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Cloning of BHV-UL25 Conserved Fragment in a Lentiviral Transfer Plasmid for the Preparation of a Monitoring Cell Line

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Abstract

Bovine herpesvirus-1 (BHV-1) is the causative agent of a domestic and wild cow disease namely infectious bovine rhinotracheitis (IBR). Severe economic damage due to IBR is possible to occur and vaccines are not completely effective against the disease. Therefore, over the recent years, the development of effective and genetic-based therapies to control viral infections, such as IBR has been remarkable. A common way to evaluate such therapeutic strategies is cloning of the viral target sequence into appropriate vectors for the preparation of cell lines expressing viral subgenomic replicons. Due to the required duties of *UL25* gene, serine protease substrate domain of this gene was cloned in pCDH-CMV-MCS-EF1-cGFP-T2A-Puro lentiviral vector at the upstream of GFP gene. The cloning accuracy was verified by restriction of enzyme digestion and sequencing. Thus, this recombinant plasmid will be available to produce lentiviral vectors with the desired gene; after infection of eukaryotic cells with such lentiviral vectors the target gene will be expressed.

Keywords: Cloning, BHV-1, Lentiviral Plasmid, UL25

1. Background

Bovine herpesvirus-1 (BHV-1) is a member of *Herpesviridae* (1) with a 135301 bp genomic dsDNA. In the genome, there are four segments including a unique long (UL) segment, a unique short (US) segment, and two inverted repeat regions, namely inverted internal repeat (IR) and terminal repeat (TR) sequences. The genome encodes for 70 proteins. Thirty-three proteins are structural, 13 of which are present in the viral envelope and 10 are capable to encode viral glycoproteins. Six genes that encode for six different glycoproteins are located in the UL segment (2). The *UL25* gene is located at 60602 - 62398 position. Its product is a tegument protein, which plays an important role in the assembly and packaging of the genome into the capsid (3).

Bovine herpesvirus-1 is classified into three subgroups including *BHV-1.1*, *BHV-1.2* and *BHV-1.3*. *BHV-1.1* is responsible for the respiratory manifestations of the disease (IBR) (4, 5).

Over the years, a great deal of research to develop anti-IBR vaccines has led to the fact that several vaccines are now available for this disease. However, none of them are completely effective. Therefore, the development of specific and adequate methods for the prevention and treatment of BHV-1 is great of necessity (6).

Since conventional therapies for the treatment of human and animal viral diseases have their limitations and alternative therapies are urgently needed, gene therapy techniques, such as RNAi technology have been used as potential therapeutic approaches. One way to evaluate the efficacy of gene therapy is to provide reporter cell lines for the expression of viral target genes. Therefore, the first step is to clone the viral target sequences into a suitable vector for transfer to the appropriate cell line.

In the previous studies, due to the conservation of the *BHV-UL25* and its functional roles, it has been frequently selected as a target. Thus, in the present study, the conserved *BHV-UL25* serine protease substrate domain was selected for cloning in a lentiviral plasmid (7).

Over the recent years, gene therapy via lentiviral

vectors (LV) has increased dramatically. Lentiviral vectors based on the human immunodeficiency virus1 (*HIV-1*) are intended as a vector with high capabilities for gene transfer studies. They can integrate and transfer genes into non-dividing cells. The pseudotyped envelope with VSV-G protein increases the types of host cells. To create such a vector, the most common method is to clone the essential virion and transgene genes into different plasmids and consequently, the co-transfection of them to the appropriate cell line. Accordingly, the possibility of generating replication-competent lentivirus will be very low (8, 9).

Lentiviruses can induce their target gene expression for a long time. In addition, they are able to transfer large nucleotide sequences. Furthermore, because of the low probability of integration into or next to the cell's oncogene, the possibility of mutagenesis and carcinogenesis following gene induction by lentiviral vectors is lower than that by other vectors (10).

Due to the mentioned reasons mentioned above, in the present study, lentiviral vectors were preferred for the cloning of the desired gene in a lentiviral transfer vector.

Among the available lentiviral plasmids, pCDH-CMV-MCS-EF1-cGFP-T2A-Puro, which has ampicillin and puromycin resistance genes, was selected for ease of access and handling. Both antibiotics are easy to achieve. Moreover, the vector has a GFP gene as a marker protein that is applicable for the evaluation of transfection efficiency. In addition, this plasmid passes strong *RSV*, *CMV*, and *EF1* promoters for expression of the desired gene.

2. Objectives

The objective of this study was to produce a pCDH-CMV-MCS-EF1-cGFP-T2A-Puro lentiviral plasmid expressing BHV-UL25 serine protease substrate domain as a transfer vector.

