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Rescue a Reassortant Human-Bovine Rotavirus and a Recombinant NSP3-EGFP Rotavirus Serotype G1

Mona Aminipour[1], Mohsen Lotfi[2], Naser Harzandi[1], Ebrahim Babapour[1], Seyed Dawood Mousavi Nasab[3,4], Mostafa Ghaderi[5*]

[1]Department of Microbiology, Ka.C, Islamic Azad University, Karaj, Iran; [2]Department of Quality Control, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran; [3]WHO Collaborating Center for Reference and Research on Rabies, Pasteur Institute of Iran, Tehran, Iran; [4]Research Center for Emerging and Reemerging Infectious Disease, Pasteur Institute of Iran, Tehran, Iran; [5]Department of Human Viral Vaccine, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

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ABSTRACT

Background: Human group A rotaviruses (RVs), with an 11-segmented double-stranded RNA genome, are a cause of viral gastroenteritis in infants and young children. Despite the availability of effective live-attenuated vaccines, RV infection is still a global health challenge. Hence, a reverse genetics system enabling the manipulation of RV segments, such as genome reassortment, is essential for developing new RV vaccines. Herein, a reverse genetics system was used to rescue a reassortant human-bovine RV, incorporating the VP7 gene segment from the human RV strain Wa (serotype G1) and a recombinant RV.

Methods: To rescue a reassortant human-bovine RV, we used a reverse genetics system in which 13 plasmid constructs were co-transfected into BHK-T7 cells. In this method, 10 plasmids were derived from bovine RV, one encoded the human RV VP7 segment of serotype G1, and two expressed the capping enzyme subunits of vaccinia virus. We also created a recombinant RV featuring enhanced green fluorescent protein (EGFP) by replacing the bovine NSP3 plasmid with an NSP3-EGFP construct, where EGFP expression was regulated by an internal ribosomal entry site.

Results: Our results showed successful rescue of the reassortant human-bovine RV and the recombinant strain, with the NSP3-EGFP construct facilitating EGFP translation across multiple RV passages. Replacing the *NSP3* gene segment of bovine RV with the release of a recombinant RV enabled EGFP expression in several RV passages.

Conclusion: Our study establishes the feasibility of using a reverse genetics system to generate a live-attenuated RV vaccine candidate, potentially enhancing future control efforts against RV infections.
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Keywords: Genotype, Rotavirus, Vaccines

Corresponding Author: Mostafa Ghaderi
Department of Human Viral Vaccine, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran; E-mail: ghaderi_viro@yahoo.com; ORCID ID: 0000-0002-8249-1231

1. INTRODUCTION

Rotaviruses (RVs) are the leading cause of viral gastroenteritis in humans, especially affecting infants and young children under the age of five. Among these infections, group A RVs are identified as the main pathogens responsible for gastroenteritis in different species, including mammals and birds[1-3]. In humans, these RVs commonly invade the intestines, causing severe acute gastroenteritis in young children. This condition poses significant health challenges and can be fatal, with annual deaths ranging from approximately 128,500 to 215,000[4-6].

RVs are classified as non-enveloped viruses with a unique triple-layered icosahedral structure. Their genome is composed of 11 segments of double-stranded RNA and falls within the RV genus, which is a part of the Sedoreoviridae family. Within the coding sequences of the RV genome, there are 12 protein expression cassettes, including six non-structural proteins (NSP1–NSP6) and six structural proteins (VP1–VP4, VP6, and VP7)[7,8]. Among these structural proteins, VP4 and VP7 are incorporated into the outer layer of the viral capsid, allowing for the classification of RV genotypes and serotypes into G and P categories, respectively[8-10].

