Iranian J Biotech. 2017 July;15(3):e1558 **DOI:10.15171/ijb.1558**

Research Article

Designing Two Individual AcMNPV Polyhedrin-Plus Bac-to-Bac Expression System in order to Express GFP and CPV-VP2 in Insect Cells

Mohammad Sadegh Hashemzadeh [1,2] , Seyed Jafar Mousavy [1*], Ruhollah Dorostkar [2], Fatemeh Fotouhi [3], Firouz Ebrahimi [1]

[1] Department of Biology, Faculty of Basic Sciences, Imam Hossein University, Tehran, Iran
[2] Applied Virology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
[3] Department of Influenza and other Respiratory viruses, Pasteur Institute of Iran, Tehran, Iran

[*] *Corresponding author:* Seyed Jafar Mousavy, Department of Biology, Faculty of Basic Sciences, Imam Hossein University, Tehran, Iran
Tel: +98 21 77104934, E-mail: jmosavi@ihu.ac.ir

Received: 20 April 2016; **Revised**: 4 September 2016; **Accepted**:5 September 2017; **Published online:** 27 September 2017

Background: The importance of viral protein-2 (VP2) of canine parvovirus (CPV) in binding to human cancer cells, production of veterinary vaccines and diagnostic kits has motivated several researches on producing this protein.
Objectives: Our purpose was to construct recombinant bacmid shuttle vectors expressing VP2 of CPV using Bac-to-Bac baculoviral expression system.
Materials and Methods: Mini-Tn7 transposones engineered in pFastBac1 donor vectors were used to construct expression cassettes of GFP and CPV-VP2. The plasmids were transferred into *E. coli* DH_{10}Bac competent cells. Site-specific transposition of the genes into bacmid was accomplished using helper plasmid. Occurrence of Transposition was confirmed via PCR using specific primers and PUC/M_{13} universal primers. The recombinant bacmid DNAs were transfected into Sf9 cells using cationic lipids to generate new recombinant baculoviruses expressing GFP and CPV-VP2. GFP and VP2 expressions were evaluated by fluorescence microscopy and western analysis, respectively.
Results: Cloning, subcloning and recombination processes of both GFP and VP2 were accomplished and verified. Accuracy of transfection process was confirmed by GFP fluorescence microscopy.VP2 expression was verified by SDS-PAGE and western analysis.
Conclusions: Two Bac-to-Bac expression systems were designed to produce recombinant VP2 and GFP in insect cells.

Keywords: Bacmid, Baculovirus, Canine Parvovirus, GFP, VP2

1. Background

The family of *Parvoviridae* is composed of small viruses that are non-enveloped and icosahedral. These viruses contain linear ssDNA (single-stranded DNA) genomes about 5000 nucleotides long. Canine parvovirus (CPV) belongs to *Parvovirus* genus of this family and first appeared in the late 1970s, as a new disease. It is prevalent in dogs around the world (1, 2, 3). CPV particles have a diameter of 25 nm and are composed of three proteins, VP1 (viral protein-1), VP2 (viral protein-2), and VP3 (viral protein-3) (4).

VP2 is the major component of the viral capsid. About 90% of the capsid protein is VP2, and 10% is VP1, which includes the entire sequence of VP2 and 154 additional residues at its N-terminus. The third protein (VP3) is generated after intracellular proteolytic cleavage of VP2 by removing approximately 25 amino acids from its N-terminus. Wild-type natural capsid is composed of 60 subunits primarily of the VP2, along with a few subunits of VP1 and VP3 (5-16). CPV has a natural affinity to cancer cells via VP2 ligands/transferrin receptors (TfRs) attachment. In fact, the VP2 protein of CPV is the main part of attachment ligands for entry into specific and cancerous cells through transferrin receptors (TfRs) (1, 17, 18). Yuan *et al*. (2001) claimed that VP2 can assemble into capsid-like structures and the expression of VP2 alone can result in assembly of a typically-sized virus like particle (VLP) for therapeutic purposes. VP2 protein of canine parvovirus binds to human cancer cells. Thus, it has the potential to be used as veterinary detection kit and for vaccine development (5-16).

One of the best systems, considered in production

of recombinant proteins, is the use of baculoviruses in insect cell expression system. The system allows proper post-translational modifications; producing biosimilars of protein of interest (19-22). Here, Bac-to-Bac baculovirus expression vector system (BEVS) was used for heterologous expression in in insect cells. The system has two components: pFastBac donor plasmid vector into which the gene(s) of interest will be cloned and has an expression cassette and baculovirus shuttle vector (bacmid). In latter, the expression cassette is transposed via recombinant pFastBac (23).

