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Original Article

Expression of Antigenic Determinants of the Haemagglutinin Large Subunit of Novel Influenza Virus in Insect Cells

(haemagglutinin / Bacmid / baculovirus)

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Abstract. The global outbreak of novel A/H1N1 spread in human population worldwide has revealed an emergency need for producing a vaccine against this virus. Current influenza vaccines encounter problems with safety issues and weak response in high-risk population. It has been established that haemagglutinin is the most important viral antigen to which antibody responses are directed, and recombinant subunit vaccines, haemagglutinin of influenza A and B viruses, have been considered in order to facilitate vaccine production. In the present study, we have focused on construction of a recombinant baculovirus encoding the large subunit of novel influenza virus A/H1N1 haemagglutinin. The full genome of haemagglutinin was cloned into pGEM-TEasy vector and sequenced. The large subunit of the haemagglutinin gene was amplified by PCR using specific primers and cloned into pFastBacHTc donor plasmid, which was then confirmed by restriction enzyme analysis and sequencing and transformed into *E. coli* DH10Bac competent cells. The bacmid DNA was transfected into insect cells to produce recombinant baculovirus. Expression of recombinant haemagglutinin in insect cells was determined by SDS-PAGE and immunoblotting. It has been shown that the recombinant haemagglutinin (rHA) obtained from the baculovirus insect cell expression system has suitable immunogenicity in human and can be considered as a candidate flu vaccine. Here we produced large amounts of the HA1 protein of novel influenza A/H1N1 (Iranian isolate) in insect cells. The immunogenicity and efficacy of the recombinant HA1 will be evaluated as a vaccine candidate and compared to the recombinant HA1 produced in a prokaryotic system.

Introduction

Influenza virus A is one of the orthomyxoviridae family members and includes eight segmented negative sense RNAs encoding external proteins (HA, NA), transmembrane protein (M_2) and internal proteins (PB_2, PB_1, PA, NP, M, NS) (Webster et al., 1992). Influenza A is classified into diverse subtypes based on the differences in surface glycoproteins, HA (16 subtypes) and NA (9 subtypes) (Munster et al., 2007). The number of HA subtypes which present in aquatic avian reservoir is higher than in other species such as horse (H1, H7) and pig (H1, H3) (Peiris et al., 2009). The influenza virus A subtypes that caused pandemic flu with considerable mortality and morbidity in 20th century were H1N1 (Spanish flu, 1918), H2N2 (Asian flu, 1957) and H3N2 (Hong Kong flu, 1968) (Hsieh et al., 2006) and a novel strain of influenza A (novel A/H1N1/2009) caused the first influenza pandemic in the 21th century (Kang et al., 2010). Novel A/H1N1 was primarily isolated from Mexico and USA, and then spread in human population worldwide (Kang et al., 2010). This virus came into existence through combined components of human, bird and swine H1N1 influenza viruses (Rambaut and Holmes, 2009; Shinde et al., 2009; Trifonov et al., 2009). From then until March 2010, almost all countries had reported confirmed cases and more than 17,700 deaths to WHO (Bautista et al., 2010). The first confirmed case from Iran was reported by Gooya et al. (2010).

In some cases, novel A/H1N1 has similar symptoms to seasonal H1N1 influenza and swine H1N1 influenza, but different epidemiological features. The virus caused

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Abbreviations: CPE – cytopathic effect, rHA – recombinant haemagglutinin.

severe disease leading to death in young adults (Kang et al., 2010).

Novel A/H1N1 has antigenic similarities to classical swine H1N1 virus and 1918-like virus. Classical swine H1N1 and 1918-like vaccines induced neutralizing antibodies that protected C57B/6 mice from lethal challenge with novel A/H1N1 (Manicassamy et al., 2010).