Studies have introduced a binary classification system for circulating RVs, identifying a total of 41 G genotypes and 57 P serotypes[11-13]. Of these genotypes, G1–G4, G9, G12, P[8], and P[4] are the most prevalent strains of circulating RVs. These genotypes account for approximately 90% of human RV infections. The primary combinations of these infections are characterized as G1P[8], G2P[4], G3P[8], G4P[8], G12P[8], and G9P8[14-16]. Among the licensed RV vaccines for human use, which are all live-attenuated, two notable options are RotaTeq and RotaSiil. RotaTeq is a pentavalent vaccine derived from bovine RV[16-21], targeting various genotypes of human RV and focusing on the VP4 and VP7 capsid antigens. The vaccine includes four G genotypes and one P genotype, specifically G1, G2, G3, G4, and P[8], effectively covering the most prevalent human RVs in circulation. In contrast, the RotaSiil vaccine comprises only G genotypes, featuring five reassortant human-bovine RV strains: G1, G2, G3, G4, and G9. This vaccine was developed in India in 2017 and achieved WHO prequalification in September 2018. Besides these two pentavalent vaccines, manufacturers have introduced four monovalent human RV vaccines: Rotarix, Rotavac, Rotavin, and Lanzhou, all of which are commercially available to combat human RV infections[22].

Traditional methods of producing RV vaccines can lead to reduced vaccine efficacy and potency due to challenges such as serial passage of the virus or the need for reassortment between human and animal RVs to establish the desired vaccine strain[23]. Recently, a new method named reverse genetics has emerged to overcome these challenges by enabling the rescue of genetically manipulated RVs. This innovative method was first described by Komoto et al. in 2006[24], demonstrating the possibility of rescuing genomic RNA for human RV VP4 using a vaccinia virus (VV) expressing T7 RNA polymerase[24]. In 2017, Kanai et al. refined the reverse genetics system to successfully rescue the simian SA11 RV strain utilizing a plasmid-based approach[11]. Since then, numerous studies have documented the rescue of various RV strains, including both monoreassortant and double reassortant strains that are genetically based on simian RVs[25-29]. In this study, we successfully rescued a reassortant human G1-bovine RV through the transfection of baby hamster kidney cells that continuously expressed T7 RNA polymerase (BHK-T7).

2. MATERIALS AND METHODS

2.1. Construction of RV genes

The complementary DNA (cDNA) genome of RV, comprising 11 segments and various accessory elements—including a T7 promoter (pT7) placed at the beginning of each gene, a ribozyme delta, and a T7 terminator at the end of the genes—was synthesized. The cDNA was then cloned into the pUC57 plasmid by Genscript, a company based in Hong Kong. Of the 11 genes synthesized, 10 were derived from bovine RV genes (specifically the WC-3 strain), while the remaining gene corresponded to the VP7 outer capsid antigen sourced from the human RV strain Wa, classified as the G1 genotype.

2.2. Cell lines

Baby hamster kidney (BHK-T7) and MA104 cells (gifted from Razi Vaccine and Serum Research Institute, Tehran, Iran) were cultured in a high-glucose version of Dulbecco's Modified Eagle's Minimal medium (DMEM) from Gibco (USA). This medium was supplemented with 10% fetal bovine serum (Gibco) and antibiotics, including penicillin at a concentration of 100 U/mL and streptomycin at 0.1 mg/mL.

2.3. Transfection and virus rescue

After preparing six-well plate monolayers of BHK-T7 cells at a density of 5×10^5 cells per well, we conducted the transfection of 11 RV plasmids along with VV capping enzyme subunits obtained from Shingen Company (Hong Kong). This process was accomplished using 10 µL of Lipofectamine transfection reagent. The plasmid concentrations were 0.7 µg each of the WC-3 and Wa rescue plasmids, as well as pWC-VP1, pWC-VP2, pWC-VP3, pWC-VP4, pWC-VP6, pSC-VP7, pWC-NSP1, pWC-NSP2, pWC-NSP3, pWC-NSP4, and pWC-NSP5. In addition, we followed transfection methods described previously in detail[11]. We also incorporated 0.7 µg of each capping subunit enzyme expression plasmid (pVV-CapS and pVV-CapL) into the study. One approach for RV rescue involved testing a plasmid carrying the T7 RNA polymerase gene to evaluate its impact on the efficiency of RV rescue (Fig. 1). The pFT7A plasmid, as previously detailed in our report[30], was co-transfected to measure T7 RNA polymerase activity in BHK-T7 cells. To create a

