International Journal of Medical Laboratory 2017;4(2):142-154.

International Journal of Medical Laboratory
Shahid Sadoughi University of Medical Sciences

Original Article

Challenging for Expression Bovine Rotavirus (RF Strain) Full-Length VP7 Protein in Prokaryotic System

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Article history
Received 21 Apr 2017
Accepted 25 May 2017
Available online 28 Jun 2017

Key words
Expression system
Rotavirus
Toxicity
VP7

A B S T R A C T

Background and Aims: Rotavirus enteritis is an acute viral infectious disease among infants. VP7 protein has a key role in attachment and entry virus into the target cell. The VP7 protein is involved in inducing the production of neutralizing antibodies that protect infants against reinfection of the virus. The aim of this study was to heterologous expression of the VP7 gene of bovine rotavirus as a fusion protein of Trx–VP7 in a genetically engineered bacteria.

Materials and Methods: Total RNA was extracted from MA104 cells infected with bovine rotavirus strain RF. BRV VP7 gene was amplified using polymerase chain reaction. Another gene was the VP7 synthetic that made in the pBSK plasmid. The pBSK-VP7 plasmid was digested using BamHI and SacI restriction endonucleases, then recombined into the prokaryotic expression vector pET32a. The pET32a-VP7 amplified and pET32a-VP7 synthetic were transformed into BL21 (DE3) competent cells of *Escherichia coli*, respectively, and induced by different concentrations of Isopropyl β-D-1-thiogalactopyranoside at 30°C and 37°C in luria bertani and terrific broth media, then analyzed using SDS-PAGE and western blotting.

Results: SDS-PAGE analysis showed that the both pET32a-VP7 amplified and pET32a-VP7 synthetic were not expressed in the BL21 (DE3) cells, But the expression of pET32a-VP7 synthetic was weak at 30° C in luria bertani media.

Conclusion: This is the first report of the production of bovine rotavirus (RF strain) Full-Length VP7 in prokaryotic system expression. VP7 is a membrane protein and has toxic domains that show high toxicity in this expression system.

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Introduction

Rotavirus enteritis is an acute viral infectious disease among infants and young children and is caused by Rotavirus [1, 2]. Rotavirus is a double-stranded RNA virus belonging to the genus Rotavirus of the family Reoviridae [3]. Rotavirus is classified into six serologically distinct types, A to F that share cross-reacting antigens detectable by serological tests such as immunofluorescence, enzyme-linked immunosorbent assay, and immunoelectron-microscopy [4-6].The rotaviral genome of 11 segments of dsRNA is contained within virus core capsid. The total genome contains about 18522 base pairs [7]. The genome of group A viruses is composed of four high molecular weight dsRNA segments (segments 1 to 4), five middle-sized segments (segments 5 to 9) including a distinct triplet of segments (segments 7 to 9) and two smaller segments (segments 10 and 11) [8]. The rotavirus genome code for structural proteins found in the virion and for nonstructural particles found in infected cells but not in mature forms of viral particles [9]. The consensus is that the protein products (VP1 to VP4, VP5, VP6, VP7, VP8) of six of the genomic segments are structural proteins which found in the virion and nonstructural proteins are coded by the outer five genomic segments [10]. Comparisons of the sequence of individual segments of different rotaviruses of group 1 have shown that changes occur through genetic shift and drift, i.e. genome reassortment and sequence changes within segments [11]. Recent studies indicate that the antigenic composition of particles may be influenced by the interactions of specific combinations of the two outer capsid proteins, by trypsin treatment of particles or by oligosaccharide addition at different sites on the outer capsid glycoprotein [12-16]. VP1 is encoded by genome segment1 that makes up the rotavirus core particles [17]. VP2 is encoded by genome segment 2; it is the most abundant structural protein found in core particles [18, 19] and is the third most abundant protein in double-shelled particles [20]. VP2 is the only structural protein shown to possess nucleic acid (dsRNA, ssRNA, and dsDNA) binding activity when evaluated by RNA overlay protein blot assay [21]. VP3 encoded by genome segment 3 is a minor structural protein that may co-migrate with the outer capsid protein VP4 in many gel systems [19]. VP6 is encoded by genome segment 6 and is the major structural protein in virus particles located on the outer surface of single-shelled particles [22, 23]. These are the outer layer proteins VP4 and VP7 that these proteins have a key role in attachment and entry virus into the target cell [24]. VP7 (mainly encoded by gene segment 9) is a component important protein on the virus surface [9, 25] and glycosylated with molecular weight 37.5 kDa [26]. From this protein used in serotyping, hence among variant serotypes can be seen the difference in VP7 [27]. Both proteins VP4 and VP7 are involved in inducing the production of neutralizing antibodies that protect infants against reinfection of the same type virus [28]. So far, at least 27 G genotypes (based on the nucleotide sequence of VP7) and 37 P genotypes (based on the nucleotide