3. Methods

3.1. Preparation of Serine Protease Substrate Fragment of BHV1-UL25

The sequence of *BHV1-UL25* serine protease substrate domain was obtained from National Center of Biotechnology Information (NCBI) database (Ac. No. AJ004801.1) and aligned with all the known gene sequences in other BHV1 strains extracted from NCBI. The alignment was performed using the online software www.ebi.ac.uk/Tools/msa/clusalo.

Afterwards, *EcoRI* and *BamHI* cleavage sites were added to the 5' and 3' ends, respectively. The His Tag sequence was added to 3' end for future monitoring tests. Finally, this sequence was sent to the Shine Gene Molecular Biotechnology Company (China) for synthesis and clone in pGH pBLUSCRIP (+) vector.

3.2. Restriction Enzyme Digestion

pCDH-CMV-MCS-EF1-cGFP-T2A-Puro lentiviral plasmid (Addgene) and pGH pBLUSCRIP (+) carried *BHV1-UL25* serine protease substrate fragment digested by *BamHI* (Roche, Germany, Cat. No: 10220612001) and *EcoRI* (Roche, Germany, Cat. No.: 10220566001) for extraction of the inserted fragment. Therefore, 7.5 μL of plasmid (2 μg/μL), 1.3 μL of each enzyme (10 U), 2.5 μL of 10X K buffer, and nuclease-free water were added to a final volume of 25 μL. After incubation at 37°C for 1 hour, 1 μL of digested plasmid was electrophoresed in a 1% agarose gel in order to confirm the restriction enzyme digestion.

3.3. Extraction of BHV1-UL25 Serine Protease Substrate Fragment from Agarose Gel

In order to purify the digested plasmid, after digestion with *EcoRI* and *BamHI* and removing the undigested plasmids and restriction enzymes, the product of digestion was extracted from agarose gel using Expin Gel SV (50 prep) kit (Gene all, Cat. No.: 000001422) according to the manufacturer's instructions.

3.4. Ligation of BHV1-UL25 Serine Protease Substrate Fragment into Lentiviral Plasmid and Transformation

For the ligation of the inserted fragment into vector 2 μL of T4 10X ligation buffer (Fermentas, Germany, Cat. No.: B69), 2 μL of 50% PEG-4000 (Fermentas, Germany), 1 μL of T4 DNA ligase (Fermentas, Germany, Cat. No.: EL0013), 150 ng of the inserted DNA, and 50 ng of plasmid were mixed with nuclease-free water up to 20 μL total volume. Heat shock protocol was applied for the transformation of ligation mixture. In summary, 10 μL of ligation product was added to 100 μL of DH5α. Subsequently, the mixture was placed in the following temperature conditions: 30 min at 4°C, 90 s at 42°C, and 5 min at 4°C. Afterwards, 900 μL of LB broth was added and the mixture was shaken at 200 rpm for 45 min and centrifuged at 6000 rpm for 3 min. Finally, the culture of pellet in LB agar +100 mg mL^{-1} ampicillin was performed and put at 37°C for 14 - 16 hours.

3.5. Colony PCR

After culturing the transformed bacteria, PCR reaction and mini preparation method of plasmid extraction was performed for 10 individual clones of each plate. PCR was carried out with the general primers of pCDH (CMV-F: AATGGGCGGTAGGCGTGTA-3'and

EF1-R: 5'-GGACTGTGGGCGATGTG-3'). The thermal cycling conditions was as follow: 95°C for 4 min, 30 cycles at 95°C for 30 s, 55°C for 35 s, 72°C for 45 s, and one cycle at 72°C for 9 min. The positive BHV1-UL25 serine protease substrate fragment that was synthetized and cloned in pUC57 vector by Shine Gene Molecular Biotechnology Company (China) and negative controls (deionized water) were employed in each test. The PCR reaction was included of 12 μL of dW, 9 μL of Taq DNA Polymerase Master Mix Red 2x -2 mM $MgCl_2$, (Amplicon, Cat. no. A180301), 1 pmol of each primer, and 2 μL of DNA template up to 25 μL total volume.

3.6. Sequencing

After electrophoresis in 0.8% agarose gel, two reactions of colony PCR-positive samples, which contained the recombinant plasmids, were sent to Bioneer Company (Korea) for sequencing.

4. Results

4.1. Extraction of BHV1-UL25 Serine Protease Substrate Fragment from Agarose Gel

One μL of digested plasmid and 1 μL of the uncut plasmid were electrophoresed in a 1% gel along with an Excel Band™ 1 KB Plus (0.1 - 10 kb) DNA ladder and the extraction of the fragment from digested pGH pBLUSCRIP (+) and the difference of size between cut and uncut plasmids were observed (Figure 1).