Among viral proteins, haemagglutinin is the most crucial envelope glycoprotein and very important component of flu vaccines (Skehel and Wiley, 2000; Xu et al., 2010). HA contains three monomers, each composed of globular head (HA1) responsible for binding to sialic acid by 2-6 α and 2-3 α receptors; and stalk (HA2). HA of this novel virus has a relative lack of glycosylation sites on the globular head (HA1) near antigenic region, like 1918 isolates and circular viruses in the early 1940s. Previous study suggested that lack of HA glycosylation affects pathogenesis of the novel A/H1N1 and helps it to be more virulent in the lung (Reichert et al., 2010).

The worldwide outbreak of novel A/H1N1 emphasizes the emergency requirement to produce a vaccine against this virus. Several studies about confronting the novel A/H1N1 have been done and a number of protective ways were suggested (Quan et al., 2007).

In the present study, novel H1N1 haemaglutinin was isolated from a confirmed clinical sample and cloned into the baculovirus expression system to produce recombinant HA1 in Sf9 insect cells. The antigenic determinants of the expressed protein were determined using immunoblotting assay. In our ongoing project we will further evaluate the biological activity, immunogenicity and protective efficacy of the recombinant HA1 in animal models as a vaccine candidate.

Material and Methods

HA gene extraction, cloning and sequencing

The clinical throat swab samples, in which the presence of swine influenza genome had been confirmed by real-time PCR according to the WHO protocol (Poon et al., 2009), were used as templates to isolate the *HA* gene. To amplify the HA full genome, one-Step RT-PCR procedure was carried out using Superscript® III RT/Platinum®Taq enzyme mix (Invitrogen, Darmstadt, Germany) and specific primers according to the recommendation of Chan et al. (Chan et al., 2006). The PCR product was cloned into the pGEM-TEasy vector (Promega, Madison, WI) and confirmed by sequencing.

HA1 gene PCR and cloning process

The large subunit of haemagglutinin gene was amplified by PCR using specific *HA1* forward 5'GCGAATTC-AATGAAGGCAATACTAG 3' (CinnaGen, karaj, Iran) and reverse 5'CACTCGAGCTACAGTCTCAATTTTG 3' (CinnaGen, karaj Iran) primers. The restriction enzyme sites for *Eco*RI and *Xho*I (Fermentas, Burlington, Canada) were considered at 5′ ends of the forward and reverse primers, respectively, as underlined. The thermocycling profile was 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 56 °C for 45 s and 72 °C for 2 min followed by the final extension at 72 °C for 10 min. Pfu DNA polymerase (Fermentas) was used as replication enzyme, as it exhibits the lowest error rate (Cline et al., 1996). Amplified *HA1* DNA was resolved on 1% agarose gel (CinnaGen, karaj, Iran), purified with Qiaquick PCR purification kit (iNtRon, Daejeon, South Korea) and subcloned into pFastBacHTc (Invitrogen, Darmstadt, Germany) donor plasmid after digestion with appropriate enzymes. The recombinant vector was confirmed by PCR, digestion, and finally sequencing.

The recombinant donor plasmid was transformed into the *E. coli* DH10Bac (Invitrogen) competent cells containing baculo-derived bacmid DNA. The recombinant bacmid was constructed by transposing between the mini-Tn7 element on pFastBac-HA1 and the att Tn7 attachment site on the bacmid. The transformants were plated onto LB agar (Himedia Laboratories, Vadhani Ind. Est., India) containing kanamycin (50 $mgml^{-1}$, Sigma-Aldrich, Steinheim, Germany), gentamicin (7 $mgml^{-1}$, Sigma-Aldrich), tetracycline (10 $mgml^{-1}$, Sigma-Aldrich), X-gal (100 $mgml^{-1}$, Sigma-Aldrich) and isopropylthio-b-galactoside (40 $mgml^{-1}$, Sigma-Aldrich) and incubated at 37 °C for 48 h. The recombinant bacmid was isolated from the overnight cultures by alkaline lysis purification and confirmed by PCR using M13 (CinnaGen, karaj, Iran) and specific *HA1* primers (Hitchman et al., 2009). The bacmid DNA was run on 0.7% agarose gel for 18 h/overnight.