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sequence of VP4) have been identified in group A rotaviruses [29]. Because diversity in VP7 protein possible access antibodies does not exist against rotaviruses different types. The first recombinant protein of VP7 was developed in 1980 [30]. Up to now, structural proteins, especially VP4 and VP7 used to produce virus-like particles according to genetic recombination methods [31, 32]. Based on previous studies, recombinant VP7 protein protects the animals against rotavirus disease by induc passive immunity [33]. Because intestinal local immunity has an important role in limiting rotavirus infection, the subunit vaccine produced by recombinant proteins can not stimulate local immunity [34]. Most treatments for bovine rotavirus diarrheal disease is supportive and there is no effective specific treatment for this disease [35]. Therefore, VP7 is a dominant candidate by designing vaccines based on recombinant protein.

The aim of this study was to the expression of bovine rotavirus (RF strain) Full-Length VP7 in the prokaryotic system as a fusion protein of pET32a/VP7-TRX and pET32a in a genetically engineered bacteria. Since the VP7 is a species-specific protein, therefore every type need to produce a specific antibody that is required for different studies such as the production of reassortant viruses and selection of these viruses. Because there is no antibody available commercially, as a result, its antibody should be achieved through the production of recombinant proteins. The findings promise to use VP7 as a developing new recombinant protein.

Materials and Methods

Plasmids and *E.coli* strains

The pET32a (Novagen, Germany) plasmid was used for cloning and expression genes of interest under the control of the Lac operons. The *Escherichia coli (E.coli)* DH5a strain was used for maintenance and propagation of plasmid, *E.coli* DH5a strain is not used for expression protein, because it has protease that breaks down the recombinant protein, so *E.coli* BL21 (DE3) strain was applied for expression protein.

Bacterial culture media

Two bacterial culture media were used in the present study luria bertani (LB) medium (1% Yeast Extract, 0.5% Tryptone, 1% sodium chloride), terrific broth (TB) medium (2/4% Yeast Extract, 1/2% Tryptone, 0.94% Potassium Phosphate, Dibasic, 0.22% Potassium Phosphate, Monobasic).

RNA Extraction cDNA synthesis and amplification

The genomic dsRNA was extracted from cultured BRV using Guanidine/phenol (SinaClon, Iran). Total RNA was extracted based on the kits instructions and was re-suspended in nuclease-free water and stored at -20°C. Rotavirus dsRNA was used as a template to synthesize cDNA copies from both viral strands. Reverse transcription was carried out using a mixture of 6µl dsRNA template and 1 µl of 100 µM of each primer. The reaction tube was heated at 97ºC for 5 min. and quickly cooled on ice, then reverse transcription mixture consisting of 4 µl of 5X incubation buffer, 2 µl of 2.5 mM dNTPs, and 1 µl reverse transcriptase was added in a

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total volume of 20 µl. The tubes were mixed and incubated at 42ºC for one hours in a thermocycler. Polymerase chain reaction (PCR) was carried out in total volume of 50 µl containing: 5 ul of cDNA synthesis reaction mixture, 5 µl of 10X PCR buffer, 1 µl of 10 µM each of primers, 1 ul of 10 mM dNTPs mix, 5 U/µl Taq DNA polymerase (SinaClone), PCR program was an initial denaturation at 94ºC 5 min.; 95ºC 30 sec, 55ºC 30 sec 72ºC one min. (35 cycles); Final extension 10 min. at 72ºC and final hold at 4ºC. Finally 5 µl of the PCR products were assayed on 2% agarose gel electrophoresis, bands were identified by DNA safe stain, The amplified PCR product and pET32a plasmid were digested by BamH1 and SacI enzymes and both of them were cleaned up. VP7 synthetic gene was pulled out from pBSK plasmid by enzymatic digestion of BamHI and SacI enzymes.

Cloning of amplified VP7 gene

The specific primers VP7 of strain RF bovine rotavirus were designed based on sequencing VP7 strain RF BRV in NCBI (Accession number No. KF729664). VP7 gene of strain RF bovine rotavirus was amplified by RT-PCR using gene-specific primers, the restriction enzymes of BamH1 and Sac1 were added at the 5' end of the forward primer and reverse primer as followes:

F: CA*GGATCC*ATTCTAATCTTCTTGACATCGA

R: CA*GAGCTC*CTACGTTCTCCCTTGGTCCT

We have chosen nucleotide position 73 to 774 of the full VP7 gene. This sequence produced 234 amino acids and is containing antigenic and neutralizing sites [1]. We have designed two constructions for cloning as follows:

Cloning of synthetic VP7 gene

A construction obtained from amplification VP7 fragment of strain RF bovine rotavirus and another bovine Rotavirus VP7 synthetic gene construction was made in pBSK plasmid by BIOMATIC Co. Germany. For the synthetic gene, we have it codon optimized. Codon optimization was compatible with *E.coli*.

