using a rabbit anti-HA PcAb and a mouse anti-VP2 mAb. As shown in Fig. 5, a reaction with the anti-HA PcAb was observed in the cytoplasm of the cells infected by each of the three recombinant marker viruses (red fluorescent signal) at 2, 8, and 16 h postinfection. These cells also showed positive staining in the cytoplasm with the anti-VP2 mAb (green fluorescent signal), in contrast to the rHY12 virus positive control and uninfected cell controls. These results indicate that the HA epitope insertion at the N-terminus of

Table 2 Analysis of the genetic stability of the inserted HA epitope tag in the three recombinant marker viruses at the 5th, 10th, and 15th passages

Virus	5th passage		10th passage		15th passage	
	Number of clones[a]	Tag insert retained[b]	Number of clones[a]	Tag insert retained[b]	Number of clones[a]	Tag insert retained[b]
rHY12-3A-2-HA	50	Yes	50	Yes	50	Yes
rHY12-3A-3-HA	50	Yes	50	Yes	50	Yes
rHY12-3A-9-HA	50	Yes	50	Yes	50	Yes
rHY12	50	No	50	No	50	No

[a]All clones were tested by plaque assay

[b]The HA epitope tag inserts were identified by RT-PCR and sequencing

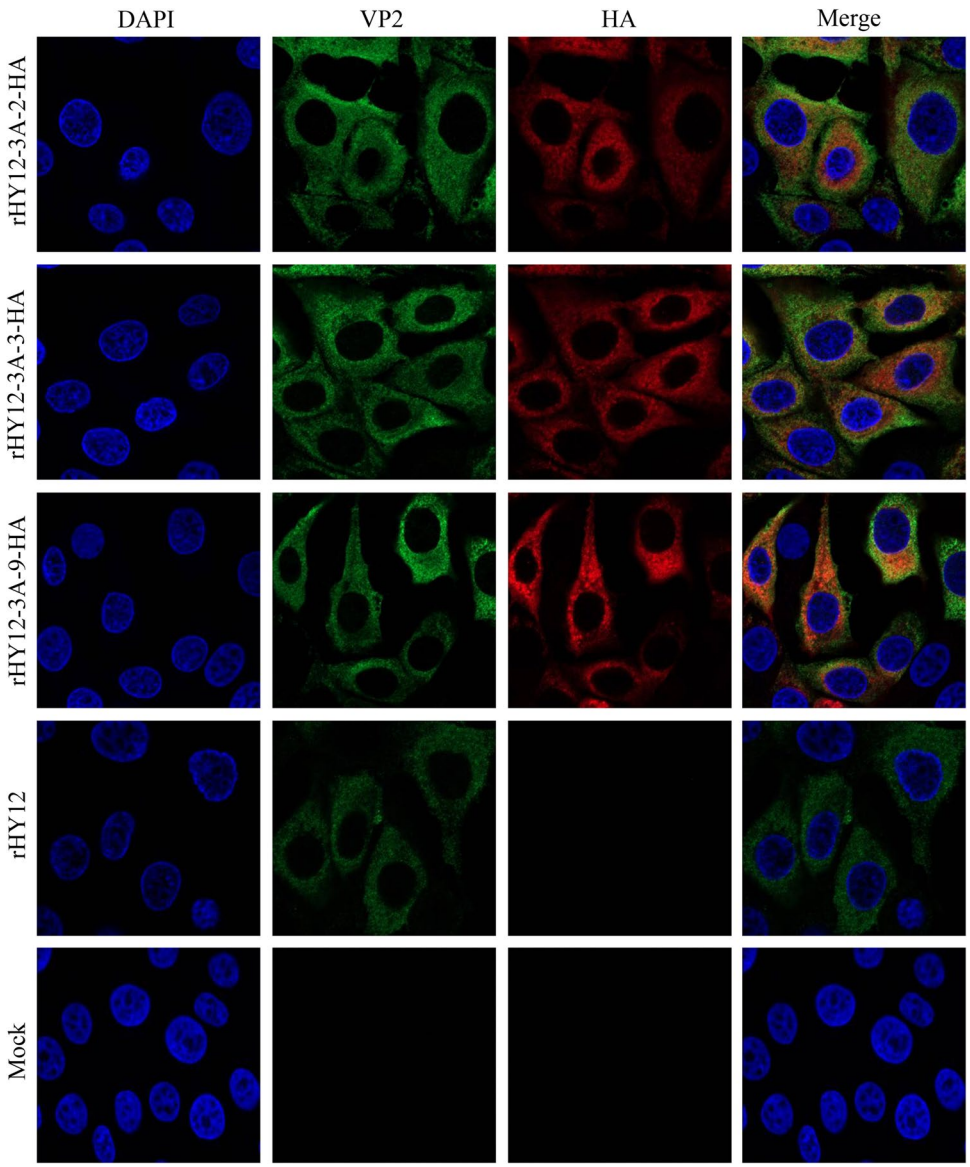

Fig. 5 Localization of the HA-tagged 3A protein in MDBK cells by confocal microscopy. A representative image showing the localization of the HA-tagged 3A protein in MDBK cells infected with the recombinant marker viruses at 8 hpi, as determined by probing the infected cells with anti-HA PcAb (red) and anti-VP2 mAb (green) is shown. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, blue). MDBK cells infected with parental rHY12 virus were used as a positive control. Mock-infected cells were used as a negative control

the HY12-encoded 3A protein can be used for monitoring the localization of 3AB and 3A proteins.

Lack of effect of the HA epitope insertion in the 3A coding sequence on viral infectivity

