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ORIGINAL ARTICLE

Construction of a single-cycle replication recombinant infectious laryngotracheitis virus lacking the glycoprotein H gene and evaluation of its role in viral entry and infectivity

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Abstract

Infectious laryngotracheitis virus (ILTV), a significant avian pathogen belonging to the subfamily *Alphaherpesvirinae*, causes severe respiratory disease, particularly in unvaccinated flocks. In this study, we produced a recombinant ILTV lacking the essential envelope glycoprotein H (gH), a key determinant of viral entry and propagation. To achieve this, we engineered an ILTV mutant with the gH gene replaced by a BleCherry fluorescent reporter cassette. This modification enabled the identification and isolation of recombinant viruses through red fluorescence. To facilitate the replication of this gH-deficient mutant, we generated a stable Vero cell line expressing gH and a green fluorescent protein (GFP) reporter. This engineered cell line proved crucial for generating the recombinant ILTV and allowing controlled single-cycle replication. The recombinant ILTV exhibited enhanced viability across a range of multiplicities of infection (MOIs), although with a significant reduction in overall viral replication. Importantly, the modified virus is unable to replicate in the absence of exogenous gH, minimizing the risks associated with viral spread and unintended infections. This novel single-cycle recombinant ILTV platform holds significant promise for various applications, including safe gene delivery and the development of improved vaccine strategies for enhanced avian health management and disease control.

Abbreviations

ILTV	infectious laryngotracheitis virus
gH	glycoprotein H
MOI	multiplicity of infection
PCR	polymerase chain reaction
GFP	green fluorescent protein
URT	upper respiratory tract
US	unique short region
UL	unique long region
IR	internal repeat
TR	terminal repeat
gB	glycoprotein B
EF1	elongation factor 1a
XTT	2:3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
CPE	cytopathic effect
HSV	herpes simplex virus
EHV	equine herpesvirus
CMV	cytomegalovirus
VSV	vesicular stomatitis virus

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Introduction

Infectious laryngotracheitis virus (ILTV), a member of the subfamily *Alphaherpesvirinae* (species *Iltovirus gallidalpha1*), causes respiratory disease in avian species. It replicates lytically in the tracheal and upper respiratory tract epithelium and can establish latent infections in the trigeminal nerve and trachea, which can reactivate, leading to recurrent outbreaks. [1]. Clinical signs include nasal discharge, gasping, conjunctivitis, tracheal mucus, dyspnea, and reduced egg production. Mortality rates can reach 80% depending on the virulence of the strain [2].

ILTV, like other members of the subfamily *Alphaherpesvirinae*, possesses a linear double-stranded DNA genome (~150 kb) containing unique short (US) and unique long (UL) sequences and inverted repeats (IRs) [3]. The virion is enveloped and contains an icosahedral capsid. Envelope glycoproteins (gB, gC, gD, gE, gG, gH, gJ, and gI) are crucial for viral attachment and entry and are major immunogens [4, 5].

Among the ILTV glycoproteins, gH, gB, and gD exhibit significant conservation among alphaherpesviruses, particularly within the C-terminal part of the extracellular domain. While gB is essential for membrane fusion and viral attachment and gD plays a crucial role in mediating viral entry and serves as a major immunogen, the precise functions of gH in ILTV remain less well understood [6].

Glycoprotein H (gH, UL 22) is an essential envelope glycoprotein that is crucial for viral entry. With an approximate molecular weight of 91 kDa, gH consists of a surface domain, a transmembrane domain, and an intramembrane domain [7]. Studies with herpes simplex virus 1 (HSV-1) have shown that gH forms a heterodimer with glycoprotein L (gL) on the viral envelope surface, and this gH/gL complex is indispensable for viral entry, promoting both cell-to-cell spread and the formation of syncytia [8]. The gH/gL heterodimer is a core component of the viral fusion machinery, mediating the fusion of the viral envelope with the host cell plasma membrane. Following receptor binding, the fusion process is orchestrated, involving the gH/gL complex in conjunction with glycoprotein B (gB). The gH/gL heterodimer might also participate in the fusion of the virion envelope with the outer nuclear membrane during viral morphogenesis [9].

Previous research utilizing a temperature-sensitive (ts) mutant of HSV-1 demonstrated that the absence of the gH gene completely abolishes viral infectivity, highlighting the critical role of gH in maintaining viral viability [10]. Furthermore, antibodies specifically targeting gH have been shown to effectively inhibit membrane fusion in syncytial virus strains. While these antibodies can prevent virus entry, they do not interfere with viral attachment to host cells, indicating that gH plays a crucial role in the post-attachment stages of viral infection [11]. However, a comprehensive understanding of gH function in other herpesviruses remains relatively limited. Comparative analysis of gH homologues of different herpesviruses has shown a low level of amino acid sequence conservation. Nevertheless, significant homology is observed within the C-terminal region of the extracellular domain [12], suggesting the potential importance of this region for functional activity.

The development of stable cell lines has significantly advanced the production of replication-defective viruses. These viruses hold immense potential as viral vectors for applications such as vaccine development and gene therapy [13]. For the successful establishment of such systems, they must have several key features: (1) a low rate of recombination between the stable cell line and the recombinant virus, (2) consistent expression of the complementing protein by the host cell, and (3) efficient complementation to enable the production of viruses capable of a single round of replication [14].

Single-cycle viruses, characterized by an incomplete genome and a defined replication phenotype, are restricted to a single round of replication within the host cell. This limitation prevents them from establishing a productive infection or generating subsequent viral progeny [15].

Recombinant viruses can be used for diverse applications, with a prominent example being their use as vaccines against viral pathogens [16]. In this study, we generated a gH-deficient recombinant of ILTV with single-cycle replication properties. This engineered virus is intended to serve as a viral vector platform for gene delivery and vaccine development.

Materials and methods

Cells and viruses

Vero (African green monkey kidney) and HEK-AD (human embryonic kidney) cell lines were obtained from the National Cell Bank of Iran (Pasteur Institute of Iran, Tehran). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (penicillin-streptomycin). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The ILTV strain used in this study was kindly provided by Dr. Arash Ghalyanchilangeroudi (University of Tehran). Virus stocks were generated through low-multiplicity infections.

Construction of gH expression vectors

A 2445-bp DNA fragment encoding the gH protein (NC_006623.1) was chemically synthesized. This fragment included an N-terminal HA epitope tag (29 bp) to enable detection by Western blotting using an anti-HA antibody. Furthermore, SwaI and BamHI restriction sites were incorporated for subsequent cloning. The gH-HA construct was then subcloned into the pCDH-CMV-MCS-EF1-copGFP-T2A-Puro vector (CD-513B-1, System Bioscience, Sydney, Australia) using the aforementioned restriction sites. This vector, which has a puromycin resistance gene and a CopGFP fluorescent protein gene under the control of a human elongation factor 1a (EF1) promoter, was chosen for its suitability for generating stable cell lines. In some experiments, the vector was linearized to facilitate the integration of the transgene into the host cell genome and subsequent selection of stable cell lines based on fluorescence-activated cell sorting (FACS) for CopGFP expression.

