

Original Article

Assessment the Efficiency of the Constructed Minigenome of Rabies Virus using PV Strain as Helper Virus

Mehdi Ajorloo PhD¹, Taravat Bamdad PhD^{1*}, Ali Reza Gholami PhD^{2,3}, Keyhan Azadmanesh PhD⁴

Abstract

Introduction: Rabies is an acute viral disease that causes encephalomyelitis in mammals and human. The only way to prevent this disease is through vaccination before or after exposure. The aim of this study is to evaluate the efficiency of the Pasteur virus (PV) minigenome, using PV strain.

Materials and Methods: Enhanced Green Fluorescent Protein (EGFP) sequence was placed between the designed necessary elements (Hammerhead, HDV ribozyme, 3' Leader, and 5' Trailer sequences), which resemble the rabies virus PV strain (PV2061) genome and anti-genome. These constructs were placed between T7 polymerase promoter and T7 polymerase terminator sequences. The accuracy of the minigenome was confirmed by the expression of EGFP using the helper virus in T7-BHK cell line.

Results: The viral necessary elements of positive and negative sense strands were evaluated for the ability of EGFP expression in the presence of the helper virus. While the positive strand showed background results, no EGFP background was observed in the negative strand application.

Conclusion: Establishment of minigenome system does not require advanced biosafety levels. Furthermore, using minigenome system eliminates many potential confounding factors that may be present in coding regions of the genome. Use of the minigenome system is easier and more feasible than the full genome rescue of the virus. This study successfully shows the efficiency of the constructed rabies virus minigenome in expression of inserted gene.

Keywords: Minigenome, rabies virus, reverses genetics

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Introduction

Rabies is an acute viral disease that causes encephalomyelitis in all the warm-blooded mammals including man. Rabies in humans can be divided into five stages, including incubation period, prodrome, acute neurologic phase, coma, and death.^{1,2} Annually, more than seventy thousand people die due to the rabies worldwide and implementing correct vaccination protocol is the only way to prevent the exposed patients from rabies.³ There are several vaccinal strains of rabies virus for vaccine production.³ Rabies virus belongs to the genus *Lyssavirus* in the family *Rhabdoviridae*. Members of this family are enveloped, negative-strand RNA viruses. Genomic length of the rabies virus is about 12 kb and encodes five proteins named nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and the RNA-dependent RNA polymerase (L).^{1,2} The genomic and antigenomic sequences are encapsidated by N protein. This structure, along with phosphoprotein and viral RNA-dependent RNA polymerase forms ribonucleoprotein (RNP) complexes.^{1,2} Genomic RNP is served as a template for the polymerase L to transcribe and produce mRNAs of viral proteins. After an accumulation of viral

proteins to a particular level, synthesis of a full-length antigenomic RNP begins. Full-length antigenomic RNP issued as a template for the production of new genomic RNPs.^{1,2} Although, both M and G were indicated unnecessary for infectious virion rescue by reverse genetics. G and M are versatile proteins involved in important structural and functional roles during infection from virion penetration to assembly and budding, as well as manipulation of host cellular pathways.^{1,4-8}

Reverse genetic engineering is a method to obtain and manipulate different strains of virus. It is believed that future generation of rabies vaccines should be based on reverse genetics technology in order to raise more efficient immunity against rabies through a single vaccination.⁹

Development of a desirable virus by genetic engineering includes certain specific stages. Proper function of virus constructed genome needs to be examined, and for this purpose minigenome of desired virus should be designed first. In the case of rabies virus, once the efficiency of minigenome is confirmed by using helper virus, the proper function of virus core proteins such as N, P, and L are evaluated. Finally, rescue of the full genome viruses should be optimized using helper plasmids.¹⁰

This study was designed as a precursor to establish a new technology for production of various vaccinal strains of rabies virus, which can be widely used in further studies.

For this purpose, Enhanced Green Fluorescent Protein (EGFP) sequences were placed between the designed necessary elements as a reporter gene, which resemble the viral genome. The accuracy of the minigenome was confirmed by the expression of EGFP using the helper virus.