To investigate whether the insertion of the HA epitope into the N-terminus of the 3A protein affects viral infectivity, different cell lines (MDBK, BHK-21, Vero and PK15 cells) were separately infected with the recombinant marker viruses or parental rHY12 virus. As shown in Fig. 6, the recombinant maker viruses were able to infect all of the cell lines, with titers similar to that of the parental rHY12 virus in each cell line, suggesting that the insertion of the HA epitope into the N-terminus of the 3A protein did not affect the host cell range of the virus.

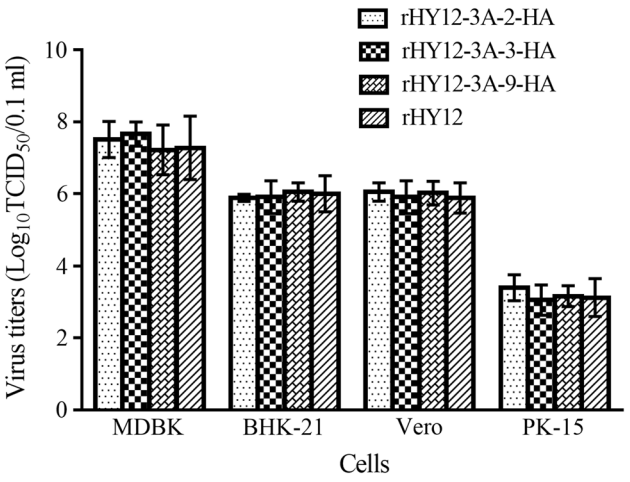

Fig. 6 Infectivity of the recombinant marker viruses on different cell lines. MDBK, BHK-21, Vero and PK15 cells were infected with the serial tenfold dilutions of recombinant marker viruses or parental rHY12 virus (10^8-10^0) with three repeats for each cell line. Error bars represent standard deviations

Antibody response to the recombinant marker viruses in mice

To determine the effect of the HA epitope insertion on the antibody response of the host to the HY12 viruses, four groups of mice were inoculated intramuscularly with the three recombinant marker viruses and parental rHY12 virus. HY12-VP2- and HA-epitope-tag-based ELISAs were then used to detect antibodies against the HY12 virus and HA epitope, respectively. As shown in Fig. 7A, the mice in all four groups produced antibodies against the HY12 virus, beginning 7 days post-inoculation (dpi). The antibodies reached peak titers at 14 dpi and maintained a nearly constant level for 42 days before decreasing. Similarly, antibodies to the HA epitope were produced beginning on 7 dpi, reached peak titers at 14 dpi, and were maintained at nearly constant levels for 42 days before decreasing (Fig. 7B). As expected, mice inoculated with the parental rHY12 virus did not produce antibodies against the HA epitope. These results indicate that the recombinant viruses harboring the HA epitope were able to elicit an immune response to the HA epitope tag.

