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Construction of an avian hepatitis E virus replicon expressing heterologous genes and evaluation of its potential as an RNA vaccine platform

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To evaluate avian hepatitis E virus (aHEV) as an RNA vaccine platform, ORF2 of aHEV was replaced by heterologous genes, such as eGFP and HA-tag, in aHEV infectious cDNA clones. eGFP and HA-tag replicons were expressed in LMH cells. To confirm expression of the heterologous protein, ORF2 was replaced with the antigenic S1 gene of IBV. The IBVS1 replicon was expressed in LMH cells. To our knowledge, this is the first investigation showing the potential as a RNA vaccine platform using an aHEV. In the future, it may be used in the development of RNA vaccines against various pathogens.

Keywords: avian hepatitis E virus; replicon; RNA vaccine platform

Avian hepatitis E virus (aHEV) has a 5′-capped, positive-sense, single-stranded RNA genome of approximately 6.6 kb and consists of 5′-non-coding regions (NCR), three open reading frames (ORFs), and a 3′ NCR. ORF1 encodes the non-structural proteins, ORF2 encodes an immunogenic capsid protein, and ORF3 encodes a phosphoprotein [1,2]. Infectious cDNA clones that can synthesize the original viral RNA are useful for virus characterization and pathogenicity research. They are also useful vaccine platforms; a replicon can be created by inserting a gene of interest into the structural gene [3]. The RNA genomes of positive-strand RNA viruses have dual functions: they act as an mRNA template for RNA-dependent RNA polymerase (RDRP) and as a genomic template for replication by the respective RDRP. Replicon RNA is readily produced by *in vitro* transcription from cDNA templates [4,5]. However, no study has reported a replicon vaccine platform using aHEV. Therefore, we explored the possibility of aHEV as a vaccine platform using an aHEV infectious cDNA clone. The ORF2 gene of aHEV was replaced by heterologous genes, such as enhanced green fluorescent protein (eGFP), hemagglutinin (HA)-tag, and infectious bronchitis virus (IBV) S1. The S1 protein of IBV is critical for antigenic neutralization and hemagglutination. IBV infection is a significant problem in the poultry industry, and novel vaccines are needed to combat emerging and variant IBV serotypes [6]. These replicons were constructed and used to transcribe the capped RNA transcripts and to transfect leghorn male hepatoma (LMH) cells. This is the first study of a replicon vaccine platform using aHEV infectious cDNA clones.

The previously constructed genotype 2 aHEV infectious cDNA pT9-aHEV-GI clone and the genotype 1 aHEV infectious cDNA pT11-aHEV-K clone were used in this study [7]. Specific primers were prepared to insert eGFP and an HA-tag into the pT9-aHEV-GI clone (Table 1). The products were subjected to fusion

polymerase chain reaction (PCR), amplified by cloning and transformation, and sequenced. Clones containing the consensus sequences of heterologous genes were inserted into ORF2 by digestion with *Bam*HI and *Kpn*I (pTGI-GFP replicon) and with *Bam*HI and *Xba*I (pTGI-HA replicon) (Fig. 1). Specific primers were prepared to insert eGFP, HA-tag, and IBV S1 into the pT11-aHEV-K clone (Table 1). IBV used K047-12, which was most similar to the QX-like IBV reported previously [8].

Table 1. Oligonucleotide primers used to construct eGFP, HA-tag, and IBVS1 replicons