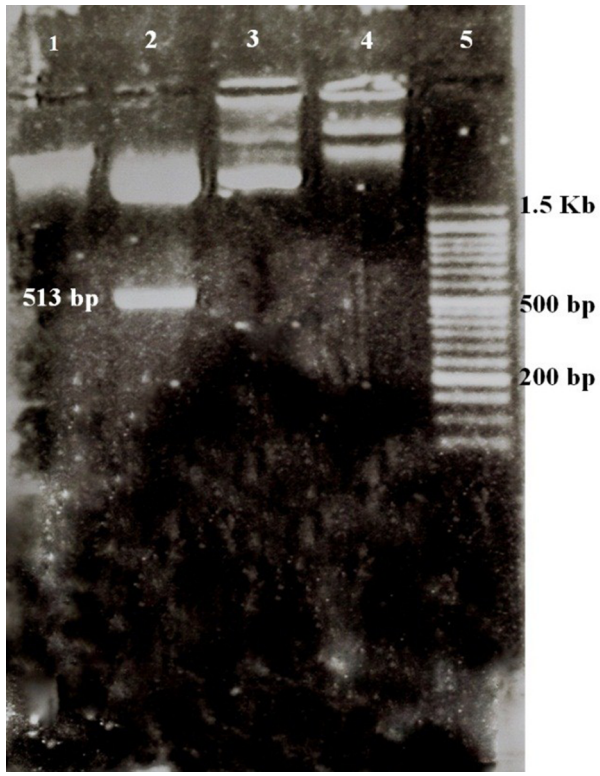

Figure 1. Extraction of digested and uncut pGH pBLUSCRIP II SK-UL25 (+) and pCDH-CMV-MCS-EF1-cGFP-T2A-Puro; (1) Digested pCDH (8197 bp); (2) Digested pGH pBLUSCRIP II SK-UL25 (+); the band above: Digested plasmid (2943 bp), bottom band: BHV1-UL25 serine protease substrate fragment (513 bp); (3) Uncut pCDH (8220 bp); (4) Uncut pGH pBLUSCRIP II SK-UL25 (3456 bp); (5) 50 bp DNA ladder

4.2. Colony PCR

After colony PCR, the presence of the *BHV1-UL25* fragment was detected in transformed colonies samples with the size of 726 bp (Figure 2). The length of the PCR product in origin pCDH (positive control) was in the range of 213 bp. Moreover, no band was detected in untransformed DH5α (negative control).

4.3. Extraction of Recombinant Plasmids Containing of BHV1-UL25-Serine Protease Substrate

One μL of extracted plasmids from two positive clones were electrophoresed on 1% agarose gel and the production of recombinant vector was confirmed by observing the desired band at 8602 bp (Figure 3).

4.4. Sequencing

Analysis of the read sequences of recombinant plasmids showed the presence of the desired fragment and the accuracy of the cloning process (Appendix in supplementary file).

5. Discussion

On the one hand, the effectiveness of existing anti-IBR vaccines or drugs has not been approved by the majority of scientists (6, 11) and on the other hand, treatment based on suppressing or changing the desired gene expression has minimal side effects compared to other treatment methods. Therefore, in recent decades, gene therapy has been welcomed as an alternative method of treatment (12).

Genetics has been involved in the treatment of herpes viruses in the recent years due to the low specificity and adverse effects of chemical drugs. New studies have been developed on the antiviral approaches of gene therapy against these viruses. Accordingly, designing and developing methods for evaluating such treatments is necessary. Hence, in the present study, the early stages of developing an approach to assessing to genetic treatments against *BHV-1* were performed.

One way to evaluate such molecules is to introduce them into cell lines that express the genes they are supposed to suppress. For this purpose, the first step is