The ligation and transformation of BRV VP7 into pET32a

PCR product of BRV VP7 amplified gene and VP7 synthetic gene were ligated into the pET32a vector and transformed into competent DH5α cells of *E.coli*. Positive recombinant pET32a/VP7 plasmids were confirmed by digested with both restriction enzymes BamHI and SacI. Afterward, the recombinant plasmids purified from positive clones which were sequenced by chain-termination sequencing.

Expressions and identification of recombinant pET32a/VP7 by SDS-PAGE and western blotting

The recombinant plasmids, pET32a/VP7 amplified and pET32a/VP7 synthetic were used for the transformation of competent E.coli strain BL21 (DE3) cells prepared using calcium chloride method. For the expression of VP7, a single colony from both plates were inoculated into two 5 ml LB mediums. After overnight incubation, 200 µl of the each culture was transferred to the flasks containing 15 ml fresh LB and TB medium with ampicillin.

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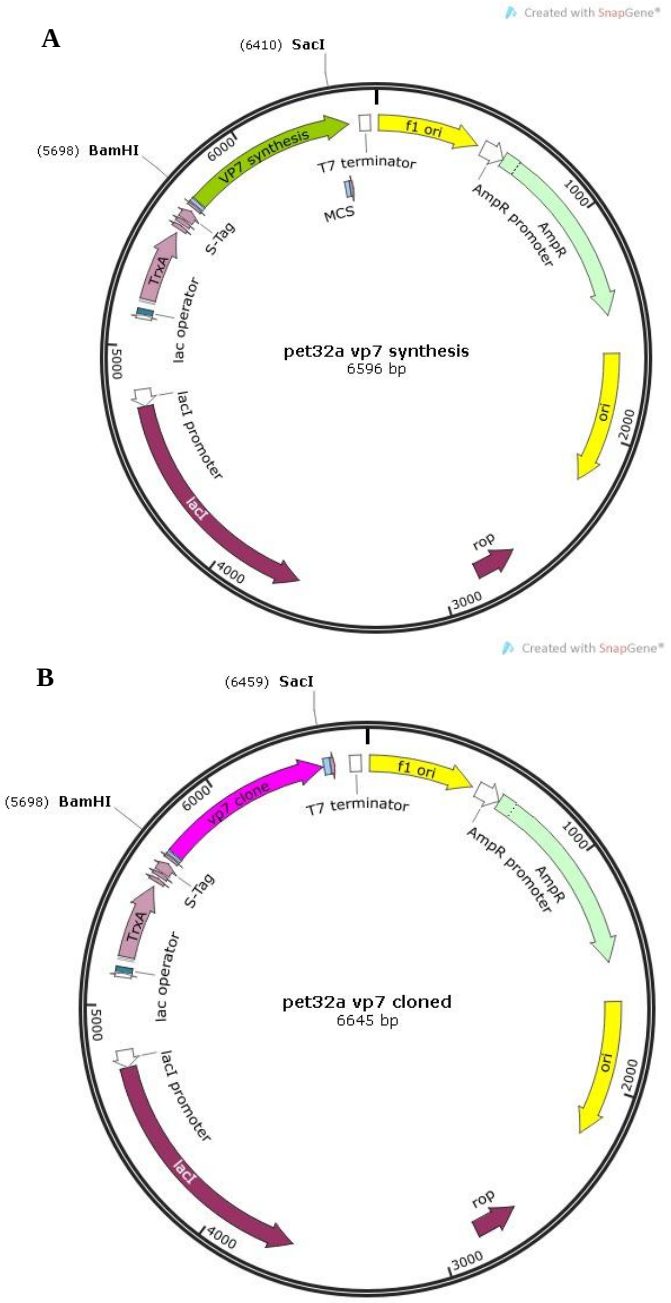


Fig. 1. A: Construction of the recombinant expression plasmid pET32a/VP7 Synthetic, B: Construction of the recombinant expression plasmid pET32a/VP7 amplified. VP7 gene was fused with TrxA gene in constructions

Additionally, 200 µl of the overnight culture of BL21/pET32a without recombinant gene was transferred to the flasks containing 15ml fresh LB and TB medium containing ampicillin. Cells were shaken at 230 rpm to a density of OD 600 0.5, then over-expression of recombinant protein was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to the final concentrations of 0.3, 0.5, 0.7 and 1 mM. The cultures were incubated at 37ºC for 6 h after of induction and each sample was collected. Pellets were washed in 100 µl of phosphate buffered