2. Objectives

Our purpose was to construct recombinant bacmid shuttle vectors expressing GFP and VP2 of CPV using Bac-to-Bac baculoviral expression vector system (BEVS). The first component of BEVS system through the construction of mini-Tn7 transposons was generated by cloning of GFP and *CPV-VP2* genes into pFastBac1 donor plasmid vector. The second component of BEVS system was created through the construction of two recombinant bacmid DNAs, expressing GFP and VP2, using site-specific transposition mechanism. These constructs can be used to produce large scale of recombinant GFP and VP2 proteins in insect cells for therapeutic aims.

3. Materials and Methods

3.1. Preparation of the Genes, Plasmids, Bacterial Strains and Cells

The *VP2* was isolated from the recombinant construct of pET-21a. The *GFP* was isolated from RAPAd® shRNA Adenoviral Expression System (Cell Biolabs, Inc). "T/A cloning vector" (RBC Bioscience, Taiwan) was used as the general vector. pFastBac1 was used as the transfer vector for subcloning , (Invitrogen, USA).

The *E. coli* strain DH5α (Invitrogen, USA) was used for transformation and amplification of recombinant vectors (such as RBC T/A cloning vector and pFastBac1 donor plasmid vector). The *E. coli* strain DH10Bac (Invitrogen, USA) containing the modified baculoviral DNA (bacmid shuttle vector) with a target site of mini-attTn7 and the helper plasmid was used as a proper strain to carry out the transposition process. The helper plasmid begets tetracycline resistance and encodes the enzymes required for transposition of the gene of interest into the bacmid.

Spodoptera frugiperda (Sf9) cells were purchased from the Cell Bank (Iranian Biological Resource Center, Iran).

3.2. Extraction of the Plasmids

After the selection of proper colonies by blue/white screening, the recombinant plasmids were extracted from 1500 µL of bacterial cell cultures using a Roche commercial kit (Germany) according to the manufacturer's instructions.

Bacmid DNAs were isolated after the selection of proper colonies by blue/white screening of DH_{10}Bac cells cultured on a LB agar plate (incubated at 37 ºC for 48 h) containing 7 $\mu g.mL^{-1}$ gentamicin, 50 $\mu g.mL^{-1}$ kanamycin, 10 $\mu g.mL^{-1}$ tetracycline, 100 $\mu g.mL^{-1}$ X-gal and 40 $\mu g.mL^{-1}$ isopropylthio-β-D-galactoside or IPTG (Invitrogen, USA). The recombinant bacmid plasmids were also extracted from 1500 µL of bacterial broth cell cultures using a Roche commercial kit (Germany) according to the manufacturer's instructions. The bacmid DNA was isolated from overnight cultures by alkaline lysis method (24).

It is notable that bacmid DNA is a high-molecular-weight plasmid (~ 135 kbp) and due care to avoid shearing is to be taken.

3.3. Designing and Synthesizing the Specific Primers for CPV-VP2 *and* GFP *Sequences*

The sequences of *VP2* region of CPV genome and *GFP* region were adapted from Genbank and the specific primers targeting these regions were designed using the Oligo software, version 3.0. The forward and reverse specific primers for *VP2* and *GFP* are presented in Table 1. The primer sequences did not show genomic cross-reactivity with other viruses, human genome and other probable interfering genomes in BLAST. It only detected a 1755 bp fragment of *CPV-VP2* ORF and a 720 bp fragment of *GFP* ORF (data not presented). The primers were synthesized by Bioneer (Korea).

3.4. Isolation and Amplification of CPV-VP2 *and* GFP

The full-lengths of *CPV-VP2* (1755 bp) as well as *GFP* (720 bp) were PCR amplified using specific primers and cloned into RBC T/A cloning vector. Template DNA (1 µL of each of the genes, separately) was added to a 50 µL total volume of PCR mixture containing 10 pmol of each forward and reverse primers, 5 mM $MgSO_4$, 0.5 mM dNTPs, 2.5 U of pfu DNA polymerase (Fermentas, Vilnius, Lithuania) and 5 µL of 10× PCR buffer.

Amplification reactions were performed in a thermocycler (Biorad, USA) under the following profiles: 5 min at 94 °C followed by 40 cycles at 94 °C for 45 sec, 67 °C for 60 sec and 72 °C for 160 sec, with a final extension step at 72 °C for 10 min, for *VP2* and 5 min at 94 °C followed by 40 cycles at 94 °C for 45 sec, 57 °C for 45 sec and 72 °C for 60 sec, with a final extension

Table 1. Indicating the amplified regions and the primer sets details, used for PCR analysis of the recombinant bacmids.

