

*Full Length Research Paper*

## Expression of infectious bovine rhinotracheitis virus glycoprotein D in bacterial cell

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**Bovine Herpesvirus 1 (BHV-1) belongs to the genus of Varicellovirus and the family of Herpesviridae which contains three main gB, gC and gD genes. In order to cloning of the coding region of gD gene of IBR virus, PCR product of the open reading frame of the gene from IBR virus isolated in Iran was amplified by PCR. A 1047bp PCR product of the gD gene with EcoRI, HindIII restriction sites were subcloned of pTZ57R/T and digested by the mentioned endonucleases. Digested insert cloned in to pET-32a and transferred in E.coli cells. For the expression of gD protein, the pET-32a recombinant vector was transformed and then induced in BL21 (DE3) strain of E.coli competent cells using IPTG. The presence of gD expressed protein was shown in immunoblotting and SDS-PAGE system. With respect to the remarkable frequency of infection to IBR in Iran and the necessity of controlling it through vaccination with recombinant vaccines of thymidine kinase, manufacturing and applying the recombinant gD protein are vital goals in recognition and distinction between infection and responses caused by vaccine.**

**Key words:** IBR virus, gD protein, pET-32a vector, protein expression, SDS-PAGE, immunoblotting.

### INTRODUCTION

Bovine herpesvirus 1 (BHV-1), a member of the *Alphaherpesvirinae* subfamily (Meurens et al., 2004), classified in the list B of the Office International des Epizooties (Winkler et al., 2000), is an important viral pathogen of cattle that causes two major disease syndromes: infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis (Chase et al., 1990; Taylor et al., 1998; Meurens

et al., 2004). Secondary bacterial infections resulting in bronchopneumonia and death are common (Winkler et al., 2000). Although IBR was thought to have been eradicated in several European countries, it still causes economic losses to the European and the U.S. beef industries (Meurens et al., 2004). In the nations where BHV-1 has not been eradicated, control and eradication programs are associated with vaccination strategy with live attenuated or inactivated vaccines (Taylor et al., 1998).

The glycoprotein C, D, E, G and I, in *UL49h* and thymidine kinase proteins are involved in viral virulence and are useful targets for diagnosis, prevention or antiviral treatment (Smith et al., 1994; Van Engelenburg et al., 1994; Young and Smith, 1995; Van Oirschot et al., 1996; Schwyzer and Ackermann, 1996; Liang et al., 1996; Liang et al., 1997; Kaashoek et al., 1998). Most herpesvirus glycoproteins are present in the viral envelope and are important for virus-host interactions and are also necessary

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**Abbreviations:** BHV-1, Bovine herpesvirus 1; IBR, infectious bovine rhinotracheitis; gD, glycoprotein D; IPTG, isopropyl β-D-1-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DMSO, dimethylsulfoxide; PVDF, polyvinylidene difluoride; DAB, diaminobenzidine; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; TBST, tris buffer saline Tween 20.

for a productive BHV-1 infection of bovine cells (Chase et al., 1990). However, *gB*, *gC* and *gD* have been known as the major BHV-1 envelope glycoproteins (Winkler et al., 2000; Meurens et al., 2004). The lack of information on isolated BHV-1 in Iran seems obvious.

The aim of this study is to clone and express BHV-1 *gD* gene in order to obtain glycoprotein B of the isolated virus in Iran. The prepared recombinant protein will be applied in future studies to design a Dot-ELISA kit for detecting and distinguishing infected and vaccinated cows. In addition, this recombinant protein can be used as an antigen to prepare monoclonal antibody.

## MATERIALS AND METHODS

### Sample, plasmids and bacterial strains

The extracted DNA from IBR virus isolated in Iran was selected to be cloned. Plasmid pTZ57R/T (Ins T/A clone PCR Cloning kit, Fermentas) and *Escherichia coli* strain JM107 (Fermentas) were used for initial cloning, sequencing and maintenance of DNA fragment. For recombinant protein production, a prokaryotic expression vector pET-32a (Novagen) was used. The recombinant pET-32a (pET-32a-*gD*) was transformed into *E. coli* BL21 (DE3) (Fermentas) as host strain. The required antibiotics were added to Luria-Bertani (LB) media according to the reference recommendation (Sambrook and Russell, 2001).

