IDENTIFICATION AND REGULATION OF EXPRESSION OF THE UL24 PROTEIN OF EQUINE HERPES VIRUS TYPE 1

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A B S T R A C T

The UL24 gene of Equine herpes virus type 1 is conserved across many herpesviruses, but its protein product has not been identified. We expressed the UL24 gene in Glutathione S-transferase (GST) Gene Fusion System. SDS-PAGE analysis revealed that the recombinant protein UL24 (pUL24) was not overexpressed in Escherichia coli BL21 host cells after induction by different IPTG concentrations, temperatures and times. That was attributed to transmembrane cytotoxic effects of the protein. The N terminus containing the first 477 bp was deleted and the remaining C terminus 342 bp was cloned into PGEX-6p-1 for construction of a recombinant plasmid PGEX-6p-1/UL24C. SDS-PAGE analysis revealed that the recombinant protein UL24C (pUL24C) was overexpressed in Escherichia coli BL21 host cells. The UL24 c-terminus protein about 11 KDa named pUL24C was purified and used to immunize guinea pig, producing polyclonal antibody. In immunoblotting experiments, this antiserum recognized a 37KDa protein in lysates from infected cells. The specificity and sensitivity of anti-pUL24C serum were detected with Agar gel immunodiffusion reaction. The pUL24C polyclonal antibody can be used for further characterization concerning the dynamic expression of UL24 protein and intracellular localization of UL24 protein in EHV-1 infected cells.

KEY WORDS: DNA polymerase, EHV-1, FHK, Neuron, RT-PCR.

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1. INTRODUCTION

Equine herpesvirus-1 (EHV-1) is an important, ubiquitous equine viral pathogen that exerts its major impact by inducing abortion storms or sporadic abortions in pregnant mares, early neonatal death in foals, respiratory disease in young horses and myeloencephalopathy (Patel and Heldens, 2005). Outbreaks of these three clinical manifestations may occur separately or concurrently within a horse population. The first isolation of EHV-1 from a neurological case was described by (Saxegaard, 1966). Since then, reports of EHV-1 neurological disease have been increasing both in frequency and severity, particularly in recent years in USA and Europe (Lunn *et al.,* 2010). Equine herpesvirus-1 myeloencephalopathy (EHM) can cause devastating losses and have a severe impact on the equine industry (Kohn *et al.,* 2006; Henninger *et al.,* 2007). The neurological symptoms occur in various

degrees from mild ataxia to paraplegia in horse. The neurological signs may be caused by vasculitis followed by hemorrhage, thrombosis, hypoxia and secondary ischemic degeneration (Lunn *et al.,* 2010).

EHV-1 is a member of the subfamily Alphaherpesvirinae with a 150 kbp double-stranded DNA genome, consisting of 80 open reading frames (ORFs), 76 of which are unique ((Patel and Heldens, 2005). Gene transcription and regulation is tightly and sequentially regulated into three phases: immediate early (IE), early (E) and late (L). The virus encodes a single IE gene, 55 E genes and 20 L genes. Six of these (IE E × 4 and L × 1) have regulatory functions and are responsible for the tightly controlled cascade of virus gene regulation (Slater *et al.,* 1993).

EHV-1 UL24 protein is encoded by ORF37. UL24 homologs are present throughout the Herpesviridae family. The Herpes simplex virus-1 (HSV-1) UL24 is a 30-kDa nuclear-associated protein that is not required for growth in cultured cells (Pearson and Coen, 2002). The UL24 homolog identified in bovine herpes virus type 1 (BHV-1) was shown to have a transcription profile similar to that of HSV-1 UL24. Deletion of the BHV-1 UL24 open reading frame (ORF) had little effect on viral replication in vitro (Whitbeck *et al.,* 1994). Although the molecular function of UL24 protein is not known, mutation of the HSV-1 gene results in the development of a syncytial plaque-forming phenotype following infection of certain cell types in vitro. Studies using HSV-1 UL24 point mutants in a murine ocular disease model suggested that the HSV-1 UL24 gene product was important for peripheral replication in corneal tissue, acute replication in sensory ganglia, and reactivation from explanted mouse ganglia. The UL24 of HSV-2 is reported as a pathogenicity determinant in murine and guinea pig disease models (Blakeney *et al.,* 2005). Inoculating three different types of cell lines with UL24 mutant HSV-2, they reported that it had no effect on viral replication or virus titers as it yielded a cytopathic effect with syncytial formation and virus titers as those produced by the wild-type virus. In a previous study of our laboratory (Kasem *et al.,* 2010) suggested that the EHV-1 UL24 has a role in neuropathogenicity of EHV-1 in the mouse model. However, the function of EHV-1 UL24 has not been completely resolved yet and the characterization of the EHV-1 UL24 protein (pUL24) remains unclear.

