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Expression of Biologically Active Measles Virus Hemagglutinin Glycoprotein by a Recombinant Baculovirus

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Abstract: In this study, one of the measles virus membrane proteins, named hemagglutinin (H) which has a key role in tropism, receptor binding, hemagglutinating activity and also induction of protective immunity against viral infection, was expressed by the baculovirus expression system using specific plasmid (pDONR221) to produce entry clone. Measles Virus (AIK-C strain) genome was extracted from infected Vero cells. H gene was amplified by specific primers during RT-PCR reaction and inserted into the specific plasmid (pDONR221) using BP recombination reaction. Recombinant baculovirus harboring H gene was consequently constructed by LR reaction. Insect cells (*Sf9*) were infected with recombinant baculovirus. In order to increase viral titer, recombinant baculoviruses were passaged four times in *Sf9* cells. Synthesis of H protein was verified by SDS-PAGE, western-blot and indirect immunofluorescence using goat polyclonal antibody against Measles Virus. The results showed that H protein was partially glycosylated, but it appeared to be active in hemagglutination assay.

Key words: Measles virus AIK-C strain, hemagglutinin, recombinant protein, pDONR221

INTRODUCTION

Measles Virus (MV) a member of Morbillivirus genus in the Paramyxoviridae family is an enveloped virus with a non-segmented negative strand RNA genome (Griffin, 2007). It has two envelope glycoproteins, the Hemagglutinin (H) and the Fusion (F) protein. H is responsible for receptor binding and hemagglutinating activity. In this regard it could be used for recombinant protein preparation in order to induce the immune system (Huang *et al.*, 2001; VanBinendijk *et al.*, 1997). MV causes a common childhood disease with high fever and a typical skin rash. Patients with measles develop profound immunosuppression, often leading to secondary infections. MV also causes postinfectious encephalitis, measles inclusion encephalitis and subacute sclerosing panencephalitis (Yanagi *et al.*, 2006). Despite the availability of effective live vaccines against Measles, it still claims lives of over 700000 infants and children around the world annually (Premenko-Lanier *et al.*, 2006). Furthermore, they are ineffective in infants under 9 to 15 months of age. This ineffectiveness of the vaccines is due to maternally acquired measles virus specific antibodies which readily neutralize this parentally administered attenuated vaccine virus (Skiadopoulos *et al.*, 2001; Moss *et al.*, 1999). As a result, the need for a measles virus vaccine to protect

children less than 15 months of age is the highest in the regions of the world where there is a high prevalence of infection with human immunodeficiency virus (Premenko-Lanier *et al.*, 2006).

The baculovirus expression system employing *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV) and *Spodoptera frugiperda* (*Sf9*) insect cells in culture is very popular for high level expression of heterologous genes (Hu, 2005). In such a system, the strong, efficient promoter of polyhedrin gene directs transcription of the foreign gene. Polyhedrin is not essential for the infection process, so it is possible to replace the polyhedrin gene with a heterologous gene while retaining the polyhedrin regulatory signals (O'Reilly *et al.*, 1992).

We constructed the recombinant baculovirus containing His-tagged Measles Virus hemagglutinin gene by Gateway baculodirect expression system and expressed the protein in insect cells (*Sf9*). The hemagglutinin was partially glycosylated and biologically active in hemagglutination assay.

MATERIALS AND METHODS

Cells and viruses: Measles Virus AIK-C vaccine strain kindly provided by Dr. Abbas Shafyi (Razi Vaccine and Serum Research Institute). Vero cells were cultivated in

Dulbecco's Modified Eagle Medium supplemented with 5% Fetal Calf Serum, L-glutamine and antibiotics, used to support virus growth. Cells were incubated until cytopathic effect was observed, then infected cells were harvested and stored at -70°C . *Spodoptera frugiperda* insect cells and linear DNA of *Autographa californica* Nuclear Polyhedrosis Virus were obtained from Invitrogen Company, USA. *Sf9* cells were cultured in Grace medium (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal calf serum, Yeastolate (GIBCO, Grand Island, N.Y.), lactalbumin hydrolysate, 50 μg of gentamycin sulfate per mL at 27°C by the procedures of Summers and Smith (1987).

RNA extraction: Infected cell cultures were centrifuged at low speed for 20 min. RNA was extracted from clarified supernatant using RNA extraction kit (RNAfast manufactured in National Institute of Genetic Engineering and Biotechnology). The extracted RNA was stored at -70°C .

