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Cloning and Expression of Coxsakievirus B3 Viral Protein-1 in *E. Coli*

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ABSTRACT

Background: Viral protein-1 (VP1) is a major capsid protein of Coxsakievirus B3 (CVB3) that plays an important role in directing viruses towards permissive cells and acts as a main antigenic site of the virus in eliciting of host immune response, hence it seems VP1 can be considered as a vaccine candidate against CVB3 infection. In this study, cDNA of VP1 was prepared, cloned into pET expression vector and the recombinant protein (VP1) was over expressed in *E. coli*. **Methods:** The viruses were grown in suspension cultures of Vero cells with an input virus multiplicity of 10-50 plaque-forming units/cell. After observing complete cytopathic effect, the total RNA (cells and virus) was prepared for RT-PCR and by using specific primers, VP1 cDNA was amplified and ligated into pET vectors (32 a and 28 a). The recombinant vector was transferred into competent *E. coli* (BL-21) and after selection of proper colony, which carried correct cDNA within the vector; cells were cultured and induced with isopropyl B-D-thiogalactopyranoside, in order to express protein (VP1). The cultures were tested for presence of VP1 by SDS-PAGE and Western-Blotting analysis. **Results:** Molecular techniques such as PCR which showed exact defined size of the VP1 (819 bp), restriction digestion and finally immunoblot analysis of over expressed protein; all confirmed the correct cloning and expression of VP1 in this research. **Conclusion:** In this research, full length of VP1 as major capsid protein of CVB3 was over expressed in *E. coli* which, can be used for further studies, including neutralizing antibody production against CVB3. *Iran. Biomed. J. 11 (3): 147-152, 2007*

Keywords: Coxsakievirus B3 (CVB3), Viral protein-1 (VP1), Entroviruses, Gene expression

I INTRODUCTION

Coxsakievirus B3 (CVB3), one of the six CVB serotypes, is a member of the genus Enterovirus within the family picornaviridae [1, 2]. The genome of CVB3, like that of other Entroviruses, is a single-stranded, sense, polyadenylated RNA molecule with 7400 nucleotides in length and a single open reading frame (ORF) , flanked by 5′ and 3′ non-translated regions. The full length protein encoded by this ORF, is subdivided into three regions: P1-P3, [3-5]. The capsids of Coxcakieviruses are composed of four structural proteins: Viral protein-1 (VP1), VP2, VP3, and VP4. VP1, VP2, and VP3 have no sequence homology, but all three proteins have the same topology [1, 6, 7]. The main structural differences among VP1, VP2, and VP3 lie in the loops that connect the β-strands and the N- and C-terminal sequences which extend from the β-barrel domain. These amino acid sequences give each picornavirus its distinct morphology and antigenicity. The C-termini are located on the surface of the virion, and the N-termini are on the interior, indicating that significant rearrangement of the P1 precursor occurs on proteolytic cleavage.VP1 is folded into eight-stranded anti-parallel β-sheets with a jelly-roll topology [8, 9]. The interaction of this protein as the main antigenic criterion with host receptor initiates structural changes which leads to penetration of the N-terminal end and lipophilic part of VP1 within cellular membrane and consequently leads to viral genome entry and host cell infection. Also, presence of several B-cell and T-cell epitopes

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within this protein facilitates the binding of antibody molecules to VP1, and hence VP1 can provoke the immune system of the body easily. Because, the major antigenic site of the virus surface is within VP1, it has been reported that neutralizing antibodies and anti-picornavirus drugs bind to the hydrophilic tunnel of VP1 and causes conformational changes which prohibit the attachment of these viruses to host cells and hence preventing the onset of infection [6, 10-12]. It has been reported that VP1 of foot-and-mouth disease (FMDV) [6, 13], poliovirus [14, 15] can elicit neutralizing antibodies and animals vaccinated with recombinant VP1 were protected from the virus infection when challenged. Since coxcakieviruses are appreciable human pathogens causing a wide spectrum of diseases, ranging from mild respiratory illness to sever myocarditis and neurological disorders [16-18] and VP1 plays critical role in onset of CVB3 infection. In order to develop vaccine against CVB3, the recombinant VP1 preparation is necessary. In this study, VP1 from CVB3 was cloned into pET expression vector and the protein was over expressed in bacteria.