To investigate the characteristics of EHV-1 UL24 protein, we expressed the pUL24C gene in Glutathione S-transferase (GST) Gene Fusion System and used the resulting protein to immunize guinea pig, producing polyclonal antibody that, detected by agar immunodiffusion and western blotting techniques. This work was supposed to facilitate the understanding of EHV-1 pUL24 function.

2. MATERIAL AND METHODS

2.1. Virus strain. EHV-1 Ab4p strain, which was kindly provided by Dr. A. J. Davison, Glasgow University, Scotland, was used.

2.2. Kits used for cloning and expression:-

Restriction enzymes (ECOR1, XHO1 and NOT 1) (TOYOBO, Japan). PEGEX-6P (GE Healthcare Bio-Sciences AB). 2x Ligation buffer. (PROMEGA. US). T4 DNA Ligase enzyme (PROMEGA. US). Competent cells (BL21). SOB medium (super optimal broth). IPTG (isopropyl-B-D-thiogalactopyranoside).

2.3. Primers used:

Orf 37 Forward primer with ECOR1:
TTCTTGGAATTCATGAAACGTAGA
CAGCGTCTGACAGCTAG
Orf 37 Reverse primer with NOT1:

TTCTTGGCGGCCGCttattttggggaaaaaag ggacgccgc

2.4. Computer analysis of EHV-1 UL24

EHV-1 ORF 37 designated as UL24 gene. It was about 819 bp and expected to encode a protein comprising 272 amino acids with a putative molecular mass of 30 KDa. A series of bioinformatics aided tools were used to analyze the intracelluar location of pUL24: PSORT II Prediction [from the website http:// psort. nibb. ac. jp/ form2. Html] (Xiang *et al.,* 2009), TargetP 1.1 [from the website http:// www. cbs. dtu. dk/ services/ TargetP] (Shen *et al.,* 2009), SignalP 3.0[from the website http:// www. cbs. dtu. dk/ services/ SignalP] (Wu *et al.,* 2010), TMHMM 2.0 server [from the website http:// www. cbs. dtu. dk/ services/] (Emanuelsson *et al.,* 2007), Predict NLS server [from the website http:// www. rostlab. org/ services/ predictNLS/] (Nair and Rost, 2003), CSS-Palm 2.0 online server [from the website http:// csspalm. biocuckoo. org/ online. Php] (Ren *et al.,* 2008).
Prediction of transmembrane helices in proteins (from the website http://www.cbs.dtu.dk/services/TMHMM/) and the Golgi predictor [from the website http:// ccb. imb. uq. edu. au/ golgi/ golgi_ predictor. Shtml] (Yuan and Teasdale, 2002). Predictions of them were based on the putative amino acid sequence of pUL24.