Primers: Primers used for RT-PCR and PCR were designed based on published MV sequences (GenBank Accession No.AF266286) and contained recombinant sites (attB1 and attB2 for Forward and Reverse primers respectively shown with Bolded nucleotides) for cloning into pDONR221 vector. The stop codon in the reverse primer was omitted in order to be in frame with C-terminal 6xHis in the BaculoDirect™ Linear DNA for tagging and purifying our recombinant protein. The sequence of the primers were as follow:

H-Forward: 5' GGGGACAAGTTTGTACAAAAAAGCA
GGCTCAATGTCACCACAACGAGACC 3'
 H-Reverse: 5' GGGGGACCAC**TTTGTACAAGAAAGCT**
GGGTCTATCTGCGATTGGTTCATC 3'

Amplification of H gene: Extracted RNA was used as template for reverse transcription reaction. The RT-PCR reaction was performed in total of 20 μL final volume using 2.5 mM of each dNTPs, 100 μmol of each primer, reaction buffer (containing 250 mM Tris HCl, 250 mM KCl, 20 mM MgCl_2 , 50 mM DTT), 40 U RNase Inhibitor, 200 U M-MuLV Reverse Transcriptase (Fermentas) and nuclease free water. Thermocycler was programmed for one cycle at 70°C for 5 min, followed by one cycle at 37°C for 5 min and one cycle at 42°C for 60 min. The reaction was stopped at 70°C for 10 min and then the microtube was chilled on ice (Verma, 1981). PCR test was performed in total of 50 μL final volume, using 1 ng cDNA, 10 μmol of each primer, 2.5 mM of each dNTPs, 2.5 mM MgSO_4 and 2.5 units of pfu DNA polymerase (Lundberg, 1991). Thermocycler was programmed for one cycle at 94°C for 5 min followed by thirty cycles at 95°C for 1 min, 50°C for

1 min, 72°C for 70 sec and one cycle of final extension at 72°C for 10 min. The PCR product were electrophoresed in 1% (w/v) agarose gel and stained by ethidium bromide and visualized by UV transilluminator.

Construction of H entry vector: The pDONR221 plasmid and Omnimax chemically competent *E. coli* (Invitrogen) were used for cloning. The full coding region sequence of H protein (AIK-C Stain) with attB sites was inserted into pDONR221 containing attP sites (Fig. 1), using the BP recombination reaction by BP clonase (Invitrogen). Bacterial cells were transformed according to the published method (Sambrook and Russel, 2001). Transformation mix was spread on a prewarmed selective plate (LB+50 μg mL^{-1} Kanamycin) and incubated overnight at 37°C . The presence of the ccdB gene as controller of cell death gene in pDONR221 allows negative selection of the donor vectors in *E. coli* following recombination and transformation. The ccdB protein interferes with *E. coli* DNA gyrase, thereby inhibiting growth of most *E. coli* strains (Invitrogen).

Colonies carrying entry clone were picked up and suspended in 5 mL of LB broth with appropriate kanamycin concentration. Plasmids were purified from the bacteria using plasmid purification kit (Roche). Plasmid constructs were confirmed by restriction endonucleases digestion and sequenced with M13 primers in both direction by a dye terminator method, using the ABI 3130XL sequencer (Griffin and Griffin, 1993). The sequencing results were compared with other sequences deposited in the Genbank by the BLAST software (Atschul *et al.*, 1997).

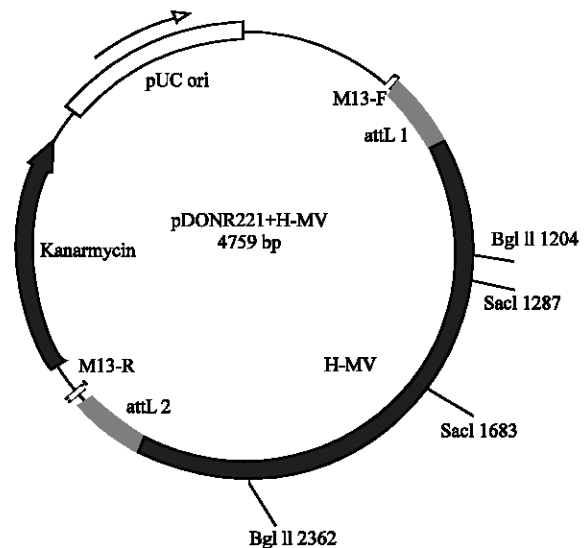


Fig. 1: Schematic presentation of entry clone construct (pDONR221 + H gene of Measles Virus) with some restriction sites