### Primers design

Primers were designed according to the published sequence for *gD* gene of IBR (accession number: AY690484.1). The forward primer, *gD* F:5-ATTATAGAATTCATGCAGGGCCGACATTG-3 contain *EcoRI* site while the reverse primer, *gD* R:5-TGATTGAAGCTTGT GCGTGATGGCCTCG-3 contain recognition site for *HindIII*. The restriction enzyme sites (underlined) were added to the primers for subsequent cloning procedure.

### Gene amplification of *gD* (encoding the glycoprotein D)

The amplification reaction was performed in 50  $\mu$ L reaction mixtures containing 0.1 mM of each deoxynucleotide, 15 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl (pH = 9), 2 mM MgCl<sub>2</sub>, 10% dimethylsulfoxide (DMSO, Sigma), 1.5 U of Taq DNA polymerase (Sigma) and 40 ng of template DNA. The polymerase chain reaction (PCR) was carried out in a PCR programmed thermocycler (Eppendorf, Mastercycler 5330, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany Co.) using the thermal profiles: initial cycle 94°C for 9 min, followed by a further 35 cycles; denaturation at 95°C for 60 s; annealing at 58°C for 60 s and extension by polymerase at 72°C for 60 s. The final cycle was run at 72°C for 7 min (Ros and Belak, 1999). The PCR product was analyzed by electrophoresis in 1% agarose gel in 1X TBE buffer and visualized by ethidium bromide stain on UV transilluminator. The PCR product was purified by high pure PCR product purification kit (Roche applied science) according to the manufacturer's recommendation.

### Cloning of *gD* gene

The PCR product was digested with *EcoRI* and *HindIII* and ligated to pTZ57R/T and pET-32a, which were digested by 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 pTZ57R/T-*gD* and pET-32a-*gD* 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 glycoprotein D

*E. coli* strain BL21 (DE3) was transformed with pET-32a-*gD* and grown in LB broth supplemented with ampicillin (100 mg/ml) at 37°C with agitation. In order to optimize the expression condition, different concentrations of isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) (0.5, 0.8, 1 and 1.5 mM) at different bacterial growth rates (OD<sub>600</sub> = 0.5, 0.7, 1) were tested for three hours and analyzed on 17% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The expressed protein was purified using Ni-NTA column (Qiagen) according to manufacturer's instructions. Quantity of the purified recombinant glycoprotein D was analyzed by Bradford methods and subsequently, its quality was assayed by SDS-PAGE 15% (2.5  $\mu$ g/well). In order to analyze the cross-reaction between fused segments of tax protein with infected sera, an *E. coli* BL21 (DE3) containing pET-32a vector was induced by IPTG.