Transfection and baculovirus production

Insect Sf9 cells were cultured in Grace's medium (Gibco, Karlsruhe, Germany) supplemented with 12% foetal bovine serum (FBS, Gibco) and incubated at 27 °C. Recombinant baculovirus was constructed by transfecting the recombinant bacmid to Sf9 monolayer cells using Cellfectin (Invitrogen) according to manufacturer's protocol and monitored daily until the cytopathic effect (CPE) appeared. The recombinant baculovirus was harvested from the cell culture medium at 72 h post transfection and stored at 4 °C or used for inoculating more Sf9 cells to amplify the virus stock.

Protein expression and determination

To produce the HA1 protein, the Sf9 cells were inoculated with the recombinant baculovirus and incubated at 27 °C for 96 hours. When CPE was completed, the cell pellet was harvested by centrifugation (11.5 *g*, 5 min) and washed three times with cold phosphate-buffered saline (pH 7.4). Expression of recombinant haemagglutinin was evaluated using SDS-polyacrylamide (Sigma-Aldrich) gel electrophoresis and immunoblotting as described previously (Calandrella et al., 2001). Briefly, the cell pellet was frozen and thawed three times and sonicated using an ultrasonicator (UIS250V) at 60% power three times for 30 s with 90 s intervals, and then suspended in lysis buffer (50 mM Tris-HCl (Sigma-Aldrich), pH 8.5, 5 mM 2-mercaptoethanol, 1 mM phe-

nylmethylsulphonyl fluoride, 100 mM KCl (Sigma-Aldrich)) and boiled for 5–10 min. Total cell proteins were separated on 12% SDS-PAGE and transferred onto nitrocellulose membrane (0.45 μm, S&S Bioscience GmbH, Whatman group, Dassel, Germany) using semi-dry electrotransfer (Applex, 016932). The blotting was performed in transfer buffer (10% methanol (Dr. Mojallali, Tehran, Iran), 24 mM Tris (Sigma-Aldrich), 194 mM glycine (Sigma-Aldrich), pH 8) at 10 V for 30 min. Nonspecific binding was blocked by incubation of the membrane in blocking solution (2.5% bovine serum albumin (Roche, Mannheim, Germany) in phosphate-buffered saline) for 1 h at room temperature. The blotted protein was reacted with primary antibody specific to novel HA (Swine-Origin Influenza A H1N1 Haemagglutinin Antibody, ProSci, Sushou, Jiangsu, China). Goat anti-rabbit antibody (Sigma-Aldrich) conjugated with HRP was used as secondary antibody. The TMB substance solution (Sigma-Aldrich) was added to visualize reacted protein bands. The Sf9 cell pellet and Page ruler (pr0602, Vivantis, Subang Jaya, Malaysia) were used in parallel as negative control and molecular weight ladder, respectively.

Results

HA isolation and sequencing

The full genome of novel influenza A/H1N1 from clinical samples was amplified by PCR using specific primers and the expected 1778 bp segment PCR product was cloned into pGEM-TEasy vector (Promega) and sequenced. Analysis of sequencing was accomplished by Chromas software (version 1.45 – Australia) and the nucleotide sequence data was deposited in GenBank database under the accession number: "HQ419001.1".

Construction and verification of Bacmid-HA1

The *HA1* gene fragment was obtained from the HA full genome using specific primers. The PCR product was assessed by running on 1% agarose gel (not shown) and cloned into pFastBacHTc between *Eco*RI and *Xho*I restriction sites. The recombinant plasmid was confirmed by digestion (Fig. 1) and sequencing. Analysis of sequencing revealed the correctness of PCR amplification and cloning of the gene of interest downstream of polyhedrin promoter and in frame with 6xHis tag.

