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Cloning, subcloning and recombination of VP2 gene of canine parvovirus into baculoviral shuttle vector enabling its expression in insect cells

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ABSTRACT

The VP2 protein of canine parvovirus (CPV) is the main part of capsid and attachment ligands for entry into specific 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. So, in this research our purpose was to construct a recombinant bacmid shuttle vector expressing VP2 of CPV using site-specific transposition mechanism in Bac-to-Bac baculovirus expression system. 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. Then the confirmed pVP2FastBac1 plasmid was transferred into E. coli DH_{10}Bac competent cells and the site-specific transposition of VP2 into bacmid shuttle vector was accomplished using the helper plasmid. Finally, the accuracy of transposition process was evaluated by a PCR panel using specific primers and PUC/M_{13} universal primers. Cloning, subcloning and recombination of VP2 gene of canine parvovirus into baculoviral shuttle vector were performed and confirmed successfully. In other words, VP2-containing recombinant bacmid was constructeed successfully. In this study, we used the Bac-to-Bac system for site-specific transposition of VP2 gene from pVP2FastBac1 to baculovirus derived bacmid shuttle vector. The constructed recombinant bacmid can express recombinant VP2 protein in insect cells.

Keywords: Canine parvovirus, VP2, expression cassette, pFastBac1, Bacmid

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INTRODUCTION

The *Parvoviridae* family consists of small, icosahedral, non-enveloped viruses that contain linear single-stranded DNA (ssDNA) genomes about 5000 nucleotides long. Canine parvovirus (CPV) belongs to *Parvovirus* genus of this family and first emerged in the late 1970s as the cause of a new disease in dogs and is now prevalent in dogs

worldwide [1,2,3]. CPV particles have a diameter of 25 nm and are composed of three proteins, viral protein-1 (VP1), viral protein-2 (VP2), and viral protein-3 (VP3) [4]. VP2 is the major component of the viral capsid. 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. The third protein, VP3, is produced after intracellular proteolytic cleavage, which removes approximately 25 amino acids from the N-terminus of VP2. A wild-type capsid contains 60 subunits primarily of the VP2, along with a few VP1 and VP3 subunits [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 aims. However, the importance of VP2 protein of canine parvovirus in binding to human cancer cells and production of veterinary detection kits for detecting the virus and also in vaccination, has motivated a lot of research on production of this protein [5-16].

One of the best systems, considered in production of recombinant proteins, is the use of baculoviruses in insect cell expression system. The recombinant baculovirus and insect cell expression system provides high levels of recombinant proteins that undergo post-translational modifications like glycosylation. Therefore, the application of such system allows large quantity production of a desirable protein, in the native conformation [19-22]. 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 [23]. In this study, we tried to generate the second component of BEVS system, through construction of a recombinant bacmid DNA encoding VP2 of canine parvovirus, using site-specific transposition mechanism. This construct can be used to produce large scale of VP2 protein in insect cell.

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). The *E. coli* strain DH10Bac (Invitrogen, USA) containing the baculovirus modified DNA (bacmid shuttle vector) with a mini-attTn7 target site and helper plasmid was used as an appropriate strain to perform the transposition process. The helper plasmid harbored by DH10Bac strains, confers resistance to tetracycline and encodes enzymes needed for transposition of the gene of interest into the bacmid.

Plasmid Extraction

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 µl of each primer with the concentration of 10 µM or 10 pmol/µl), 5 mM MgSO4, 0.5 mM dNTPs, 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 gene into 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 gene into 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 into pFastBac1 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 to NEBcutter software pattern. Finally, the accuracy of the VP2 gene ORF in recombinant pFastBac1 was evaluated and confirmed by sequencing process and the analysis of sequencing results was accomplished by Chromas software, version 1.45 (data not shown).

Construction of a recombinant bacmid

The VP2-containing recombinant pFastBac1 donor plasmid was transferred into the *E. coli* DH_{10}Bac competent cells. After the transformation process, incubation for 4-6 hours was accomplished for site-specific transposition of the VP2 expression cossette from the transposing vector into the bacmid shuttle vector, leading to *lacZ* gene disruption. The presence of helper plasmid is required in this process. The transformed cells were cultured on a LB agar plate containing kanamycin (50µg/ml), gentamicin (7µg/ml), tetracycline (10µg/ml), X-gal (100µg/ml) and isopropylthio-β-galactoside (IPTG, 40µg/ml) and incubated at 37ºC for 16h. The bacmid DNA was isolated from the overnight cultures by alkaline lysis purification method according to the general and current protocols.