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saline (PBS) three times and 300 µl of sodium dodecyl sulfate (SDS) sample buffer was added and then samples were heated at 100°C for 5 min. 20 µl of each sample was electrophoresed on a 12% SDS-polyacrylamide gel. The same process as outlined above was utilized at 30ºC. After electrophoresis, according to make the proteins accessible to antibody screening, the protein was transferred (100 V, 1 h) onto a nitrocellulose paper and non-specific binding was blocked by 2.5% skim milk in (PBS) for overnight. Subsequently, anti-His antibody (1:2500 dilution in tris-buffered saline (TBS) was added and incubated at room temperature for 2 h. the paper was washed 3 times with TBST and according to the manufacturer's instructions 3,3'-Diaminobenzidine substrate kit (Abcam, UK) was used for the detection of VP7. This study was approved by Ethics Committee of Ahvaz Jundishapur university of medical sciences, Ahvaz, Iran.

Result

In this study by using PCR bovine rotavirus VP7 gene amplified (Fig. 1). PBSK/VP7 was digested by both BamH1 and Sac1 and VP7 gene synthetic was pull out from vector, The PCR product and synthetic gene sizes were, respectively, 750 bp and 714 bp (Fig. 2). The size was 750 bp and the synthetic gene was 714 bp and observed by agarose gel electrophoresis (Fig. 3).

Identification of the recombinant pET32a/VP7 cloned and pET32a/VP7 synthesis plasmids

The recombinant pET32a/VP7 amplified and pET32a/VP7 synthetic plasmids were digested by both restriction enzymes of BamH1 and Sac1. The results confirmed that the VP7 gene had been cloned into the pET32a vector. The cloned VP7 gene and synthesis VP7 introduced into pET32a vector and produced the recombinant pET32a/VP7 cloned and pET32a/VP7 synthesis plasmids (Fig. 4).

Analysis the recombinant pET32a- VP7 plasmids

The recombinant pET32a/VP7 amplified and pET32a/VP7 synthesis plasmids sequencing performed Takapouzist company (Iran) confirmed that both DNA encoding VP7 cloned and synthesis genes inserted in the correct position. Alignment of the sequence indicated that recombinant DNA was 100% homologous with bovine rotavirus VP7 gene.

Both pET32a/VP7 amplified and Synthesis plasmids were transformed into expression host *E.Coli* strains BL21 (DE3) cells. After IPTG induction with final concentrations of 0.3, 0.5, 0,7 and 1 mM for 6h both the recombinant glycoprotein VP7 amplified and synthesis were not express in the total lysates (Figs. 5, 7, 8) but pET32a/VP7 synthesis plasmid transformed in BL21 (DE3) with final concentration of 0,3 mM IPTG for 6h in LB was expressed very low (Fig. 6), VP7 was expressed very low at about 39 kDa on SDS–PAGE. Analysis the recombinant pET32a/VP7 amplified and pET32a/VP7 synthesis plasmids by SDS-PAGE technique showed that pET32a/VP7 synthesis was lowly expressed in BL21 (DE3) cells (Fig. 9).

Western-blotting

The VP7 reaction was determined by anti-His tag antibody. A weak band with an approximate size of 46 KD appeared on nitrocellulose membrane (Fig. 10).

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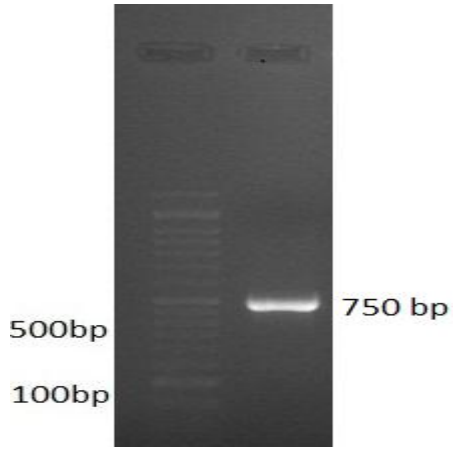