Authors' affiliations: ¹Department of Virology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran, ²Human Rabies Vaccine Laboratory, Production and Research Complex, Pasteur Institute of Iran, Tehran, Iran, ³WHOCC for Reference and Research on Rabies, Pasteur Institute of Iran, Tehran, Iran, ⁴Department of Virology, Pasteur Institute of Iran, Tehran, Iran.

***Corresponding author and reprints:** Taravat Bamdad PhD, Department of Virology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran. Tel: +98-21-82884525, Fax: +98-21-82883581, E-mail: bamdad_t@modares.ac.ir.

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Materials and Methods

Design of positive and negative senses of rabies virus minigenome

To construct the positive sense rabies virus minigenome, 3' leader and 5' trailer sequences of rabies virus PV strain (PV2061) were placed on both sides of the reporter gene (EGFP). These sequences are the essential elements both in virus replication and transcription processes. These structures were placed between the sequences of the hammer head and HDV ribozyme.^{11,12} These constructs were placed between T7 polymerase promoter and T7 polymerase terminator sequences. Three extra G residues were put downstream of T7 promoter to facilitate efficient transcription initiation of the T7 RNA polymerase.¹³

Restriction enzyme sites were also embedded on both sides of this construct. Finally the construct was sent to (GENE-RAY Company, Hong Kong, China) for synthesis and cloned into the pGH Bluescript vector (GENE-RAY Company, Hong Kong, China), (Figure 1 and 2A).

Minigenome of the rabies virus was also designed in a negative sense. A modified sequence of the hammerhead and HDV ribozyme were placed in the negative sense minigenome structure.¹⁴ This construct was sent to GENE-RAY Company for synthesis and cloned into the pGH Bluescript vector (Figure 2B and 3).

The pCEP4 plasmid (Invitrogen, Carlsbad, California, USA) was also used as a vector for minigenome insertion. The CMV promoter in the pCEP4 vector was removed by *SalI* (Fermentas) before cloning the synthesized constructs of any senses into this vector. Verification of cloning was done by enzymatic digestion with *SalI*.

Preparation of T7-BHK cell line

The T7-BHK cell line expressing T7 RNA polymerase permanently was obtained from the Department of Cell Bank in Pasteur Institute of Iran. These cells were cultured in DMEM (Dulbecco minimal essential medium) cell culture media supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C with 5% CO₂ supply.

Adjustment of G418 antibiotic dose

T7-BHK cell line is resistance to G418 antibiotic (Sigma-Aldrich). To adjust lethal dose of G418 for the cell, both BSR (a clone of BHK cell line) and T7-BHK cell lines were cultured in a 24-well cell culture plate at a concentration of 2.5×10^5 per well and different doses of the drug were added to each well (50, 100, 200, 400 µg/mL). The media of cell culture were replaced with new media every other day.

Confirmation of T7-BHK cell line T7 polymerase activity

To confirm the T7 polymerase activity of T7-BHK cell line, a construct with EGFP under the control of T7 promoter¹⁵ was

transfected in both BSR and T7-BHK cell lines by Lipofectamin 2000 (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instruction (Figure 4).

Determination of positive sense rabies virus minigenome function in infected cells with helper virus

Twelve hours before infection, the constructed positive sense minigenomes was cloned into the pGH Bluescript and pCEP4 vectors were transfected in both BSR and T7-BHK cell lines. Transfected cells along with uninfected negative control cells were infected by rabies virus PV strain with a multiplicity of infection (MOI) equal to 3. EGFP expression was examined in both infected and uninfected cells; 24 – 72 hours post infection.

Determination of negative sense rabies virus minigenome functions in infected cells with helper virus

Minigenome of the rabies virus was used in a negative sense. The constructed pCEP4/negative sense minigenome plasmid was transfected in both BSR and T7-BHK cell lines, 6, 12, 18 and 24 hours before infection with rabies virus PV strain.