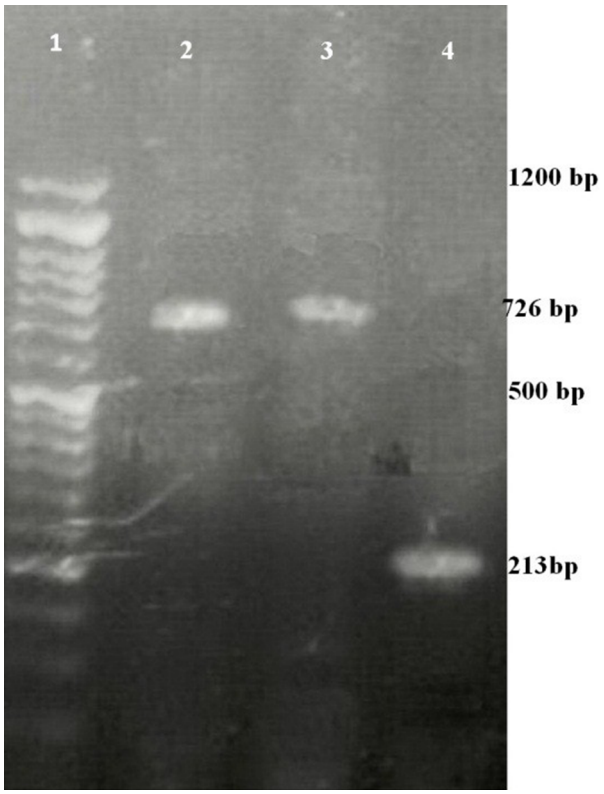

Figure 2. Colony PCR for detection of BHV1-UL25 serine protease substrate; (1) Ladder (50 bp); (2) and (3) PCR products (726 bp); (4) Positive control (213 bp)

the cloning of these target sequences into an appropriate plasmid for transfection into a particular cell line (13).

IBRV UL25 is a nuclear gene that is conserved among members of the *Herpesviride* family. Its necessity for the assembly and addition of the tegument layer to the virion encouraged the authors of the present study to select it. In a study performed by Bowman et al., using the UL-25 gene cloning in pET41 plasmid and its expression in cells, the researchers showed the role of UL25 in the formation of a stable capsid and the proper packaging of the viral genome (2). Non-expression of *BHV-1 UL25* occurred in MDBK cells. Several works have been conducted to study the sequence and structure of the *UL25* gene since then, all of which have shown this gene is conserved among all subfamilies of *Herpesviridae* (2).

UL25 is a DNA packaging tegument protein and its main role is considered to be DNA encapsidatoin. The placement of the serine protease substrate domain in UL25 indicates that this is the cleavage site of serine proteases that facilitate DNA encapsidatoin, and its conservation indicates it has an important role for the virus life cycle (14).

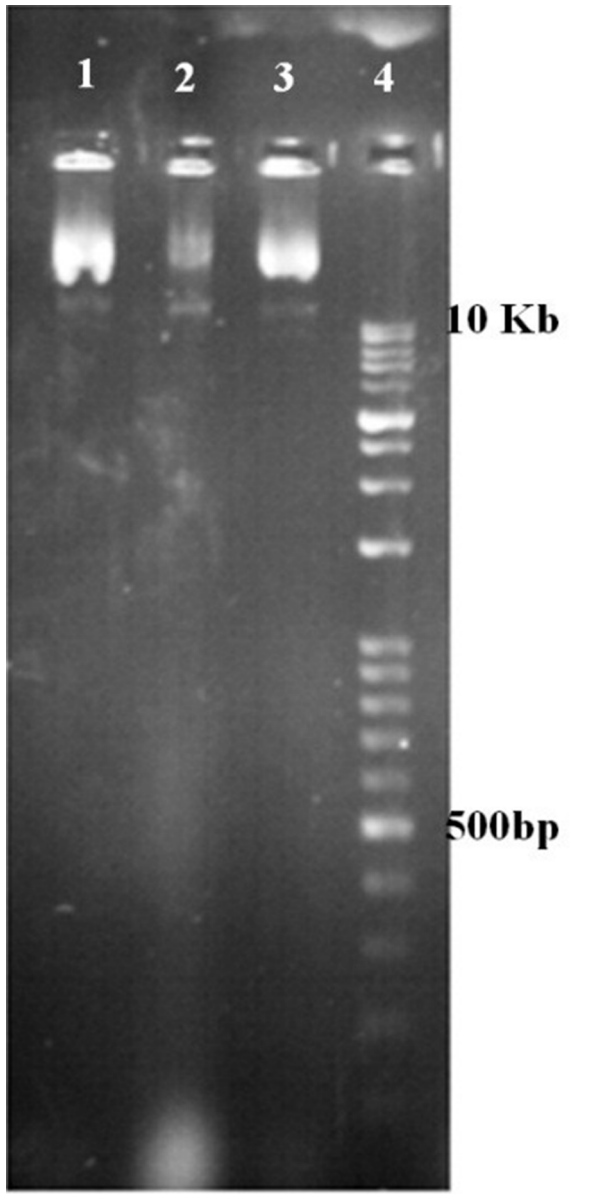

Figure 3. Extraction of BHV1-UL25 serine protease substrate-pCDH-CMV-MCS-EF1-cGFP-T2A-Puro; (1) - (3) Recombinant pCDH-CMV-MCS-EF1-cGFP-T2A-Puro plasmids in different concentrations (8520 bp); (4) 1 kb ladder