MATERIALS AND METHODS

Materials. Materials were obtained from the following sources: Vero cells, National Cell Bank of Iran (NCBI C101); *Coxsakievirus B3 (CVB3)* Nancy strain from ATCC (American type tissue culture, USA) ATCCH VR-30; *E. coli BL-21(DE3)*, pET28a, pET32a vector from Novagen (Germany); RPMI, FCS, Agarose, Gibco (Scotland); Isopropanol chloroform, $CaCl_2$, Merck (Germany); Random Hexamer, Reverse Transcriptase, Tag, plaque-forming units (PFU) polymerase, *NcoI, HindIII*, T4 Ligase, Ampicillin, kanamycin, Roche Diagnostic (Germany); Miniperep kit (for purification of DNA), Qiagen (Germany) and Rainbow marker, Pharmacia (USA); LB, Scharlau (Spain).

Viral RNA preparation. RPMI 1640 medium supplemented with 10% FCS was used for growth and maintenance of Vero cell cultures. Cells at 80% confluency were infected with CVB3 in medium containing 1% FCS by 10^5 PFU. Infected cells were pelleted after 24 h by centrifugation at 1200 ×g for 10 min and total RNA was extracted by RNX solution.

DNA recombinant technology. Extraction of plasmid, digestion, isolation, ligation, transformation, identification, PCR and so on were performed as described in standard literature [19]. cDNA was synthesized by reverse transcriptase M-MULV enzyme (Roche Diagnostic manual). VP1 cDNA was amplified by PCR using set of forward and reverse primers. The forward primer contained *NcoI* restriction site and reverse primer had *HindIII* site after stop codon. The forward and reverse sequences of the primer were respectively, 5′ - TTG CCA TGG GCC CAG TGG AAG- 3′ and 5′ - TGT AAG CTT TTA TTG CCT AGT AGT GGT AAC TC- 3′.

Construction of expression vector. In this study, pET28a and pET32a were used as expression Vectors [20]. VP1 cDNA digested by *NcoI and HindIII* restriction enzymes and by T4 ligase, ligated to the same site in the vector to form pET-VP1.

Expression of recombinant VP1. Competent *E. coli BL21 (DE3)* cells were transformed with pET28a and pET32a expression vector harboring VP1 cDNA (pET-VP1). *E. coli* cells were grown in shaker flasks at 37ºC, in LB broth medium containing 50 µg/ml ampicillin (for pET28a) and 50 µg/ml kanamycin (for pET32a) until OD = 0.6. Then 1 mM of IPTG (isopropyl B-D-thiogalacto-pyranoside) was added to the medium, to induce VP1 expression. At zero, 1, 2 and 4 hours after induction, cells were centrifuged at 3200 ×g for 10 minute and used for further studies.

Production of rabbit antiserum against VP1. Immunization of rabbits was carried out as described [21]. Due to the lack of pure VP1, rabbits were injected by *Coxsakievirus B3* as a source of VP1 for antibody production.