Generation of a stable gH-GFP-expressing cell line

To generate stable cell lines, Vero cells were seeded in 24-well plates and transfected with the pCDH-CMV-gH-EF1-CopGFP-T2A-Puro vector using TurboFect (Invitrogen, CA). After incubation for 14 days, puromycin was added to a final concentration of 5 μg/mL. At 48 hours post-transfection, GFP expression was assessed via fluorescence microscopy. After 14 days of selection, GFP-positive cell clusters were isolated, and single-cell clones were expanded in 96-well plates. Clones with high GFP expression were identified using a gradual puromycin dilution strategy and further expanded in 75-cm² flasks. Stable Vero cell lines were maintained for up to 10 passages, and GFP expression was confirmed at passage 10. The generation of the stable HEK-AD cell line followed an identical protocol to that described for the stable Vero cell line. Specifically, an antibiotic concentration of 0.5 μg/mL was employed for selection, and the resulting stable HEK-AD cell line was validated.

DNA preparation and polymerase chain reaction (PCR) amplification

To confirm the stable integration of the gH gene into the Vero cell genome, genomic DNA was isolated from both stably transfected and untransfected Vero cells using a commercial DNA extraction kit (QIAGEN, Maryland, USA). PCR amplification was performed using primers specific for the gH gene (gH-Fw, 5′-GTCTGTAGGTCATTCCAG TGC-3′; gH-Re, 5′-GCGGGACACTTTTTAACTTT-3′). As an internal control, PCR amplification of the GAPDH gene was performed in all samples, using the following primers: GAPDH-Fw, 5′-CTCTGCTCCTCCTGTTCGAC-3′; GAPDH-Re, 5′-TTAAAAGCAGCCCTGGTGAC-3′. PCR products were then separated by electrophoresis in a 1% agarose gel.

Immunoblot analysis

Cells were lysed in RIPA buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl) and centrifuged at 15,000 × *g* for 10 minutes at 4°C. The protein concentration in the supernatant was determined using a bicinchoninic acid (BCA) assay (Sigma-Aldrich, Maryland, USA).

For Western blot analysis, 50 μg of protein was separated by SDS-PAGE (10–12%) and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was probed with an anti-HA.11 primary antibody (1:5000 dilution; 90150; BioLegend, California, USA) followed by a peroxidase-conjugated anti-mouse IgG secondary antibody (BioLegend). Protein bands were visualized using ECL Western Blotting Substrate (Thermo Fisher Scientific). β-actin was used as a loading control and was detected using an anti-β-actin antibody (A2228; Sigma–Aldrich, Maryland USA) and the same secondary antibody.

Flow cytometry assay

For the quantitative assessment of gH-GFP expression in the stable Vero cell line, a flow cytometry assay was used. The percentage of GFP-positive cells containing gH integrated into the cell genome was estimated by observing the intensity of GFP expression in the cells. Both the stably transfected and untransfected Vero cells were cultured, trypsinized, collected, and washed with phosphate-buffered saline (PBS). The cells were then resuspended in PBS and subjected to analysis using a CyFlow flow cytometer (Partec, Germany).

In vitro ILTV characterization and propagation

Vero cells were infected with ILTV at an MOI of 3 in 10-cm dishes. At 96 hours postinfection, cytopathic effects were observed, and the cells were harvested. The cell supernatant was collected, aliquoted, titrated, and stored at -70°C.

For DNA extraction, cells were pelleted, resuspended in lysis buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$), and subjected to three freeze-thaw cycles. After centrifugation, the supernatant was treated with RNase A and DNase I to remove RNA and DNA. Proteinase K and 0.1% SDS were added for protein digestion, and after incubation,

DNA was extracted using phenol-chloroform and ethanol precipitation.

The gH gene was amplified by PCR using the primers gH-Fw (5′-GTCTGTAGGTCATTCCAGTGC-3′) and gH-Re (5′-GCGGGACACTTTTTAACTTT-3′), and PCR products were analyzed by electrophoresis on a 1% agarose gel.

Titration of wild-type progeny viruses

Viral titers were determined by plaque assay [17]. Vero cells were seeded in 6-well plates and infected with serial dilutions of the wild-type virus. After a 2-hour incubation at 37°C, the inoculum was removed, and the cells were overlaid with DMEM supplemented with 2% fetal bovine serum (FBS), antibiotics (penicillin-streptomycin), and 1.5% carboxymethyl cellulose. The plates were incubated at 37°C for 72–96 hours, allowing plaques to form. The plaques were then counted, and the average plaque count was calculated to determine the viral titer.

Generation of gH-deleted arms and BleCherry-containing shuttle vectors

The gH gene sequence in the pUC57 vector contained two NcoI restriction sites at positions +1234 and +2067. To generate homologous recombination arms, the gH sequence between these sites was removed by NcoI digestion and subsequent blunt-end repair using *Pfu* DNA polymerase [18].

The BleCherry expression cassette, including the CMV promoter, BleCherry gene, and BGH polyA signal, was amplified by PCR from the pSL-Homo-F1-CMV-BleCherry-pA-Homo-F2 vector (kindly provided by Dr. Abdoli [19]) using the primers Ble-Fw (5′-CATGCCTGCTATT GTCTTC-3′) and Ble-Re (5′-CTAGTTATTAATAGTAAT CAATTACGG-3′). This PCR product was then ligated to the modified pUC57 vector containing the gH homologous arms via blunt-end cloning.

The successful construction of the shuttle vectors was confirmed by double restriction enzyme digestion with BglII and SalI.

Generation of a ΔgH-Red recombinant ILTV

To generate a ΔgH-Red recombinant ILTV, Vero cells in 6-well plates were transfected with the pUC57-ARM1-CMV-BleCherry-PolyA-ARM2 shuttle vector using TurboFect (Invitrogen, California, USA). BleCherry expression was confirmed by fluorescence microscopy at 24 hours post-transfection. The cells were then infected with wild-type ILTV at an MOI of 1 and incubated at 37°C with 5% CO_2 for 72 hours. The viral supernatant was collected and used to infect Vero cells cultured in 60-mm plates. After 2 hours of adsorption, the inoculum was removed, and the cells were overlaid with a medium containing 1.5% carboxymethylcellulose, 2% FBS, and 1% antibiotics. Plaque purification was performed, and fluorescent plaques were identified and isolated. The virus was released from the plaques by three freeze-thaw cycles. This plaque purification process was repeated three times. Finally, the isolated ΔgH-Red recombinant ILTV was propagated and stored at -70°C.

DNA extraction of ΔgH-Red recombinant and wild-type ILTV and PCR amplification

To confirm the generation of the ΔgH-Red recombinant viruses, viral DNA was extracted from both recombinant and wild-type ILTV viruses. PCR amplification was performed using primers targeting the UL22 gene (Fw-homo UL22, 5′-ATGTCCTTTACTCATTTCCT-3′; Re-homo UL22, 5′-TTA AAGTTCGCTCTCCTCGTC-3′). The PCR products were then separated on a 1% agarose gel for analysis.

Viral growth kinetics

Single-step growth kinetics of wild-type ILTV and ΔgH-Red recombinant ILTV were determined in confluent monolayers of stable Vero cells [20]. Briefly, the cells were infected at a multiplicity of infection (MOI) of 0.1 for 1 hour at 37°C. After adsorption, the inoculum was removed, and the cells were washed and overlaid with DMEM supplemented with 2% FBS. At 12, 24, 36, 48, and 72 hours postinfection (hpi), the infected cells were harvested, and viral titers were determined by plaque assay. The experiment was performed in triplicate.