2.5 Cloning of PGEX-6p-1 expression plasmid

The entire EHV-1 UL24 open reading frame (ORF) was expressed in bacteria as a glutathione S-transferase (GST) fusion protein. For this, the entire EHV-1 UL24 ORF coding region was amplified by long PCR using a forward primer that introduced an EcoRI site directly upstream of the start codon, allowing for an in-frame fusion with the GST open reading frame and a reverse primer that introduced an NotI site directly downstream of the UL24 termination codon. The 845-bp EcoRI- NotI PCR-generated fragment containing complete EHV-1 UL24 ORF was ligated into pGEX-6P-1 (GE Healthcare Bio-Sciences AB), generating the pGEX-6P-1-UL24 expression vector. Subsequently, PCR, restriction enzyme digestion and DNA sequencing (TaKaRa) tests were performed to ensure correct insertion. Strong outside transmembrane helices in UL24 protein was strongly predicted in N-terminus which make the recombinant protein UL24 (pUL24) was not overexpressed in Escherichia coli BL21 after induction by different Isopropyl 1-β-D-galactopyranoside (IPTG) concentration, temperatures and times. On result of expression get occur we changed our strategy cloning the C-terminus (starting from amino acid residue 160 to 272) was ligated into pGEX-6P-1 (GE Healthcare Bio-Sciences AB), generating the pGEX-6P-1-UL24C expression vector. Subsequently, PCR, restriction enzyme digestion and DNA sequencing (TaKaRa) tests were performed to ensure the correct insertion.

2.6 Expression and purification of recombinant UL24C protein

Expression of the GST-UL24C fusion protein was induced by the addition of Isopropyl 1-β-D-galactopyranoside (IPTG) to a culture of BL21 cells transformed with the pGEX-6P-1-UL24C plasmid. The positive recombinant plasmids were transformed to Escherichia coli BL21 for expression by the addition of IPTG. The temperature and duration of IPTG and its working concentration were optimized to maximize the expression of pUL24C. To obtain a highly expressed level of UL24C protein, we tried optimizing expression conditions by using different temperatures (25, 30, 37°C), different IPTG concentrations (0.1, 0.5 m M), and different incubation times (4, 6, overnight).
The recombinant pUL24C was purified

under denaturing condition. The induced cells were centrifuged at 10,000 rpm for 10 min, and resuspended the pellet in 10 ml of ice cold STE Buffer with the addition of lysozyme (0.1 mg/ml) and incubated at room temperature for 30 min. Just before sonication, 100 ml of 1 M DTT and 1.4 ml of 10% Sarkosyl was added. The cell lysate was then sonicated for a total time of 5 min. Centrifuged at 16,000 rpm for 20 min to pellet debris. Supernatant was transferred to a 50-ml conical tube and the pellet was discarded. 4 ml of 10% Triton was added and top up with STE Buffer to 20 ml then incubation at room temperature for 30 min. The lysate was added to 1 ml bed of prepared Glutathione Sepharose in Phosphate Buffer saline (PBS) and incubated at room temperature for 1 hr with agitation. The beads were washed with 50 ml of 1 X PBS, eluted with 1 ml fractions of 10 x elution buffer (50 mM Tris.Cl, pH 9.0 and 20 mM GSH). The purity of pUL24 was tested by SDS-PAGE.

2.7 Guinea pigs immunization

For preparation of polyclonal antibodies (Chang *et al.,* 2010), Guinea pigs were first immunized intramuscularly with a mixture of 0.1 mg denatured recombinant pUL24C and an equal amount of Titer Max Gold adjuvant (Sigma, Shanghai, China). Two weeks later, 0.1 mg purified fusion pUL24C and an equal amount of Titer Max Gold adjuvant were used for secondary immunity. At last, sera were collected 15 days later. Control pre-immune serum was obtained from the non-vaccinated healthy guinea pigs.

2.8 Western blot analysis

The purified fusion pUL24C was suspended in 100 µl of PBS, and mixed with the same volume of sample buffer (2x SDS sample buffer). Then separated by SDS-PAGE and electro transferred to nitrocellulose membranes (Millipore). Blots were blocked with 5 % skimmed milk in Phosphate-buffered saline- Tween (10x PBS, D2W, 0.25 % Tween 20) and incubated for 1 h with guinea pigs antisera against the UL24C gene products at dilutions of 1: 2 000 in PBS-T. Bound antibody was detected with Horse Radish Peroxidase (HRP) conjugated anti-guinea pigs antibodies (Bethyl®) and visualized by chemiluminescence (Amersham®) recorded on X-ray films. The obtained guinea pig polyclonal anti-serum against pUL24 was analyzed by 12% SDS-PAGE.