Primer Pairs	Sequence	Fragment Size* (bp)	Amplicon
pUC/M_{13} F VP2 specific R	5′-GTTTTCCCAGTCACGAC-3′ 5′-TTAATATAATTTTCTAGGTGCTAGT-3′	3400	Tn7 R + Polyhedrin promoter + VP2 gene
VP2 specific F pUC/M_{13} R	5′-ATGAGTGATGGAGCAGTTCAAC-3′ 5′-CAGGAAACAGCTATGAC-3′	2450	VP2 gene + Tn7 L
pUC/M_{13} F pUC/M_{13} R	5′-GTTTTCCCAGTCACGAC-3′ 5′-CAGGAAACAGCTATGAC-3′	4000	Tn7 R + Polyhedrin promoter + VP2 gene + Tn7 L
VP2 specific F VP2 specific R	5′-ATGAGTGATGGAGCAGTTCAAC-3′ 5′-TTAATATAATTTTCTAGGTGCTAGT-3′	1750	VP2 gene
pUC/M_{13} F GFP specific R	5′-GTTTTCCCAGTCACGAC-3′ 5′-TTACTTGTACAGCTCGTCC-3′	2400	Tn7 R + Polyhedrin promoter + GFP gene
GFP specific F pUC/M_{13} R	5′-ATGGTGAGCAAGGGCGAG-3′ 5′-CAGGAAACAGCTATGAC-3′	1350	GFP gene + Tn7 L
pUC/M_{13} F pUC/M_{13} R	5′-GTTTTCCCAGTCACGAC-3′ 5′-CAGGAAACAGCTATGAC-3′	3000	Tn7 R + Polyhedrin promoter + GFP gene + Tn7 L
GFP specific F GFP specific R	5′-ATGGTGAGCAAGGGCGAG-3′ 5′-TTACTTGTACAGCTCGTCC-3′	720	GFP gene

* The Sizes of all fragments have been calculated and reported approximately, according to the bacmid DNA sequence data.

step at 72 °C for 10 min, for *GFP*. PCR products were analyzed by electrophoresis using 1-2% (w/v) agarose gel, stained with safe view (Kiagene, IRI).

3.5. Cloning of VP2 *and* GFP *into RBC T/A Cloning Vector*

The PCR product was extracted from low melting agarose gel using a DNA extraction kit (Vivantis-Korea). The purified amplicons were adenylated in a one-step procedure according to general protocols (24). The adenylated amplicons were subsequently cloned into RBC T/A cloning vector (RBC Bioscience, Taiwan). Blue/ white screening allowed distinguishing colonies with plasmids containing the amplicon. The presence of inserts was checked by both PCR and enzyme restriction analysis (*Eco*RI/ *Xba*I).

3.6. Determination of the Orientation of the Genes

Gene orientation within cloning vector was determined by PCR using universal M13 forward (Invitrogen, USA), and specific forward and reverse primers. It is notable that the universal PUC/M13 flanking sites are located in RBC vector (RBC Bioscience, Taiwan). *Bam*HI/*Eco*RI restriction sites were chosen for subcloning of *VP2* into pFastBac1 donor plasmid vector and *Eco*RI/*Xba*I restriction sites for *GFP*. The two genes were cloned in opposite directions.

3.7. Subcloning of VP2 *and* GFP *into pFastBac1 Donor Plasmid Vectors*

The *VP2* and *GFP*were digested with corresponding enzymes. The amplicons were gel-purified using Vivantis DNA extraction kit (Korea). The purified products were ligated into pFastBac1 donor plasmid vectors (Invitrogen, USA), digested using the same restriction enzymes.

The presence of the *VP2* and *GFP* in expression cassette of pFastBac1 donor vectors containing mini-Tn_7 transposones were evaluated by DNA sequencing.

3.8. Construction of Two Individual Recombinant Bacmid DNAs Encoding VP2 *and* GFP

The *VP2* and *GFP* containing recombinant pFastBac1 donor plasmids were transferred into the *E. coli* DH_{10}Bac competent cells, separately. For site-specific transposition of *VP2* or *GFP* into the bacmid shuttle vector, the cells containing the expression cassettes were incubated with transposing cassette for 4-6 h. The transposition causes the disruption of the *lacZ* operon. The presence of the helper plasmid that encodes the transposase is required. The transformed cells were cultured on a LB agar plate containing kanamycin (50 $\mu g.mL^{-1}$), gentamicin (7 $\mu g.mL^{-1}$), tetracycline (10 $\mu g.mL^{-1}$), X-gal (100 $\mu g.mL^{-1}$) and IPTG (40 $\mu g.mL^{-1}$). The plates were incubated at 37 ºC for 16 h. The bacmid DNA was isolated from the overnight cultures by alkaline lysis purification method.