Following transformation of *E. coli* DH10Bac with the pFastBacHTc-HA1 plasmid, the transposed colonies were visible on agar as large white colonies among the blue ones containing the non-recombinant bacmid. The bacmid-HA1 was confirmed through PCR using specific primers and/or M13 primers. The bacmid DNA contains M13 forward and reverse priming sites flanking the mini-att Tn7 site within the LacZ a-complementation region. PCR was done using M13 forward and reverse primers for 3400 bp PCR product (Fig. 2), *HA1*-specific forward primer and M13 reverse primers for 1600 bp PCR products, *HA1*-specific reverse primer and M13-forward primer for 2600 bp PCR products (not shown). Non-recombinant bacmid amplification using M13 primers resulted in a 300 bp fragment (Fig. 2).

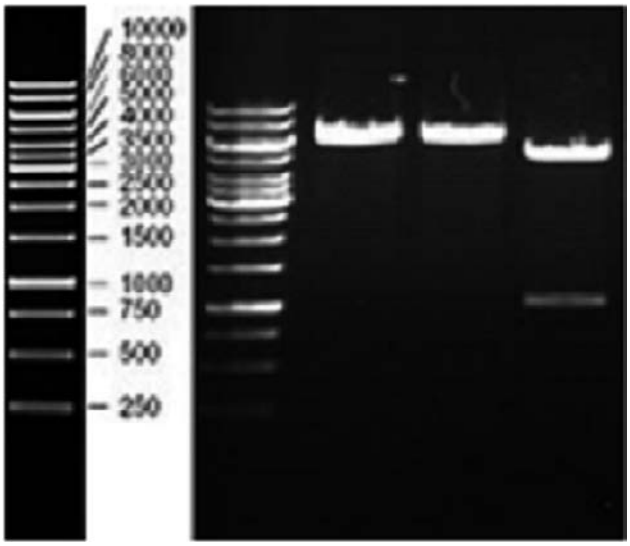

Fig. 1. Restriction enzyme analysis of pFastBacTHc-HA1; from left to right: 1 kb gene ruler; linear plasmid digested with *Eco*RI; linear plasmid digested with *Xho*I; double-digested plasmid.

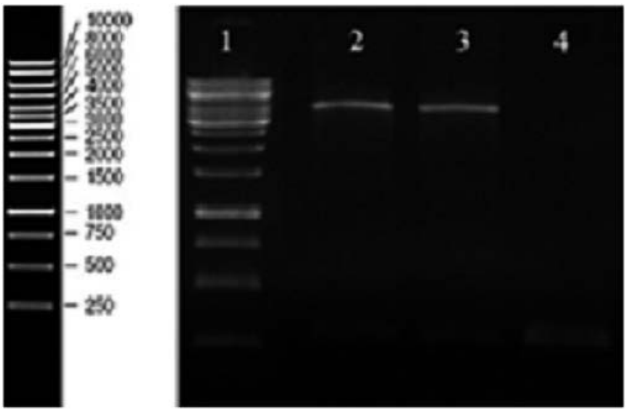

Fig. 2. Confirmation of recombinant bacmid DNA by PCR using M13 primers. Lane 1, 1 kb gene ruler; lanes 2 and 3, DNA of 3,400 bp PCR product of recombinant bacmids; lane 4, DNA of 300 bp PCR product of non-recombinant bacmid

Transfection and protein expression

The recombinant baculovirus was constructed by transfection of recombinant bacmid in Sf9 monolayer cells using Cellfectin reagent (Fotouhi et al., 2008). Three days post-transfection, the cells stopped division and displayed typical CPE, i.e. swollen cells and peripheral nuclei. The healthy control cells continued to divide and form a confluent normal cell monolayer, and wild-type baculovirus-inoculated cells showed visible and specific inclusion bodies (Fig. 3). After three generations, the recombinant baculovirus harbouring the *HA1* gene of novel influenza virus was collected and used as virus stock to express recombinant haemagglutinin protein.