2.9. Gel diffusion reaction

Gel diffusion reaction was used to detect the reactivity and specificity of the purified UL24 anti-serum (Lunn *et al.,* 2010). One gram of agar was dissolved in 100 ml normal saline. It was heated, cooled down to 55°C, and then poured into the plates to a thickness of 2 mm. After subsequent solidification with cooling, the agar was perforated with 3 mm diameter holes that may hold about 100 µl solution. Twenty microliters each of the pre-immune serum, 1:2, 1:4, 1:8, 1:16 and 1:32 diluted anti-serum was added into the peripheral apertures. At last, 20 µl-purified pUL24 was added into the central aperture. The plate was incubated at 37°C for 24 hr before observation.

3. RESULTS

3.1. Prediction of subcellular localization of EHV-1 pUL24

Computer analysis of the subcellular localization of EHV-1 pUL24 suggested that the pUL24 was mainly located in nucleus (70.6%) of infected cells. However, according to the prediction, EHV-1 pUL24 contained no potential N-terminal signal peptides, ER retention motif in the C-terminus, peroxisomal targeting signal in the C-terminus, 2nd peroxisomal targeting signal and possible vacuolar targeting motif.

EHV-1 pUL24 contained potential mitochondrial targeting peptide, transmembrane region and nuclear localization signal (NLS). Further, Golgi prediction results indicated pUL24 was not a Golgi type II membrane protein (Golgi localised transmembrane protein) since the index values of a Golgi protein should be greater than the threshold (20.005) while the index values of pUL24 was 0.

3.2. Expression and purification of UL24 recombinant protein

Recombinant plasmids containing the encoding region of EHV-1 UL24 were constructed for expression. The constructed recombinant plasmid pGEX-6p-1/UL24 was transformed into E. coli BL21 for expression. After incubation at different temperature and IPTG concentrations, the cultures were analyzed by SDS-PAGE. The recombinant protein UL24 (pUL24) was not overexpressed in Escherichia coli BL21. Prediction of transmembrane helices in proteins (from the website http://www.cbs.dtu.dk/services/TMHMM/) showed strong outside transmembrane helices in UL24 protein in N-terminus which make the recombinant protein UL24 (pUL24) was not overexpressed in Escherichia coli BL21 host cells after induction by different IPTG concentration, temperatures and times due to transmembrane cytotoxic effects. We changed our strategy via cloning the C-terminus starting from amino acid residue 160 to 272. Other recombinant plasmids containing the encoding region of EHV-1 UL24 C-terminus were constructed for expression. Schematic diagrams of the cloning strategy of EHV-1 UL24C were shown in Figure 1. PCR, Restriction enzyme digestion and DNA sequencing were used to confirm the correctness of insertion the constructed recombinant plasmids pGEX-6p-1/UL24C that was transformed into *E. coli* BL21 for expression. The expression level of synthesized pGEX-6p-1/UL24C at 25° was slightly more than at 30°C, and no observable difference was seen between 30 and 37°C. While incubation time was increased, the expressed protein was increased also. The different concentrations of IPTG showed no apparent increase in the expressed protein. The fusion protein was highly expressed after induction at 25°C with 0.1 mM IPTG. SDS-PAGE of cell lysates revealed a major protein band of the expected 37 kDa. However, the corresponding band of pUL24C was absent in the cultures of pGEX-6p-1/UL24C before induction (Fig. 2). Purification of EHV-1 pUL24C was performed under denaturing condition since most of pUL24C were expressed as insoluble inclusion bodies (IB) in *E. coli*. Eluent containing 10% Sarkosyl in STE Buffer was used for purification. The purified pUL24C was dissolved finally in elution buffer. SDS-PAGE analysis demonstrated the purity of pUL24C after washing (Fig. 3 lane 3). Immunogenicity of the purified pUL24C was detected by Western blotting assay. As shown in Figure 4, the EHV-1/UL24C anti-serum can specifically recognized a 37 KDa band (Fig. 4), which corresponded to the theoretical molecular mass of GST/UL24C.