Cell viability assay

Cell viability was assessed using a 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay. Vero, stably transfected Vero, HEK-AD, and stably transfected HEK-AD cells were seeded at a density of 10^4 cells per well in 96-well plates and infected with wild-type ILTV or ΔgH-Red recombinant ILTV at MOIs of 10, 1, 0.1, 0.01, and 0.001. After a 1-hour adsorption period, the inoculum was removed, and the cells were incubated with DMEM containing 2% FBS for 72 hours at 37°C. Cell viability was then determined by adding XTT reagent (Roche, Basel, Switzerland) to each well and measuring the absorbance at 450 nm using a microplate reader (BioTek Instruments) after a 4-hour incubation at 37°C. All data points represent the mean of three independent experiments.

Virus infection assay

To determine the infectivity of recombinant ILTV viruses, a plaque assay was performed. Cell monolayers were inoculated with wild-type and ΔgH-Red ILTV at a multiplicity of infection (MOI) of 0.1. After a 1-hour adsorption period, the inoculum was removed, and cells were overlaid with DMEM containing 2% FBS. Virus replication was allowed to proceed for 72 hours. For wild-type ILTV, cells were fixed with methanol and stained with crystal violet to visualize plaques. For ΔgH-Red ILTV, plaques were visualized by fluorescence microscopy using a BEL Engineering microscope.

Statistical analysis

Data were analyzed by two-way ANOVA using GraphPad Prism 9 software. Results are presented as the mean ± standard deviation (SD). Statistical significance was defined as $p < 0.05$.

Results

Generation of a stable cell line expressing gH-GFP

To generate a stable Vero cell line expressing the gH gene and GFP, a two-step approach was employed. First, the gH coding sequence was synthesized and subcloned into the pCDH-CMV-MCS-EF1-CopGFP-T2A-Puro expression vector. Second, Vero cells were transfected with this construct and selected with puromycin (5 µg/mL) to generate stable colonies expressing both gH and GFP. Single-cell clones were isolated, expanded, and further purified through limiting dilution. The stable integration of the gH gene into the Vero cell genome was confirmed by the fact that continuous expression of GFP was observed (Fig. 1A-D).

To confirm the generation of stable Vero cell lines expressing gH, genomic DNA was isolated from both stably transfected and untransfected Vero cells. PCR amplification was performed using primers specific for the gH gene and GAPDH as an internal control. A 1080-bp band, corresponding to the expected size of the gH amplicon, was observed only in the stable cell line, while no amplification was detected in control Vero cells. GAPDH amplification (183 bp) was observed in both cell lines, confirming the integrity of the genomic DNA (Fig. 1E).

Western blot analysis was performed to assess gH protein expression. An anti-HA antibody was used to detect the HA tag fused to the gH protein. The results demonstrated the presence of HA-tagged gH protein in the stable cell line but not in control Vero cells, confirming the successful expression of the integrated gH gene (Fig. 1F).

Continuous expression of gH-GFP was monitored by fluorescence microscopy in stable Vero cells over 10 passages. Consistent GFP fluorescence was observed in all passages, indicating stable and sustained expression of the integrated gene (Fig. 1G).

Flow cytometry analysis was performed to determine the percentage of GFP-positive cells in the stable cell line. Approximately 70% of the stable cell population exhibited GFP fluorescence, indicating that a high percentage of cells had successfully integrated and expressed the gH gene (Fig. 1H).

Characterization of wild-type ILTV and plaque assay

Viral titers were determined by plaque assay on Vero cells. Serial dilutions of the virus were used to infect Vero cell monolayers, and plaques were visualized by crystal violet staining. Representative images of ILTV-induced cytopathic effects (CPEs) and plaque morphology are shown in Fig. 2A and B. The average titer of the ILTV stock was 5×10^7 plaque-forming units (PFU)/ml based on three independent plaque assays (Fig. 2C).

To characterize wild-type ILTV, Vero cells were infected, and progeny virus was harvested. Viral DNA was extracted, and PCR amplification using gH-specific primers yielded a 1080-bp fragment, confirming the presence of the ILTV gH gene (Fig. 2D). As a control, the gH gene was also amplified from a commercial live-attenuated ILTV vaccine, resulting in a similar 1080-bp fragment. All PCR experiments were performed in triplicate.

Generation of ΔgH-Red recombinant ILTV

To generate a recombinant ILTV lacking the gH gene, homologous recombination was used to replace the gH gene in the wild-type ILTV genome with a CMV-BleCherry-BGH poly-A cassette. Figure 3A illustrates the genomic location of gH in wild-type ILTV and the strategy for its deletion. The steps involved in generating the ΔgH-Red recombinant ILTV in stable GFP-expressing Vero cells are shown in Fig. 3B.

Vero cells were transfected with a shuttle vector containing the CMV-BleCherry-BGH poly-A cassette flanked by homologous arms (pUC57-ARM1-CMV-BleCherry-PolyA-ARM2). BleCherry expression was monitored by fluorescence microscopy (Fig. 4A and B). Double digestion with BglII and SalI confirmed the correct cloning of the shuttle vector by generating two fragments of 3623 bp and 2673 bp (Fig. 4E).