It is notable that bacmid DNA is a high-molecular-weight plasmid (~ 135 kbp) and we must take care not to shear it. Over-drying, mechanically resuspension and storing the purified bacmid DNA at -20°C (as repeated freezing and thawing) may shear the DNA and gentle tapping of the bottom of the tube and storing the purified bacmid DNA at +4°C is recommended.

Analysis of recombinant bacmid DNA

The evaluation of VP2 gene existence in bacmid DNA is not performed as other plasmids. The enzymatic digestion analysis is not convenient and a PCR only by using specific primers is not sufficient. So, the transposition process accuracy and/or VP2 gene existence in bacmid DNA was evaluated by a PCR panel using both VP2 specific primers and PUC/M_{13} universal primers (Table 1). In fact, the PCR using specific primers shows the accuracy of DH_{10}Bac transformation by recombinant pFastBac1 and the PCR using PUC/M_{13} universal primers indicates the accuracy of

recombination through site-specific transposition mechanism. The evaluation of VP2 orientation in recombinant bacmid is also indicated by the PCR using both VP2 specific and PUC/M_{13} universal primers.

Table 1. Details of amplified region and primer sets used for PCR analysis of recombinant bacmid

Primer Pairs	Sequence (5' to 3')	Fragment Size (bp)	The Amplified Region
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

- All the Fragment Sizes are Expressed Approximately and Has Been Calculated According to the Sequence date of the Bacmid DNA

Polymerase chain reaction programs

PCR reaction was performed in a tube containing 5 µL of 10x PCR buffer, 1 µL of dNTP mix (0.2 mM for each), 1.5 µL of MgCl2 (1.5 mM), 1 µL of each primer with the concentration of 10 µM or 10 pmol/µl (10 pmol for each), 1-2 µL of template DNA, 1 unit of *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania) and water nuclease-free up to 50 µL final volume. Amplification reactions were performed in Biorad thermocycler (USA) and the PCR program included the following steps for specific primers: denaturation at 94ºC for 5 min, followed by 30 cycles of 94ºC for 45 sec (denaturation), 67ºC for 60 sec (annealing), 72ºC for 80 sec (extension), and a final extension at 72ºC for 10 min. For PUC/M_{13} universal primers the program included the following steps: denaturation at 94ºC for 5 min, followed by 30 cycles of 94ºC for 45 sec (denaturation), 63ºC for 45 sec (annealing), 72ºC for 300 sec (extension), and a final extension at 72ºC for 7 min. Finally, the PCR program included the following steps for VP2 specific and PUC/M_{13} universal primers: denaturation at 94ºC for 5 min, followed by 30 cycles of 94ºC for 45 sec (denaturation), 63ºC for 45 sec (annealing), 72ºC for 210 sec (extension), and a final extension at 72ºC for 10 min. PCR products were analyzed by electrophoresis using 1% (w/v) agarose gel, stained with safe view (Kiagene, IRI).

RESULTS

Isolation and amplification of CPV-VP2 gene by 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 gel electrophoresis (Fig. 1).

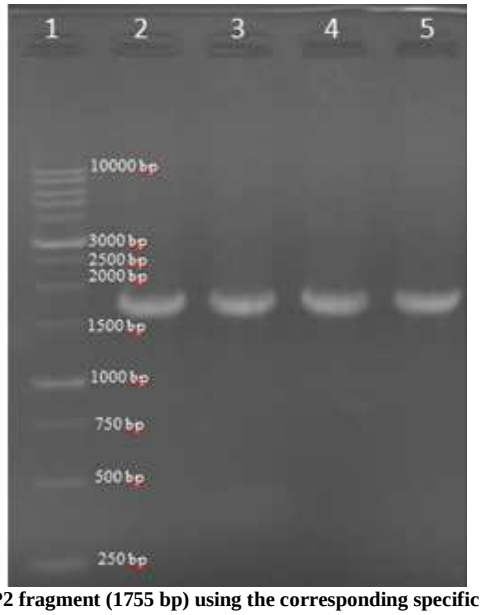

Fig. 1: Gel-based analysis of amplified VP2 fragment (1755 bp) using the corresponding specific primers, Lane 1: 1 Kb DNA size marker (Yekta Tajhiz Azma, Iran) Lane 2-5: Demonstrating expected 1775 bp bond as the result of CPV-VP2 amplification by pfu DNA polymerase