To obtain the appropriate MOI, BSR and T7-BHK cell lines were infected with serial dilution of virus equal to MOI of 0.01, 0.05, 0.1, 1, and 3. Expression of EGFP was evaluated in transfected cells without infection as negative control 24 – 72 hours post infection.

Results

Adjustment of G418 antibiotic dose

BSR and T7-BHK cell lines treated with 50, 100, 200, 400 µg/mL of G418 were observed for two weeks. After 2 weeks, the BSR cells showed sensitivity to G418 in 100 µg/mL concentration and more. No significant destruction in T7-BHK cells was observed in 100 µg/mL concentration or more as illustrated in Figure 4. The best lethal dose showing T7BHK resistance to G418 was determined in this experiment at 400 µg/mL (Figure 5).

Confirmation of T7-BHK cell line T7 polymerase activity

After 48 hours, BSR and T7-BHK cell lines that were transfected by T7 promoter EGFP construct were examined under fluorescent microscope for expression of EGFP. Results showed that EGFP was only expressed in T7-BHK cells and no expression was observed in BSR cells as demonstrated in Figure 6.

Determination of positive sense minigenome functionality in infected cells with rabies virus PV strain as helper virus

Expression of EGFP in T7-BHK cells as well as BSR cells was observed 24 – 72 hours post infection (Figure 7).

Results of this experiment showed EGFP expression in uninfected cells at a low level.