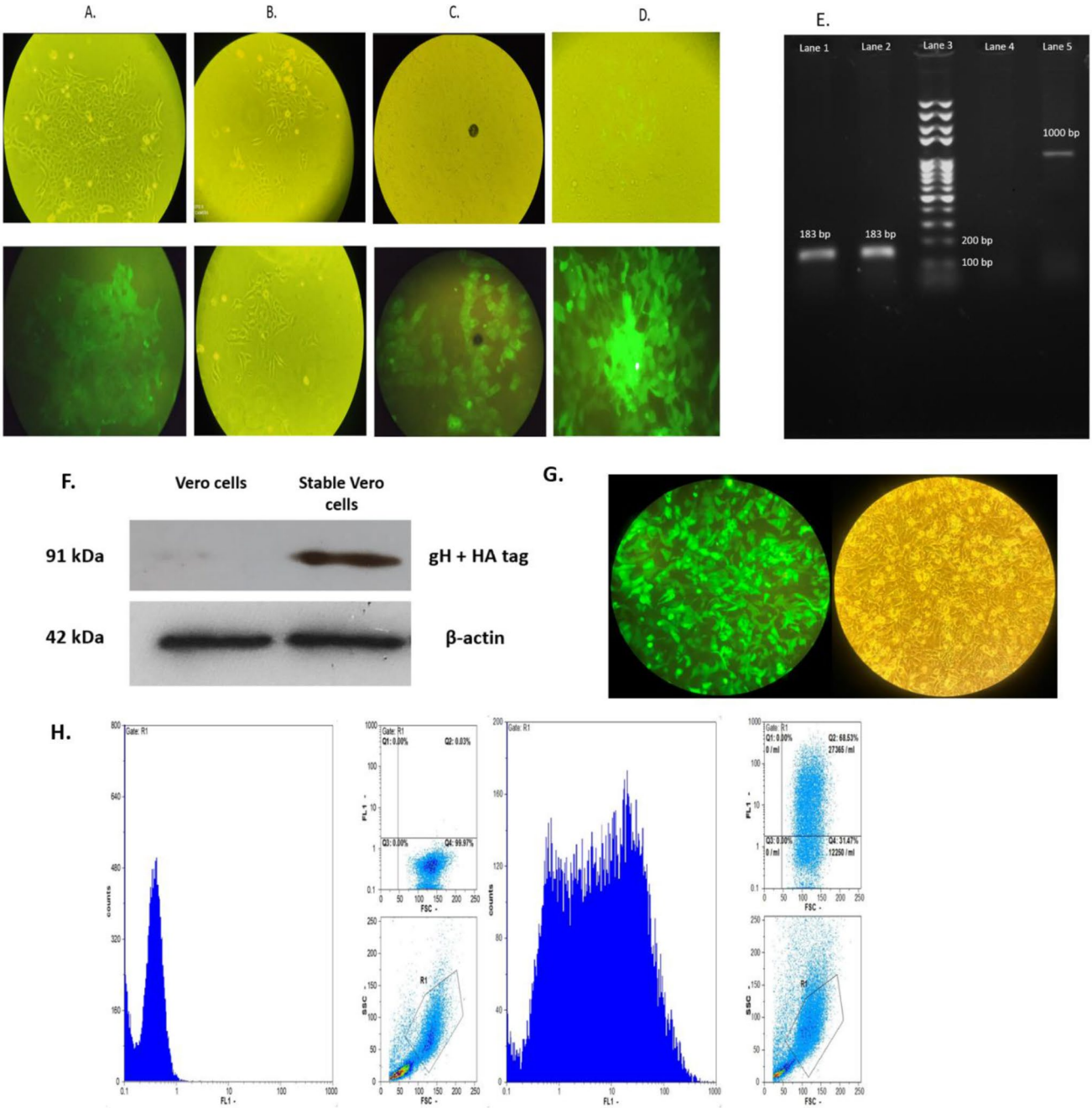

Fig. 1 Generation of stable gH-expressing Vero cells. **A** represents the formation of GFP-positive gH-expressing colonies, **B** and **C** represent single cells separated from colonies and generated new colonies, and **D** represents the expansion of GFP-positive colonies in the T75 flask. PCR analysis of stable and Vero cells is shown in C. Lane 1: Vero cells with a 183-bp amplified GAPDH fragment as an internal control; Lane 2: stable Vero cells with a 183-bp amplified GAPDH fragment as an internal control; Lane 3: 10 kb marker; Lane 4: no detected amplified gH fragment in Vero cells; Lane 5: stable Vero cells with a 1080 bp amplified fragment of gH integrated into the genome. Immunoblot analysis with an HA tag monoclonal antibody is shown in **F**, and the results revealed an approximately 91 kDa band in stable Vero cells, with beta-actin being the housekeeping protein. Constant expression of gH-GFP in stable Vero cells after 10 passages is shown in **G**. Quantification via flow cytometry analysis in **H** revealed that 70% of the population of stable Vero cells was GFP positive and that gH and GFP were integrated into the cell genome

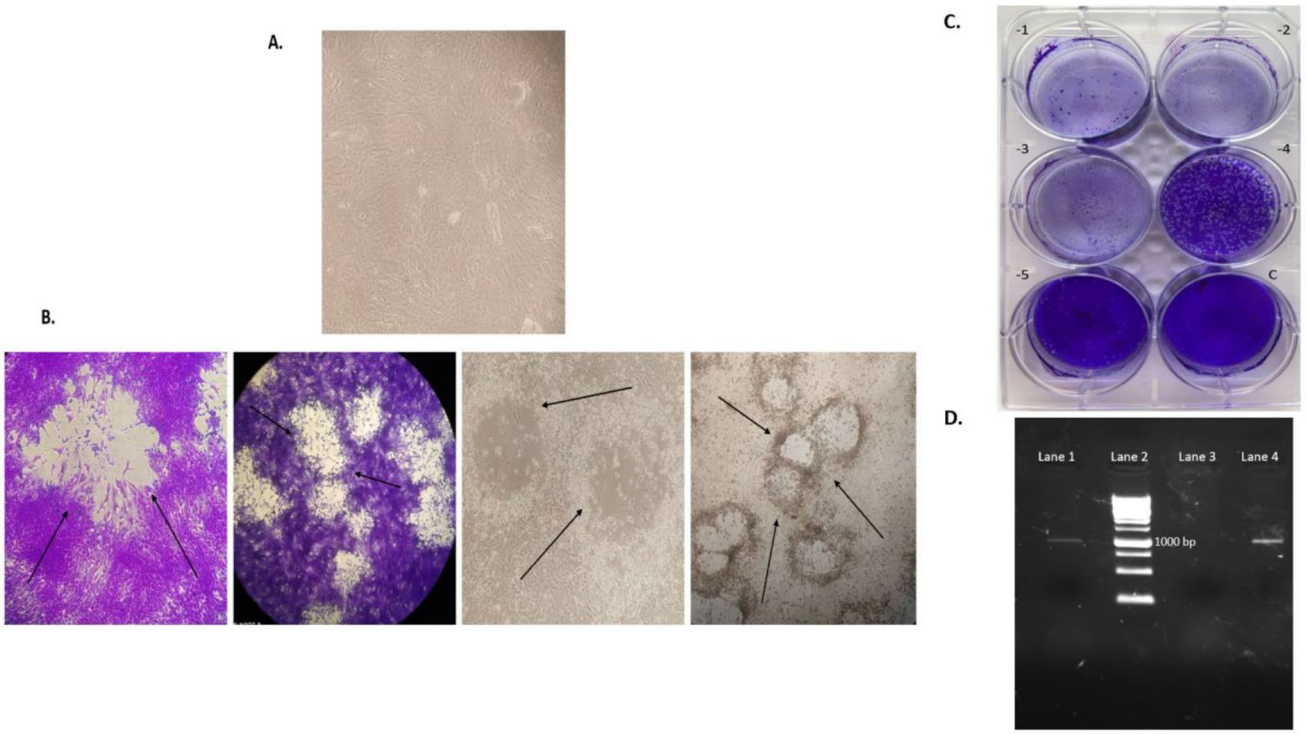

Fig. 2 Characterization and titration of the ILTV. Cytopathic effects and plaque formation of ILTV in Vero cells compared with those in control cells are shown in **A** and **B**. Plaque assay plates with various dilutions of ILTV are shown in B, and the titre of wild-type ILTV was 5×10^7 (pfu/ml). Characterization of ILTV via PCR analysis is shown in C, and the results confirm ILTV via amplification of a 1080 bp fragment in Lane 1. Lane 2 is a 10 kb ladder, and Lane 3 is an NTC. Additionally, Lane 4 represents the 1080 bp fragment amplified by the gH primer in the extracted viral genome of the commercial live attenuated ILTV vaccine

