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Expression of Bovine Leukemia Virus Tax Protein in Bacterial Cell

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Abstract: In order to cloning of the coding region of Tax gene of Bovine Leukosis Virus (BLV), PCR product of the open reading frame of the gene from BLV-FLK cell line and the buffy coat of infected cow were amplified by PCR. A 927 bp PCR product of the Tax gene with XhoI, BamHI restriction sites were subcloned of pCR 4-TOPO and digested by the mentioned endonucleases. Digested insert cloned in to pET-32(a) and transfected in *E.coli* cells. For the expression of Tax protein, the pET-32(a) recombinant vector was transformed and then induced in BL21 (DE3) strain of *E.coli* competent cells using IPTG, the presence of Tax expressed protein was shown in immunoblotting and SDS-PAGE system. With considering the significant prevalence of infection with BLV in Iran and the need for controlling the infection or disease through vaccination, the application of Tax recombinant protein for vaccine production is of great goals of this study in near future.

Key words: pET-32(a) vector, protein expression, immunoblotting, SDS-PAGE, *E. coli*

INTRODUCTION

Bovine Leukemia Virus (BLV) and Human T-cell Leukemia Virus type1 (HTLV-1) are members of the *Deltaretrovirus* genus in the *Retroviridae* family (Uchiyama, 1997; Blattner, 1999; Twizere *et al*., 2000; Willems *et al*., 2000; Yoshida, 2001). These viruses infect either T or B lymphocytes and lead to hematologic or neurologic disorders (Johnson *et al*., 2001; Nagai and Jacobson, 2001). In addition to the structural genes required for the synthesis of the viral particle (gag, pol and env), these viruses also contain a region x located and the 3′ end of their genome. This region encodes a series of proteins involved in the regulation of viral expression (Tax, rex, r3 and g4 for blv virus) (Jensen *et al*., 1991; Twizere *et al*., 2000). Among these, the Tax protein is a 34-38 kDa transcription activator, which increases the synthesis of all viral mRNAs (Willems *et al*., 1987; Hollsberg, 1999; Twizere *et al*., 2000). Transactivation by Tax requires 21 bp imperfect repeats located in the 5' Long Terminal Repeat (LTR). In fact, Tax does not bind directly to DNA but interacts with the CREB/ATF cellular proteins and increases their affinity for the 21 bp enhancer elements (Adam *et al*., 1994; Kettmann *et al*., 1994; Boros *et al*., 1995; Adam *et al*., 1996). Although, some limited variation might be compatible with function, Tax is an essential gene that is absolutely required for infectivity *in vivo* (Willems *et al*., 1993; Twizere *et al*., 2000).

Viral transactivator protein, Tax, is being considered the most potential vaccine candidates in human retrovirus infection, Human T-cell Leukemia Virus type 1 (HTLV-1), because this molecule can induce protective immunity (Kannagi *et al*., 1992) and protective effects of vaccination with bovine leukemia virus Tax DNA in sheep showed by Usui *et al*. (2003).

In this study, the Tax gene of BLV from Iranian isolated virus was cloned and expressed as thioredoxin 6xHis-Tax fusion protein in *E. coli*. The preparing recombinant protein will be applied in near future for designing vaccine for control of infectious in Iran.

MATERIALS AND METHODS

This study was conducted from April 2007 to February 2008 in Faculty of Veterinary Medicine Islamic Azad University of Shahrekord.

Sample, plasmids and bacterial strains: The extracted DNA from buffy coat of one of the BLV infected cows, which had previously shown positive molecular and serological results based on PCR and ELISA was selected to be cloned (Momtaz and Hemmatzadeh, 2003; Momtaz *et al*., 2008). Plasmid pCR 4-TOPO(TOPO T/A Cloning kit, Invitrogen) and *E. coli* strain JM107 (Fermentas) were used for initial cloning, sequencing and maintenance of DNA fragment. For recombinant protein production, a prokaryotic expression vector pET-32(a) (Novagen) was used. This vector can express a fusion protein with the 109 aa Trx-Tag thioredoxin, a six histidin tag (6×His) and a T7 terminator sit. The recombinant pET-32(a) (pET-32-Tax) is transformed into*E. coli* BL21

(DE3) (Fermentas) as host strain. The required antibiotics were added to LB media according to the reference recommendation (Sambrook and Russell, 2001).

Primers design: Primers were designed according to the published sequence for Tax gene of BLV (accession number: AY700378.1). The forward primer, Tax F: 5- GTC GGA TCC GCA AGT GTT GTT GGT TGG GG -3 contain BamHI site. Reverse primer, Tax R:5- GAC CTC GAG TCA AAA AAG GCG GGA GAG CC -3 contain recognition site for XhoI. The restriction enzyme sites (underlined) were added to the primers for subsequent cloning procedure.