Figure 1. Schematic figure of designed positive sense minigenome

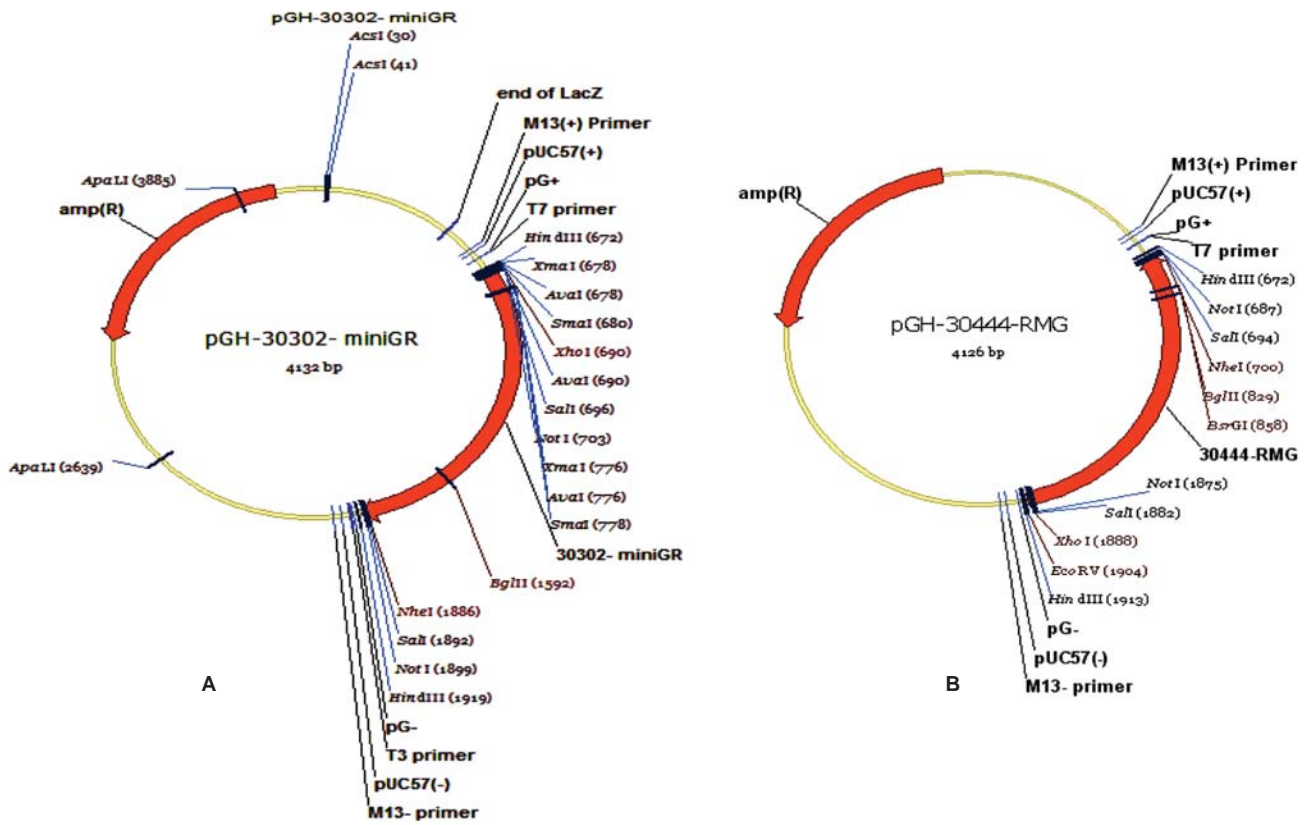


Figure 2. pGH Bluescript vectors. A) Contains positive sense minigenome; B) Contains negative sense minigenome

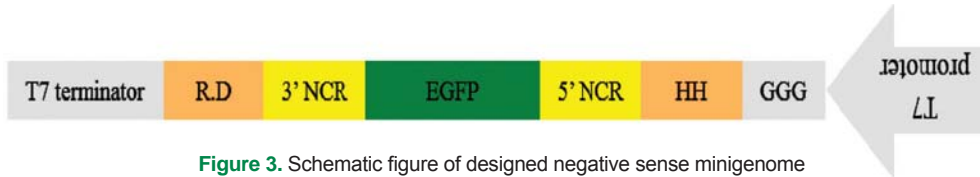


Figure 3. Schematic figure of designed negative sense minigenome



Figure 4. Schematic figure of a construct to determine T7-BHK cell line function

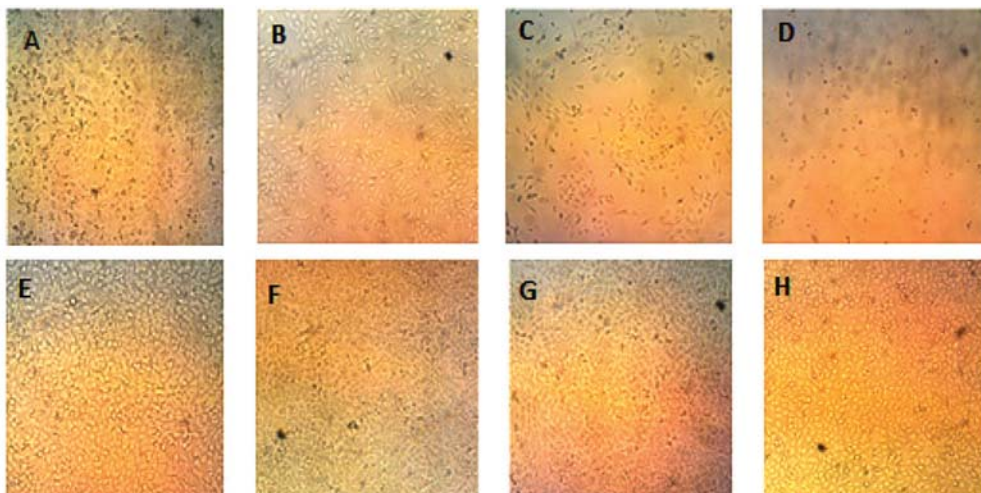


Figure 5. Adjustment of G418 antibiotic doses. For adjustment of G418 doses, both BSR (a clone of BHK cell line) and T7-BHK cell lines were cultured in a 24 well culture plate and different doses of drug were added to each wells (50, 100, 200, 400 µg/mL). A) BSR (50 µg/mL); B) BSR (100 µg/mL); C) BSR (200 µg/mL); D) BSR (400 µg/mL); E) T7-BHK (50 µg/mL); F) T7-BHK (100 µg/mL); G) T7-BHK (200 µg/mL); H) T7-BHK (400 µg/mL).