recombinant RV that includes a coding sequence for the enhanced green fluorescent protein (*EGFP*) gene, we edited the plasmid encoding the NSP3 sequence to insert the EGFP sequence between nucleotides 967 and 968. This insertion used an internal ribosomal entry site (IRES) from the foot-and-mouth disease (FMD) virus to facilitate the re-initiation of translation for the downstream EGFP sequence. This modified plasmid was synthesized by Shingen Company. After transfection, BHK-T7 cells were maintained in DMEM medium without fetal bovine serum for 48 hours. Subsequently, MA104 cells and trypsin (0.3 µg/ml) were introduced to the transfected BHK-T7 cells, and viral replication was monitored until cytopathic effects were observed following co-culture. The 11 T7 plasmids contained full-length segments of 11 individual RV genes flanked by the pT7 and the hepatitis delta virus (HDV) ribozyme, along with two cytomegalovirus (CMV) plasmids corresponding to capping enzyme subunits of VV. A plasmid related to the T7 RNA polymerase enzyme was also used in one of the transfections. To generate a reassortant human-bovine RV, BHK-T7 cells were co-transfected with the 13 or 13+1 T7 and CMV plasmids (Fig. 1).

2.4. Assessment of rescued reassortant RV

To analyze the reassortant RV rescued through reverse genetics, we used MA104 cells for the propagation of the rescued RV in DMEM medium containing 0.3 µg/mL trypsin. RV isolation was performed through plaque purification, and virus titers were determined on MA104 cells. For plaque formation analysis, MA104 cell monolayers were infected with the rescued RV at a multiplicity of infection (MOI) of 0.01. After applying an agarose overlay in DMEM medium with trypsin (0.3 µg/mL), the cell monolayers were incubated in a CO_2 incubator at 37°C until the visualization of virus cytopathic effects. Afterwards, agarose overlayed with separately isolated virus plaques were prepared. RV titrations were tested in 96-well plates in the presence of trypsin at 0.3 µg/mL. To assess viral genome, viral RNA was extracted from infected cell lysates using TRIzol solution. To determine the presence of the human RV VP7 gene in the rescued RVs, RT-PCR assays were carried out with specific primers. Briefly, 6 µL of extracted RNA was added to RT mixtures (containing specific primers and dNTPs) and incubated at 90°C for 10 min before being placed on ice for 2 min. Then, the second reaction buffer (containing 10× reverse transcription buffer, RTase enzyme, RNase inhibitor, and DEPC-treated water) was added, and the mixture was incubated at 50°C for 60 min. Finally, the RT reaction mixture was incubated at 85°C for 5 min to inactivate the RT reaction. Afterwards, PCR amplification was performed for each sample of RV isolation. The PCR reactions were adjusted in a 50-µL volume containing 8 µL of synthesized cDNA, 25 µL of 2× PCR master mix, and 10 mM of each specific primer (forward: 5'-ACG GGA TCA CTA GAC GCT GTA-3' and reverse: 5'-ATC CGT TCC CAT TGA GAT CCA TT-3') for detection of the VP7 sequence of human RV.