3.9. Analysis of Recombinant Bacmid DNAs

The transposition process accuracy and/or the existence of genes in bacmid DNAs were evaluated by PCR panel using both *VP2* (or *GFP*) specific primers and PUC/M_{13} universal primers (Table 1). PCR using specific primers showed the accuracy of DH_{10}Bac transformation by recombinant pFastBac1 and PCR using PUC/M_{13} universal primers. Orientations of the genes in recombinant bacmid were checked by PCR using *VP2* (or *GFP*) specific and PUC/M_{13} universal primers.

3.10. Polymerase Chain Reaction

PCR was accomplished in a microtube containing 5 µL of 10× *Taq* PCR buffer, 1 µL of dNTPs (0.2 mM for each), 1.5 µL of $MgCl_2$ (1.5 mM), 1 µL of each primer with the concentration of 10 µM for each, 1-2 µL of template DNA, 1 unit of *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania) and nuclease-free ddH_2O up to 50 µL final volume. Amplification reactions were performed in Biorad thermocycler (USA) and the PCR program included the following steps for all the amplicons [94 ºC: 5 min, followed by 30 cycles of 94 ºC: 45 sec; annealing ºC: 60 sec; 72 ºC: based on 30 sec for each kbp, and a final extension at 72 ºC: 10 min], except annealing temperatures that were different. For VP2, GFP, PUC/M_{13} universal primers, VP2/GFP specific and PUC/M_{13} universal primers annealing temperatures were 67, 57, 63, and 63/57 ºC, respectively. PCR products were evaluated by electrophoresis using 1-2% (w/v) agarose gel.

3.11. Cell Culture and Transfection of Sf9 Cells

Sf9 cells were grown in Grace's insect cell culture medium (Invitrogen, Catalog no. 11595-030) supplemented with 10% heat-inactivated fetal bovine serum or FBS (Gibco, USA), 1% non-essential amino acids and 2mM L-Glutamine (Bio-idea, Iran) and maintained in a non-humidified incubator at 26-28 °C. For the highest transfection efficiency, un-supplemented (no FBS) Grace's medium (pH 6.2-6.4) was used without antibiotics and serum.

To produce recombinant baculovirus, the pre-cultured Sf9 cells (8×10^5 cells/well in 6-well tissue culture plates and in the log phase with greater than 95% viability and 70% confluency) were transfected by 1-3 µg isolated recombinant bacmid DNA using a cationic lipid named Cellfectin II reagent (Invitrogen, USA) according to the manufacturer instruction. Cell density, DNA concentration, Cellfectin II concentration, DNA/Cellfectin II ratio and incubation time were optimized. The transfected cells were incubated at 27 ± 0.5 ºC for 72 h to let baculovirus assembly and release into the culture medium. The recombinant baculovirus generation was monitored every day by visualization of the cytopathic effects (CPEs). The evaluated signs of infection included: a 25-50% increase in cell diameter, increased size of cell nuclei, cessation of cell growth, granular appearance, detachment from the plate or flask and cell lysis. The culture medium was collected 72 h post-infection (hpi), clarified by low-speed centrifugation for 10 min at 1500 ×*g* and subjected to multiple rounds of virus propagation. These viral stocks were stored at +4 ºC in dark. FBS was added to a final concentration of 2%.

3.12. Evaluation of VP2 Expression in Insect Cells

The infected cells were lysed with 100 µL of 1× SDS-PAGE buffer (62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS) and boiled for at least 3 min after adding the sample buffer (10% w/v SDS, 10 mM 2-mercapto-ethanol, 20% v/v glycerol, 0.2 M Tris-HCl, pH 6.8, 0.05% w/v bromo-phenol-blue). The proteins in the samples were separated and examined by SDS-PAGE and Coomassie staining. Uninfected Sf9 cells, which similarly treated were used as a negative control.

3.13. Evaluation of GFP Expression by Fluorescence Microscopy

The infected cells were monitored daily by visualization of green fluorescent protein (GFP) expression using inverted fluorescence microscope (Model no. CKX41; Olympus, Center Valley, PA, USA).

4. Results

4.1. Amplification and cloning of CPV-VP2 *and* GFP

Gel-based analysis of amplified *VP2* and *GFP* fragments using the corresponding specific primers confirmed the expected 1755 bp and 720 bp amplicons respectively, using 1-2% (w/v) agarose gel electrophoresis (Supplementary Figs. 1a and 1b).

The fragments were cloned into RBC T/A cloning vectors, separately after the extraction from low melting agarose gels and the accuracy of cloning process in these vectors were confirmed using PCR (Supplementary Figs. 2a and 2c) and enzymatic digestion analysis (Supplementary Figs. 2b and 2d).