### Immunoblot analysis

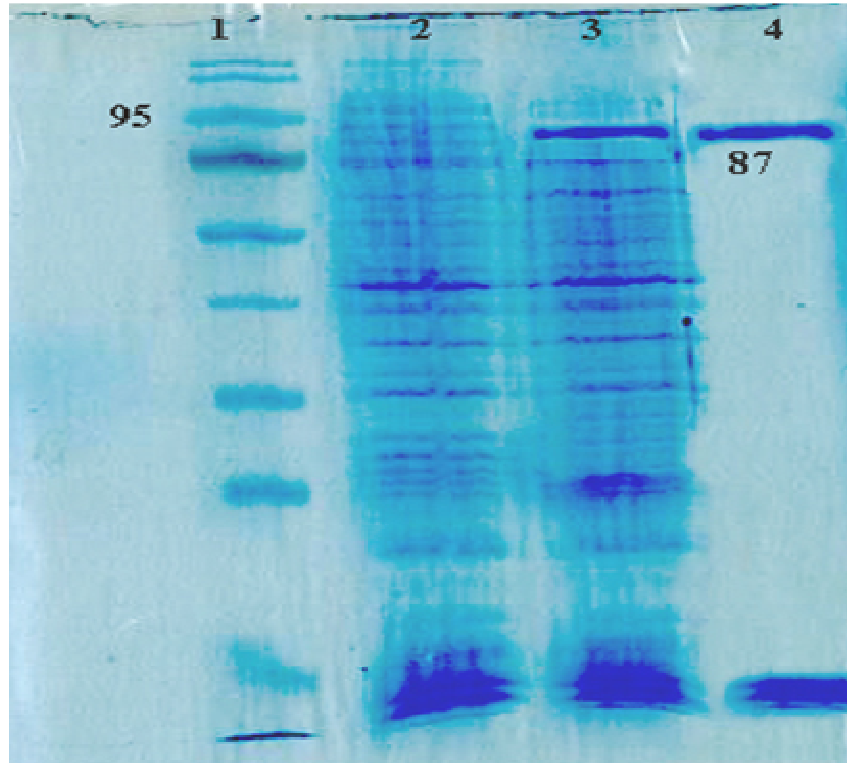
For western blot analysis, 0.5  $\mu$ g of purified recombinant glycoprotein D was used per well. As a negative control, the bacterial lysate from induced *E. coli* BL21 (DE3) contain pET-32a vector was analyzed by western blot. The gel was blotted on to polyvinylidene difluoride (PVDF Membrane, Roche Diagnostics GmbH) membrane using transfer buffer containing 25 mM Tris (pH = 8.3), 192 mM glycine and 20% methanol at 55v for 1 h at 4°C. The blotted membrane was blocked with 3% (w/v) bovine serum albumin (BSA) in tris buffer saline tween20 (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 IBR-infected cow serum, diluted 1: 25, respectively. Negative serum from apparently health cow that had negative results in PCR and enzyme-linked immunosorbent assay (ELISA) was used as control. After reaction with the primary 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 three times with TBST and reactions were developed by diaminobenzidine (DAB) solution (Sigma).

## RESULTS

The recombinant plasmid (pET-32a-*gD*) 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 (data not shown).

Expression of pET-32a-*gD* in *E. coli* BL21 (DE3) was induced and the expressed protein was purified by Ni-NTA column (Figure 1). The result showed that the best conditions for recombinant *gD* protein expression can be achieved when 1 mM of IPTG and OD<sub>600</sub> = 1 for 3 h was used.

To determine the reactivity of recombinant protein *gD*,



**Figure 1.** Expression of recombinant gD protein and its purification. Lane 1, protein marker; lane 2, pET-32a-*gD* before induction; lane 3, pET-32a-*gD* after induction; lane 4, purified gD recombinant protein.

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

## DISCUSSION

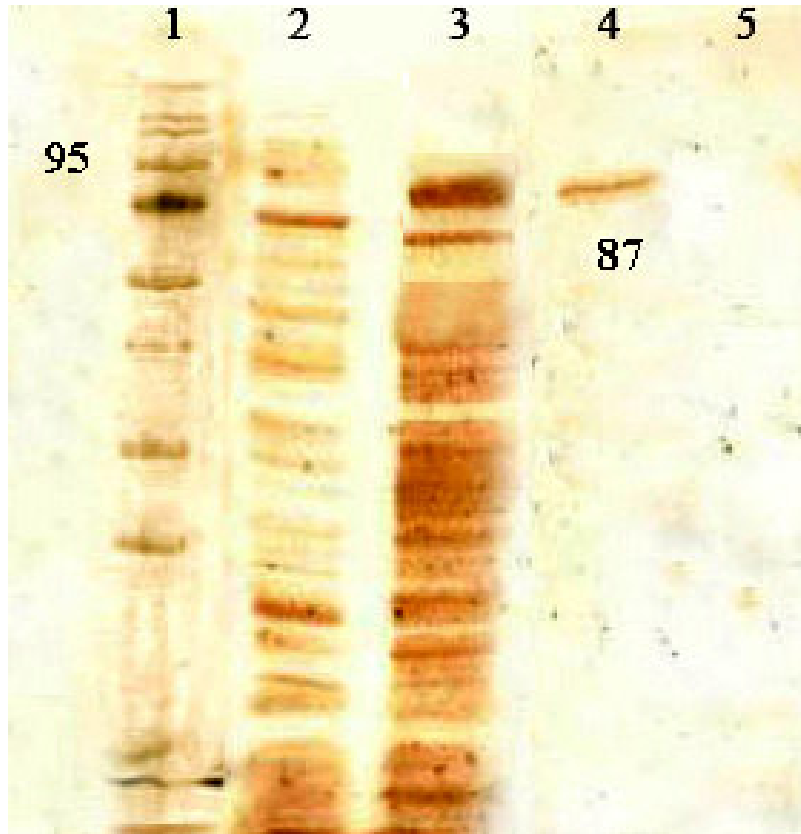
Herpes virus infections have been reported from all continents and most countries in the world. Because of the certain nature of these kind of latent infections and diseases caused by the causative agent of the infection, non-appearance of significant clinical signs during the existence of the latent virus in the body as well as complicated immunohistochemical, histopathological and laboratory diagnosis of these diseases has made herpes virus infections to have a worldwide spread in different countries. The results of many studies conducted on these viruses in humans and animals have provided a wide-spread field for studying genetic variability, laboratory diagnosis, epidemiological studies and finally, appropriate strategies for preventing herpes virus infections. Among