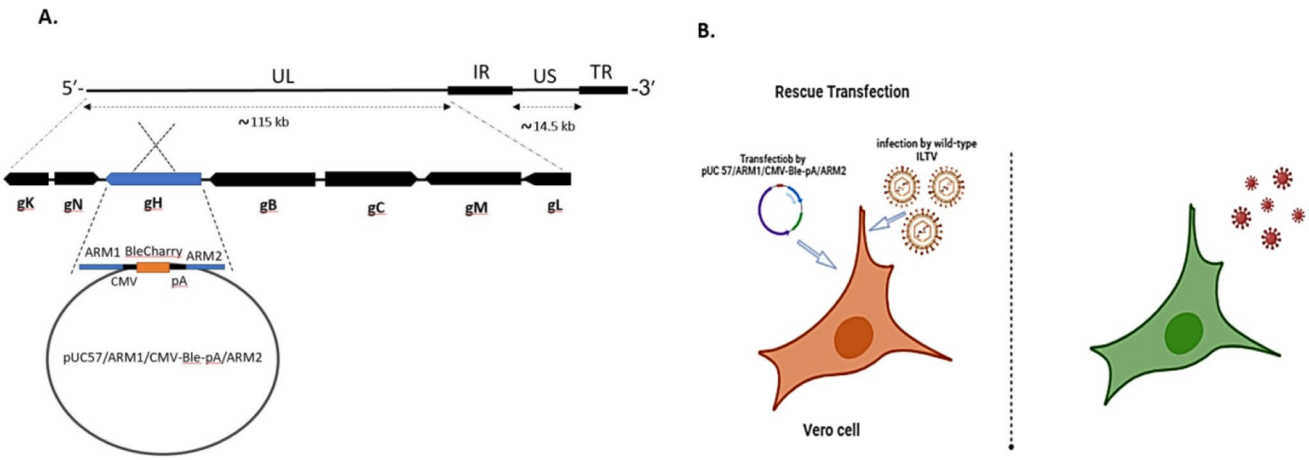

Fig. 3 Schematic diagram of the deletion of gh in the genome of ILTV and the generation of ΔgH- Red recombinant ILTV. **A**. Schematic diagram of the ILTV showing the regions modified by the homologous recombination shuttle vector. The pUC57-ARM1-CMV-BleCherry-PolyA-ARM2 shuttle vector generated the ΔgH-Red recombinant ILTV by deleting the gH gene and inserting the BleCherry expression cassette into the gH locus. **B** Schematic diagram showing the steps involved in the formation of recombinant gH-deleted ILTV in Vero cells and the generation of a single cycle of ΔgH-Red recombinant ILTV in a stable Vero cell line

Cells expressing BleCherry were infected with wild-type ILTV. Following the appearance of CPEs, the culture supernatant was harvested and used to infect stable gH-expressing Vero cells. Plaques were selected using a 1.5% carboxymethyl cellulose overlay.

Red plaques, indicative of recombinant virus, were observed (Fig. 4C and D). Three rounds of plaque purification were performed to isolate the ΔgH-Red recombinant ILTV.

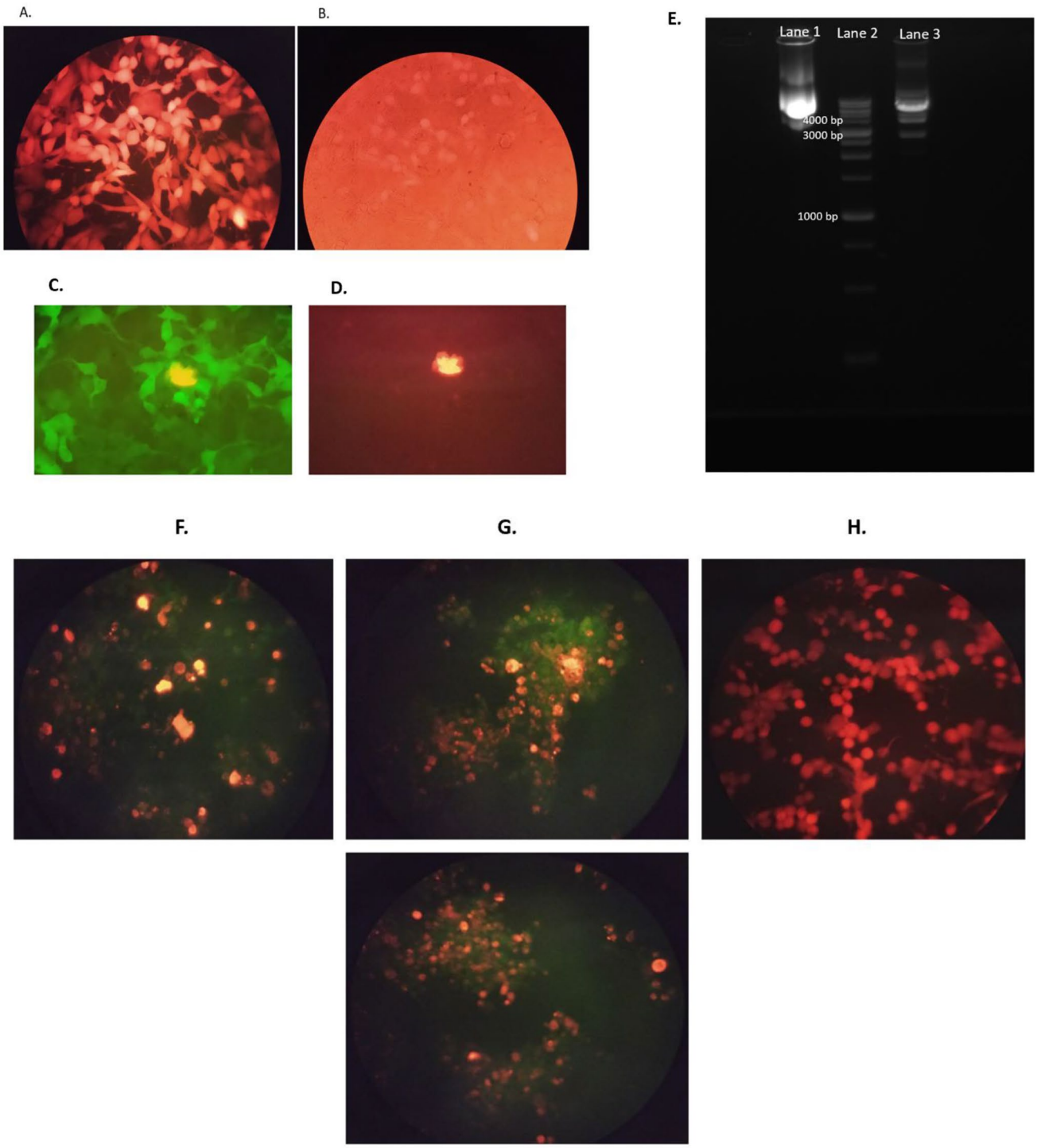

Fig. 4 Generation of ΔgH- Red recombinant ILTV. **A** and **B**. Vero cells were transfected with the homologous recombination shuttle vector and the BleCherry expression cassette. **E** Confirmation of the cloning of the shuttle vector via double digestion with *Bgl*II and *Sal*I and generation of two fragments of 3623 and 2673 bp in Lane 3 in comparison with the undigested shuttle vector, with 6299 bp in Lane 1 and Lane 2 representing a 10 kb ladder. **C** and **D**. Analysis of BleCherry-positive infected cells. The red stain shows single-cell BleCherry expression by ΔgH-Red recombinant ILTV. **F** Plaque purification was performed, and an increase in ΔgH- Red recombinant ILTV was detected. G Single plaque showing BleCherry expression and H a monolayer of stable cells infected with red recombinant ILTV and BleCherry

The titer of the isolated ΔgH-Red recombinant ILTV was determined by plaque assay on stably transfected Vero cells (Fig. 4F, G, and H). The titer of the recombinant virus was found to be 4×10^5 pfu/ml, which was lower than that of wild-type ILTV. This result was confirmed by three independent titrations.

Confirmation of the generation of ΔgH- Red recombinant ILTV

To confirm the generation of ΔgH-Red recombinant ILTV, genomic DNA was extracted from purified virions of both ΔgH-Red recombinant ILTV and wild-type ILTV. PCR amplification using primers specific for the homoUL22 gene in wild-type ILTV yielded a 2415-bp fragment. In contrast, amplification in the ΔgH-Red recombinant ILTV genome resulted in a 3605-bp fragment. This increase in fragment size is attributable to the insertion of the BleCherry cassette within the gH gene during homologous recombination (Fig. 5).