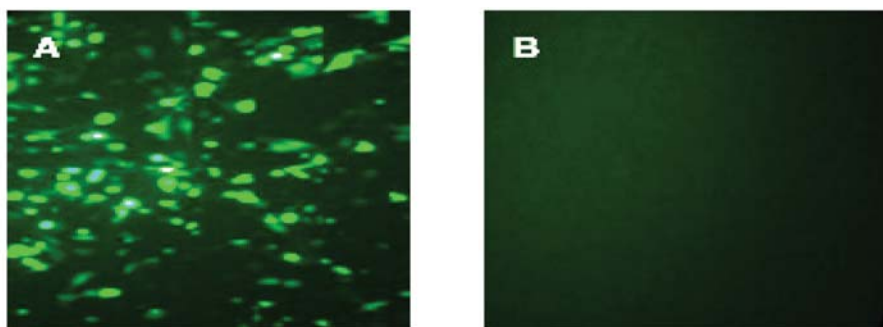


Figure 6. Confirmation of T7 polymerase activity in T7-BHK cell line. **A)** Expression of EGFP in T7-BHK cells; **B)** No expression of EGFP in BSR cells.

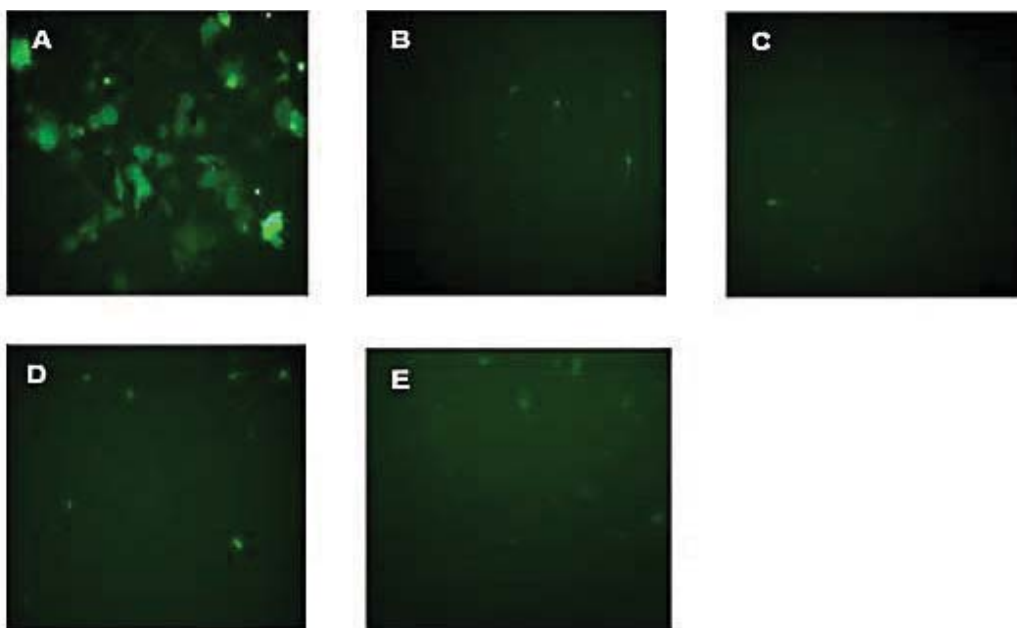


Figure 7. Determination of positive sense minigenome/PGH vector function in infected cells with rabies helper virus. Slight EGFP expression by positive sense minigenome is seen in uninfected BSR cells which might be due to the cryptic promoter. **A)** T7-BHK cells transfected with EGFP construct as a positive control; **B)** BSR cells transfected with minigenome and uninfected with rabies virus; **C)** BSR cells transfected with minigenome and infected with rabies virus; **D)** T7-BHK cells transfected with minigenome and uninfected with rabies virus; **E)** T7-BHK cells transfected with minigenome and infected with rabies virus.