Confirmation of cloning of VP2 gene into 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).

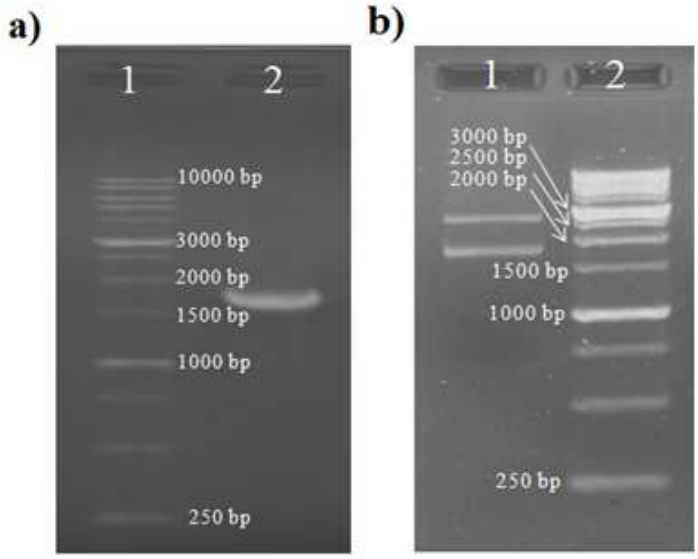

Fig. 2: a) Confirmation of cloning of VP2 gene into RBC T/A cloning vector by PCR. Lane 1: 1 Kb DNA size marker (Yekta Tajhiz Azma, Iran). Lane 2: VP2 gene expected fragment (1775 bp). b) Enzymatic digestion analysis of VP2-containing recombinant vector (*Eco*RI/*Xba*I). Lane 1: revealed expected 1775 bp VP2 fragment and 2728 bp linearized vector. Lane 2: 1 Kb DNA size marker (Yekta Tajhiz Azma, 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 (Yekta Tajhiz Azma, Iran). Lane 3 : Negative PCR resulted, using the universal M13 forward and specific reverse primers

Confirmation of VP2 gene subcloning into pFastBac1 donor plasmid vector

The VP2 fragment, digested using *Bam*HI/*Eco*RI restriction sites (Fig. 4a), was purified and subcloned into pFastBac1 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).