Fig. 2. PCR amplification products of BRV VP7gene with 750 bp size

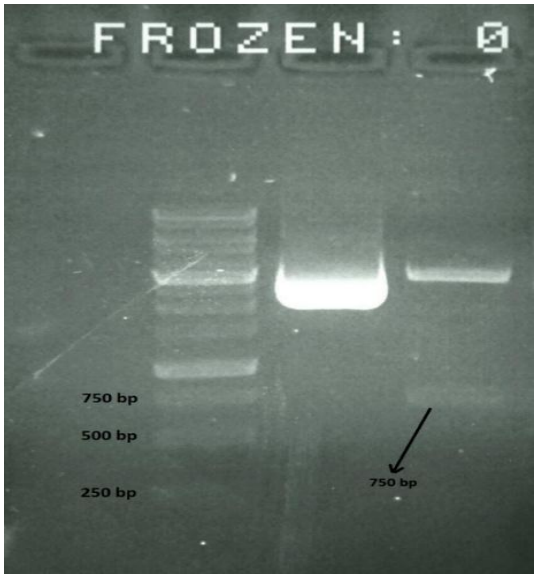


Fig. 3. Extracted VP7 gene synthetic from pBSK plasmid

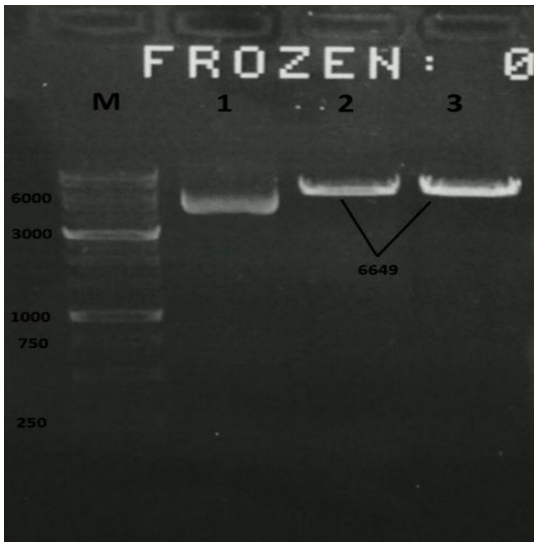


Fig. 4. Identification of the recombinant pET32a/VP7 amplified and pET32a/VP7 synthesis plasmids. Lane M: DNA ladder 1kb; Lane 1*:* pET-32a plasmid; Lane 2: pET32a/VP7 amplified; Lane 3: pET32a/VP7 synthetic.

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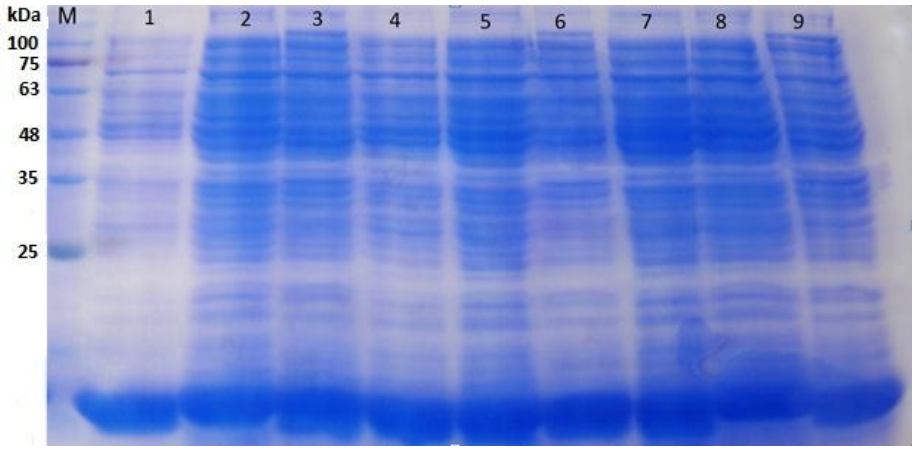


Fig. 5. Induction of expression pET32a/VP7 by different concentration of IPTG in *E.Coli* BL21 cells. Protein in the whole cell lysates was separated using SDS-PAGE. Line:M) protein marker, Lane:1) BL21-pET32a, Lane:2) BL21-pET32a/VP7 amplified induced with 0.3 mM IPTG in LB medium, Lane:3) BL21-pET32a/VP7 amplified induced with 0.5 mM IPTG in LB medium, Lane:4) BL21-pET32a/VP7 amplified induced with 0.7 mM IPTG in LB medium, Lane:5) BL21-pET32a/VP7 amplified induced with 1 mM IPTG in LB-medium, Lane:6) BL21-pET32a/VP7 amplified induced with 0.3 mM IPTG in TB medium, Lane:7) BL21-pET32a/VP7 amplified induced with 0.5 mM IPTG in TB medium, Lane:8) BL21-pET32a/VP7 amplified induced with 0.7 mM IPTG in TB medium, Lane:9) BL21-pET32a/VP7 amplified induced with 1 mM IPTG in TB medium. All samples were induced with a temperature of 30°C for 6 hours.