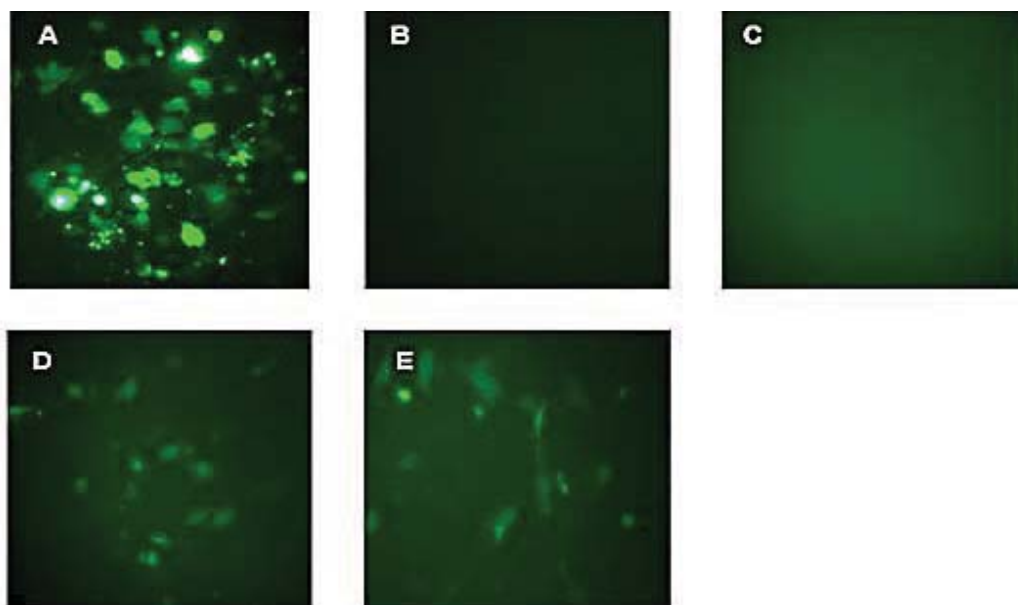


Figure 8. Determination of positive sense minigenome/pCEP4 vector function in infected cells with rabies virus. There is EGFP expression in images A, D and E. Expression of EGFP is absent in conditions B and C. Low amount of EGFP expression was observed in T7BHK cells in the presence and absence of helper virus. **A)** T7-BHK cells transfected with EGFP construct as a positive control; **B)** BSR cells transfected with minigenome but uninfected with rabies virus; **C)** BSR cells transfected with minigenome and infected with rabies virus; **D)** T7-BHK cells transfected with minigenome and uninfected with rabies virus; **E)** T7-BHK cells transfected with minigenome and infected with rabies virus.

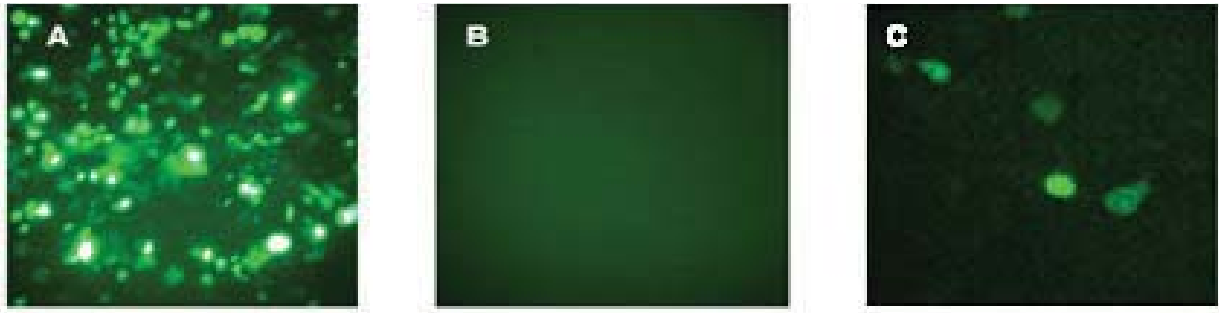


Figure 9. Determination of negative sense minigenome/pCEP4 vector function in infected cells with rabies virus. No expression of EGFP in T7-BHK cells transfected with minigenome and uninfected with rabies virus determined the reverse genetics system has been worked. **A)** Expression of EGFP in T7-BHK cells transfected with EGFP construct as a positive control; **B)** No expression of EGFP in T7-BHK cells transfected with minigenome and uninfected with rabies virus; **C)** Expression of EGFP in T7-BHK cells transfected with minigenome and infected with rabies virus.