4.2. Determination of VP2 *&* GFP *Orientations in pFastBac1*

The orientations of the genes in the cloning vectors determined by PCR panel using the universal PUC/M13 forward and specific forward primers, and the universal

PUC/M13 forward and specific reverse primers, respectively. The first PCR using the universal PUC/M13 forward and specific forward primers was positive for *VP2* and this was negative for *GFP* (Supplementary Figs. 3a and 3b).

The fragments of *VP2* and *GFP*, digested using the restriction sites of interest (Supplementary Figs. 4a and 4d) were purified (Supplementary Figure 4e) and subcloned into pFastBac1 donor plasmid vectors. Presence of the genes of interest in expression cassette of pFastBac1 was confirmed by enzymatic digestion (Supplementary Figs. 4b, 4c and 4g) and PCR (Supplementary Figs. 4c and 4f). Triple digestion using *Eco*RV and *Hind*III enzymes and also double digestion using *Bam*HI and *Eco*RI were accomplished for *VP2*. Double digest was carried out using *Eco*RI and *Xba*I for *GFP* and the fragments produced were analyzed and confirmed according to NEBcutter software pattern. Finally, the accuracy of the *VP2* (or *GFP*) ORF in recombinant pFastBac1 was confirmed by DNA sequencing . The orientation of the genes of interest was opposite and due to the orientations obtained, we chose *Bam*HI/*Eco*RI restriction sites for subcloning of *VP2* into pFastBac1 donor plasmid vector and *Eco*RI/*Xba*I for *GFP*.

4.3. Analysis of Recombinant bacmid DNA Shuttle Vectors for VP2 *and* GFP

The transformation of *E. coli* DH_{10}Bac cells was accomplished successfully by *VP2* and *GFP* containing recombinant pFastBac1 donor plasmid vectors. The site-specific transposition of *VP2* (or *GFP*) expression cassettes from the transposing vectors into the bacmid shuttle vectors were performed with the presence of helper plasmid and selected on LB agar. PCR was performed using PUC/M_{13} universal primers to ensure the occurrence of (Supplementary Figs. 5a and 5b).

A PCR was carried out using *VP2* (or *GFP*) specific and PUC/M_{13} universal primers to ensure the transposition in recombinant bacmids. The bacmid DNA includes PUC/M_{13} forward and reverse priming sites, flanking the Tn_7 mini-att site within the region of *LacZ* α-complementation. The results indicated the accuracy of recombination through site-specific transposition mechanism and proper orientation of *VP2* (or *GFP*) in the recombinant bacmid. The panel of PCR was performed using PUC/M_{13} universal forward and reverse primers, *VP2* (or *GFP*) gene specific forward and reverse primers, *VP2* (or *GFP*) gene specific forward primer and PUC/M_{13} universal reverse primer and finally *VP2* (or *GFP*) gene specific reverse primer and PUC/M_{13} universal forward primer, respectively(Supplementary Figs. 6a and 6b).

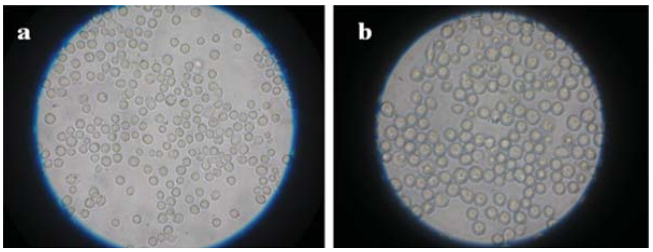

Figure 1. a) Untransfected Sf9 culture (negative control), **b**) The Sf9 cells transfected with recombinant bacmid DNA showing the typical CPEs.

4.4. Transfection of Sf9 Cells and CPE Observation

The extracted recombinant bacmid DNAs were transfected into Sf9 cells using Cellfectin II reagent (Invitrogen, USA) and untransfected Sf9 culture (as the negative control) continued to divide and formed a confluent normal cell monolayer, quite different from the infected cells (Figs. 1a and 1b).

4.5. Expression of Recombinant VP2 in Insect Cells

Total proteins of the cell lysates were evaluated on 4-12% polyacrylamide gel (SDS-PAGE). In Figure 2a, the intended protein band with predicted molecular weight of about 64 kDa was detected, representing the recombinant VP2 expression.

Western analysis using anti-VP2 monoclonal antibodies (AbCam, USA) confirmed the specificity of the detected band and also represented the expression accuracy of the protein of interest (Fig. 2b).