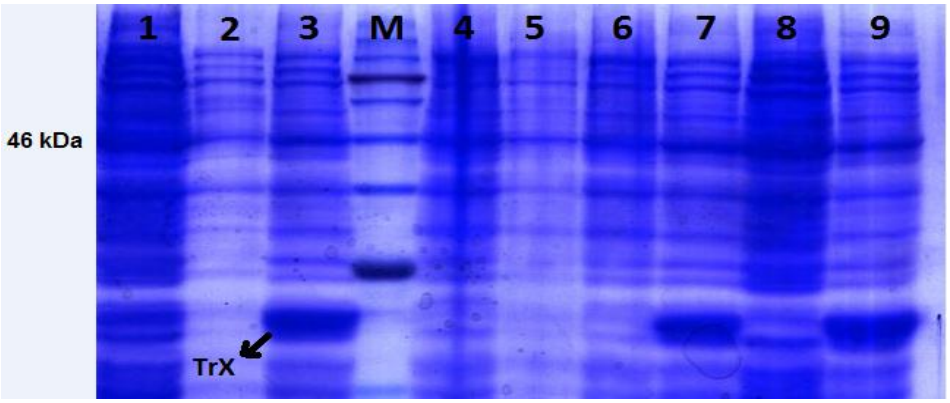


Fig. 6. Induction of expression pET32a/VP7 by different concentration of IPTG in *E.Coli* BL21 cells. Protein in the whole cell lysates was separated using SDS-PAGE. Line:M) protein marker, Lane:1) BL21-pET32a/VP7 synthetic induced with 0.3 mM IPTG in LB medium, Lane:2) BL21-pET32a/VP7 synthetic induced with 0.5 mM IPTG in LB medium, Lane:3) BL21-pET32a, Lane:4) BL21-pET32a/VP7 synthetic induced with 0.7 mM IPTG in LB medium, Lane:5) BL21-pET32a/VP7 synthetic induced with 1 mM IPTG in LB-medium, Lane:6) BL21-pET32a/VP7 synthetic induced with 0.3 mM IPTG in TB medium, Lane:7) BL21-pET32a/VP7 synthetic induced with 0.5 mM IPTG in TB medium, Lane:8) BL21-pET32a/VP7 synthetic induced with 0.7 mM IPTG in TB medium, Lane:9) BL21-pET32a/VP7 synthetic induced with 1 mM IPTG in TB medium. All samples were induced with a temperature of 30°C for 6 hours.

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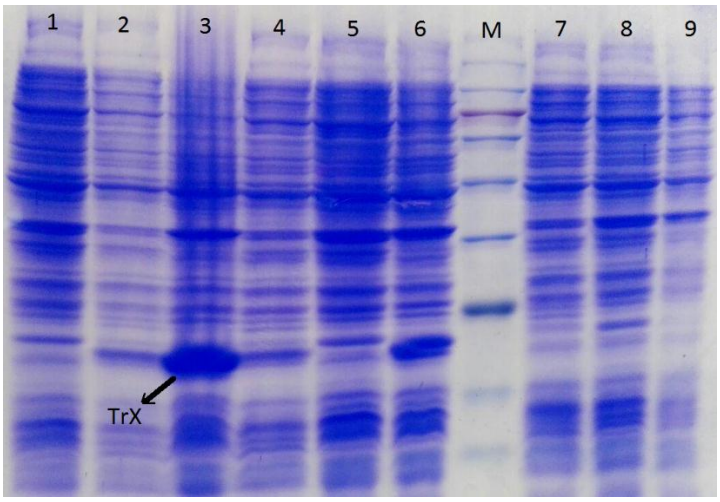


Fig. 7. Induction of expression pET32a/VP7 by different concentration of IPTG in *E.Coli* BL21 cells. Protein in the whole cell lysates was separated using SDS-PAGE. Line:M) protein marker, Lane:1) BL21-pET32a/VP7 amplified induced with 0.3 mM IPTG in LB medium, Lane:2) BL21-pET32a/VP7 amplified induced with 0.5 mM IPTG in LB medium, Lane:3) BL21-pET32a, Lane:4) BL21-pET32a/VP7 amplified induced with 0.7 mM IPTG in LB medium, Lane:5) BL21-pET32a/VP7 amplified induced with 1 mM IPTG in LB-medium, Lane:6) BL21-pET32a/VP7 amplified induced with 0.3 mM IPTG in TB medium, Lane:7) BL21-pET32a/VP7 amplified induced with 0.5 mM IPTG in TB medium, Lane:8) BL21-pET32a/VP7 synthetic induced with 0.7 mM IPTG in TB medium, Lane:9) BL21-pET32a/VP7 amplified induced with 1 mM IPTG in TB medium. All samples were induced with a temperature of 37°C for 6 hours.