Immunoblot analysis. For characterization of expressed VP1 protein, the cell pellet was resuspended in 4 ml lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 5 mM benzamidine and 0.5 mM PMSF) supplemented with protease inhibitor and lysozyme. The bacterial suspension was incubated at room temperature for 30 min for lysing of the cells. After the cells were completely lysed, 5 ng/ml deoxyribonuclease I was added to digest any associated DNA. This step reduces viscosity of the extract. After 10 min, the lysate was centrifuged at 26,000 ×g for 30 min to

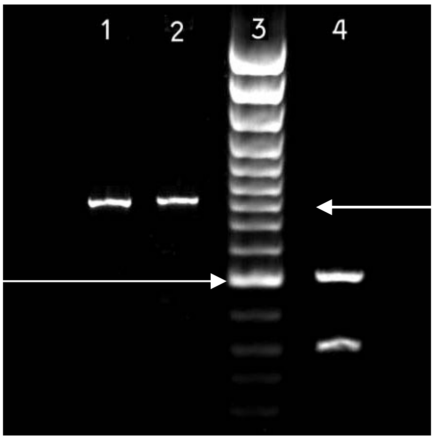

Fig. 1. PCR product of *coxcakiviruse* type B3 and Digestion with EcoRI. Lanes 1 and 2, CVB3 VP1 gene (819 bp); Lane 3, Puc18/Taq1 marker; lane 4, Digestion of PCR product with EcoRI.

remove the insoluble cell debris. Then, the extracts were electrophoresed (in SDS-12.5% poly-acrylamide gel) and transferred into a poly-vinylidene fluoride (PVDF) membrane (Roche Diagnostic, Germany). PVDF sheet was blocked with 3% BSA in TBS-T solution (20 mM Tris-HCl, pH 7.5, 150 mm NaCl, and 0.05% Tween 20). Then, anti-VP1 polyclonal antibody was added at a dilution of 1:500 in TBS-T solution for 1 h. A second incubation with HRP anti-rabbit Ig antiserum (1:2000) in TBS-T was carried out and the third incubation of 5-10 min was done with Diaminobenzine (DAB) solution. (0.5 mg/ml DAB, 0.1% H_2O_2).

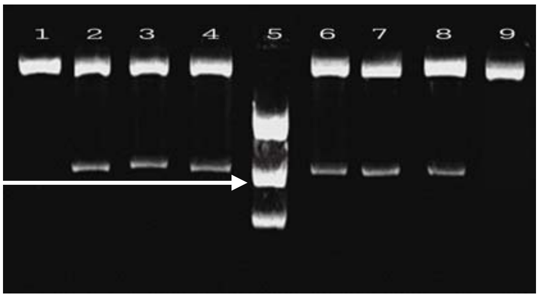

Fig. 2. Digestion products PET28a & PET32a with *HindIII* and *NcoI*. Lane 1, PET28a without insert; Lane 9, PET32a without insert; Lanes 2, 3 and 4, clones in PET28a; Lanes 6, 7 and 8, clones in PET32a; Lane 5, pUC18/Taq1 marker.

RESULTS

Molecular cloning of VP1. Total RNA was extracted from infected Vero cells and cDNA was synthesized by Reverse Transcriptase enzyme. Specific restriction sites, *HindIII* and *NcoI*, for unidirectional cloning and having stop codon were introduced into the amplified gene. *Pfu* DNA polymerase with proofreading activity was used to amplify cDNA (Fig. 1). Cutting the cDNA with EcoRI, has two fragments of 313 and 515 bp, correctly. The eluted cDNA was ligated into *HindIII*, *NcoI*-site of both *pET28a* and *pET32a* vectors. Restriction digestion and subsequent agarose gel electrophoresis of the clone indicated the presence of a 819-bp fragment (Fig. 2). To confirm the ligation, VP1 recombinant plasmid was digested with *NcoI* and *HindIII*. The recombinant plasmids were also confirmed by direct PCR (Fig. 3). DNA sequence analysis confirmed its correct orientation when compared to the sequence of VP1 (data not shown).

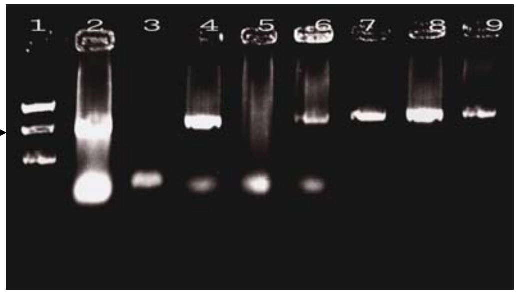

Fig. 3. Clones analysis with Direct PCR. Lane 1, pUC18/Taq1 marker; Lane 2, posetive control; Lane 3, negetive control; Lane 5, PET without insert; Lanes 4 and 6-9 clones.