Gene amplification of Tax (encoding the Tax protein): PCR was performed in a 50 μL total volume containing 1 μg of template DNA, 2 μM of each primer, 2 mM $Mgcl_2$, 200 μM dNTP, 1×PCR buffer and 1 unit of Taq DNA polymerase (Sigma). The following conditions were used for amplification: Initial denaturation at 94°C for 4 min, followed by 33 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 50 sec. The program followed by a final extension at 72°C for 6 min. The PCR product was analyzed by electrophoresis in 1% agarose gel in 1×TBE buffer and visualized by ethidium bromide staining on UV transilluminator. The PCR product was purified by High pure PCR product purification kit (Roche applied science) according to the manufacturer recommendation.

Cloning of *Tax* gene: The 927 bp PCR product of Tax gene was digested by BamHI and XhoI restriction enzymes and ligated to pCR 4-TOPO and pET-32(a), which were digested by the same restriction enzymes, using T4 DNA ligase (Invitrogen) at 14°C over night. *E.coli* JM107 and *E. coli* BL21(DE3) competent cells were prepared by calcium chloride method and were used for transformation of pCR 4-TOPO-Tax and pET-32(a)-Tax vectors, respectively. The transformed bacteria were selected by screening the colonies on LB media containing antibiotic. The suspected colony was further analyzed by restriction enzyme digestion and PCR (Sambrook and Russell, 2001).

Expression and purification of recombinant Tax protein: *E.coli* BL21(DE3) was transformed with pET-32(a)-Tax and grown in LB broth supplemented with Ampicillin (100 mg mL^{-1}) at 37°C with agitation in order to optimize the expression condition, different concentrations of IPTG (0.5, 0.8, 1, 1.5 mM) at different bacterial growth rates (OD_{600} = 0.5, 0.7, 1) were tested for 3 h and analyzed on 17% SDS-PAGE. The expressed protein was purified using Ni-NTA column (Qiagene) according to manufacture instructions. Quantity of the purified recombinant Tax protein was analyzed by Bradford methods and subsequently it's quality was assayed by SDS-PAGE 15% (2.5 μg $well^{-1}$). In order to analyse, the cross-reaction between fused segment of Tax protein with infected sera, an *E.coli* BL21(DE3) containing pET-32 (a) a vector was induced by IPTG.

Immunoblot analysis: For western blot analysis, 0.5 μg of purified recombinant Tax protein was used per well. As a negative control, the bacterial lysate from induced *E. coli* BL21(DE3) contain pET-32(a) vector was analyzed by westen blot. The gel was blotted on to Polyvinylidine Difluoride (PVDF Membrane, Roche Diagnostics GmbH) membrane using transfer buffer containing 25 mM tris (pH = 8.3), 192 mM glycine and 20% methanol at 55 V for 1 h at 4°C. The blotted membr ane was blocked with 3% (w v^{-1}) BSA in TBST buffer (0.5 M NaCl, 0.02 M Tris pH = 8.5, 0.05% Tween 20) for 1 h at Room Temperature (RT). Membrane was incubated for 2 h at 37°C with BLV-infected cow serum, diluted 1:25, respectively. Negative serum from apparently health cow that had negative results in PCR and ELISA was used as control. After reaction the primery antibody, the blotted membranes were washed three times with TBST and incubated with peroxidase conjugated anti-bovine IgG (Sigma) at a 1:2500 dilution in TBST. The blots were then washed 3 times with TBST and reaction were developed by Diamino Benzidine (DAB) solution (Sigma) (Sambrook and Russell, 2001).

RESULTS

The recombinant plasmid (pCR 4-TOPO-Tax) was sequenced by specific primers and Sanger sequencing method (Macrogen, Korea). The sequencing result was confirmed by comparing with databases and using Basic Local Alignment Search Tool (BLAST) software.

Expression of pET-32(a)-Tax in *E.coli* BL21(DE3) induced and the expressed protein was purified by Ni-NTA column (Fig. 1). The result showed that the best conditions for recombinant Tax protein expression can be achieved when 1 mM of IPTG and OD_{600} = 0.7 for 3 h was used.

To determine the reactivity of recombinant protein Tax, the purified recombinant protein was assayed by western blotting method. The 5 infected cattle serum (which had previously shown positive serological result based on ELISA and AGID) were used. A negative serum from disinfected cattle used as a control. Figure 2 illustrated the specific interaction between positive sera and purified recombinant Tax protein. There was no reaction between the expressed pET-32(a) in *E. coli* BL21(DE3) and BLV infected sera (Lane 5 in Fig. 2).