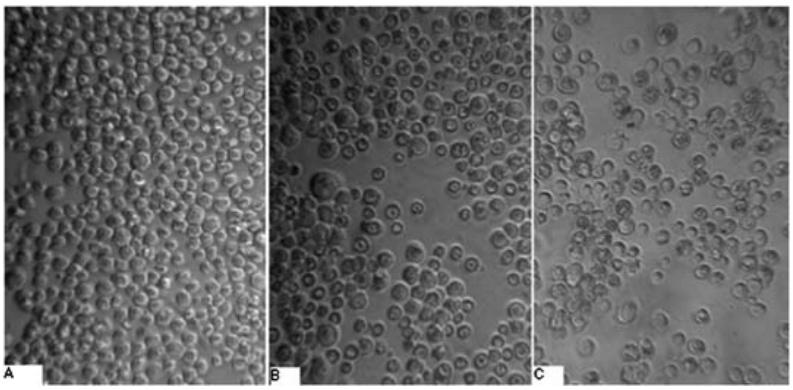

Fig. 3. Sf9 cell appearance (a): uninfected cells; (b): cells infected with recombinant baculovirus; (c): cell infected with wild-type baculovirus

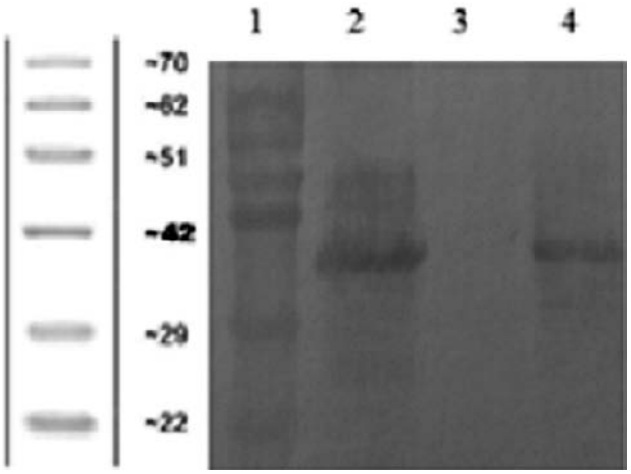

Fig. 4. Western blot analysis of HA1 expressed in Sf9 cells. Lane 1: protein marker with relative molecular weights indicated. Lanes 2 and 4: lysates from Sf9 cells infected with recombinant baculovirus. Lane 3: lysate of the uninfected cells.

The Sf9 cells were inoculated with the recombinant baculovirus (MOI around 5) and incubated at 27 °C for 96 h. SDS-PAGE of total protein followed by immunoblotting using polyclonal anti-HA1 antibody demonstrated one polypeptide band with the predicted MW of about 35–40 kDa (Fig. 4).

Discussion

The novel influenza virus (A/H1N1) was responsible for the 2009 flu pandemic. This virus is a sort of triple re-assortment between swine, bird and human viruses (Shinde et al., 2009; Smith et al., 2009). It has been confirmed that haemagglutinin is the most important viral antigen to which antibody responses are directed.

Nucleotide and phylogenetic analysis of novel A/H1N1 haemagglutinin revealed that the novel virus has closer correlations with the swine-origin H1N1 subtypes than other H1N1 viruses (Ahn and Hyeon, 2010).

Influenza disease caused by the novel H1N1 is of moderate severity but differs from seasonal flu. Pathological and immunological data showed that those over 60 years are rather out of danger, likely due to the presence of cross-neutralizing antibodies (Peiris et al., 2009).

The presence of cross-neutralizing antibodies in human sera against different H1N1 viruses was evaluated by Ikonen et al. (2010). They applied HI test for sera collected from individuals born between 1909 and 2005 and found out that more than 96 % of persons born between 1909 and 1919 had antibodies against the 2009 pandemic virus. Surprisingly, most cases who were younger than 67 (born after 1944) lacked these antibodies. Sequence analysis of the *HA* gene of the pandemic virus revealed close relation to that of the Spanish and 1976 swine influenza viruses. Due to different changes occurred on the surface of the HA molecule in antigenic epitopes, especially near sialic acid receptor binding sites, seasonal H1N1 viruses did not induce considerable protective immunity against 2009 H1N1 infection (Ikonen et al., 2010). Strains of the 2009 H1N1 viruses are antigenically homogeneous, and the A/California/7/2009 strain that was selected for pandemic influenza vaccines worldwide is antigenically similar to nearly all isolates that have been examined to date (Bautista et al., 2010).

