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International Journal of Pharmaceutical Research & Allied Sciences, 2016, 5(3):343-349

Research Article

ISSN : 2277-3657
CODEN(USA) : IJPRPM

Generation of mini-Tn7 transposone by cloning of CPV-VP2 into eukaryotic expression cassette, in *E. coli*

Mohammad Sadegh Hashemzadeh[1], Seyed Jafar Mousavy[*2], Ruhollah Dorostkar[3], Fatemeh Fotouhi[4] and Firouz Ebrahimi[5]

[1]Ph.D Candidate in Nanobiotechnology, Department of Biology, Faculty of Basic Sciences, Imam Hossein University, Tehran, Iran
[2]Assistant Professor of Biochemistry (Ph.D), Department of Biology, Faculty of Basic Sciences, Imam Hossein University, Tehran, Iran
[3]Assistant Professor of Virology (Ph.D), Applied Virology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
[4]Associate Professor of Virology (Ph.D), Department of Influenza and other Respiratory viruses, Pasteur Institute of Iran, Tehran, Iran
[5]Assistant Professor of Clinical Biochemistry (Ph.D), Department of Biology, Faculty of Basic Sciences, Imam Hossein University, Tehran, Iran
[*]Corresponding E-mail: jmosavi@ihu.ac.ir

ABSTRACT

The VP2 protein of canine parvovirus (CPV)is the main part of capsid and attachment ligands for entry intospecific and cancerous cells through transferrin receptors (TfRs). Expression of VP2 alone results in assembly of a typically-sized virus like particle (VLP) in insect cells for therapeutic purposes. Towards this goal, the first step is to construct an expression cassette in pFastBac1 donor vector for transposition of VP2 into bacmid shuttle vector using Bac-to-Bac baculoviral system. So, in this research, we generated new recombinant pVP2FastBac1 enablingsite-specific transposition of VP2 into bacmid. The full-length of CPV-VP2 gene (1755 bp) was isolated by PCR amplification using specific primers and cloned firstly into RBC T/A cloning vector and then subcloned into the corresponding restriction sites of pFastBac1 donor plasmid vector. Then the accuracy of cloning process in these vectors was evaluated by PCR and enzymatic digestion analysis. Successful cloning of CPV-VP2 gene into eukaryotic expression cassette of pFastBac1 donor vector was confirmed by PCR and enzymatic digestion. In other words, mini-Tn7 transposone was generated successfully. In this study, the mini-Tn7 transposone containing CPV-VP2 gene was constructed. It is required for transposition of the interest gene into bacmid DNA in order to express it in insect cell.

Keywords: Canine parvovirus, VP2, expression cassette, pFastBac1

INTRODUCTION

Canine parvovirus (CPV) is a member of the *Parvovirus* genus in the family of *Parvoviridae*. This family are spherical, nonenveloped, T=1 icosahedral viruses that infect a wide range of natural hosts. CPV first appeared in the late 1970s and is a natural pathogen of dogs [1,2,3] and encapsidates a single-stranded DNA genome of approximately 5 kb. CPV particles have a diameter of 25 nm and are composed of three proteins, VP1, VP2, and VP3 [4]. VP2 is the major component of the viral capsid and contains 584 amino acid residues. About 90% of the

protein in the capsid is VP2, and 10% is VP1, which contains the entire VP2 sequence and 154 additional residues at its N terminus. In full (DNA-containing) capsids, some VP2 proteins can be converted to VP3 by proteolytic cleavage of approximately 12 to 15 amino acids from the N terminus. A wild-type capsid contains 60 subunits primarily of the VP2, along with a few VP1 and VP3 subunits [5-16]. The VP2 protein of CPVis the main part of attachment ligands for entry intospecific 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 [5-16].