3.3 Verification of the character of polyclonal antibody against EHV-1 pUL24

The reactivity and specificity of polyclonal antibodies against EHV-1 pUL24.C obtained from immune guinea pigs was detected by Western blotting assay. As shown in Figure 4, the purified anti-pUL24C serum reacted strongly with an approximate 37 KDa protein, which represented EHV-1 pUL24C. Agar diffusion reaction was performed to determine the immunoreactivity of anti-pUL24C serum with purified pUL24C. The obtained result suggested the highest titer of the agar diffusion reaction of anti-pUL24C serum with pUL24C was 1:16 Pre-immune serum used as a negative control didn't show

Hgg

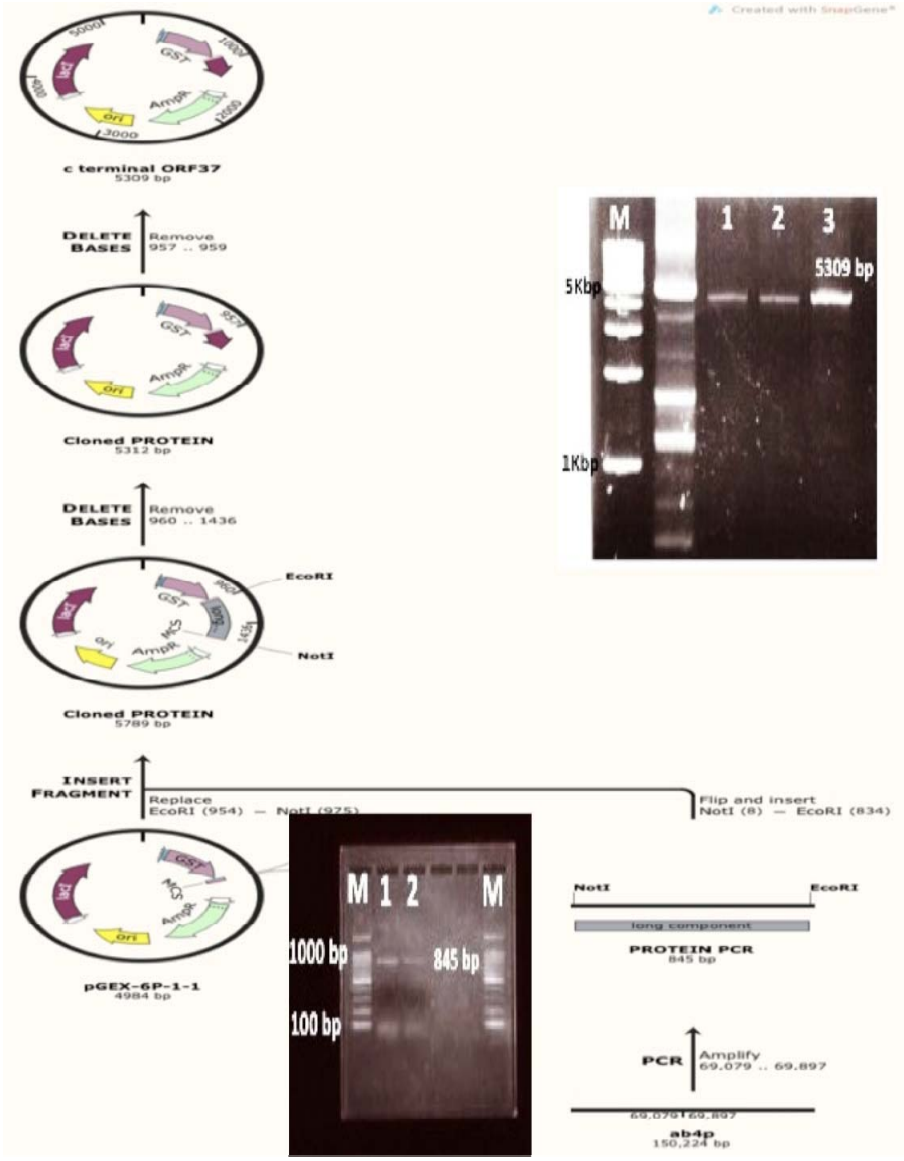

Fig. 1: Schematic diagram of the UL24 ORF cloned into the pPGEX-6p-1 cloning vector and construction of the recombinant expression plasmid pPGEX-6p-1 /UL24.C.