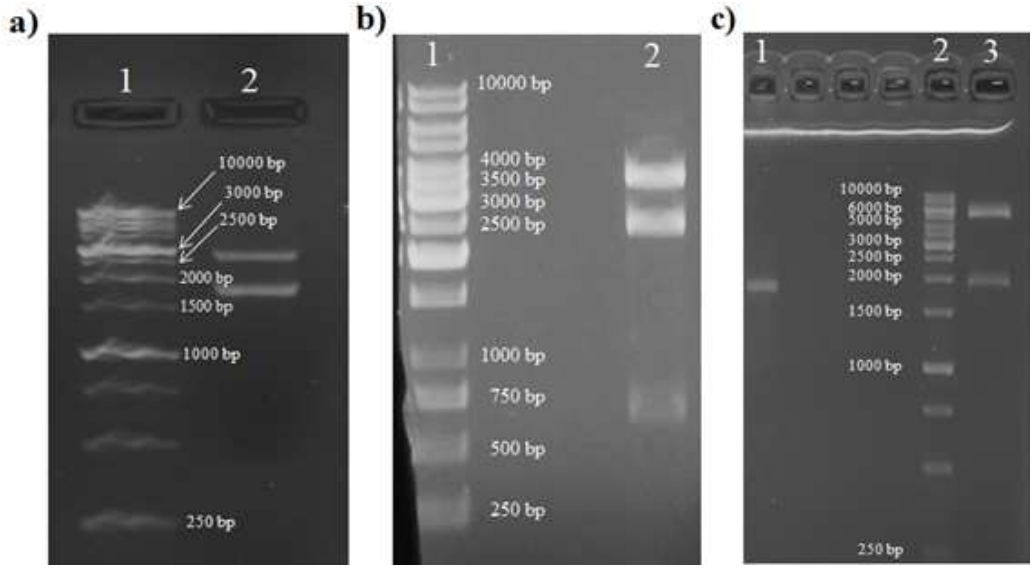

Fig. 4: a) Enzymatic digestion of recombinant RBC vector with determined VP2 orientation by *Bam*HI/*Eco*RI restriction enzymes. Lane 1: 1 Kb DNA size marker (Yekta Tajhiz Azma, Iran). Lane 2: revealed expected 1775 bp VP2 fragment and ~ 2700 bp linearized vector. b) Enzymatic triple digestion of recombinant 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

Analysis of recombinant bacmid DNA construct

After the verification of VP2 gene existence in expression cossette of pFastBac1, the transformation of *E. coli* DH_{10}Bac cells was accomplished successfully by VP2-containing recombinant pFastBac1 donor plasmid vector. Subsequently, the site-specific transposition of VP2 expression cossette from the transposing vector into the bacmid shuttle vector was performed with the presence of helper plasmid. After plating the cells on LB agar, the colonies containing recombinant bacmid were visible as large white colonies among the blue ones harboring the unaltered bacmids. The selected white colonies were restreaked onto a LB agar plate to ensure if they have true white phenotype. Since verification of the high molecular weight recombinant bacmid DNA is not convenient by digestion, at first a PCR using PUC/M_{13} universal primers was performed to ensure that recombination has been doen in selected colonies. All the white colonies showed a 4000 bp fragment, indicating the successful recombination in them. The results of amplification in non-recombinant bacmids of blue colonies (as the negative control) using M13/pUC primers showed a 300 bp fragment, indicating the lack of recombination performance (Figure 5).

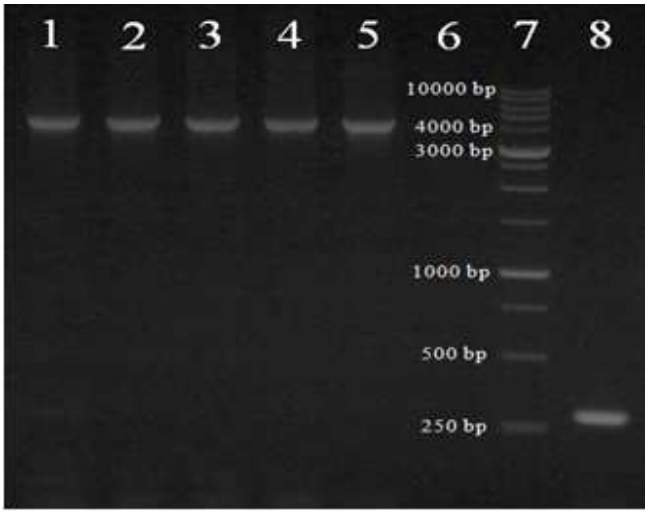

Fig. 5: Primary verification of recombination process through transposition mechanism by PCR, using PUC/M13 universal primers. Lanes 1-5: The expected fragment (~4000 bp) including Tn7 R + Polyhedrin promoter + VP2 gene + Tn7 L produced by PCR of recombinant bacmids extracted from white colonies. Lane 6: Negative control of PCR (without template). Lane 7: 1 kb DNA size marker (Yekta Tajhiz Azma, Iran). Lane 8: The expected fragment (~300 bp) produced by PCR of non-recombinant bacmid extracted from a blue colony as a negative control

In the next step, a PCR panel was performed using VP2 specific and PUC/M_{13} universal primers to ensure that proper transposition of the gene of interest has been doen in recombinant bacmids.