As to date, the virus-like particles has been expressed in different hosts. One of the best systems, considered in production of VLPs, is the use of baculoviruses in insect cell expression system [19,20]. There are various methods for construction of recombinant baculoviral vectors. One method is the use of Bac-to-Bac baculovirus expression vector system (BEVS) with an efficient site-specific transposition mechanism to generate recombinant baculovirus. This system has two major components. The pFastBac donor plasmid vector into which the gene(s) of interest will be cloned and has an expression cassett. The second component is the baculovirus shuttle vector (bacmid) into which the expression cassett will be transposed via recombinant pFastBac, constructed [21]. In the present study, we tried to generate the first component of BEVS system, through the construction of mini-Tn7 transposone by cloning of CPV-VP2 into pFastBac1 donor plasmid vector.

MATERIALS AND METHODS

Bacterial Strains, Plasmids

The *E. coli* strain DH5α (Invitrogen, USA) was used for transformation and amplifying recombinant vectors (such as RBC T/A cloning vector and pFastBac1 donor plasmid vector). The VP2 gene was isolated from the recombinant construct of pET-21a. For cloning of VP2 gene, in order to change the restriction sites of flanking regions of the gene of interest, "T/A cloning vector" (RBC Bioscience, Taiwan) was used as the general vector and for subcloning of the gene of interest, pFastBac1 was used as the transfer vector (Invitrogen, USA).

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

Design and synthesis of specific primers

The VP2 region sequence of CPV genome was adapted from genbank after alignment of the nucleotide sequences of available CPV strains (obtained from NCBI database) and specific primers targeting this region were designed using the Allele ID software, version 7.0 (Premier Biosoft International, Palo Alto, CA, USA).The forward oligonucleotide primer for VP2 gene sequence was 5′-ATGAGTGATGGAGCAGTTCAAC-3′ as well as the reverse oligonucleotide primer for this region was 5′-TTAATATAATTTTCTAGGTGCTAGT-3′. None of the primer sequences showed genomic cross-reactivity with other viruses, human genome and other probable interfering genomes in a BLAST search analysis and only detected a 1755 bp fragment of CPV-VP region ORF (results not presented). The primers were synthesized by Bioneer Company (Korea).

Isolation and amplification of CPV-VP2 gene

In this study, the full-length of CPV-VP2 gene (1755 bp) was isolated by PCR amplification using specific primers and cloned firstly into RBC T/A cloning vector. Towards this goal, 1 µl of extracted recombinant plasmid (pET-21a) was added to a 50 µl total volume of PCR mixture containing 10 pmol of each forward and reverse primers (1 µlof each primer with the concentration of 10 µM or 10 pmol/µl), 5 mM MgSO4, 0.5 mMdNTPs, 2.5 unit of pfu DNA polymerase (Fermentas, Vilnius, Lithuania) and 5 µl of 10X PCR buffer.

Amplification reactions were performed in a thermocycler (Biorad, USA) under the following profile: 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. PCR products were analyzed by electrophoresis using 1% (w/v) agarose gel, stained with safe view (Kiagene, IRI).

Cloning of VP2 geneinto RBC T/A cloning vector

The PCR product was extracted from the low melting agarose gel by using a DNA extraction kit (Vivantis-Korea) and after one-step adenylation according to general protocols, subsequently cloned into RBC T/A cloning vector (RBC Bioscience, Taiwan). After the blue/ white screening of colonies, despite of ampicillin resistance marker existence, the accuracy of cloning process in this vector was evaluated by PCR and enzymatic digestion analysis (*Eco*RI/*Xba*I).

Determination of the gene orientation

In order to select proper restriction sites for subcloning of the gene of interest, it was important to determine the gene orientation after the cloning process. For this purpose, we used PCR technique by the universal M13 forward (Invitrogen, USA, Catalog no. N540-02) and specific forward and reverse primers. It is notable that the universal PUC/M13 flanking sites are located in RBC vector (RBC Bioscience, Taiwan). After the determination of gene orientation, we chose*Bam*HI/*Eco*RI restriction sites for subcloning of VP2 gene into pFastBac1 donor plasmid vector.