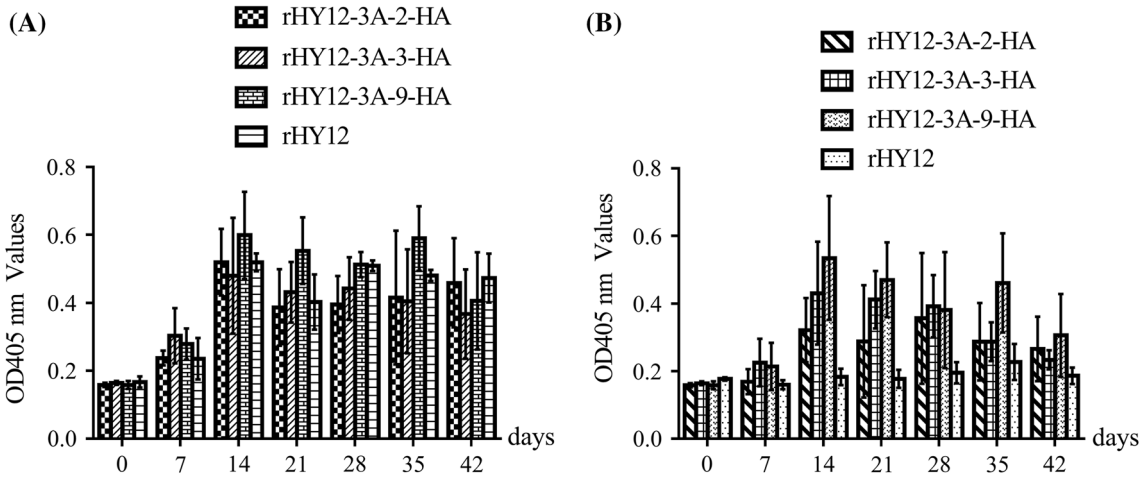

Fig. 7 Antibodies detected in mice infected with the recombinant marker viruses. Anti-HY12 and anti-HA antibodies were detected by ELISAs. The sera were collected from mice inoculated with the parental rHY12 and three recombinant marker viruses at 0, 7, 14, 21, 28, 35, and 42 days. (A) HY12-VP2-protein-based ELISA. (B) HA-epitope-tag-based ELISA. The data are the average of three experiments with the standard deviation