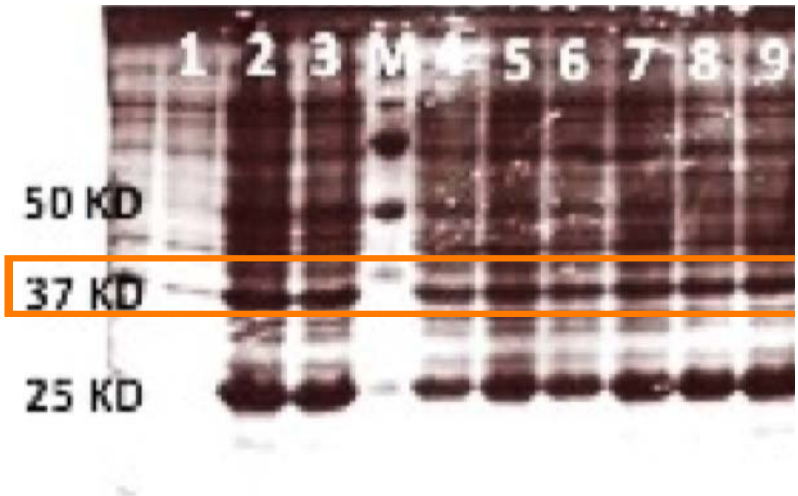

Fig. 2: Expression pPGEX-6p-1/UL24.C protein produced in *E. coli* BL21. pPGEX-6p-1/UL24. C protein was expressed in *E. coli* BL21. M represented standard protein molecular weight markers. Lane1, the recombinant pPGEX-6p-1/UL24C cultures before induction in *E. coli* BL21, Lane2, the culture of pPGEX-6p-1 after induction in *E. coli* BL21 at 25°C and 0.1 IPTG ; Lane3, the culture of pPGEX-6p-1 after induction in *E. coli* BL21 at 25°C and 0.5 IPTG; Lane 4 and 6, the culture of pPGEX-6p-1 after induction in E. coli BL21 at 30°C and 0.1 IPTG; Lane5 and 7, the culture of pPGEX-6p-1 after induction in *E. coli* at 30°C and 0.5 IPTG; Lane8, the culture of pPGEX-6p-1 after induction in *E. coli* at 37°C and 0.1 IPTG and Lane 9, the culture of pPGEX-6p-1 after induction in *E. coli* at 37°C and 0.5 IPTG.