cattles, herpes virus infections, the bovine herpes virus type 1 and different diseases caused are significant and hence, many widespread researches have been made on diagnosis, control and prevention methods for these diseases using serologic and molecular biological methods.

Among BHV-1 structure genes, the ones coding for glycoproteins *gB*, *gC* and *gD* are considered as major and relatively protected genes and today most molecular biological diagnostic methods have been designed based on PCR, cloning and on the basis of detection of these genes (Yan et al., 2008). *gB* and *gD* gene plays main role in penetrating BHV-1 virus into a host cell and it acts as major viral antibody against protective immune response in natural infections due to virus. Those antibodies acting against glycoprotein B are considered as a main diagnostic arm in most diagnostic serum tests for infections with BHV-1 (Gao et al., 1994; Kramps et al., 1994; McGeoch and Cook, 1994; Ros and Belak, 1999; Ros and Belak, 2002).

Anyway, one of the main goals of this examination which was tracing of the coding gene of gD protein of IBR in this virus, was achieved for the first time in Iran and the presence of the corresponding gene was confirmed with the help of sequencing of the fragment.

With respect to the point that primers applied for the identification of the *gD* gene in this study were involved in the main part of encoding frame of the gene, from the



**Figure 2.** Western blot analyses against recombinant gD protein by IBR-infected serum. Lane 1, protein marker; lane 2, western blotting pET-32a-*gD* before induction; lane 3, western blotting pET-32a-*gD* after induction; lane 4, western blotting purified gD recombinant protein by infected sera; lane 5, western blotting reaction between the expressed pET-32a and positive serum.

beginning, the primers were designed for cloning and gene expression of *gD* in a way that the amplified fragment could be cloned in different vectors such as cloning and expression vectors.

The second goal of this study was cloning of the mentioned gene in each of the cloning vector (pTZ57R/T vector) and expressing vector (pET-32a). The cloning of this gene in the cloning vector after sequencing and comparing resulted sequences to other known sequences of the *gD* gene available in Genbank indicates the success in cloning the gene into the related vector. Such vectors have the capacity to be proliferated in the competent bacterial cells, digested because of several sites for restriction enzymes, extracted and inserted into the expressing vectors. The last finding was derived by cloning the coding gene of *gD* protein of IBR in the expressing vector of pET-32a for the first time in Iran and the presence of expressing protein was confirmed through SDS-PAGE and immunoblotting system.

With respect to the remarkable frequency of infection to IBR in Iran and the necessity of controlling it through vaccination with recombinant vaccines of thymidin kinase, manufacturing and applying the recombinant *gD*

protein are vital goals in recognition and distinction between infection and responses caused by vaccine. As the amplified fragment by PCR involves all the domains of *gD* and were placed in the expressing frame based on first designs of primers and has been successfully cloned in the expression vector of pET-32a, the expression of this gene and the prepared recombinant protein will be applied in the near future for designing Dot-ELISA kit for detection of antibodies against *gD* antigen of IBR in infected and vaccinated cows.

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