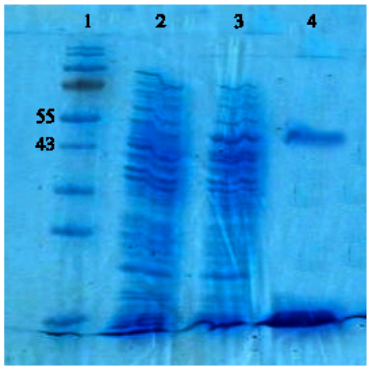

Fig. 1: Expression of recombinant tax protein and its purification (Lane 1, Protein marker, Lane 2, pET-32(a)-Tax befor induction; Lane 3, pET-32(a)-Tax after induction; Lane 4, purified tax recombinant protein)

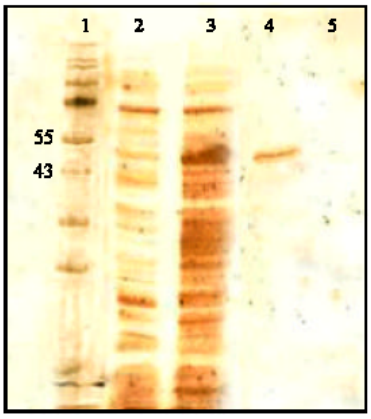

Fig. 2: Western blot analyses against recombinant tax protein by BLV-infected sera (Line 1, Protein marker; Lane 2, Western blotting pET-32(a)-Tax befor induction; Lane 3, Western blotting pET-32(a)-Tax after induction; Lane 4, Western blotting by infected sera after purification protein; Lane 5, Western blotting reaction between the expressed pET-32(a) and positive serum)

DISCUSSION

Evaluation of different aspects of BLV serodiagnosis methods is one of the objectives of control programs for BLV infection. Results of many studies having been conducted on Human Immune Deficiency Virus (HIV) have provided a widespread field for studying genetic variability, laboratory diagnosis, epidemiological studies and finally, appropriate strategies for preventing retrovirus infections. Among cattle retrovirus infections, the bovine enzootic leukosis virus is significant and hence, many widespread researches have been made on diagnosis, control and prevention methods for these diseases with using serologic and molecular biologic methods (Grover and Guillemain, 1992; Bunger *et al*., 1994; Bicka *et al*., 2001).

Among BLV structure genes, the ones coding for gag, pol and env genes are considered as major and relatively protected genes with a less gene variability and because of the same natural feature today molecular biological diagnostic methods have been designed based on PCR, cloning and sequencing based on detection of BLV provirus and/or the above mentioned structure genes. In addition to the mentioned 3 structure genes, complicated retroviruses also encode regulatory genes such as Tax, Rex, R III, C IV genes involving in virus replication, which increases the stability and strength of their pathogenesis (Jensen *et al*., 1991; Twizere *et al*., 2000).

Tax gene encodes a protein, which is necessary for virus replication and results in glands inflation. On one hand, this gene induces replication in several cellular genes such as IL-2 generating gene (Yoshida, 2001). Tax acts as a vector for α and β polymerase enzymes (Jeang *et al*., 1990) and recent studies reveal that it plays a role in oncogenesis of *Retroviruses*, particularly that of blood cell tumor (Twizere *et al*., 2000).

Tax protein has several identifying epitope for cytotoxic T-lymphocytes and it can induce response in these cells (Kannagi *et al*., 1992; Usui *et al*., 2003). Because of the significance of this gene in retroviruses, today applying Tax gene in designation of DNA vaccines particularly in human infections such as disease caused by HTLV-1 have been considered and it was recognized that vaccination against infection due to HTLV-1 with using Tax recombinant protein can induce a strong response by cytotoxic T lymphocytes (Usui *et al*., 2003).

The present study is conducted with the goal of detection and cloning of Tax gene and in near future the recombinant vector of this gene will be used for designation of bovine leukosis vaccine. One of the main goals of this study, i.e., Tax gene detection in BLV virus, in samples infected with the said virus proved to be true in Iran. The presence of this gene was confirmed thanks to nucleotide sequencing of the relevant fragmen.

Since, the primers having been used for diagnosis and detection of Tax gene in this study include most part of open reading frame of the said gene, therefore, at the beginning, the primers were designed for cloning and expression of Tax gene to enable cloning for the amplified fragment in different vectors such as cloning vectors and expressing vectors.

The second goal of this study was the cloning of Tax gene in each pCR4-TOPO cloning vector and pET-32(a) expressing vector and this gene cloning was performed in cloning vector after nucleotide sequencing and a comparison made on the resulting gene sequence with other recognized Tax gene sequences available in Genebank revealed the success of this gene cloning in relevant vector. Such a vector is able to be propagated in competent bacteria and also, because of the presence of multiple enzymatic restricting sites, it is able to splice Tax gene fragment as well as to purify and insert it in expressing vectors and this finding was also obtained through cloning for Tax protein coding gene belongs to BLV virus in pET-32(a) expressing vector for the 1st time in Iran and the presence of expressing protein was confirmed through SDS-PAGE and immunoblotting system.

With considering the significant distribution of infection with BLV in Iran and the need for controlling the infection or disease through vaccination, the application of Tax recombinant protein for vaccine production is of great goals of this study in near future. Since the gene fragment amplified in PCR include all domains corresponding to Tax protein and is located in open reading frame based on initial designation of primers and also it has been successfully cloned and expressed in pET-32(a) expressing vector, therefore, preparing Tax recombinant protein can become an accessible goal in near future.

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