Viral growth analysis

To assess viral replication, three independent growth kinetic experiments were performed. Vero cells were infected with wild-type ILTV, while gH-expressing Vero cells were infected with ΔgH-Red recombinant ILTV.

Compared to wild-type ILTV, ΔgH-Red recombinant ILTV exhibited significantly reduced replication kinetics in gH-expressing Vero cells over a 72-hour period (Fig. 6A). Statistical analysis revealed a significant decrease in ΔgH-Red replication at 36 hours postinfection compared to wild-type ILTV ($p \leq 0.0001$). Similar significant differences were observed at 48 and 72 hours postinfection ($p \leq 0.001$).

These results demonstrate that while ΔgH-Red recombinant ILTV can enter and initiate infection in gH-producing Vero cells, its replication capacity and viral titers are significantly lower than those of wild-type ILTV.

Viability of cells infected with ΔgH-Red recombinant ILTV

Cell viability was assessed 72 hours postinfection using the XTT assay. Stable complementing Vero and HEK-AD cell lines were generated using a recombinant ΔgH-Red ILTV. Parental Vero and HEK-AD cell lines were infected with the wild-type ILTV at five different multiplicities of infection (MOIs: 0.001, 0.01, 0.1, 1, and 10). As shown in Fig. 6B and C, infection with recombinant ΔgH-Red ILTV resulted in significantly higher cell viability compared to wild-type ILTV infection at all MOIs ($p \leq 0.0001$). This effect was more pronounced in gH-producing Vero cells than in gH-producing HEK-AD cells.

The observed increase in cell viability with the recombinant virus is probably attributable to its restricted replication cycle. Since the recombinant virus lacks the gH gene, it cannot spread efficiently to neighboring cells, limiting the extent of viral replication and cytopathic effects.

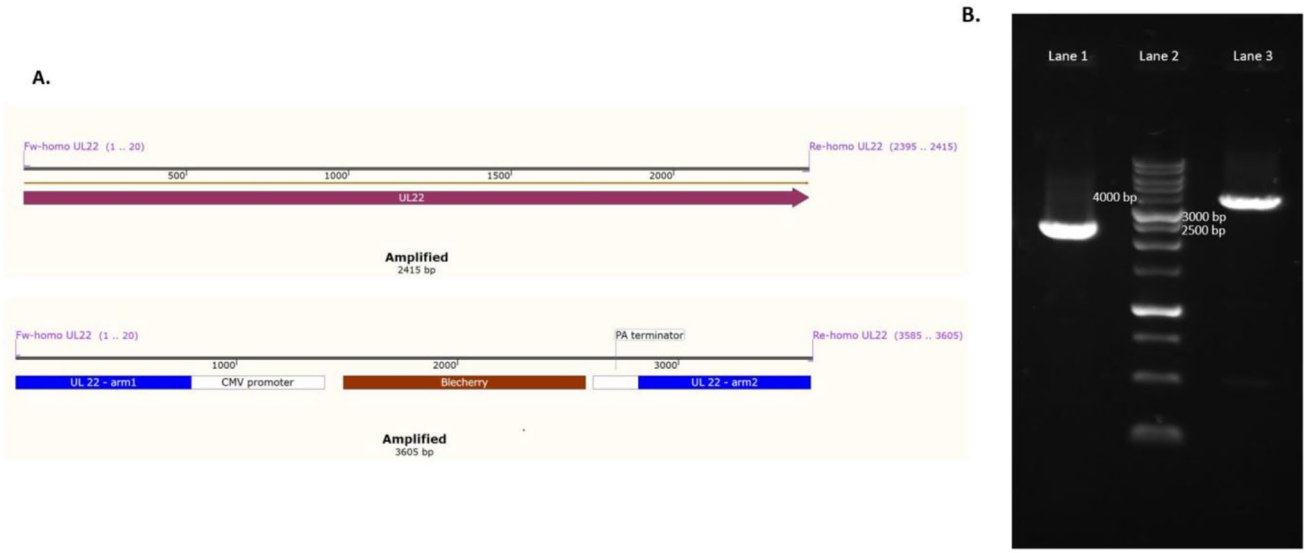

Fig. 5 Generation of ΔgH- Red recombinant ILTV was confirmed. **A** Schematic diagram of the PCR product with homo UL22 primers in the viral genomes of wild-type ILTV (2415 bp fragment) and ΔgH-Red recombinant ILTV (3605 bp fragment). **B** The PCR products were run on a 1% agarose gel. Lane 1 shows a fragment of 2415 bp on the wild-type ILTV genome amplified with homo UL22 primers, Lane 2 represents a 10 kb ladder, and Lane 3 shows a fragment of 3605 bp on the ΔgH- Red recombinant ILTV genome amplified with homo UL22 primers. The increase in the size of the amplified fragment was due to the insertion of the BleCherry cassette arms of homologous recombination