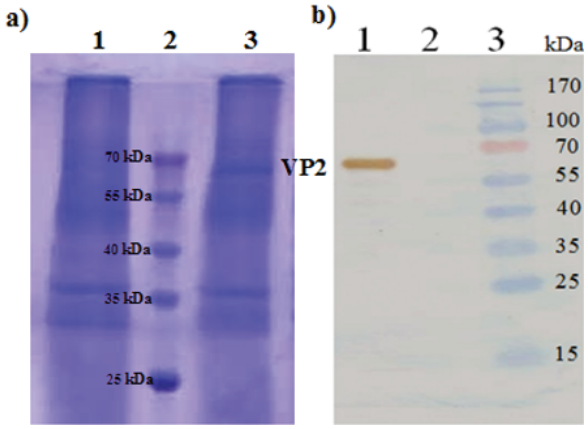

Figure 2. a) SDS-PAGE result of the proteins extracted from Sf9 cells infected by recombinant baculoviruses, Lane 1: Uninfected Sf9 cells (negative control), Lane 2: Protein molecular weight marker (MyBioSource, USA), Lanes 3: The expected stained band (~64 kDa) corresponding to expressed VP2, **b)** Western blot result of VP2 protein, Lane 1: The VP2 protein confirmed by anti-VP2 monoclonal antibody, Lane 2: BSA (as negative control), Lane 3: Protein molecular weight marker (MyBioSource, USA).

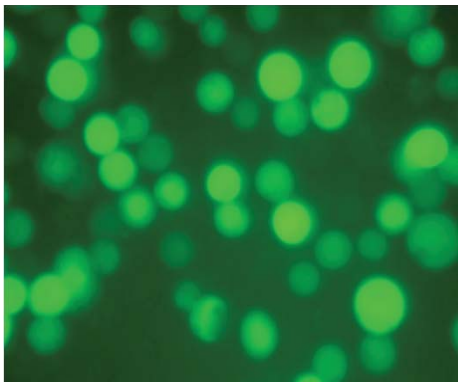

Figure 3. Fluorescence microscopy of Sf-9 cell lines infected with recombinant baculovirus expressing GFP.

4.6. GFP Expression in Insect Cells by Fluorescence Microscopy

The results of fluorescence microscopy showed successful expression of green fluorescent protein (GFP) in insect cells (Fig. 3).

5. Discussion

Bac-to-Bac baculoviral expression vector system (BEVS) is an efficient and fast method to produce recombinant baculoviruses (23, 25-26).

Expression of recombinant genes in pFastBac vector is under the control of either polyhedrin (pH) or p10 promoter for high level expression in insect cell lines. The flanking sites of the expression cassette (that contains the inserted gene) in the pFastBac vector are the right and left arms of Tn7 (Tn7R and Tn7L), and also this cassette is containing a resistance gene (gentamicin) and a SV40 poly A signal that forms a mini Tn7 transposon (23, 25-26).

The DH10Bac *E. coli* strain is being used as the host for recombinant pFastBac vector. This cells harbor a bacmid shuttle vector (derived from baculovirus) with a mini-attTn7 (attachment site for Tn7R and Tn7L) and a helper plasmid. After the construction of recombinant pFastBac, once this expression plasmid is transferred into DH10Bac cells, the transposition happens between the mini-attTn7 target site on the bacmid and the mini-Tn7 element on the pFastBac to produce a recombinant bacmid. The presence of transposition proteins encoded by the helper plasmid is necessary for this reaction. As soon as the transposition reaction is performed, one can extract the recombinant bacmid (with high molecular weight) and transfect it into the cultured insect cells to produce a new recombinant baculovirus. This generated virus can be used for primary expression evaluations. After the amplification and titeration of the baculoviral stock, the produced high-titer seed can be used for infection of the insect cells and expression of the recombinant protein of interest in large scale (23, 27-33).

Generation of recombinant baculovirus using Bac-to-Bac baculoviral expression system has some advantages over the traditional method that works on the basis of homologous recombination. The Bac-to-Bac requires less time to generate (2 weeks as opposed to 4-6 weeks), no need for plaque selection due to working based on blue/white selection, rapid and simultaneous isolation of several recombinant baculoviruses This method allows quick and simultaneous, and suitable for the expression of various proteins in structural and functional studies (23, 25-26). Considering these advantages, here Bac-to-Bac baculoviral system was set up to express VP2 of canine parvovirus in large-scale in insect cells.

6. Conclusions

In this research, we designed two individual AcMNPV polyhedrin-plus Bac-to-Bac expression system in order to express GFP and CPV-VP2 in insect cells. The recombinant baculoviruses generated here were able to express recombinant VP2 protein (and GFP as the control) in Sf9 cells for the therapeutic aims.