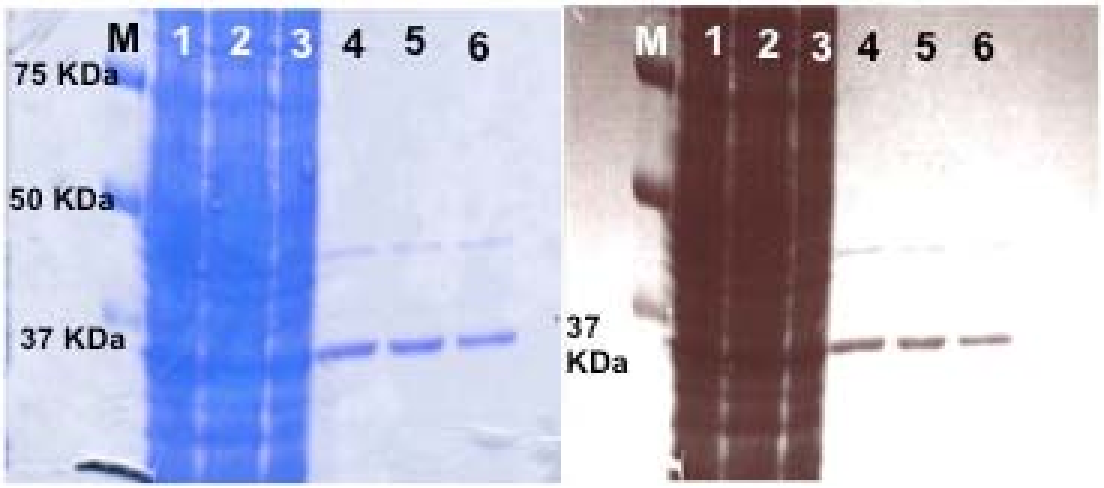

Fig. 3: Purification of pGEX-6p-1/UL24C. SDS-PAGE analysis of the purity of recombinant pUL24C. M represented standard protein molecular weight markers. Lane 1&2, the pellet of expressed pUL24C after induction in E. coli BL21; Lane 3, the purified pUL24C.

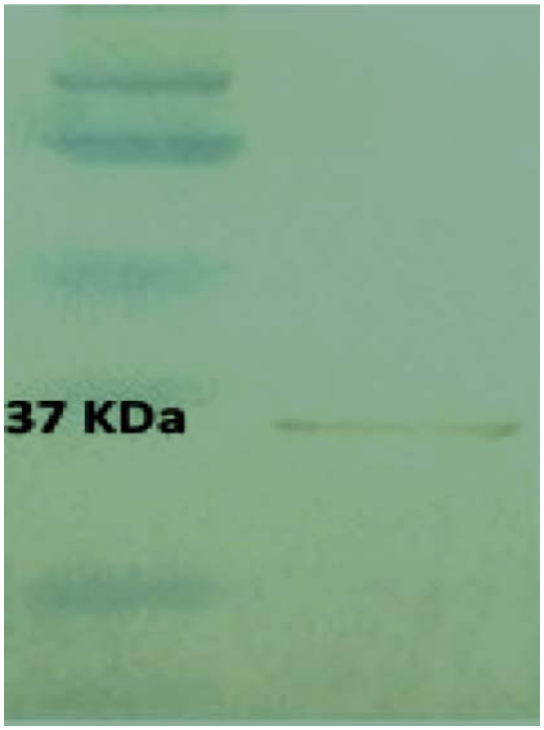

Fig. 4: Showing immunoreactivity of the recombinant UL24C protein. The immunoreactivity of the recombinant UL24C protein was analyzed by western blotting assay with the Guinea pig UL24C. M: Protein Molecular Weight marker; Lane 1, the purified GST/UL24C.

any antigen-antibody complexes.

4. Discussion

We have attempted to isolate and purify the complete EHV-1/UL24 amino acid sequence as a fusion protein form. The protein of interest was not detected on the SDS-PAGE. The expression of the EHV-1/UL24 was unfavorable in *E. coli* expression systems due to transmembrane cytotoxic effects confirmed by Prediction of transmembrane helices in proteins (http://www.cbs.dtu.dk/services/TMHMM/). Strong outside transmembrane helices in UL24 protein N-terminus make the recombinant protein UL24 (pUL24) was not overexpressed in Escherichia coli BL21 host cells after induction by different IPTG concentration, temperatures and times.

The expression of complete duck enteritis virus (DEV) UL24 amino acid sequence as a fusion protein form though recombinant plasmids, such as protocaryon expression vectors (pET32a, pET28a and PGEX-4T-1), was not detected on the SDS-PAGE. It seemed that the expression of the gene sequence was unfavorable in E. coli expression systems or cytotoxicity (Renyong *et al.,* 2009). The UL24 gene of HSV-1 was expressed in insect cells from a recombinant baculovirus (Pearson and Coen, 2002). However, as the UL24 gene product of HSV-2 was expressed in a BL21 strain of E. coli from a recombinant plasmid pGEX-4T-UL24 (Hong-Yan *et al.,* 2001), there might be a difference between HSV-2 and EHV-1.