The constructed minigenome was digested by *Sall*, which resulted in elimination of cryptic promoter and cloned into the modified pCEP4 vector. The efficiency of the cloning was verified by restriction enzyme analysis.

Twelve hours after transfection with minigenome construct, BSR and T7-BHK cells were infected by PV strain. No expression of EGFP was observed in both infected and uninfected BSR cells, However, both infected and uninfected T7-BHK cell expressed EGFP (Figure 8).

Determination of negative sense minigenome function in infected cells with rabies virus PV strain

Six, 12, 18, and 24 hours after transfection negative sense minigenome plasmid, BSR and T7-BHK cells were infected by rabies virus PV strain with different MOIs. The best expression of EGFP was observed 18 hours after infection with MOI of 0.01 in negative sense minigenome transfected T7-BHK cell line. No expression of EGFP was observed in uninfected T7-BHK cells, as well as infected and uninfected BSR cell lines (Figure 9).

Discussion

Vaccination is considered one of the greatest medical achievements in life science and public health. It also had great achievements in the declining mortality rate caused by RNA viruses.¹⁶ In this regard; forward genetics through developing attenuated virus strains by subcultures was functional. However, conventional approaches and forward genetics have considerable restrictions for developing new vaccines. In 1981, when polio virus was first generated from RNA, reverse genetics was introduced to virology.¹⁷ Since then, reverse genetics systems allowed inducing specific mutation in a gene of a virus or analogues genome and producing efficient virus from cDNA.¹⁸ Reverse genetics is now being used for different applications such as investigating pathogenesis and generating novel vaccines, as well as recombinant viruses.¹⁹ The first study in rabies virus reverse genetics and generation of infectious rabies virus from cloned cDNA was done by Schnell, et al. in 1994 in which, the full genome of rabies virus under the T7 promoter was rescued by N, P and L constructs.⁶ In this study EGFP sequence was placed between 5'NCR and 3'NCR sequences of Pasteur strain of rabies virus and this structure was placed between the sequences of hammerhead and HDV ribozyme under the control of T7 promoter. In a study by Ying Huang, et al. recombinant full-length genomic cDNA was similarly flanked by

a hammerhead ribozyme (HamRz) and the hepatitis delta virus ribozyme (HdvRz). The coding G-L region was replaced with a green fluorescent protein (GFP) gene.¹⁹ Another platform using bacterial artificial chromosome (BAC) has also been employed to reverse genetics technology, majorly for positive sense RNA viruses.²⁰⁻²² However, it has recently been employed to a negative sense ssRNA virus.²³