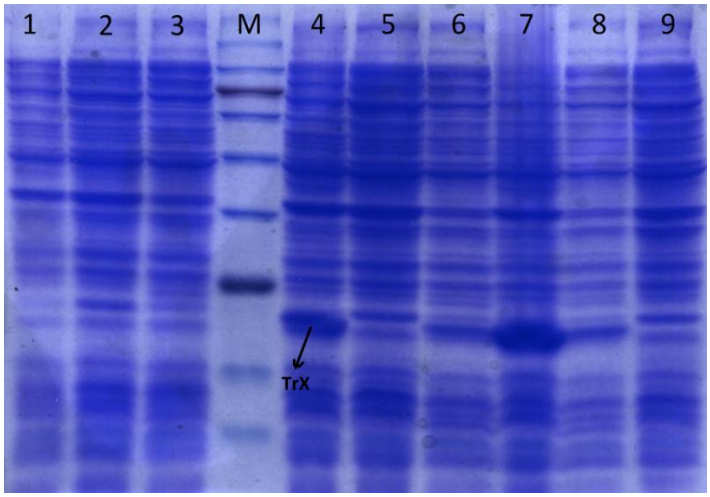


Fig. 8. Induction of expression pET32a/VP7 by different concentration of IPTG in *E.Coli* BL21 cells. Protein in the whole cell lysates was separated using SDS-PAGE. Line:M) protein marker, Lane:1) BL21-pET32a/VP7 synthetic induced with 0.3 mM IPTG in LB medium, Lane:2) BL21-pET32a/VP7 synthetic induced with 0.5 mM IPTG in LB medium, Lane:3) BL21-pET32a/VP7 synthetic induced with 0.7 mM IPTG in LB medium, Lane:4) BL21-pET32a, Lane:5) BL21-pET32a/VP7 synthetic induced with 1 mM IPTG in LB-medium, Lane:6) BL21-pET32a/VP7 synthetic induced with 0.3 mM IPTG in TB medium, Lane:7) BL21-pET32a/VP7 synthetic induced with 0.5 mM IPTG in TB medium, Lane:8) BL21-pET32a/VP7 synthetic induced with 0.7 mM IPTG in TB medium, Lane:9) BL21-pET32a/VP7 synthetic induced with 1 mM IPTG in TB medium. All samples were induced with a temperature of 37° C for 6 hours.

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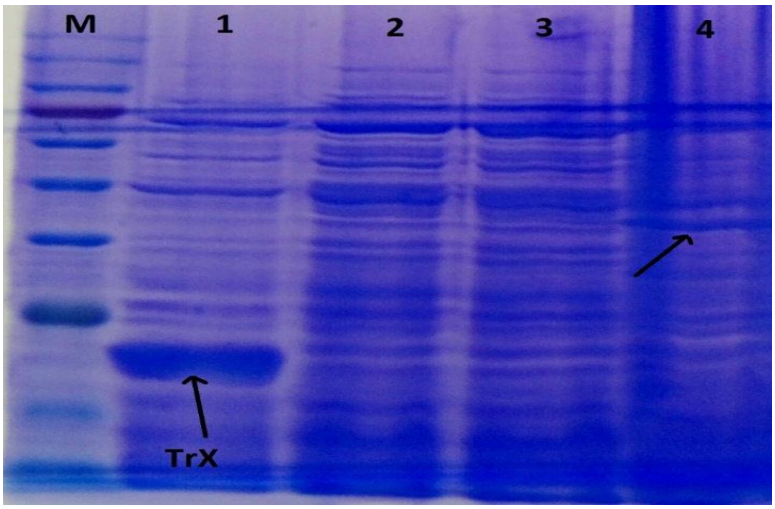


Fig. 9. Induction of expression pET32a/VP7 Synthetic in different times with IPTG in *E.Coli* BL21 cells. Protein in the whole cell lysates was separated using SDS-PAGE. Line:M) protein marker, Lane:1) BL21-pET32a, Lane:2) BL21-pET32a/VP7 synthetic induced with 0.3 mM IPTG in LB at temperature of 30° C for 2 hours, Lane:3) BL21-pET32a/VP7 synthetic induced with 0.3 mM IPTG in LB at temperature of 30° C for 4 hours., Lane:4) BL21-pET32a/VP7 synthetic induced with 0.3 mM IPTG in LB at temperature of 30° C for 6 hours.