Discussion

Viral vectors are commonly used tools for delivering genetic materials into cells and have been extensively studied in the context of gene therapy and the development of vectored vaccines based on adenoviruses and retroviruses [10, 25]. Bovine enterovirus is widely dispersed among the animal population but has a low morbidity rate [9]. Furthermore, several characteristics of this virus make it a good candidate for developing live viral vectors for the expression of foreign antigens. First, BEV generally infects animals via the enteric or respiratory tract, where mucosal immunity is the primary means of protection against foreign pathogens. Second, BEV is resistant to acidic conditions (pH 3) [36], and this can be used to distinguish enteroviruses from other picornaviruses. Third, BEV has been shown to have oncolytic properties and thus may be useful as therapeutic agents [27]. Therefore, BEVs could be used as potential viral vectors for delivering genetic material into animals. In this study, we used a reverse genetics platform to explore the feasibility of inserting an HA epitope into a non-structural protein of BEV strain HY12 and discovered three sites in the 3A protein that were suitable for the insertion of the HA epitope. The enterovirus genome contains a single large ORF that encodes four structural proteins (VP1, VP2, VP3, and VP4) and seven non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D). 3A is a small protein derived from the precursor 3AB protein, which plays a key role in viral replication. Analysis indicated that the N-terminal sequence for 3A is relatively conserved, containing a hydrophilic domain and a hydrophobic domain [5]. Early studies showed that insertion of foreign epitopes within the 3A proteins did not affect the rescue of the viruses such as FMDV (O/HN/CHA/93) with a herpes simplex virus (HSV) glycoprotein D or flag epitope inserted and had no detrimental effects on viral replication *in vitro* [16]. Similar findings were also reported for poliovirus, where no detrimental effects on viral biology or replication were observed after small insertions near the N-terminus of the 3A protein [30]. We have employed an established reverse genetics platform and successfully rescued recombinant marker viruses harboring an HA epitope tag at amino acid positions 2, 3 and 9 of the 3A protein, indicating that the insertions of the HA epitope in those position affect neither the rescue nor the replication characteristics of the HY12 recombinant marker viruses. The successful rescue of the recombinant HY12 marker viruses, confirmed by IPMA, Western blot and sequencing analysis, indicates that these positions are likely the ideal insertion sites for exogenous tags or antigenic peptides, which is also a subject for future investigation. In addition to the 3A protein, we also tried to insert the HA epitope tag in the non-structural proteins 2A and 2C. However, the insertion of the HA epitope in those proteins did not allow recombinant marker viruses to be rescued (data not shown), suggesting that HY12-encoded 2A (amino acid 47/48 and 50/51) and 2C (amino acid 6/7) are not suitable for the insertion of exogenous antigens.

It has been reported that the proteolytic sites for processing the polyprotein are highly conserved among enteroviruses [35]. The proteolytic sites between 2C and 3A in HY12 virus is FQ/GP. The insertion of an HA epitope tag in this study did not alter the 2C/3A consensus cleavage sequence, since it was inserted after the second proline. Successful rescue of the recombinant marker virus and the detection of 3AB precursor and 3A protein by Western blot confirmed that the insertion of HA epitope did not change the consensus 2C/3A proteolytic sites.

Evaluation of the propagation dynamics and morphology of the three recombinant marker viruses indicated that all three positions were suitable for HA epitope insertion without affecting viral replication or morphology when compared to the parental rHY12 virus in MDBK cells. More importantly, the inserted HA epitope was stably retained during viral passage *in vitro*, and no effect on the viral host range was observed in different cell lines, suggesting that the N-terminus of the 3A protein was an ideal position for the insertion of foreign genetic material.

A previous study has shown that the prevalence of preexisiting antibodies against EV-E is generally lower than that against EV-F in cattle populations [32]. Since HY12 is an EV-E virus, it may be more suitable as a live viral vaccine vector than EV-F. In the present study, we detected anti-HA antibodies and anti-HY12 antibodies in ICR mice inoculated with the recombinant viruses. Mice inoculated with the parental HY12 virus produced antibodies only against the HY12 virus, whereas most mice inoculated with the three recombinant marker viruses were able to produce relatively high antibody titers against both the HY12 virus and the HA epitopes. These results indicate that HY12 viruses can be used as a viral vector to deliver exogenous tags or antigenic peptides and therefore may be an ideal system to generate recombinant viruses for immunization against diseases.

In conclusion, we successfully generated and rescued recombinant HY12-3A-2-HA, HY12-3A-3-HA, and HY12-3A-9-HA viruses and demonstrated that the insertion of the HA epitope in the N-terminus of the HY12-encoded 3A protein had no effect on the biological properties of the recombinant viruses, thus providing an ideal platform for future investigation of marker viral vaccines and exploration of interactions of viral 3A protein with cellular and viral proteins.

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Compliance with ethical standards

Conflicts of interest The authors declare that there are no conflicts of interest.

Ethical approval The handling of mice and the procedures used for this study were done following a standard protocol reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Jilin University (approval no JLU-20150226), in strict compliance with the requirements of the Animal Ethics Procedures and Guidelines of the People's Republic of China.

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