In the present study, for permanent expression of the target genes, the transduction was performed by a lentiviral vector. Plasmids were also used for gene expression induction after the transfection although the duration of gene expression was low (15). Lentiviral vectors could infect almost all types of cells and induce remarkable sufficient gene expression in their host cells for a long time. Furthermore, owing to their potent

to integrate into the host chromatin, their desire to eliminate all pathogenic genes in the vector, and finally, because there is no interference with the pre-existing antiviral reactions of immune system, lentiviral vectors LVs possess an effective delivery. Moreover, LVs can transmit large nucleotide sequences (3000 bp) and the probability of mutagenesis and carcinogenesis is low after their application.

Given the reasons mentioned above, lentiviral vectors were used to express the target genes in the desired cells and the first step for the preparation of these vectors is the cloning of the desired gene in the transfer vector in lentiviral packaging systems (16, 17).

Among the available lentiviral plasmids, pCDH-CMV-MCS-EF1-cGFP-T2A-Puro, which has ampicillin and puromycin resistance genes, was selected for ease of access and handling. Both antibiotics are easy to achieve. On the other hand, the vector has a GFP gene as a marker protein, which is applicable to the diagnosis of transfection efficiency (18, 19). This plasmid passes strong *RSV*, *CMV*, and *EF1* promoters for expression of the desired gene and GFP. The multiple cloning sites in this plasmid include the cleavage sites of the most restriction enzymes. Jafarzade et al. firstly increased the efficiency of LVs (pCDH-CMV-MCS-EF1-cGFP-T2A-Puro) and epEGFP-N1 for induction their desired gene in 293T cells (20). Ranjibar et al. amplified the Cop-GFP from the pCDH plasmid for developing a phagemid for production of reporter mammalian-cell (21).

There are some applications of RNAi against the members of *Herpesviridae*. For example, siRNAs were designed by Jia and Sun for suppression of gamma *Herpesvirinae* ORF-45 to demonstrate its performance. BHK-21 cells expressed it were developed to evaluate these siRNAs (22).

Wiebusch et al. designed siRNAs for HCMV-UL54 and produced *U373* cells expressing *UL54* sub-replicons after cloning the target gene segments into appropriate plasmids and transfecting them (23).

Zhe et al. targeted *HSV-1-UL39* by their siRNAs. They evaluated siRNAs by designing, cloning, and providing an ICP6 reporting system (24).

Wilkes and Kania suppressed *FHV-1-gD* by siRNA molecules decrease viral titer (25). Specific evaluation of these two siRNAs was performed with gene cloning and plasmid transfusion into Crandell-Rees cat kidney cells (25).

Narute et al. designed two anti *BHV1-UL25* siRNAs. However, in their study, they did not provide a proprietary evaluation reporter system (7).

There are some researches in which lentiviruses mediated RNAi have been applied for other viruses *Herpesviridae* family; Wang et al. suppressed LMP2A with lentivirus-mediated RNAi in GT38 cells (26). Song et al. used lentivectors for expression of IBRV gD shRNAs and with TCID50 assay showed the reduction in viral titer. They transfected pcDNA3-gD plasmid and recombinant LVs expressing shRNA into 293T cells and RNAi efficacy was confirmed utilizing Western blot (6). Amjadimanesh et al. applied BHV1-UL25 shRNAs and showed that they had considerable antiviral attributes in challenge with virus and MDBK cells expressed UL25 (27).

5.1. Conclusions

The findings revealed that the *BHV1-UL25* target fragment was successfully cloned in the desired plasmid and in the future studies; it will be accessible for transfection of the appropriate cell lines using lentiviral packing systems to produce specific reporter cells.

Supplementary Material

Supplementary material(s) is available here [To read supplementary materials, please refer to the journal website and open PDF/HTML].

Footnotes

Authors' Contribution: Azam Mokhtari and Monireh Kazemimanesh: Conceiving and design the study. Behnaz Saffar: Conceiving and design the study. Sahand Shams: Performing the experiments and analyzing the samples. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

Conflict of Interests: Azam Mokhtari and Behnaz Saffar are employees of Shahrekord University. Monireh Kazemimanesh is employee of Pasteur Institute. Sahand Shams was dvm student at Shahrekord University.

Data Reproducibility: The data presented in this study are uploaded during submission as a supplementary file and are openly available for readers upon request.

Ethical Approval: IR.SKU.REC.1400.045.

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