Construction of recombinant baculovirus: Recombinant baculovirus harboring H gene was constructed by LR reaction between entry clone and BaculoDirect Linear DNA containing thymidine kinase locus of Herpes Virus flank to the polyhedrin promoter which substitute by foreign gene and could help to selection after recombination, in the presence of ganciclovir. The reaction was performed in a total volume of 10 μL using 2 μL entry clone (50-150 ng/reaction), 5 μL BaculoDirect Linear DNA (150 ng/reaction), 1 μL TE buffer, PH 8.0 and 2 μL LR Clonase II mix. The tubes containing reaction mix were incubated at 25°C for 18 h, then 1 μL of the 2 $\mu\text{g } \mu\text{L}^{-1}$ proteinase K was added to each reaction and the tubes incubated for additional 10 min at 37°C.

Transfection of the *Sf9* cells: The BaculoDirect™ Linear DNA Expression System uses Gateway® Technology to facilitate direct transfer of the foreign gene into the baculovirus genome without the need for additional cloning or recombination in bacterial or insect cells. Transfection of recombinant baculovirus into cultured *Sf9* cells was achieved using cellfectin. LR recombination reaction were composed of two mixture: mixture A containing 5 μL LR recombination reaction and 100 μL unsupplemented Grace's Insect Medium and mixture B containing 6 μL cellfectin reagent and 100 μL unsupplemented Grace's Insect Medium. The two mixtures were combined and incubated for 45 min at RT. Then 800 μL unsupplemented Grace's Insect Medium was added to transfection mix and mixed gently. The *Sf9* cells were washed with fresh unsupplemented Grace's Insect Medium and the entire transfection mix were added onto the cells and incubated for 5 h at 27°C. Then transfection mixture were removed and 2 mL of complete Grace's insect medium with antibiotics (100 U mL⁻¹ penicillin and 100 $\mu\text{g mL}^{-1}$ streptomycin) and 100 μM ganciclovir were added to each well (Invitrogen). The cells incubated at 27°C until CPE appeared and then were harvested and pelleted by centrifuge at 3000 rpm for 5 min. The supernatant which was the P1 viral stock was stored at 4°C and protected from light. The pellet was washed 2 times with PBS and kept for detection of recombinant protein. In order to increase viral titer, recombinant baculoviruses were passaged 4 times in *Sf9* cells.

Immunofluorescence assay: Recombinant baculovirus protein expression was assayed in acetone-fixed infected *Sf9* cells with a 1:5 dilution goat antibody specific for measles virus (prepared from Razi Vaccine and Serum Institute) and rabbit anti goat fluorescein isothiocyanate-conjugated antibody (Koma Biotech Inc.).

Polyacrylamide gel electrophoresis and immunoblot:

Cellular proteins were analysed using electrophoresis lysis mix containing 0.5M Tris-hydrochloride pH: 6.8, 2% SDS, 10% Glycerol, 2% β -mercaptomethanol, 1% bromophenolblue and boiled for 5 min. Samples were applied to 10% acrylamide gels (acrylamide/bisacrylamide weight ratio, 50:1.6) and subjected to electrophoresis at 140 V, 4 h by the methods of Laemmli. Protein bands were detected by coomassie blue staining.

Hemagglutination assay: Cells expressing H protein were serially twofold diluted in 96-well round-bottom microdilution plates in 25 μL volumes. A 1% suspension of monkey erythrocytes was prepared in PBS and added to each well in 50 μL volumes. The plates were kept at 4°C overnight and the results were recorded after 16 h.

RESULTS AND DISCUSSION

Amplification of H gene: In order to obtain Measles Virus H gene with attachment sites for recombination reaction, PCR was performed using specific primers containing attachment sites. As it shown in Fig. 2 DNA band of approximately 1950 base pair was achieved (Lane 2). In control reaction no DNA band was detected (Lane 1).

Analyzing the entry vector: After BP reaction and purification of H containing plasmid (Entry Clone) from bacteria, they were digested by Bgl II restriction endonuclease since H gene has two restriction sites for Bgl II, but pDONR221 has no restriction site for this enzyme. Therefore only two fragments with approximate molecular weight of 1100 and 3500 base pair appeared on agarose gel (Fig. 3 Lane 2, Lane 4).