Acknowledgments

We thank the Applied Virology Research Center in Baqiyatallah University of Medical Sciences, for financial and moral support and services provided during this study as a main part of Ph.D thesis.

References

1. Hueffer K, Parker JS, Weichert WS, Geisel RE, Sgro JY, Parrish CR. The natural host range shift and subsequent evolution of canine parvovirus resulted from virus-specific binding to the canine transferrin receptor. *J Virol*. 2003;**77**:1718-1726. DOI: 10.1128/JVI.77.3.1718-1726.2003.
2. MacLachlan NJ, Dubovi EJ. Parvoviridae. In: Maclachlan NJ, Dubovi EJ, editors. Fenner's veterinary virology. 4th ed. *London*. Academic;2011:507.
3. Hoelzer K, Parrish CR. The emergence of parvoviruses of carnivores. *Vet Res*. 2010;**41**:39-51. DOI: 10.1051/vetres/2010011.
4. Wu H, Rossmann MG. The canine parvovirus empty capsid structure. *J Mol Biol*. 1993;**233**(2):231-244. DOI: 10.1006/jmbi.1993.1502.
5. Decaro N, Desario C, Parisi A, Martella V, Lorusso A, Miccolupo A, Mari V, Colaianni ML, Cavalli A, Di Trani L, Buonavoglia C. Genetic analysis of canine parvovirus type 2c. *Virology*. 2009;**385**:5-10. DOI: 10.1016/j.virol.2008.12.016.
6. Battilani M, Ciulli S, Tisato E, Prosperi S. Genetic analysis of canine parvovirus isolates (CPV-2) from dogs in Italy. *Virus Res*. 2002;**83**:149-157. DOI: 10.1016/S0168-1702(01)00431-2.
7. Wang HC, Chen WD, Lin SL, Chan JP, Wong ML. Phylogenetic