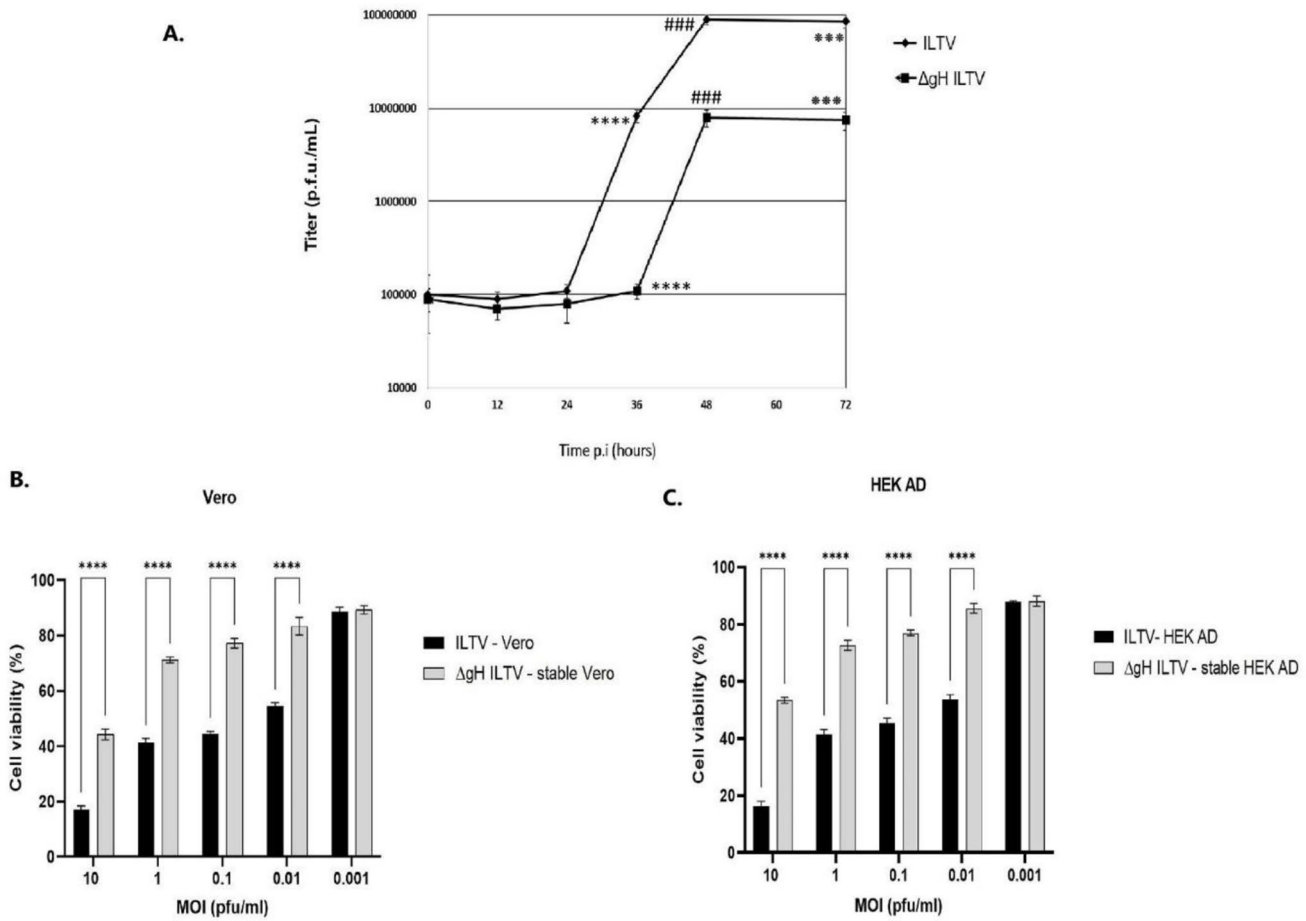

Fig. 6 *In vitro* growth kinetics and cell viability of ILTV and ΔgH recombinant ILTV. For characterization of viral growth in **A**, Vero and stable Vero cells were infected with ILTV and ΔgH recombinant ILTV at an MOI of 0.1. The supernatant was collected at 12, 24, 36, 48 and 72 h post infection, and titrations were performed via plaque assays. **B**, Cell viability in Vero and HEK-AD cells was determined via the XTT assay. Vero cells and stable Vero cells were infected with ILTV and ΔgH recombinant ILTV at MOIs of 10, 1, 0.1, 0.01 and 0.001, and XTT analysis was used to determine cell viability. Similar conditions were used for HEK-AD and stable HEK-AD cells, and the results are shown in C. (*p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001)

At an MOI of 0.001, the effect of the recombinant virus on cell viability was minimal, and no significant difference was observed between cells infected with the recombinant virus and wild-type ILTV.

The higher cell viability observed in stably transfected HEK-AD cells infected with the recombinant virus compared to stably transfected Vero cells may be explained by the higher replication capacity of wild-type ILTV in Vero cells. Vero cells, which are deficient in interferon production, support robust viral replication, leading to more-pronounced cytopathic effects compared to HEK-AD cells.

gH of ILTV is essential for viral entry and replication

Wild-type ILTV efficiently replicated in Vero cells, resulting in extensive plaque formation within 48 hours (Fig. 7A). In contrast, ΔgH-Red ILTV exhibited restricted replication in Vero cells, with only single-cell infections observed and no plaque formation evident after 96 hours (Fig. 7B). However, ΔgH-Red ILTV replicated efficiently in Vero cells stably expressing gH, forming plaques similar to those induced by wild-type ILTV (Fig. 7C), albeit with reduced viral growth. Importantly, ΔgH-Red ILTV recovered from gH-expressing cells failed to infect naïve Vero cells, indicating that this recombinant virus is replication-deficient. These findings underscore the critical role of gH in ILTV replication *in vitro*.

Discussion

One of the challenges associated with live attenuated ILTV vaccines is the potential for reversion to virulence and shedding in the flock. Therefore, the aim of this study was to construct a recombinant ILTV lacking the glycoprotein H gene, exhibiting single-cycle replication capability, with the

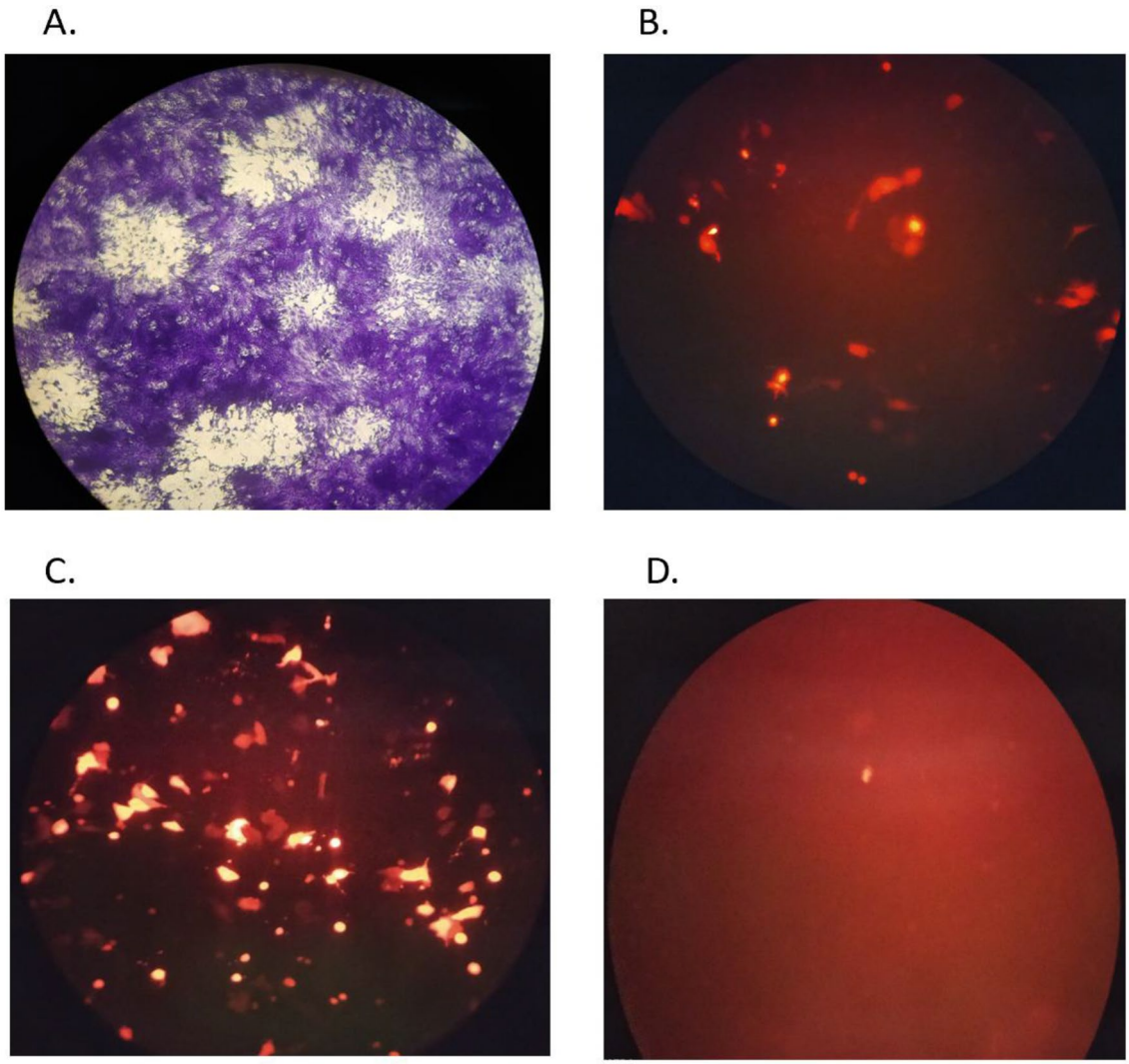

Fig. 7 Infection of Vero cells with ΔgH-Red recombinant ILTV. Vero cells were infected with wild-type ILTV (**A**) or ΔgH-Red recombinant ILTV (**B**). Stable Vero cells were also infected with ΔgH- Red recombinant ILTV (**C**). Recovered virus was collected from stable Vero cells and used to infect naïve Vero cells (**D**). The infected cells appear red, as all viruses express BleCherry. The cells were inspected with a fluorescence microscope (BEL ENGINEERING, MONZA, Italy) and stained with crystal violet, and images were taken with a camera (Nikon SMZ-745T). The scale bar represents 100 µm