Immunofluorescence test: Immunofluorescence analysis of infected *Sf9* cells with recombinant baculovirus containing H gene using measles virus antiserum and fluorescein isothiocyanate conjugated rabbit anti goat antibody showed that the entire infected cells were stained indicating the presence of viral expressed proteins (Fig. 4B). Control uninfected cells processed similarly did not show any fluorescence (Fig. 4A).

Expression of recombinant H proteins in *Sf9* cells: *Sf9* cells cultivated in 6-well microdilution plates (Fig. 5A) were infected with recombinant (Fig. 5B) or non-recombinant virus at a multiplicity of infection 5 PFU per cell. At specific times (4, 5, 6 and 7 days) following infection, cells showing CPE were washed twice with PBS, lysed with SDS-electrophoresis sample buffer applied to polyacrylamide gels (10%) and subjected to

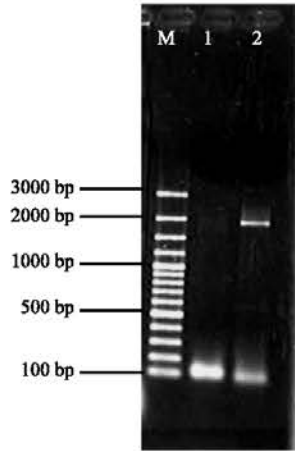


Fig. 2: Amplification of H gene (Measles virus AIK-C strain) on 1% agarose gel. Lane M: DNA Molecular weight Marker (0.07-12.2 Kbp). Lane 1: Negative control Lane 2: Amplification of H gene with specific primers (1950 bp)

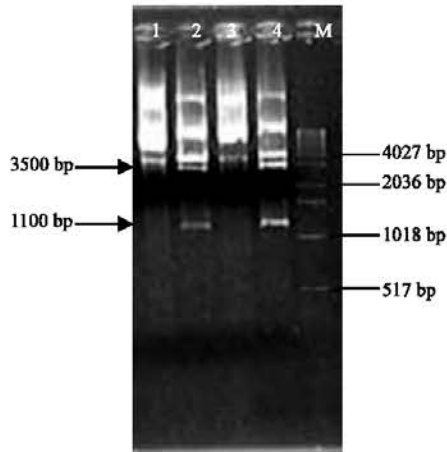


Fig. 3: Digestion of entry clones by BglIII restriction endonuclease on 0.7% agarose gel. Lane 1: Undigested entry clone 1, Lane 2: Digested entry clone Lane, Lane 3: Undigested entry clone 2, Lane 4: Digested entry clone 2. Lane M: DNA Molecular weight Marker (0.07-12.2Kbp)

electrophoresis. As it is shown in Fig. 6B many protein bands observed in the gel which some of them were H specific proteins as appeared by immunoblot (Fig. 6A).

Two species of H protein appeared to be synthesized: 80 and 60 kD which probably were glycosylated and non-glycosylated forms of H protein respectively. Lower molecular weight bands were most probably degradation products of the H protein.

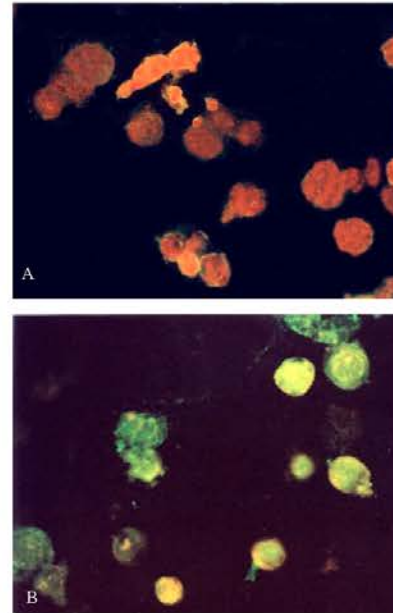


Fig. 4: Immunofluorescence of uninfected (A) and Infected *Sf9* Cells with recombinant baculovirus harboring H gene of Measles Virus (B)