Subcloning of VP2 geneinto pFastBac1 donor plasmid vector

The VP2 fragment, digested using *Bam*HI/*Eco*RI restriction sites, was purified by low melting agarose gel using a DNA extraction kit (Vivantis-Korea). The purified product was ligated intopFastBac1 donor plasmid vector (Invitrogen-USA) that was digested using the same restriction sites. After transforming *E. coli* competent cells by ligated products and blue/ white screening of colonies, despite of gentamicin resistance marker existence, the presence of gene of interest in expression cossette of pFastBac1 donor vector was evaluated by PCR and enzymatic digestion. Triple digestion using *Eco*RV and *Hind*III enzymes and also double digestion using *Bam*HI and *Eco*RI were accomplished and the fragments produced were analyzed according toNEBcutter software pattern. The final confirmation was performed via sequencing (data not shown).

RESULTS

Isolation and amplification of CPV-VP2 geneby pfu DNA polymerase

Gel-based analysis of amplified VP2 fragment using the corresponding specific primers confirmed the expected 1755 bp amplicon using 1% (w/v) agarose gelelectrophoresis (Fig. 1).

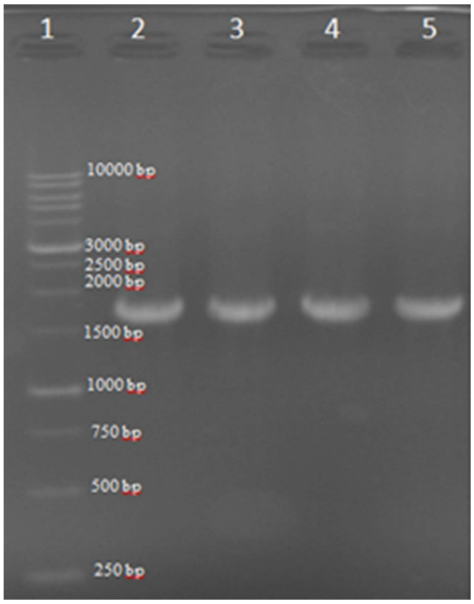

Fig. 1: Gel-based analysis of amplified VP2 fragment (1755 bp) using the corresponding specific primers, Lane 1: 1 Kb DNA size marker (YektaTajhizAzma, Iran) Lane 2-5: Demonstrating expected 1775 bp bond as the result of CPV-VP2 amplification by pfu DNA polymerase

Confirmation of cloning of VP2 geneinto RBC T/A cloning vector

The fragment produced, was cloned into RBC T/A cloning vector after extraction from low melting agarose gel and the accuracy of cloning process in this vector was confirmed using PCR (Fig. 2a) and enzymatic digestion analysis (Fig. 2b).

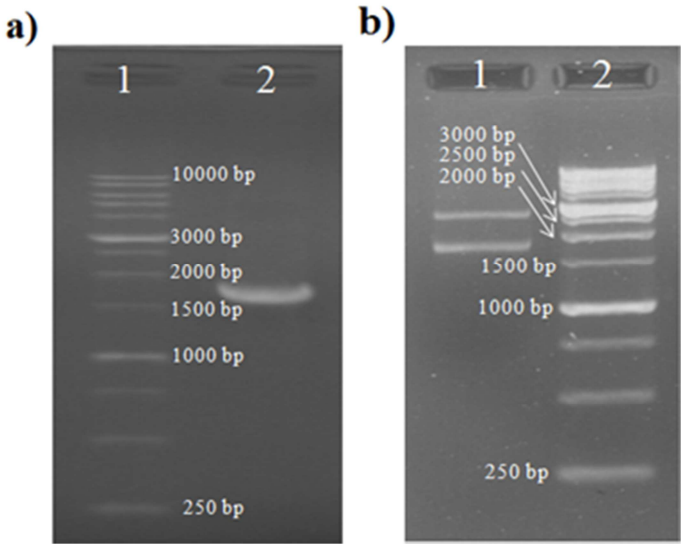

Fig. 2: a) Confirmation of cloning of VP2 gene into RBC T/A cloning vector by PCR. Lane 1: 1 Kb DNA size marker (YektaTajhizAzma, Iran). Lane 2: VP2 gene expected fragment (1775 bp). b) Enzymatic digestion analysis of VP2-containing recombinant vector (*Eco*RI/*Xba*I). Lane 1: revealed expected 1775 bp VP2 fragment and 2728 bp linearized vector. Lane 2: 1 Kb DNA size marker (YektaTajhizAzma, Iran)