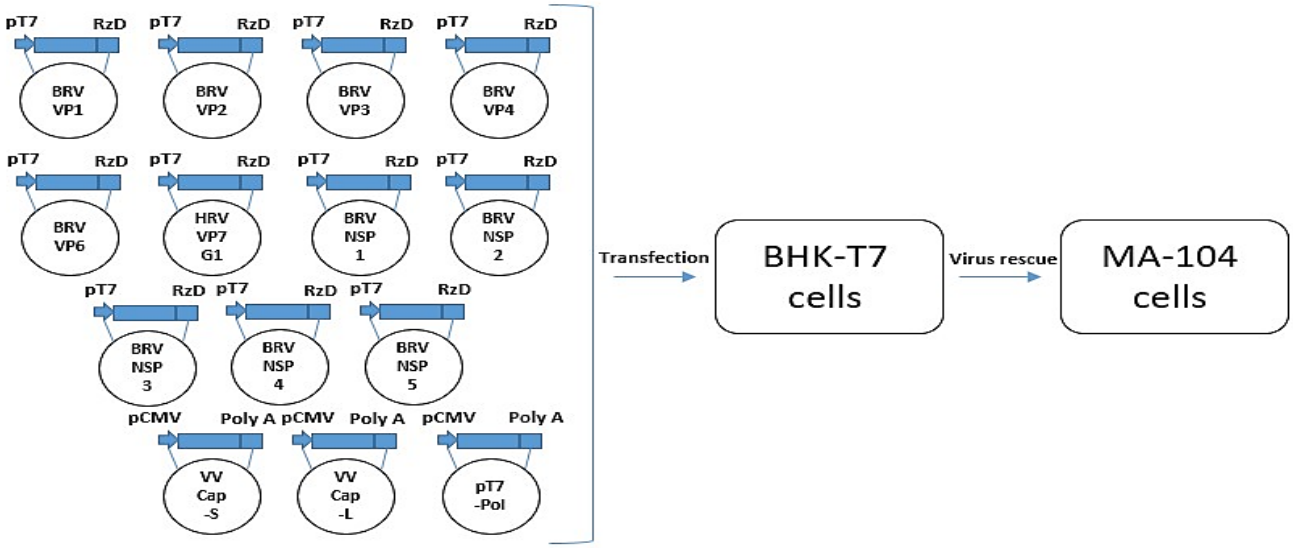

Fig. 1. Schematic presentation for RV reverse genetics using a 13-plasmid RG to produce a reassortant human-bovine RV. The 11 T7 plasmids represent the full-length segments of 11 individual RV genes, each flanked by a pT7 promoter and an HDV ribozyme. Also, two CMV plasmids encode subunits of a capping enzyme derived from VV. One of the transfections utilized a plasmid encoding T7 RNA polymerase. BHK-T7 cells were co-transfected with either 13 or 13+1 T7 and CMV plasmids to generate the reassortant human-bovine RV.

The PCR amplification was carried out under the following conditions: primary denaturation at 95°C for 6 min, followed by 30 amplification cycles consisting of denaturation at 95°C for 50 s, annealing at 57°C for 35 s, extension at 72°C for 55 s, and a final extension step for 8 min. To detect the EGFP sequence in the recombinant rescued RV, PCR amplification was performed using a set of primers: 5'-ATG GTG AGC AAG GGC GAG GAG-3' and 5'-TTA CTT GTA CAG CTC GTC CAT G-3' as forward and reverse primers, respectively. The PCR cycle conditions for this amplification were as follows: primary denaturation at 95°C for 6 min, followed by 35 amplification cycles consisting of denaturation at 95°C for 50 s, annealing at 58°C for 35 s, extension at 72°C for 90 s, and a final extension step for 6 min. The amplified PCR products for VP7 gene sequences were resolved by electrophoresis on 1% agarose gels in Tris-acetate-EDTA buffer. The products were then gel-purified using PCR Clean-up (QIAquick Gel Extraction), and the sequences were analyzed.

2.5. Data analysis

Statistical differences between the peak titers achieved by the viruses were evaluated using an unpaired Student's t-test with Welch's correction. Titers were presented as means ± standard deviations with 95% confidence intervals (GraphPad Prism, version 8).

3. RESULTS

3.1. Reassortant RV release

To establish a reverse genetics system for rescuing a reassortant human-bovine RV, we based our approach on a previous report[11] for the rescue of different RVs. Specifically, we used the VP7 gene sequence from the Wa strain (genotype G1) of human RV and selected the other 10 gene sequences from the WC-3 strain of bovine RV, as available on NCBI (https://www.ncbi.nlm.nih.gov/nuccore/EF990699.1). The necessary flanking sequence elements included the pT7 sequence before the first nucleotide of the RV genes, HDV ribozyme sequences, and the T7 terminator at the end of each RV gene sequence (Fig. 2A). To achieve RV rescue, we transfected 11 different RV plasmids into BHK-T7 cells using two methods: one with equivalent concentrations and the other, which has been published by Kanai et al.[11]. After 48 h post-transfection, we added the MA-104 cells to increase RV replication. Five days after plasmid transfection into BHK-T7 cells—during which we observed cell degradation—we subjected the cells to two cycles of freezing and thawing, followed by inoculation of the cell lysates into MA-104 cells. We observed that the start of cytopathic effects occurred one day after virus adsorption, with complete cytopathic effects observed by the third day of virus passage in MA-104 cells. Our results indicated a significant difference between transfection of only RV plasmids and the transfection of RV plasmids in conjunction with an expression plasmid of T7 RNA polymerase. In the latter case, cell lysis and virus rescue were evident around the fourth day after transfection.

