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Original Article

Baculoviral Expression of Influenza A Virus (H1N1 New Caledonia) Neuraminidase in Insect Cells

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Abstract

Background and Aims: Each year, the influenza virus causes moderate to severe infections with a high prevalence throughout the world. Accordingly, an influenza vaccine that ensures protection with only a single dose would be a much more cost effective approach to influenza prophylaxis. Generation of Influenza non-replicating virus-like particles (VLP) in baculoviral expression system is an attractive method for achieving this goal. One of the main components of such particles is Neuraminidase surficial glycoprotein that has important role to elicit humoral and cellular immune responses.

Materials and Methods: In this study, the NA coding region amplified from the human influenza virus [A/New Caledonia 20/1999/ (H1N1)] was used to construct the NA recombinant bacmid into *E.coli* DH10Bac cells.

Results: After verification of the new recombinant bacmid, it was transfected into the Spodoptera frugiperda (Sf9) insect cell line to generate recombinant baculovirus expressing the NA gene. The expression of NA in insect cells was confirmed by SDS-PAGE and western blot analysis.

Conclusion: The recombinant baculovirus expressing the NA gene can be used in construction of influenza VLP when co-infect along with the other monocistronic baculoviruses expressing influenza Hemagglutinin and Matrix antigens. Moreover, the NA protein expressed in insect cells might be fully glycosylated and therefore is appropriate to be use in influenza vaccinology projects.

Keywords: Influenza virus; SF9 cells; Baculovirus; Neuraminidase

Introduction

Influenza A virus belongs to the orthomixovirideae which is classified based on the antigenic properties of their two distinct surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA). To date sixteen HA subtypes and nine NA subtypes have been recognized serologically. Influenza A viruses are highly contagious pathogens that are able to infect a large variety of animals as well as humans. Influenza A viruses because of their segmented RNA genome and zoonotic nature are able to maintain or increase their epidemic or pandemic disease potential through mutation of antigenic determinants (antigenic drift) and by exchange and reassortment of one or more entire gene fragments (antigenic shift) between different virus strains. This rapid evolution in

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Influenza genome causes the need of annually updating vaccine formulations to include new viral antigens (1).

The disease is seasonal and occurs in cold regions during the winter, but can also appear in tropical areas during rainy seasons. The influenza pandemic is not limited to seasons and can occur during any time of the year. The major epidemiological health approach towards preventing the disease and improving outcome is by using vaccination individuals with inactivated virus prepared from A and B influenza viruses that are expected to prevail that year. Importantly, most approaches consider the license for humans in the production of a subunit or inactivated vaccine. In addition, the creation of a vaccine with the least side effects for human is emphasized major goal of vaccine design. Early studies have shown that immune protection against influenza virus infection is primarily mediated by antibody responses against the HA, although antibodies to the neuraminidase protein can also be protective alone in animal challenge studies in a different manner than antibodies to the hemagglutinin protein. (2).

A number of new influenza vaccine strategies, including cell-culture derived inactivated virus vaccines, DNA vaccines, recombinant subunit vaccines, influenza virosomes and immunostimulatory complexes, as well as virus-like particles (VLP) are being explored and evaluated (3,4). Because of high immunogenity, and being able to stimulate both the humoral and cellular arms of the immune system, many researchers have focused on VLP approach. The recombinant baculovirus and insect cell expression system provides high levels of recombinant protein that undergoes post-translational modification like glycosylation (5, 6). Therefore, application of such system allows large quantity production of a desirable protein, in native configuration as a vaccine.

In this study NA gene was isolated from Influenza A (H1N1 New Caledonia) and amplified and then a clone of NA recombinant bacmid was constructed. This construct as a monocistronic baculovirus can be used to produce influenza VLPs when co-infects along with the other monocistronic baculoviruses expressing structural proteins such as HA, M1 and M2. NA recombinant baculovirus also can be used to produce large scale of NA protein in insect cells, suitable for influenza vaccine research.

Methods

Cell culture and virus propagation

Madin-Darby Canine Kidney (MDCK) epithelial cells were cultivated in DMEM supplemented with 5% fetal calf serum (FCS). The cells were infected with the H1N1 strain of influenza virus, New Caledonia and the virus titer was determined using a hemagglutinin test as previously described (7).

Amplification of NA gene by RT-PCR

Total RNA was extracted from 250 µl of the supernatant of infected MDCK cells, containing 512 HAU/ ml (Hemagglution Unite) using the RNX^{TM} kit (CinnaGen, Iran). To perform RT-PCR, 50 µl of the extracted RNA was first converted into cDNA using the reverse transcriptase enzyme and random hexamer primer (8).