Determination of VP2 orientation

VP2 orientation in the cloning vector determined by a PCR panel using the universal M13 forward and specific forward primers, and the universal M13 forward and specific reverse primers, respectively. The first PCR using the universal M13 forward and specific forward primers was positive (Fig. 3) and due to the orientation obtained, we chose*Bam*HI/*Eco*RI restriction sites for subcloning of VP2 gene into pFastBac1 donor plasmid vector.

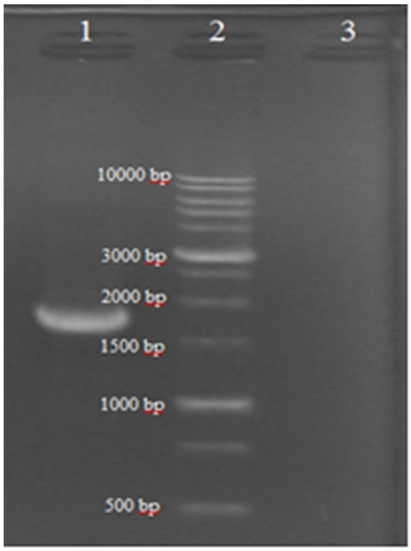

Fig. 3: A panel of PCR for determination of VP2 orientation in recombinant RBC vector. Lane 1: Positive PCR resulted, using the universal M13 and specific forward primers. Lane 2: 1 Kb DNA size marker (YektaTajhizAzma, Iran). Lane 3 : Negative PCR resulted, using the universal M13 forward and specific reverse primers

Confirmation of VP2 genesubcloning into pFastBac1 donor plasmid vector

The VP2 fragment, digested using *Bam*HI/*Eco*RI restriction sites(Fig. 4a), was purified and subcloned intopFastBac1 donor plasmid vector, as described previously in the methods section. Then, presence of the gene of interest in expression cossette of pFastBac1 donor vector was confirmed by enzymatic digestion (Figures 4b & 4c) and PCR (Fig. 4c). Triple digestion using *Eco*RV and *Hind*III enzymes and also double digestion using *Bam*HI and *Eco*RI were accomplished and the fragments produced were analyzed and confirmed according to NEBcutter software pattern. Finally, the accuracy of the VP2 gene ORF in recombinant pFastBac1 was confirmed by sequencing process and the analysis of sequencing results was accomplished by Chromas software, version 1.45 (data not shown).