3.2. Rescue of EGFP-containing recombinant RV

To evaluate the recombinant RV containing an *EGFP* sequence integrated within the *NSP3* gene, we utilized the FMD IRES sequence to facilitate the translation of

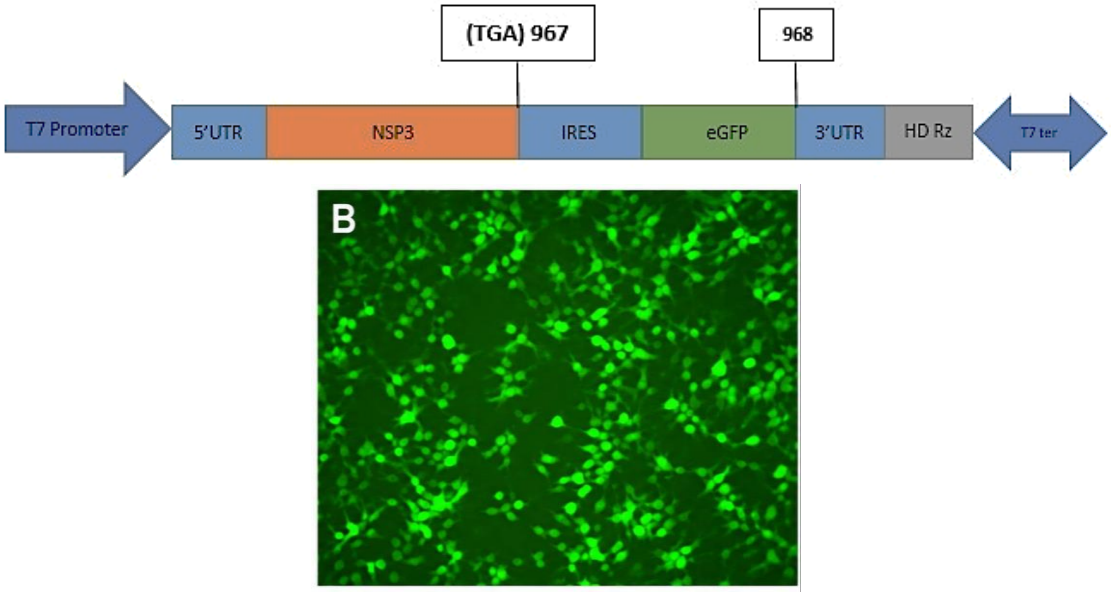

Fig. 2. (A) A schematic representation of the recombinant RV that encodes the NSP3-IRES-eGFP sequence (rRV-NSP3-IRES-eGFP); (B) fluorescent expression of rRV-NSP3-IRES-eGFP observed after 24 h of infection of MA104 cells with the recombinant RV. The MA104 cells were infected with rRV-NSP3-IRES-eGFP at a MOI of 1.

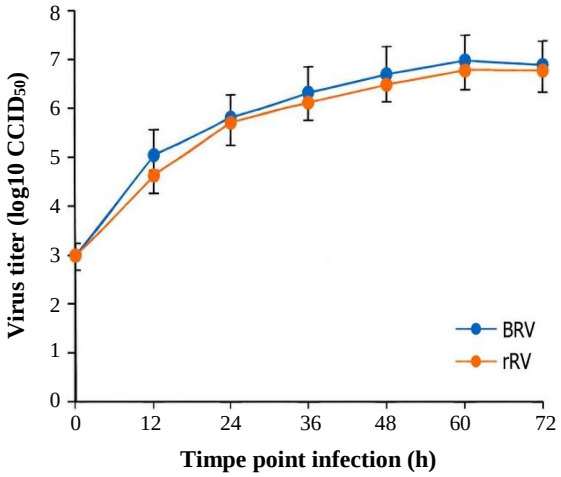

Fig. 3. Replication kinetics of bovine rotavirus (BRV) and rRV-NSP1-IRES-eGFP (rRV). MA-104 cells were infected with BRV and rRV at an MOI of 0.01. The virus titers ($CCID_{50}$) were calculated or frozen cell collections at one to five days post-infection. Data are shown as the mean and standard deviation of duplicate samples.