Primers	Primer sequence (5′ to 3′)
GI-*Bam*HI3538F3	GCGCAGTTCTGGCACCACATTGAG
GI-eGFP_4706_R	CTCACCATCCCACCCCACTTTCCTGGCACT
GI-eGFP_4698_F	GGGGTGGGATGGTGAGCAAGGGCGAGGA
GI-eGFP_*Kpn*I_R	GCGGTACCTTACTTGTACAGCTCGTCCATG
GI-HA-tag_*Sbf*I_R	TTAAGCGTAATCTGGAACATCGTATGGGTACATCCCACCCCACTTTCCTGGCACTCCAGAC
GI-3′-NCR_F	CGATGTTCCAGATTACGCTTAATGCTGTGGTTTTGGGGCTTAGGTTAAT
GI-T18*Sbf*IR4	GCCCTGCAGGTTTTTTTTTTTTTTTTTACTATGCCCGAAATGGGAGG
K-*Age*I2848F3	CGCGCCGATTATCCAACTA
K_HA-tag_*Bam*HI_R	TTAAGCGTAATCTGGAACATCGTATGGGTACATCCCACCCCACTATCCTGACACTCCAGAC
K_3′NCR_F	CGATGTTCCAGATTACGCTTAATAAGACTATGGATTGGGGTGTAAATTG
F_ORF2-GFP	TCAGGATAGTGGGGTGGGATGGTGAGCAAGGGCGAGGAGCTGTTC
R_*Fse*I-GFP	GCGGCCGGCCCTATTACTTGTACAGCTCGTCCATGC
F_ORF1-*Bcl*I	GCAGTCAAATCCCGGGTCGATTA
R_ORF1-GFP	CATCCCACCCCACTATCCTGA
IBV47S1_F	GCGCAGTTCTGGCACCACATTGAG
IBV47S1_R	ACGTCTAAAACGACGTGTCT
K_ORF1-IBV47_R	CCAACATCCCACCCCACTATCCTGACA
K_3NCR-IBV47_F	TAGACGTTAAGACTATGGATTGGGGTG
K-T18*Bam*HIR4	GCGGATCCTTTTTTTTTTTTTTTTTACTATGCCCGAGATGGGA

eGFP, enhanced green fluorescent protein; HA, hemagglutinin; IBV, infectious bronchitis virus.

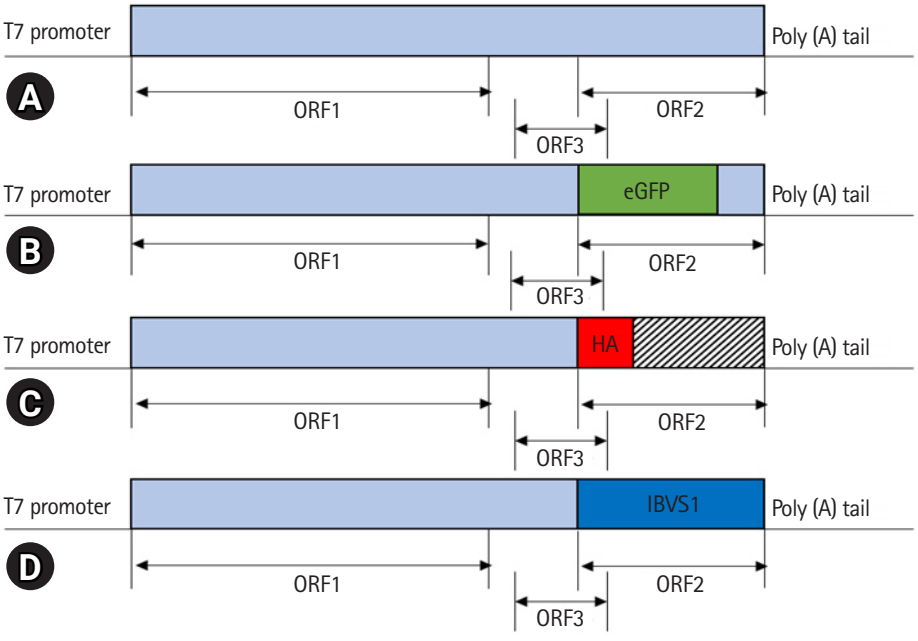

Fig. 1. Schematic diagram of construction of the avian hepatitis E virus (aHEV) enhanced green fluorescent protein (eGFP), hemagglutinin (HA)-tag, and infectious bronchitis virus (IBV) S1 replicons. (A) Infectious clone backbone of pT11-aHEV-K or pT9-aHEV-GI. (B) eGFP replicon. The front of open reading frame 2 (ORF2) was replaced with eGFP (pTK-GFP and pTGI-GFP). (C) HA replicon. ORF2 was replaced with an HA-tag (pTK-HA and pTGI-HA). (D) IBVS1 replicon. ORF2 was replaced with IBVS1 K047-12 (pTK-IBVS1).