PCR was performed to amplify the NA gene using High fidelity Taq polymerase and specific primers designed as below:

NA1 Forward:
5'CGGTCGACTTTAAAATGAACCCAAAT C3', NA1 Reverse:
5'ATCTCGAGCTACTTGTCAATGGTGAAC 3'. The underlined bases represent the XhoI and SalI restriction enzyme sites, respectively. Amplification reaction was performed in a thermocycler (Master Cycler, Eppendorf) under the following profile: 3 min at 95°C followed by 30 cycles at 95°C for 1 min, 50°C-58°C (gradient) for 1 min and 72°C for 1 min, with a final extension step at 72°C for 10 min. PCR products were analyzed using 1% (w/v) agarose gel electrophoresis stained with ethidium bromide and purified by the clean up PCR product kit (Fermentas, Lithuania).

Recombinant bacmid construction

The first step towards the production of the recombinant baculovirus is cloning the target gene in the plasmid donor pFastBac1. XhoI and SalI restriction enzymes were used to

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digest the pFastBac1 and NA amplicones. The digested PCR product was purified using a DNA purification kit (Fermentas, Lithuania), and then the digested NA gene was ligated into the pFastBac1 vector using T4 DNA ligase. The recombinant pFastBac1/NA plasmid was confirmed with restriction map and sequencing. Then it was transformed into the *E.coli* DH10Bac cells. In this host the NA segment from the pFastBac1/NA donor vector entered the bacmid through homologous recombination resulting in the NA recombinant bacmid creation. Following the transformation, the white and blue colony screening method was used to identify positive clones and PCR was carried out to confirm NA recombinant bacmid. In bacmid structure the M13 universal sequences flanks both sides of the transposon region in LacZ so the direct and reverse M13 primers were used to identify recombinant bacmids from the nonrecombinant ones (9).

Transfection of Sf9 cells to produce recombinant NA baculovirus

The Spodoptera frugiperda Sf9 insect cell line was cultured in Grace's media (Gibco) supplemented with 10% fetal bovine serum (Gibco) to 60% confluency. The monolayer then transfected with the pFastBac1/NA recombinant bacmid using Cellfectin reagent according to the manufacturer's instructions (Invitrogen, USA). The transfected cells were incubated at 27 ºC for 72 hours. Recombinant baculovirus production was monitored daily by visualization of the cytopathic effects (CPE). The culture medium was collected, clarified by centrifugation at 1000 rpm and subjected for multiple rounds of virus propagation.

Expression of recombinant protein

The cultured Sf9 cells were inoculated with recombinant baculovirus at a MOI of 10 and incubated at 27ºC for 96 h. The cells (pellet) were collected after centrifugation. To prepare cell lysate, the pelleted cells were washed three times with cold PBS, resuspended in cell lysis buffer (50 mM Tris-HCl, pH 8.5, 5 mM 2-mercaptoethanol, 100 mM KCl, 1 mM PMSF) and sonicated three times for 10 sec by 3 min intervals, at 50% power using ultrasonicator (UP 400s). After centrifugation at 12000 rpm for 15 min at 4ºC, the clarified supernatant was stored at -20ºC for analysis.

Determination of NA protein

The cell lysate of the Sf9 infected cells with recombinant baculovirus were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoreses (SDS-PAGE) and stained with coomassie brilliant blue. For further characterization, the separated proteins on SDS-PAGE were electrotransferred to nitrocellulose membrane The membrane were blocked at room temperature for 1.5h with 1% bovine serum albumin in Tris-buffered saline (20 mM Tris-HCl, 500 mM NaCl, pH 7.4) containing 0.1% tween 20 (TBS-T). The blotted proteins were reacted with anti-NA monoclonal antibody (SinoBiological-China) diluted 1:200 in TBS-T for 2h at room temperature. The appropriate secondary antibody, rabbit anti-mouse IgG conjugated with HRP (Sigma) diluted 1:2000 in TBS-T, was added and incubated for 2 h at room temperature. The protein bounds were visualized by staining the membrane with diaminobenzidine (Sigma) (10)

Results

Construction of the recombinant bacmid DNA

Figure 1 shows the amplification of full length NA gene (1420bp). The best annealing temperature for NA amplification was 50-52ºC. The fidelity of the NA open reading frame (ORF) in pFastBacNA clone was confirmed by sequencing. Analysis of sequencing was accomplished by Chromas software (version 1.45 - Australia). The Bac-to-Bac system was used In order to construct a bacmid expressing the NA gene (Invitrogene). This system possesses a donor plasmid known as pFastBac1 and a baculovirus shuttle vector known as bacmid that is located in *E.coli* DH10Bac as host. Following the transformation of pFastBac1NA and plating of the cells on agar, the transposed colonies were visible as large white colonies among the blue ones harboring the unaltered bacmid.