The bacmid DNA contains M_{13} forward and reverse priming sites, flanking the Tn7 mini-att site within the LacZ α-complementation region. The panel of PCR was done using PUC/M_{13} universal forward and reverse primers, VP2 gene specific forward and reverse primers, VP2 gene specific forward primer and PUC/M_{13} universal reverse primer and finally VP2 gene specific reverse primer and PUC/M_{13} universal forward primer, respectively. PCR of nonrecombinant bacmid extracted from a blue colony as the negative control generated an expected ~300 bp fragment using PUC/M_{13} universal forward and reverse primers (Figure 6).

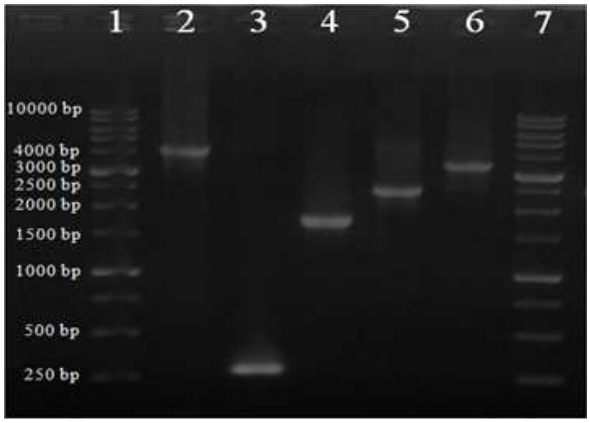

Fig. 6: The panel of PCR performed to confirm proper transposition of VP2 into the bacmid extracted from a white colony. Lanes 1and 7: 1 kb DNA size marker (Yekta Tajhiz Azma, Iran). Lane 2: The expected PCR product generated using PUC/M13 universal primers (~4000 bp) including Tn7 R + Polyhedrin promoter + VP2 gene + Tn7 L fragments. Lane 3: The expected PCR product generated using PUC/M13 universal primers (~300 bp) by the non-recombinant bacmid extracted from a blue colony as a negative control. Lane 4: The PCR product generated using VP2 gene specific forward and reverse primers (1755 bp) including VP2 gene fragment. Lane 5: The PCR product generated using VP2 gene specific forward and PUC/M13 universal reverse primers (~2500 bp) including VP2 gene + Tn7 L fragments. Lane 6: The PCR product generated using PUC/M13 universal forward and VP2 gene specific reverse primers (~3500 bp) including Tn7 R + Polyhedrin promoter + VP2 gene fragments

DISCUSSION

The expression of eukaryotic genes using baculovirus expression vectors takes advantages of their protein synthesis machinery and facilitates proper folding and post-translational modifications including glycosylation, acetylation, olygomerization and proteolysis. The Bac-to-Bac Baculovirus Expression System provides a rapid and efficient method to generate recombinant baculoviruses. This method was developed by researchers at Monsanto, and is based on site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in *E. coli*. 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 gene(s) of interest is controlled by the Autographa californica 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 a SV40 polyadenylation signal to form a mini Tn7 [23-25]. 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 transferred 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 [23,26-32].

Using the Bac-to-Bac Baculovirus Expression System to generate a recombinant baculovirus provides the following advantages over the traditional method using homologous recombination: a) Requires less than 2 weeks to identify and purify a recombinant baculovirus as compared to the 4-6 weeks required to generate a recombinant baculovirus using homologous recombination. b) Reduces the need for multiple rounds of plaque purification as the recombinant virus DNA isolated from selected colonies is not mixed with parental, non-recombinant virus. c) Permits rapid and simultaneous isolation of multiple recombinant baculoviruses, and is suited for the expression of protein variants for structure/function studies [23-25].

CONCLUSION

In this study our purpose was to construct a recombinant bacmid DNA encoding viral protein-2 (VP2) of canine parvovirus using site-specific transposition mechanism. This recombinant baculoviral verctor was constructed successfully under the control of polyhedrin promoter. We used the Bac-to-Bac system for site-specific transposition of VP2 gene from pVP2FastBac1 to baculovirus derived bacmid shuttle vector. The recombinant bacmid constructed here will transfect into the cultured Sf_9 (Spodoptera frugiperda) insect cell line to produce VP2 protein for therapeutic aims.

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