In the current study, hammerhead and HDV ribozyme were applied in upstream and downstream of the construct to facilitate release of the 3' Leader, EGFP and 5' Trailer sequences and gene expression. It has been mentioned that a productive infection might be initiated both from positive sense and the complementary negative sense transcripts.¹⁵ Therefore, in two independent attempts; we designed genome constructs in both positive and negative strand forms. However, to evaluate the possibility to skirt antigenome synthesis step we have studied the genome sense construct. Experiment with the positive sense strand revealed low expression or non-specific expression in control of EGFP from the positive sense strand in BSR cell, which was due to the cryptic promoter. Therefore, the experiment was repeated by negative strand. Cryptic promoters are short sequences on DNA that bind to RNA polymerase and start the transcription process. Cryptic promoters do not have a well-defined promoter initiation site sequence like TATAA box and the initiation mechanisms are not well-characterized.^{24,25} In a study of generating non-mammalian rhabdovirus by reverse genetics, both positive and negative sense minigenome were used and the background was observed in the case of positive sense minigenome, similar to the result of the present study.²⁴ According to the same study, we have inserted three extra guanine residues downstream to T7 RNA polymerase sequences to facilitate efficient initiation of T7 RNA polymerase transcription. However, in that study, unlike our experiments, bacterial chloramphenicol acetyltransferase gene (CAT) was used as a reporter gene and cells were infected with a recombinant vaccinia virus expressing T7 polymerase. If the T7-BHK (hamster kidney cell line expressing stably T7 polymerase) is successfully infected with the Pasteur virus, then the core protein of virus will transcribe the EGFP sequence to mRNA resulting in EGFP protein expression. However, re-analysis of plasmid sequence resulted in the manifestation of a cryptic CMV promoter contributing to false gene expression in negative controls. Occurrence of a cryptic promoter has been demonstrated previously as causative agent for problematic gene expressions in reverse genetics studies on RNA viruses of sense genome such as Dengue and Japanese

encephalitis.²⁵ A similar sequence to CMV promoter (1350bp) was detected and removed from pCEP4 vector by *Sall*, as confirmatory approach. Consistent with this, the remaining plasmid backbone was used as a vector for the minigenome construct, which resolved the problem as shown in Figure 7. Nevertheless, EGFP expression was observed to a certain amount in BHK cells expressing T7 polymerase in the absence of helper virus, which might be due to using positive sense minigenome. A modified sequence of the hammerhead and HDV ribozymes has been introduced with greater self-cleavage ability capable of generating exact 5' and 3' ends identical to the virus.¹⁴ Thus, using these modified sequences in our system may also enhance the rescue efficiency of the minigenome.

Development of a full-length infectious virus might be the ultimate goal, however, minigenome systems generally serve as an initial step. Minigenome system allows analysis of the roles of cis-acting and trans-acting elements on the non-coding regions (NCRs) of the virus genomes. These regions are important in virus transcription; replication and genome packaging.¹⁸ Similar concepts have been respected in our work. However, it has been shown in this study that control of transcription using a promoter such as T7 RNA polymerase, cryptic promoters, as well as sense expression system could have interference with appropriate results.

For the production of a minigenome system, the coding genes of the genome are removed and replaced with a detectable reporter gene, such as EGFP. Production of this reporter gene is easily detected and the authenticity of the construct is confirmed. Another advantage of using minigenome system is when a researcher works in the case of high-containment viruses, establishment of minigenome system does not require advanced biosafety levels.¹⁸ Furthermore, using minigenome system eliminates many potential confounding factors that may be present in the coding regions of the genome.²⁶ On the other hands, many RNAs are transcribed under the T7 promoter, so using positive sense minigenome may lead to direct translation of all these positive sense RNAs.

The first rabies virus vaccine was developed 130 years ago. Since then, substrate for virus propagation and production technology has changed, but the concept of the vaccine has remained the same. The goal is to develop more affordable vaccines that confer long-lasting immune response for rabies. Although dog immunity duration still remains a global demand, by virtue of reverse genetics, certain studies on replication deficient viruses have shown promising results.^{9,27,28} Use of the minigenome system is easier and more feasible than the full genome rescue of the virus. Furthermore, after confirmation of the minigenome authenticity it will be possible to analyze and produce natural or modified full genome of the virus for a new generation of vaccine. By this method, it could be possible to induce changes in the genome of the virus for production high yield and cheaper vaccine. Reverse genetic is the only method that could be used for production of natural negative sense virus or a manipulated one. Based on previous experiments, this study successfully demonstrates an efficient and certain method of the constructing rabies virus minigenome with a helper virus, which is a fundamental approach for rescue of a PV strain of rabies virus. Co-transfection of the constructed minigenome with helper plasmids involved in virus replicatory machine remains to be implemented.

Abbreviations

PV Virus: Pasteur Virus; EGFP: Enhanced Green Fluorescent Protein; NCR: Non Coding Region; RNP: Ribonucleoprotein

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