Here, we showed that EHV-1 UL24

partial protein (C-terminus) could be efficiently expressed in E. coli. The determined recombinant plasmid pGEX-6p-1/UL24.C was transformed into Escherichia coli BL21 for prokaryotic expression. So as to lower the rate of cell growth at a lower temperature and simultaneously the incubation time was increased to overnight to increase the cell mass. The rate of protein synthesis is decreased with a slower rate of cell growth and thus sufficient time for proper protein folding is provided, increasing the possibility of the protein to be present in the native form (Kothari *et al.,* 2006). The optimal expression condition of recombinant pUL24C was induced by 0.1 mM IPTG at 25°C overnight. A GST fusion pUL24C approximately 37 KDa was collected and purified.

The guinea pig polyclonal UL24.C in our work was detected by Western blotting. The purified anti-pUL24C serum reacted strongly with an approximate 37 KDa protein, which represented EHV-1 pUL24C. It can be widely used for identification features of EHV-1 UL24 gene product. The titer of agar diffusion reaction reached 1:16, which suggested the extractive anti-pUL24C, was specific and sensitive to pUL24. A guinea pig antiserum produced against EHV-1 was able to detect this recombinant UL24 partial protein from *E. coli* by Western blot analysis with high avidity. So the rapid immunoassay for detection and quantification of EHV-1 UL24 partial protein specific antibodies in immunized hosts would be developed using this recombinant polypeptide. Some of the important attributes of a candidate molecule for vaccine development are that they should be highly conserved among various members of the same species; they should be expressed on the surface of pathogens so that antigen-presenting cells can easily recognize them, and they should be immunogenic (Khushiramani *et al.,* 2007).

These results, along with the good yield and purity of polyclonal antibody against EHV-1UL24 protein, encourage further studies on the more detailed immunological properties of UL24 protein, such as the ability to induce EHV-1 neutralizing antibodies and the production of anti-UL24 monoclonal antibodies, which together might be useful for the development of new diagnostic methods.

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معرفة وتنظيم التعبير عن بروتين UL24 لفيروس الهربس الخيلي-1

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الملخص العربي

إن جين UL24 لفيروس الهيربس الخيلي-1 تم دراسته وسط فيروسات الهيربس المختلفة ولكن لم يتم التعرف علي المنتج البروتيني لهذا الجين. في هذه الدراسة تم التعبير عن هذا الجين في نظام GST. لقد أوضح تحليل SDS-PAGE أن البروتين المؤتلف لهذا الجين لم يتم تعبيره في بكتيريا الايشيريشيا كولاي BL21 بعد التحضين في ظروف مختلفة من تركيز IPTG ودرجات الحرارة والوقت ويرجع ذلك الي التأثير السمي الخلوي لهذا البروتين. تم حذف النهاية N التي تحتوي على أول 477 قاعدة مزدوجة كما تم استنساخ النهاية C المتبقية التي تحتوي علي 342 قاعدة مزدوجة في بلازميد PGEX-6p-1 لانشاء بلازميد مؤتلف PGEX-6p-1/UL24C. وقد أوضح تحليل SDS-PAGE أن البروتين المؤتلف للنهاية C لهذا الجين تم تعبيره في بكتيريا الايشيريشيا BL21. تم تنقية هذا البروتين المؤتلف (حوالي 11 كيلو دالتون) واستخدامه لتكوين أجسام مناعية في خنازير غينيا. لقد أثبتت تجربة Immunoblotting وجود هذه الاجسام المناعية عند حجم 37 كيلو دالتون. تم تحديد حساسية وخصوصية هذه الاجسام المناعية باستخدام اختبار الترسيب المناعية. يمكن استخدام هذا السيرم المحضر المضاد لهذا الجين لمزيد من التوصيف مثل التعبير الديناميكي لبروتين هذا الجين وكذلك التواجد الخلوي لهذا البروتين داخل الخلايا المعدية بالفيروس.

(مجلة بنها للعلوم الطبية البيطرية: عدد 26(2):10- 20, يونيو 2014)