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The products were subjected to fusion PCR, amplified by cloning and transformation, and sequenced. Clones containing the consensus sequences of heterologous genes were inserted into ORF2 by digestion with *Age*I and *Bam*HI (pTK-HA replicon, and pTK-IBVS1 replicon) and with *Fse*I and *Bcl*I (pTK-GFP replicon) (Fig. 1). The plasmid DNAs from each replicon were linearized with *Xba*I (pTGI-GFP and pTGI-HA replicons) or *Bam*HI (pTK-GFP, pTK-HA, and pTK-IBVS1 replicons) and purified by phenol/chloroform extraction and ethanol precipitation. Capped RNA transcripts were synthesized as described previously [7] and used directly for the *in vitro* transfection. LMH chicken liver cells were transfected with the 500 ng of capped RNA transcripts of the pTGI-GFP, pTGI-HA, pTK-GFP, pTK-HA, and pTK-IBVS1 replicons in 24-well plates using a VIROMER mRNA Kit (Lipocalyx, Germany). To confirm insertion of GFP, we performed IFA using a rhodamine-conjugated anti-GFP IgG (Novus Biologicals, USA) at 5 days post-transfection. The pTGI-HA and pTK-HA replicons were reacted with FITC-conjugated anti-HA-tag IgG (Novus Biologicals) 5 days after transfection. The pTK-IBVS1 replicon was reacted with a primary anti-IBV chicken IgG, followed by a secondary FITC-conjugated rabbit anti-chicken IgG (Sigma, USA) for 5 days, treated with DAPI (Vector Laboratories, USA), and visualized by fluorescence microscopy (Olympus, Japan).

After transfection with the pTGI-GFP and pTK-GFP replicons, expression was monitored daily in LMH cells (Fig. 2A). LMH cells transfected with RNA transcripts synthesized using pTGI-GFP proliferated and expressed eGFP. IFA using an anti-GFP antibody conjugated to rhodamine confirmed that eGFP was expressed in LMH cells (Fig. 2B and C). Also, the expression of pTGI-HA and pTK-HA was detected using a FITC-conjugated antibody (Fig. 2D and E). LMH cells were transfected with RNA transcripts synthesized using the pTK-IBVS1 replicon. After 5 days, IFA using chicken anti-IBV antiserum and a FITC-conjugated secondary antibody confirmed expression of the S1 gene in LMH cells (Fig. 2F). These results indicate that replicons lacking the ORF3 and ORF2 proteins were expressed in LMH cells, resulting in synthesis of ORF3 and ORF2 proteins.

Because GFP-producing cells could be observed in damp cells, green fluorescence was used as a marker of proliferating cells. Expression of eGFP did not prevent cell division and was