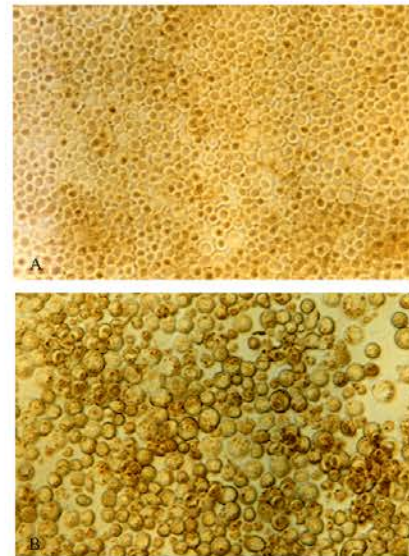


Fig. 5: Uninfected (A) and infected *Sf9* cells with recombinant baculovirus harboring H gene of Measles Virus (B). The infected cells in B showed CPE as rounding and detachment from the surface

At least 9% of the total stained proteins were H proteins as was shown by densitometric scanning of the electrophoretic gel (data not shown). Production of H gene products in insect cells remained stable even after four passages of the purified recombinant virus in culture.

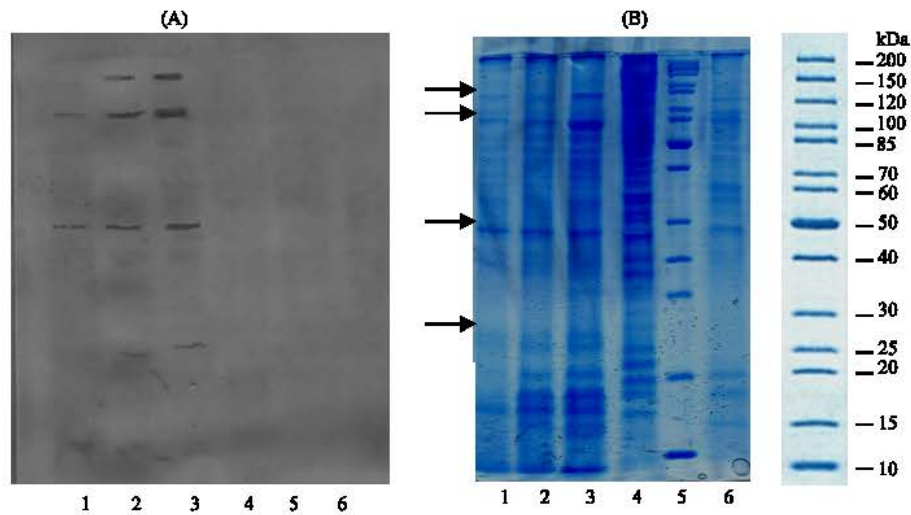


Fig. 6: Immunoblot (A) and Coomassie blue-stained gel (B) of total proteins produced in *Sf9* cells infected with either wild-type or recombinant H virus. *Sf9* cells were infected with non recombinant or recombinant virus containing the H gene of measles virus. Total proteins were solubilized by lysing the cells in sample buffer at 120 h postinfection after 2, 3 and 4 passage (Lane 1, 2, 3 respectively). Lane 4: Uninfected *Sf9* cell proteins. Lane 5: Low molecular weight protein marker. Lane 6: Non-recombinant baculovirus proteins. Immunoblot assay prepared from a gel which was a duplicate of the one shown in panel B. The immunoblot was obtained by electrophoretic transfer of proteins from gel to nitrocellulose. The H recombinant protein products are indicated with arrows