analysis of canine parvovirus *VP2* gene in Taiwan. *Virus Genes*. 2005;**31**:171-174. DOI: 10.1007/s11262-005-1791-0.
8. Chinchkar SR, Mohana Subramanian B, Hanumantha Rao N, Rangarajan PN, Thiagarajan D, Srinivasan VA. Analysis of *VP2* gene sequences of canine parvovirus isolates in India. *Arch Virol*. 2006;**151**:1881-1887. DOI: 10.1007/s00705-006-0753-8.
9. Doki M, Fujita K, Miura R, Yoneda M, Ishikawa Y, Taneno A, Kai C. Sequence analysis of *VP2* gene of canine parvovirus isolated from domestic dogs in Japan in 1999 and 2000. *Comp Immunol Microbiol Infect Dis*. 2006;**29**:199-206. DOI: 10.1016/j.cimid.2006.05.001.
10. Jeoung SY, Ahn SJ, Kim D. Genetic analysis of *VP2* gene of canine parvovirus isolates in Korea. *J Vet Med Sci*. 2008;70:719-722. DOI: 10.1292/jvms.70.719.
11. Kang BK, Song DS, Lee CS, Jung KI, Park SJ, Kim EM, Park BK. Prevalence and genetic characterization of canine parvoviruses in Korea. *Virus Genes*. 2008;**36**:127-133. DOI: 10.1007/s11262-007-0189-6.
12. Phromnoi S, Sirinarumitr K, Sirinarumitr T. Sequence analysis of *VP2* gene of canine parvovirus isolates in Thailand. *Virus Genes*. 2010;**41**:23-29. DOI: 10.1007/s11262-010-0475-6.
13. Zhang R, Yang S, Zhang W, Zhang T, Xie Z, Feng H, Wang S, Xia X. Phylogenetic analysis of the VP2 gene of canine parvoviruses circulating in China. *Virus Genes*. 2010;**40**:397-402. DOI: 10.1007/s11262-010-0466-7.
14. Gallo Calderon M, Wilda M, Boado L, Keller L, Malirat V, Iglesias M, Mattion N, La Torre J. Study of canine parvovirus evolution: comparative analysis of full-length *VP2* gene sequences from Argentina and international field strains. *Virus Genes*. 2012;**44**:32-39. DOI: 10.1007/s11262-011-0659-8.
15. Dogonyaro BB, Bosman AM, Sibeko KP, Venter EH, van Vuuren M. Genetic analysis of the VP2-encoding gene of canine parvovirus strains from Africa. *Vet Microbiol*. 2013;**165**:460-465. DOI: 10.1016/j.vetmic.2013.04.022.
16. Soma T, Taharaguchi S, Ohinata T, Ishii H, Hara M. Analysis of the VP2 protein gene of canine parvovirus strains from affected dogs in Japan. *Res Vet Sci*. 2013;**94**:368-371. DOI: 10.1016/j.rvsc.2012.09.013.
17. Hueffer K, Parrish CR. Parvovirus host range, cell tropism and evolution. *Curr Opin Microbiol*. 2003;**6**:392-398. DOI: 10.1016/S1369-5274(03)00083-3.
18. Chang SF, Sgro JY, Parrish CR. Multiple amino acids in the capsid structure of canine parvovirus coordinately determine the canine host range and specific antigenic and hemagglutination properties. *J Virol.* 1992;**66**:6858-6867.
19. Ikonomou L, Schneider YJ, Agathos SN. Insect cell culture for industrial production of recombinant proteins. *Appl Microbiol Biotechnol*. 2003;**62**:1-20. DOI: 10.1007/s00253-003-1223-9.
20. Wen Z, Ye L, Gao Y, Pan L, Dong K, Bu Z, Compans RW, Yang C. Immunization by influenza virus-like particles protects aged mice against lethal influenza virus challenge. *Antiviral Res*. 2009;**84**(3):215-224. DOI: 10.1016/j.antiviral.2009.09.005.
21. Anderson D, Harris R, Polayers D, Ciccarone V, Donahue R, Gerald G, Jessee J, Luckow V. Rapid generation of recombinant baculovirus and expression of foreign genes using the Bac-to-Bac baculovirus expression system. *Focus*. 1995;**17**(2):53-58.
22. Luckow VA, Lee SC, Barry GF, Olins PO. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. *J Virol*. 1993;**67**(8):4566-4579.
23. Xiang X, Yang R, Yu S, Cao C, Guo A, Chen L, Wu X, Cui W, Cenis JL. Construction of a BmNPV polyhedrin-plus Bac-to-Bac baculovirus expression system for application in silkworm, *Bombyx mori*. Appl *Microbiol Biotechnol*. 2010;**87**(1):289-295. DOI: 10.1007/s00253-010-2476-8.
24. Sambrook J, Russell D. *Molecular Cloning: A Laboratory Manual*. 3th Edition, USA, Cold Spring Harbor; 2001. DOI: 10.1002/abio.370050118.
25. Hitchman RB, Locanto E, Possee RD, King LA. Optimizing the baculovirus expression vector system. *Methods*. 2011;**55**(1):52-57. DOI: 10.1016/j.ymeth.2011.06.011.
26. Yue WF, Li XH, Wu WC, Roy B, Li GL, Liu JM, Wu XF, Zhou JY, Zhang CX, David WC, Miao YG. Improvement of recombinant baculovirus infection efficiency to express manganese superoxide dismutase in silkworm larvae through dual promoters of Pph and Pp10. *Appl Microbiol Biotechnol*. 2008;**78**(4):651-657. DOI: 10.1007/s00253-007-1337-6.
27. Aucoin MG, Mena JA, Kamen AA. Bioprocessing of baculovirus vectors: a review. *Curr Gene Ther*. 2010;**10**(3):174-186. DOI: 10.2174/156652310791321288.
28. Sandhu KS, Naciri M, Al-Rubeai M. Prediction of recombinant protein production in an insect cell-baculovirus system using a flow cytometric technique. *J Immunol Methods*. 2007;**325**(1-2):104-113. DOI: 10.1016/j.jim.2007.06.005.
29. Hitchman RB, Possee RD, Crombie AT, Chambers A, Ho K, Siaterli E, Lissina O, Sternard H, Novy R, Loomis K, Bird LE, Owens RJ, King LA. Genetic modification of a baculovirus vector for increased expression in insect cells. *Cell Biol Toxicol*. 2010;**26**(1):57-68. DOI: 10.1007/s10565-009-9133-y.
30. Sokolenko S, George S, Wagner A, Tuladhar A, Andrich JM, Aucoin MG. Co-expression vs. co-infection using baculovirus expression vectors in insect cell culture: Benefits and drawbacks. *Biotechnol Adv*. 2012;**30**(3):766-781. DOI: 10.1016/j.biotechadv.2012.01.009.
31. Griffiths CM, Page MJ. Production of heterologous proteins using the baculovirus/insect expression system. *Methods Mol Biol*. 1997;**75**:427-440. DOI: 10.1385/0-89603-441-0:427.
32. Fraser MJ. The baculovirus-infected insect cell as a eukaryotic gene expression system. *Curr Top Microbiol Immunol*. 1992;**158**:131-172. DOI: 10.1007/978-3-642-75608-5_6.
33. Kidd IM, Emery VC. The use of Baculoviruses as expression vectors. *Appl Biochem Biotechnol*. 1993;**42**:137-159. DOI: 10.1007/BF02788049.