Expression of VP1. *pET-VP1* recombinant vectors were transferred into *E. coli BL-21(DE3)* strain which grown on LB medium containing ampicillin at 37°C and induced to express VP1 using 1 mM-IPTG. 6xHis-tagged recombinant VP1 was found to be overproduced using IPTG as an inducer. A band corresponding to a 31-kDa 6xHis-tagged VP1 was observed in SDS-PAGE of total lysate of *E. coli* BL-21 (pET-VP1) culture after induction with IPTG (Fig. 4). The protein was confirmed by Western-Blotting analysis, using polyclonal antibody (Figs. 5 and 6). The 31 kDa protein induced in bacteria reacted with antibody prepared by injecting CVB3 into female rabbit.

DISCUSSION

Coxsakievirus B_3 (CVB_3), a member of the Picornavirus family, is a human pathogen [1]. This virus, is causative agent for at least 50% of acute myocarditis, and 25% of dilated cardiomyopathy

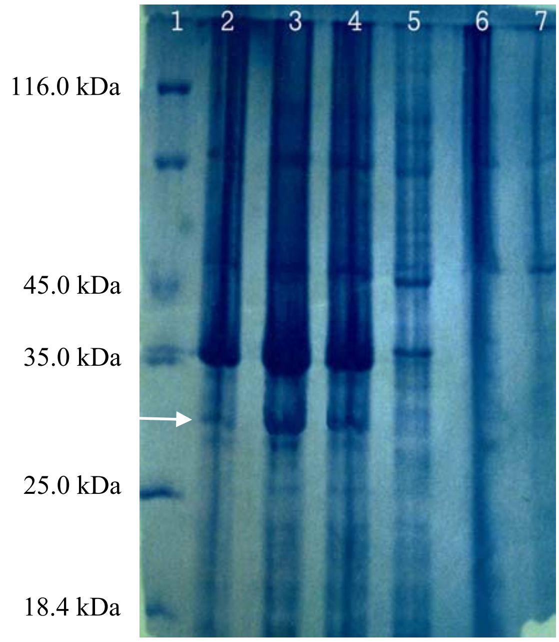

Fig. 4. SDS-PAGE analysis of total cell lysate of *E. coli* BL21 DE3 containing pET28a showing the expression of VP1 (Lanes 2-5). Lysate samples were prepared from 1 ml of cell suspension induced with IPTG. The cells were harvested and resuspended in 100 µl 2 × SDS gel loading buffer, heated to 100°C for 4 min, and loaded onto 10% polyacrylamide gel. Cell lysate of *E. coli* BL21 DE3 containing pET28a, Lane 2, 4 h; Lanes 3, 2 h; Lane, 4, 1 h; Lane 5, 0 h after induction with IPTG; Lanes 6 and 7, *E. coli* without plasmid; Lane 1, protein marker.