In this study, novel H1N1 haemaglutinin was isolated from a confirmed H1N1 clinical sample, cloned to pGEM-TEasy vector and sequenced. The result of sequencing was well-matched with other studies and revealed about more than 99 % identity with the A/California/7/2009 HA sequence. Based on HA amino acid sequences, Kilander et al. (2010) reported that substitution of D to G in position 222 (D222G) is considerably frequent in fatal and severe cases in Norway. Antigenic site studies predict this substitution to be located in the receptor binding site of the HA1 protein and it may affect the cellular tropism of the virus and could make a variety of cells in the lower respiratory tract more accessible for the novel virus. The HA sequence used in this study had D in 222 position, although our data about the patient's clinical status is incomplete.

The global outbreak of novel A/H1N1 emphasizes the emergency need for producing a vaccine against this virus. The protective ability of the live attenuated 2009

H1N1 vaccine and a seasonal trivalent inactivated vaccine (s-TIV; B/Florida/4/2006, A/Brisbane/59/2007 [H1N1], A/Brisbane/10/2007 [H3N2]), or live attenuated influenza vaccine (s-LAIV; B/Florida/4/2006, A/South Dakota/6/2007 [H1N1], A/Brisbane/10/2007 [H3N2]) were compared against the novel A/H1N1 infection in mice and ferrets. It was revealed that unlike the live attenuated 2009 H1N1 vaccine, the seasonal influenza vaccines did not create complete protection against 2009 pandemic infection in any of the experimental animals (Chen et al., 2011).

Production of current influenza vaccines, such as live attenuated virus and conventional inactivated virus, is confronted with some safety, efficacy and technical issues (Quan et al., 2007). Recombinant subunit vaccines containing HA of influenza A (H3N2 and H1N1) and B viruses have been considered as alternative vaccines to overcome some of the drawbacks (Safdar et al., 2006). Vander Veen and his colleagues developed a novel A/H1N1 vaccine based on recombinant HA using an alphavirus replicon expression system, which was determined as an effective and protective swine vaccine in pigs (Vander Veen et al., 2009).

Since the mid 1980s, insect cell baculoviruses have been extensively used for production of recombinant proteins (Fotouhi et al., 2008; Hitchman et al., 2009). Different baculo-derived recombinant HAs expressed in insect cells have been investigated as flu vaccines in some clinical trials and their immunogenicity in human was confirmed (Treanor et al., 2001, 2007).

In our previous study, the large subunit of influenza virus A/New Caledonia 20/1999/H1N1 haemagglutinin comprising 333 amino acids was expressed in a prokaryotic system (Farahmand et al., 2011) and in insect cells (not published). The protein expression was evaluated by Western blotting using specific monoclonal antibodies. As expected, the protein of interest expressed in the prokaryotic system has a molecular weight about 35 kD, while in insect cells an estimated 45–50 kD protein band corresponding to the glycosylated protein was observed.

In the present study, the HA1 of novel A/H1N1 (1-333) was expressed in insect cells and probed with specific polyclonal antibody to determine its immunological activity. Study of glycosylation sites and epitope mapping using computer software predicts that the novel H1N1 HA is similar to the 1918 Spanish flu virus. The result of Western blotting in this study was consistent with the predicted molecular weight (about 40 kD).

In our ongoing project, the immunogenicity and efficacy of recombinant HA1 produced in insect cells will be further evaluated as a vaccine candidate and compared to the recombinant HA1 produced in a prokaryotic system.

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