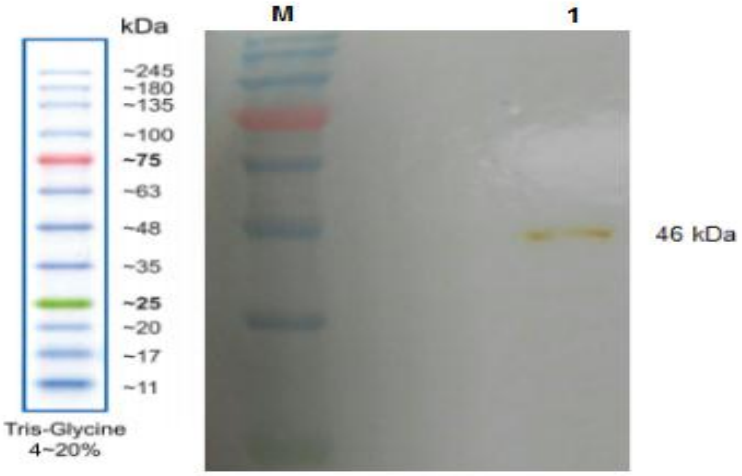


Fig. 10. Western blot analysis of the recombinant VP7 protein expression. Lane M, molecular weight marker; Lane 1, Recombinant VP7 protein with molecular weight 46 kDa.

Discussion

Rotaviruses are one of the important viruses that cause of gastroenteritis in infants and other young animals such as bovine [36]. There is required to type specific antibodies such as virus selection in reassortment, virus rescue and reverse genetics of rotavirus for virus selection in rotavirus studies. Because there is no antibody available commercially to type specific viruses, therefore, its antibody should be achieved through the production of recombinant proteins. Recombinant proteins in the bacterial system have many advantages in comparison with eukaryotic expression systems. Because of cheapness, easiness, and quickness, the prokaryotic system is suitable for recombinant protein expression. Eukaryotic

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systems have some problems such as weak secretion, long and costly methods [37]. Also eukaryotic systems which are able to process most of the post-translational modifications (disulfide bridges, N and O-glycosylation, phosphorylation) important for protein structure or function [38].

Eukaryotic and viruses genes are expressed weakly in prokaryotic cells rather than eukaryotic cells that can be because of prokaryotic prefer to use rare codons [39]. The VP7 protein is an integral membrane glycoprotein is located in the endoplasmic reticulum it seems this motif has a role in aggregation and toxicity [40].

We choose near full length of VP7 open reading frame from amino acid position of 9 to 258. Major type-specific neutralizing immunodominant epitopes has been shown that span this region [41].

E.coli BL21 (DE3) is one of the most common hosts for the expression of recombinant proteins that can be grown effectively in the simplest mediums [42-44]. In pET32a system, expression of target gene is under the control of strong bacteriophage T7 transcription, controlled by the Lac operons. Other effective features of pET32a plasmid can be noted such as it has a specific amino acid sequence of 6 histidines (6His.tag) located in '5 cloning sites, At last, it is added to the N-terminal. This sequence was used for purification of recombinant protein using affinity chromatography. [45].

The Thioredoxin (Trx) tag is used predominantly to improve the solubility and stability of proteins expressed in a bacterial cell, where it also assists in the refolding of proteins requiring a reducing environment. It also provides a useful epitope tag and has a molecular weight of approximately 12 kDa. The Trx is immediately downstream of the T7 promoter and ribosome binding site (rbs), followed by an internal His-tag S-tag and enterokinase sequences, and an open reading frame of VP7 was cloned downstream of it [46]. In this study different temperatures and concentrations of the inductor and two different cultures were examined, In these situations to observed interested protein, we checked inclusion bodies and supernatants phases. In this study, Trx tag was only used as a positive control in pET32a vector that expression of Trx 19 kDa.

Trx expression was stimulated with different concentrations of inducer at 30°C as well, but Trx-VP7 synthetic fusion protein was not observed in 46 kDa region. To confirm expression or absence of expression of interested protein western blot analysis was used. The interesting and important case was that Trx protein was expressed alone in test samples containing plasmid Trx-VP7. This case was observed several times during the protein expression. To confirm that there is no intact pET32a plasmid in the bacterial host, We have extracted plasmid from expression strain and recombinant plasmid were confirmed. We also sequenced recombinant plasmid three times, also we confirm the correctness of the inframe Trx-VP7. More interesting than other, it was that at other temperature conditions we've seen it again.

Suocheng et al. reported that bacterial growth at 37°C makes some proteins accumulate in the form of inclusion bodies. While growth at 30°C leads to activation of protein solution [13]. So in this study, two temperature 37°C and 30°C

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were used for protein expression. In this study the expression of VP7 using the expression system pET (pET32a) in BL-21 (DE3) strain at 30ºC in LB with 0.3 mM IPTG as an inducer for 6 hours.

Conclusions

Low expression levels or no expression at all can also be caused by toxicity of the target protein. VP7 is a membrane protein and has hydrophilic domains, The expression of hydrophobic heterologous proteins (usually membrane proteins) is often toxic to the bacterial host.

Conflict of Interest

The authors have declared no conflict of interest.

Acknowledgements

The results described in this paper were part of a grant with No: 93139, which was supported by Health Research Institute, Infectious and Tropical Diseases Research Center, Ahvaz Jundishapur University of Medical Sciences.

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