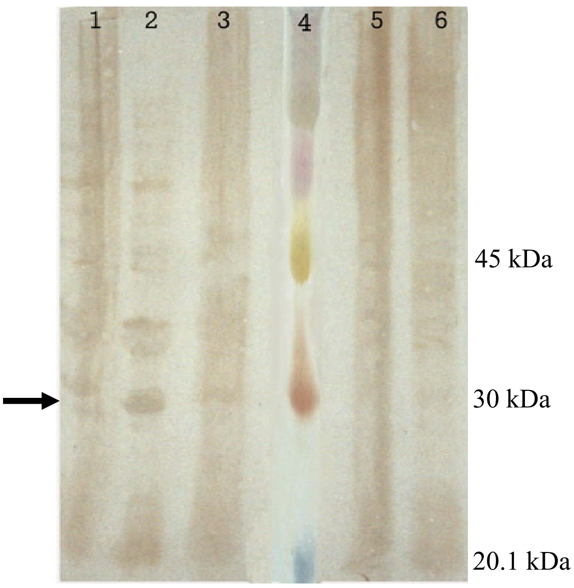

Fig. 5. Western-Blot of VP1 in PET28a. Cell lysate of *E. coli* BL21 DE3 containing pET28a. Lane 1, 4 h; Lane 2, 2 h; Lane 3, 1 h after induction with IPTG; Lanes 5 and 6, *E. coli* without plasmid; Lane 4, protein marker.

cases [22]. Despite the immense accumulation of molecular data, so far there are no virus specific preventive or therapeutic procedures available that protect human against heart diseases induced by Coxsakievirus.

Amongst CVB_3 proteins, VP1 is major capsid protein, and several B-and T-cell epitopes are located within this protein. VP1 plays important role in directing the virus for infecting the permissive cell [23] and inducing cell damage by cytopathic effect, apoptosis [24] and virus lytic cycle. It has been observed that expression of poliovirus recombinant VP1 in HeLa cells causes cell damage [18]. Several studies showed that mutations in specific sites of VP1, causes an attenuated phenotype [25-27], which can be considered as vaccine candidate.

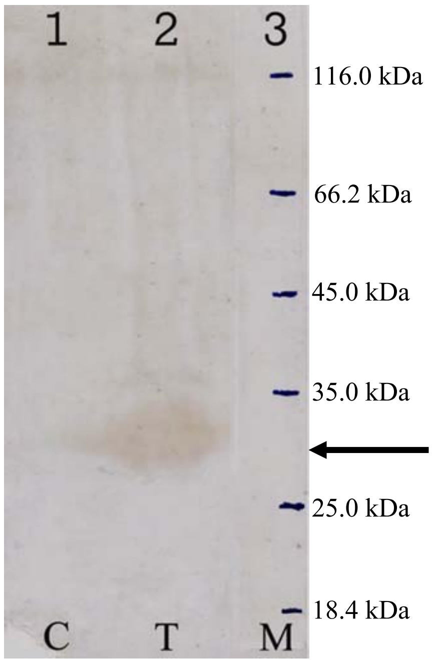

Fig. 6. Western-Bblot of VP1 inserted in PET28a. Lane 1, supernatant from *E. coli* clone without insert; Lane 2, VP1 protein without fusion; Lane 3, Protein molecular weight marker.

Protective effects of capsid proteins against viral challenge were studied by several groups and it was observed that VP1 and to some extent VP_3 are good candidates for DNA [28-31] and recombinant protein vaccines [15, 16] against entrovirus infections. Therefore, to study the preventive effect of VP1, in activating immune system against CVB3 infection, it seems necessary to prepare recombinant VP1. In this study, cDNA of VP1 gene prepared from CVB3, was first cloned in pET 32a, and then expressed as a fusion protein of 20 kDa. In the next step, the cDNA was digested from PET32a using restriction enzymes and subcloned in the some direction with same restriction site in PET28a where in this case there was no fusion protein along with VP1. SDS-PAGE analysis and Western-Blotting all confirmed the presence of full length VP1 in this study. Full length VP1 from poliovirus and FMDV have been cloned and expressed, but in this study we over expressed VP1 from CVB3. Since VP1 is one of the structural proteins of the CVB3 capsid, it is almost impossible to prepare pure VP1 by solubilizing CVB3 capsid and use for neutralizing antibody studies. In this study, full length of recombinant protein was obtained and confirmed by molecular techniques, and this protein can be used for future studies, particularly in eliciting the animal immuno response against CVB3 infection in order to prepare vaccine for CVB3, and also for better understanding the pathogenesis of CVB3 infection.

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