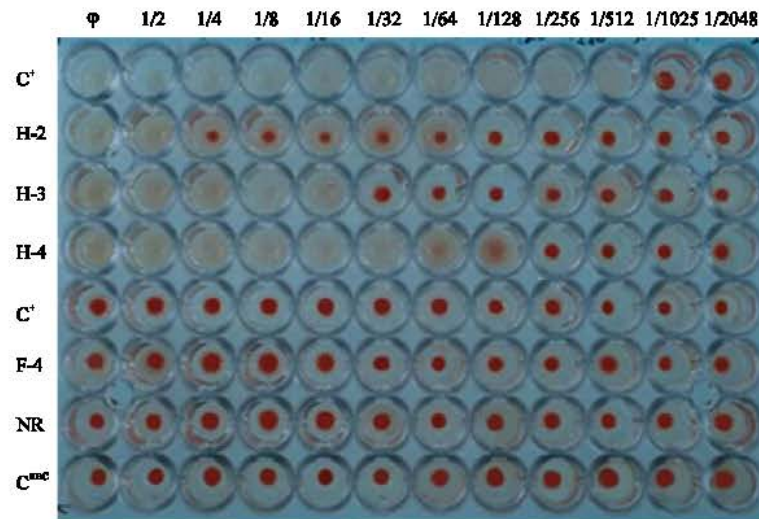


Fig. 7: Hemagglutination test in different passage of recombinant baculovirus containing H gene of Measles Virus. C⁺ Edmonston Strain of Measles Virus. H2, H3, H4: HA of *Sf9* cells from different passage of recombinant baculovirus containing H gene. C⁻: HA in uninfected *Sf9* cells. F: HA in *Sf9* cells extract that infected by recombinant baculovirus containing F gene of Measles Virus. NR: *Sf9* cells extract that infected by non-recombinant baculovirus. C^{RBC}: Control of Red Blood Cells

Biological activity of recombinant H protein:
Recombinant H protein was biologically active in hemagglutination assay. Hemagglutination of African

Green Monkey erythrocytes by *Sf9* cells extract which contained recombinant H protein was evident and a reciprocal dilution of 2, 16 and 128 was obtained after

two, three and four passages of recombinant baculovirus (Fig. 7). Partial hemadsorption was also observed on the surface of infected *Sf9* cells (data not shown).

Surface antigens of measles are the main targets for recombinant expression to develop recombinant vaccines. The simplest method is expression of proteins in prokaryotes. However, the lack of proper conformation and post-translational changes such as glycosylation restricts application of these systems to research (Hu *et al.*, 1994). An alternative method of expression systems for measles virus proteins is mammalian system using virus expression systems like vaccinia virus (Kidokora *et al.*, 2002), canarypox (Taylor *et al.*, 1992) and Semliki forest virus (Bouche *et al.*, 1998a). Major drawback for virus vectors in mammalian expression system is high cost and more importantly, safety issues regarding unexpected consequences of these systems on human body, but can be applied to diagnostic procedures (Bouche *et al.*, 1998b). Insect cell expression systems are promising alternatives in search for a suitable expression vectors. There are only two other attempts in producing glycoprotein H in insect cell expression system. Vialard and coworkers used β -galactosidase gene for screening recombinants baculovirus containing measles H protein. In their system consequent plaque purification was required (Vialard *et al.*, 1990). The use of β -galactosidase gene for screening baculovirus recombinants has previously been developed by Kidokora *et al.* (2002). This approach lacks required criteria as a candidate for vaccine developments. The large β -galactosidase-fused protein will initiate production of undesired antibody development without offering any significant advantage in downstream processing of the recombinant protein, namely purification of the H protein. Takehara *et al.* (1992) used traditional baculovirus vectors in order to construct an expression vector for recombinant measles proteins. To examine the expression and purification efficiency of the newly developed expression system, we sought recombinant expression and activity of H protein of measles using BaculoDirect™ system. In this system the resulting recombinant baculovirus DNA is transfected directly into insect cells to generate recombinant virus. In another word, cloning and expression of desired gene was performed without plaque purification or selection in bacteria., thus facilitating its faster screening (Invitrogen). In this study, the 1950 bp fragment resulted from RT-PCR was used to construct a modified baculovirus with two defined recombination sites. *Sf9* cells transfected with the newly synthesized recombinant baculovirus containing the measles virus hemagglutinin gene, were shown to synthesize the H protein under control of the polyhedrine promoter. Advantage of our approach compared with previous attempts is our high yield of recombinant protein

with similar authentic viral protein as was shown in immunofluorescent and western-blot experiments. In comparison with the prokaryotic expression system, the post-translational modification of H protein has been reported to occur (Hu *et al.*, 1994). On the other hand due to presence of the His-tag at the c-terminal of the recombinant H protein, purification of this protein was improved compared to conventional methods (Lund and Salmi, 1981).

According to present results the antigenic sites were present in the recombinant H protein. Levels of protein expression were higher than previous studies (Takehara *et al.*, 1992), since H protein could be detected on SDS-gel stained with coomassie blue. The highest level of protein expression was observed at day 5 post infection. Although, the H membrane protein was shown to be only partially glycosylated, it could still agglutinate the African Green Monkey red blood cells indicating that it was biologically active.

In conclusion, the above expression system developed in this study may be helpful for the investigation of the function and structure of the target protein.

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