the *EGFP* gene via a reinitiating mechanism. Our findings indicated that a recombinant reassortant human-bovine RV can be successfully rescued, with EGFP expression controlled by the IRES sequence (Fig 2B). To compare the replication kinetics between the parental rescued reassortant human-bovine RV and the recombinant rescued RV, we performed replication cycle tests on both viruses in duplicate. Following the infection of MA104 cells at an MOI of 0.1, we plotted multiple-step growth curves for both the reassortant human-bovine RV and the recombinant rRV-NSP3-IRES-eGFP. Our findings revealed that both the bovine and recombinant RV showed comparable average replication characteristics, with values of 5.8 and 5.68, and standard deviations of 1.4 and 1.28, respectively (Fig. 3). To further examine the stability of the foreign genes incorporated into the NSP-3 genome segment of the bovine RV, we performed serial passages of the virus. The evaluation of the inserted *EGFP* gene indicated that these foreign genes within the NSP-3 segment can remain stable through at least nine cycles of RV replication.

3.3. Molecular confirmation of human RV VP7 gene incorporation into the bovine RV backbone

To validate the presence of the human RV sequence within the backbone of the bovine RV as a reassortant RV, we conducted an RT-PCR assay to detect the G1 RV serotype in the isolated samples. The RT-PCR results confirmed that the VP7 sequence of the human serotype G1 was incorporated into the bovine RV genome (Fig. 4A). Additionally, sequencing results substantiated the presence of the human RV VP7 gene in the isolated samples. The RT-PCR findings also showed a positive result for the eGFP sequence in the rescued rRV-NSP1-IRES-eGFP during the ninth passage of this virus (Fig. 4B).

4. DISCUSSION

This study successfully rescued both reassortant and non-reassortant RVs from various genotypes, including strains such as simian RV SA11, human RVs, murine-like RVs, and more recently, bovine RVs. These studies demonstrate the effectiveness of a reverse genetic system for rescuing RVs. Additionally, different recombinant RVs incorporating foreign gene sequences have also been successfully created using reverse genetics. However, till now, no reverse genetic system has been reported specifically for rescuing reassortant human-bovine RVs.