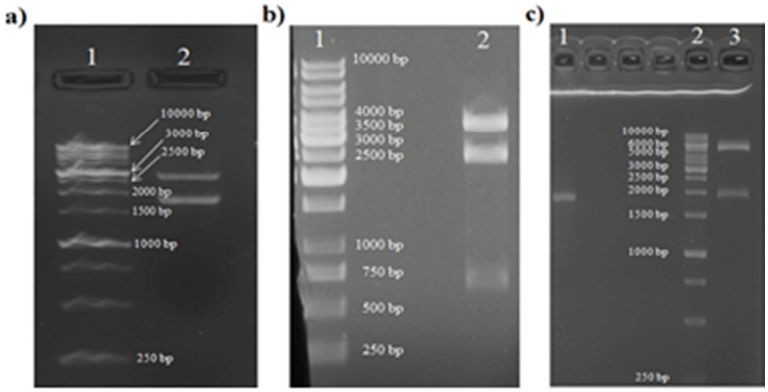

Fig. 4: a) Enzymatic digestion of recombinant RBC vector with determined VP2 orientation by *Bam*HI/*Eco*RI restriction enzymes. Lane 1: 1 Kb DNA size marker (YektaTajhizAzma, Iran).Lane 2: revealed expected 1775 bp VP2 fragment and ~ 2700 bp linearized vector. b) Enzymatic triple digestion of recombinant pFastBac1 vector (*Eco*RV/*Hind*III). Lane 1: GeneRuler 1 Kb DNA ladder (Fermentas, Vilnius, Lithuania). Lane 2: Expected ~ 600 bp, ~ 2500 bp and ~ 3500 bp fragments. c) PCR and Enzymatic double digestion of recombinant pFastBac1 vector (*Bam*HI/*Eco*RI). Lane 1: VP2 gene expected fragment (1775 bp) obtained from confirmatory PCR on recombinant pFastBac1 vector. Lane 2: GeneRuler 1 Kb DNA ladder (Fermentas, Vilnius, Lithuania). Lane 3: revealed expected 1775 bp VP2 fragment and ~ 5000 bp linearized vector

DISCUSSION

The Bac-to-Bac Baculovirus Expression System facilitates rapid and efficient generation of recombinant baculoviruses. Based on a method developed by Luckow et al., 1993, the Bac-to-Bac Baculovirus Expression System takes advantage of the site-specific transposition properties of the Tn7 transposon to simplify and enhance the process of generating recombinant bacmid DNA. The first major component of the system is a pFastBac vector into which the gene(s) of interest will be cloned. Depending on the pFastBac vector selected, expression of the gene(s) of interest is controlled by the Autographacalifornica multiple nuclear polyhedrosis virus (AcMNPV) polyhedrin (PH) or p10 promoter for high-level expression in insect cells. This expression cassette is flanked by the left and right arms of Tn7, and also contains a gentamicin resistance gene and an SV40 polyadenylation signal to form a mini Tn7 [21-23]. The second major component of the System is the DH10Bac *E. coli* strain that is used as the host for pFastBac vector. DH10Bac cells contain a baculovirus shuttle vector (bacmid) with a mini-attTn7 target site and a helper plasmid. After the generation of recombinant pFastBac construct, once the pFastBac expression plasmid is transformed into DH10Bac cells, transposition occurs between the mini-Tn7 element on the pFastBac vector and the mini-attTn7 target site on the bacmid to generate a recombinant bacmid. This transposition reaction occurs in the presence of transposition proteins supplied by the helper plasmid. Once the transposition reaction is performed, we can isolate the high molecular weight recombinant bacmid DNA and transfect the bacmid DNA into insect cells to generate a recombinant baculovirus that can be used for preliminary expression experiments. After the baculoviral stock is amplified and titered, this high-titer stock can be used to infect insect cells for large-scale expression of the recombinant protein of interest [21,24-30].

A number of pFastBac vectors are available for use with the Bac-to-Bac baculovirus expression system: 1), pFastBac1 that has strong AcMNPVpolyhedrin (PH) promoter for high level protein expression and large multiple cloning site for simplified cloning. 2), pFastBacHT with strong PH promoter for high-level protein expression and N-terminal 6xHis tag for purification of recombinant fusion proteins using metal-chelating resin and a TEV protease cleavage site for removal of the 6xHis tag following protein purification. Vector supplied in three reading frames for simplified Cloning. 3), pFastBac Dual with two strong baculovirus promoters (PH and p10) to allow simultaneous expression of two proteins and two large multiple cloning sites for simplified cloning. The pFastBac vectors and their corresponding expression control plasmids also contain the ampicillin resistance gene to allow for selection in *E. coli* using ampicillin [24-30].

CONCLUSION

In this study our aim was to construct a mini-Tn7 transposone containing CPV-VP2 gene and it was successfully accomplished. This construct is required for transposition of the gene of interest into bacmid DNA in order to express it in insect cell.

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

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