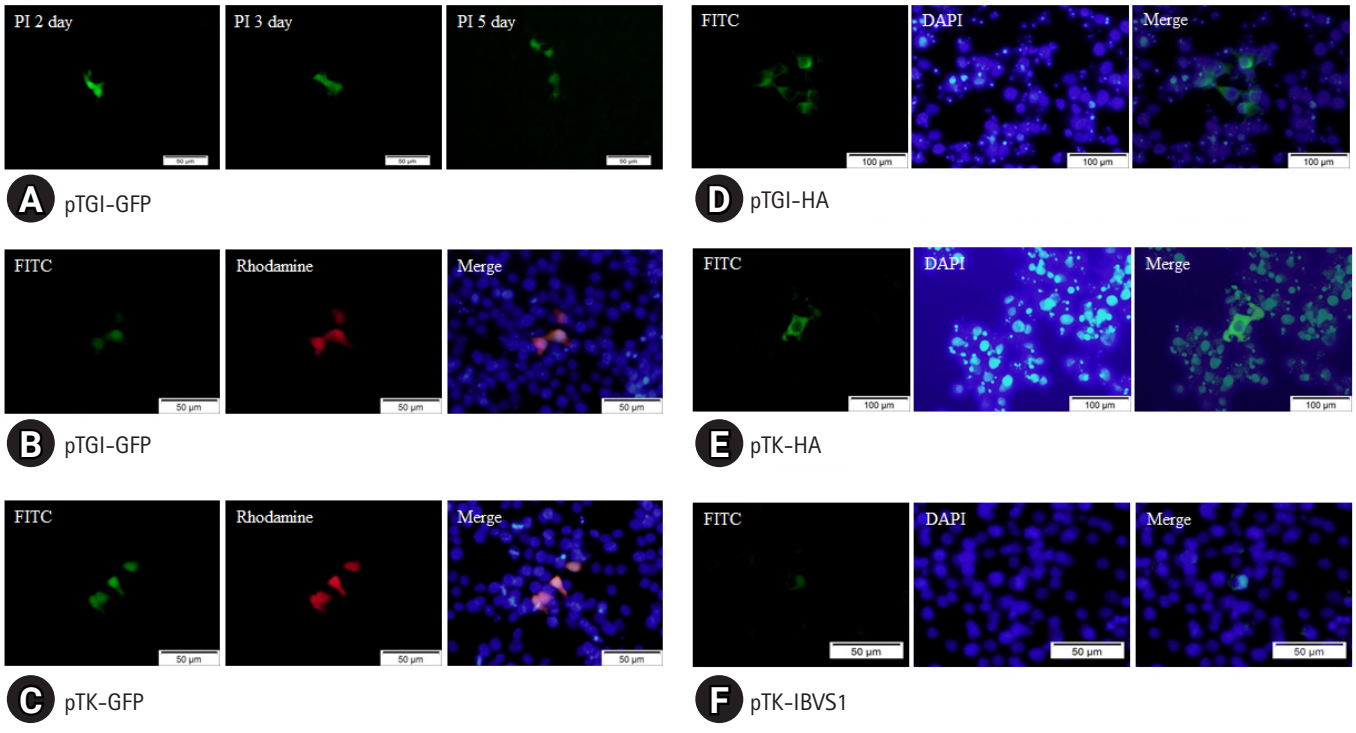

Fig. 2. Expression of the heterologous gene replicons in leghorn male hepatoma (LMH) cells. LMH cells were transfected with RNA transcripts of pTGI-GFP, pTK-GFP, pTGI-HA, pTK-HA, and pTK-IBVS1. (A) pTGI-GFP. Transfected cells were observed in damp cells at 2, 3, and 5 days post-transfection; enhanced green fluorescent protein (eGFP) expression was evident in proliferating cells. (B) pTGI-GFP and (C) pTK-GFP. eGFP expression was visualized by FITC (green) and rhodamine (red) staining; blue, DAPI-stained nuclei. (D) pTGI-HA and (E) pTK-HA. Blue, DAPI staining of nuclei; green, FITC staining of HA-tag reporter protein. (F) pTK-IBVS1. Blue, DAPI staining of nuclei; green, FITC staining of IBV spike protein. HA, hemagglutinin; IBV, infectious bronchitis virus.

persistent in cell division, like HEV [9]. Since its introduction almost 20 years ago as a short immunoreactive tag, the hemagglutinin epitope YPYDVPDYA (HA tag) from influenza virus A has been used to track proteins of interest within cells, to isolate them, and to coprecipitate their possible partners [10,11]. Using the HA-tag replicons, HA-tag protein cloned into aHEV was expressed in LMH cells. The S1 gene of IBV K047-12, which is similar in length to the ORF2 gene, was amplified and replaced with ORF2 of aHEV clone pT11-aHEV-K to construct the pTK-IBVS1 replicon. The aHEV pT11-aHEV-K clone was used because it had higher intracellular expression and infectiousness than aHEV pT9-aHEV-GI. This pTK-IBVS1 replicon was expressed in LMH cells, indicating that the aHEV replicon platform can self-amplify in LMH cells. However, further *in vivo* studies are needed to verify the potential of the aHEV replicon as RNA vaccine platform.

Although ORF3 encodes only 82 amino acids, it has been postulated to have numerous functions. Recombinant ORF3 reacts with the cytoskeleton and with non-glycosylated recombinant ORF2 protein [12]. Additionally, it reportedly exerts regulatory effects by binding to multiple cellular proteins containing SH3 domains and by activating mitogen-activated protein kinase [13]. A segment of the aHEV genome containing most of the ORF3 gene and the complete or partial ORF2 gene was removed, and a heterologous gene was inserted in-frame with the ORF2 methionine initiation codon (Fig. 1). Therefore, replicons were lacking ORF3 and ORF2. As the eGFP replicon of HEV lacking ORF3 and ORF2 proteins was expressed *in vitro*, aHEV replicons lacking ORF3 and ORF2 proteins were expressed *in vitro* [9]. Therefore, ORF3 and ORF2 are not required for expression of heterologous genes *in vitro*.

We developed RNA-based aHEV replicons capable of expressing heterologous genes in LMH cells. To our knowledge, this is the first report of a replicon vaccine platform using aHEV infectious cDNA clones.

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