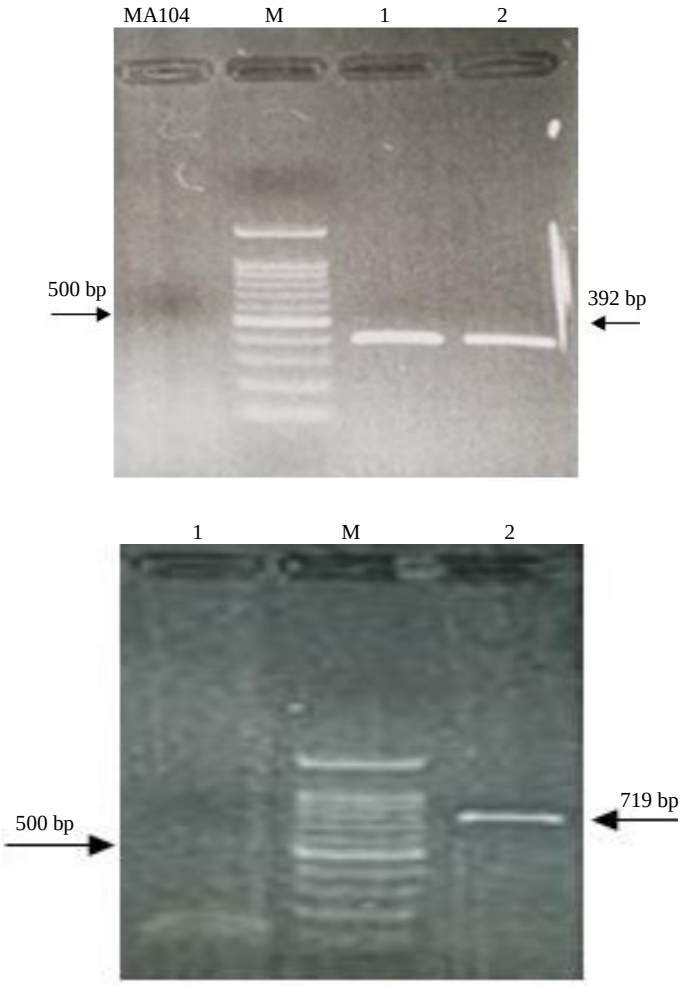

Fig. 4. (A) VP7 PCR product serving as a control for MA104 cells, with lanes 1 and 2 representing the positive control and the rescued reassortant RV, measuring 392 bp in length; (B) PCR amplification of the *EGFP* gene, which is integrated into the NSP3 segment of rRV-NSP3-IRES-eGFP, with a band of 719 bp in length.

In this research, we established a reliable reverse genetic system for rescuing a reassortant human-bovine RV. The rescued reassortant RV effectively integrates the VP7 gene sequence from the human RV Wa strain into the genetic framework of the bovine RV WC-3 strain. Our findings confirmed that this effective reverse genetic system can aid in creating a recombinant human-bovine RV that incorporates the complete *EGFP* gene sequence as an introduced foreign gene.

Similar to previous studies that utilized helper expression plasmids to enhance reverse genetic systems for RV rescue, we employed two subunits of VV capping expression plasmids to cap the nascent viral genomic RNA transcripts[11,29]. However, we did not use the *FAST* expression plasmid, which has previously been shown to be efficient for RV rescue[11]. In contrast, recent findings have suggested that using high concentrations of NSP-2 and NSP-5 can improve RV rescue without helper expression plasmids such as VV capping expression plasmids and the *FAST* expression plasmid[25]. Our documentation indicates that maintaining consistent plasmid concentration is suitable for the transfection and rescue of RV. In line with earlier reports advocating for enhanced efficacy in RV rescue through increased expression of T7 RNA polymerase, we co-transfected a T7 RNA polymerase expression plasmid into BHK-T7 cells. Echoing the findings of Qin and colleagues[31], our results highlighted that high levels of T7 RNA polymerase expression are crucial for successful RV rescue.

Research has shown that isolated RVs exhibit variations in genome size across different genomic segments. This discovery has led to various studies aimed at inserting or replacing foreign nucleotide sequences, especially in genomic segments like NSP1 and NSP3[8,11,31-35]. In our investigation, we successfully inserted an *EGFP* sequence as a foreign gene into the NSP3 segment of a rescued reassortant human-bovine RV. To achieve the expression of this foreign gene, different researchers employed several strategies, including genomic deletions, use of stop codons, and virus peptidase techniques[35]. Here, we also integrated the FMD IRES sequence to facilitate downstream expression.

With the observation of EGFP fluorescence, our findings suggest that the expression of *EGFP*, embedded within NSP3 and regulated by the FMD IRES, can be quite effective. While there has been a growing interest in utilizing RV as a viral vector, challenges remain regarding the stable maintenance of foreign gene sequences within its genome[36]. A key objective of our work was to ensure stable expression of the *EGFP* gene across multiple passages of the virus. We observed that fluorescence from *EGFP* remained consistent across six cycles of RV replication. However, we experienced a gradual loss of fluorescence from cycles seven to nine. Moreover, it is important to note that there are limitations regarding the length of foreign gene sequences that can be included in RV genomes, which warrants further research.

5. CONCLUSION

In this study, we successfully rescued a reassortant human-bovine RV, incorporating the VP7 gene sequence from the human RV strain Wa. By using a RV similar to the RotaTeq vaccine—known for its safety—as a viral vector. We highlight its promising potential for future vaccine development aimed at stimulating effective immunity against various viral antigens.

DECLARATION

Acknowledgments

Not applicable.

Generative AI and AI-assisted technologies

During the preparation of this manuscript, the authors used OpenAI to assist with grammatical editing and improving the clarity and organization of the text.

Ethical approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

MA, ML, and MGH: conceived the original idea and drafted the initial version of the manuscript; NH, EB, SDMN, MGH: critically revised the manuscript for scientific content and clarity of writing.

Data availability

All relevant data can be found within the manuscript.

Competing interests

The authors declare no competing interests.

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Supplementary information

The online version does not contain supplementary material.

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