potential to develop a vaccine to overcome the challenges of controlling this virus. According to previous studies, glycoprotein H is a crucial component of herpesviruses, exhibiting structural conservation across various herpesviruses. Its conserved features suggest a significant role in viral fusion processes, with specific domains and glycosylation sites maintained throughout the family *Orthoherpesviridae* [21]. In this study, we successfully deleted the gH gene from the wild-type ILTV genome and replaced it with the BleCherry fluorescent protein gene, allowing the visualization of viral infection dynamics. Concurrently, we established stable cell lines expressing gH-GFP proteins in both Vero and HEK-AD cells. This dual approach not only enabled us to create a gH-deficient recombinant virus but also provided a robust system to study the functional importance of gH for viral infectivity.

Previous research has shown that the absence of gH coding sequences in gH-defective HSV-1 leads to a non-viable virus; however, this deficiency can be compensated by the introduction of gH *in trans* through expression in suitable host cells [22]. In our study, we established a stable Vero cell line in which the gH gene was integrated into the cellular genome under antibiotic selection pressure. The successful integration was confirmed through the use of a fluorescent marker, allowing the identification and amplification of GFP-positive colonies. This system will facilitate the study of the functional role of gH and also provide a platform for future studies on viral pathogenesis and vaccine development.

The ΔgH-Red recombinant ILTV was generated in stably transfected Vero cells using homologous recombination techniques. During virion assembly, this recombinant virus acquired gH *in trans* from the stable cell line, allowing the packaging and release of virions via the Golgi apparatus [23]. Although these recombinant viruses formed fully developed virions, they were replication-deficient due to the absence of the gH gene [24]. This suggests that the ΔgH-Red recombinant can enter host cells and initiate a single cycle of replication but cannot produce progeny virions. This limitation positions the recombinant virus as a potential candidate for vaccine development, as it can elicit robust antibody responses and stimulate cellular immune responses without the risk of uncontrolled viral replication [25].

In similar studies, Publicover et al. explored the potential of using single-cycle vesicular stomatitis virus (VSV) vectors as vaccines [26]. Their research demonstrated that the elimination of the glycoprotein G gene resulted in a single-cycle virus that could only replicate in cells in which the G glycoprotein was transiently expressed. Those single-cycle defective viruses were shown to induce strong cytotoxic T lymphocyte (CTL) and humoral responses, demonstrating their potential as a vaccine vector.

Previously, Forrester et al. found that gH is essential for the infection process, facilitating the entry of virions into host cells and their subsequent transmission [27]. Likewise, studies on equine herpesviruses 1 and 4 have demonstrated that gH is crucial for mediating interactions between the viral envelope and the host cell membrane, affecting cell entry, membrane fusion, intracellular trafficking, and immune evasion [28]. Our finding that the ΔgH-Red recombinant strain of ILTV exhibited a replication-deficient phenotype in Vero cells lacking gH expression suggests a similar role of gH in ILTV replication. Although the recombinant virus could penetrate stable Vero cells, it was unable to produce progeny virions or form plaques, underscoring the importance of gH for viral entry and replication.

In a study by Gompels and Minson, who used electron microscopy to investigate HSV-1 infection in Vero cells, it was found that genetically defective viruses, despite resembling wild-type viruses, exhibited an altered capacity to produce virions [29]. Such defective viruses can penetrate host cells but are unable to form new functional virions [30].

The use of gH-deleted ILTV as a vaccine has a number of advantages over the use of plasmid DNA or replicating viral vectors. The modified virus is expected to retain its ability to elicit a strong protective immune response against the target pathogens, and its genetic stability reduces the likelihood that it will acquire mutations that could lead to either serious side effects or a loss of effectiveness. In contrast to live attenuated vaccines, recombinant ILTV is much less likely to revert to virulence, making it a safer option. Moreover, this virus can be manipulated in various ways to become a multifunctional tool to target other pathogens. The lack of horizontal transmission of the vaccine strain makes it very unlikely that the vaccine itself will cause outbreaks in the flocks [31].

In our investigation, we also examined the transfer of viruses from the supernatant of gH-expressing Vero cells infected with recombinant ΔgH-Red ILTV to normal Vero cells. The results indicated that while the recombinant virus could enter the cells, it was incapable of extensive replication and propagation. This observation aligns with the findings of Malicoat et al., who developed a single-cycle infectious SARS-CoV-2 virus replicon particle system designed to mimic viral replication while minimizing biosafety risks [32]. Their system effectively restricted viral replication to a single cycle, further supporting the concept of using single-cycle viruses for vaccine development.

In contrast to the wild-type ILTV, ΔgH-Red recombinant ILTV demonstrated impaired growth kinetics, presumably due to its ability to replicate or spread to adjacent cells [33]. Both stable cell lines produced in this study exhibited higher survival rates when infected with the ΔgH-Red recombinant ILTV than when infected with wild-type ILTV due to the inability of the recombinant virus to undergo multiple rounds of infection.

The one-step growth studies performed in primary chicken kidney cells have shown that the first infectious progeny viruses are generated starting approximately 8 to 12 hours postinfection, with peak production occurring between 24 and 72 hours postinfection [34]. This is consistent with earlier investigations by Desai et al., which focused on the replication kinetics of HSV-1 deficient in the gH gene, revealing significantly lower titres for recombinant viruses lacking the H gene when compared to the parent strain [10]. In the current study, the titres of both intracellular and supernatant viruses were measured for each viral type, demonstrating lower titres for the recombinant virus in comparison to the parental virus, underscoring the critical role of glycoprotein H in facilitating viral entry and replication. The reduction in viral replication was also found to be associated with enhanced cell survival.

The deficient recombinant virus strain described here appears to be a promising candidate for use as a viral vector. Further research is needed to assess its suitability for vaccine development and therapeutic applications.

Conclusion

In this study, a recombinant ILTV strain was engineered through genetic manipulation. Previous studies have emphasized the crucial role of gH in the infection cycle

of alphaherpesviruses. To provide the gH-deletion mutant with gH *in trans*, a stable Vero cell line expressing gH fused with GFP was established, and this engineered cell line provided the necessary gH for the packaging of recombinant viruses. However, these recombinant viruses exhibited a replication-deficient phenotype, restricted to a single cycle of replication. Consequently, they lack the capacity to infect additional cells, produce progeny, or spread to other cells. The generation of viruses with these characteristics holds significant promise for their potential application as viral vectors.

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Data availability All of the data generated or analysed during this study are included in this published article.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval This study did not involve any human or animal subjects, hence ethical approval is not applicable.

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