Since verification of the high-molecular weight recombinant bacmid DNA by digestion is not

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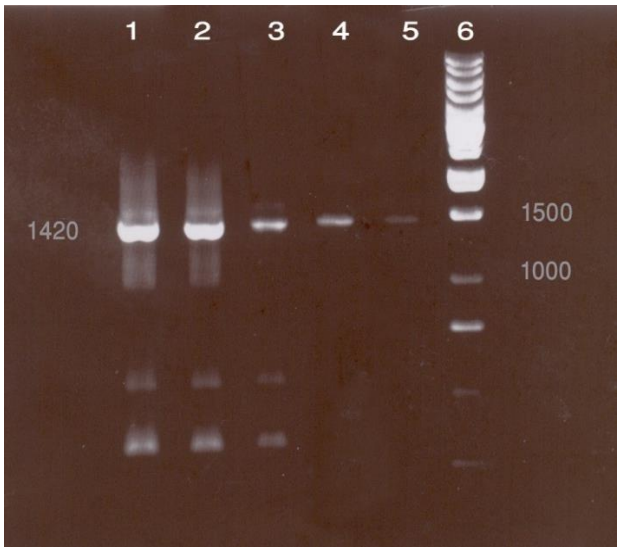


Fig. 1. Gel electrophoresis of Gradient PCR carried out to optimize annealing temperature for NA gene amplification (1420bp). Lanes 1-5, temperatures from 50°C- 58°C. Lane 6, 100 bp molecular weight DNA marker (Fermentas).

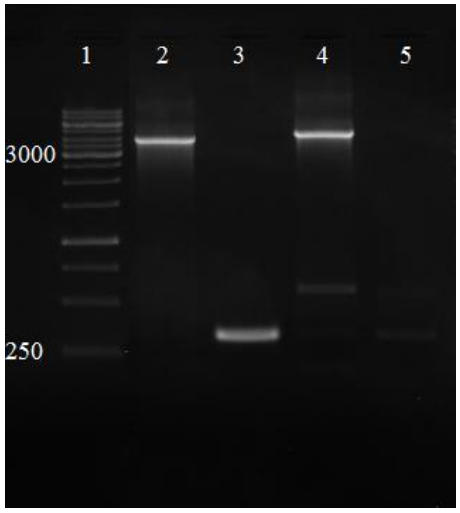


Fig. 2. A panel of PCR carried out to confirm the NA recombinant bacmid
Lane 1, 1Kbp molecular weight DNA marker (Fermentas).
Lane 2, PCR product using M13 specific primers on the NA recombinant bacmid extracted from white colony (3850 bp)
Lane 3, PCR product using M13 specific primers (303bp) on non-recombinant bacmid extracted from blue colony as a negative control.

convenient, PCR was performed using M_{13}/pUC and NA-specific primers to ensure proper transposition of the target gene in the recombinant bacmid. The bacmid DNA contains M_{13} forward and reverse priming sites flanking the mini-att Tn7 site within the LacZ α-complementation region. PCR was done using M_{13}/pUC primers on NA recombinant bacmid, Gus control bacmid and nonrecombinant bacmid as negative control. As respected, it resulted in the amplification of 3850 bp, 4200 bp and 303 bp bands respectively (fig. 2).

Transfection of Sf9 cells

The Sf9 cells were transfected with the isolated recombinant bacmid DNA using Cellfectin. The transfected cells displayed typical cytopathic effects, i.e. low cell density, division stop, enlarged cells and poor adherence to the substrate, indicating that the virus production was taking place. The mock-transfected Sf9 cultures continued to divide and form a confluent normal cell monolayer (fig. 3).

SDS-PAGE and western blot analysis

To analyze the NA recombinant protein, the Sf9 cells were infected with recombinant baculovirus and harvested after 72 h. Total protein of the cell lysates was visualized on 12% polyacrylamide gel and analyzed by western blotting using monoclonal anti-NA antibody. SDS-PAGE analysis demonstrated an exact polypeptide bands with predicted molecular weight of about 45 KDa representing the fully glycosylated forms of NA (fig. 4).

Discussion

The expression of eukaryotic proteins using baculovirus expression vectors takes advantages of their protein synthesis machinery and provides proper maturation and post-translational modifications including glycosylation, acetylation, and olygomerization. In addition, the insect cell cytoplasmic environment provides appropriate disulfide bridge assembly (11, 12). The first report concerning the use of a baculovirus expression system to produce neuraminidase

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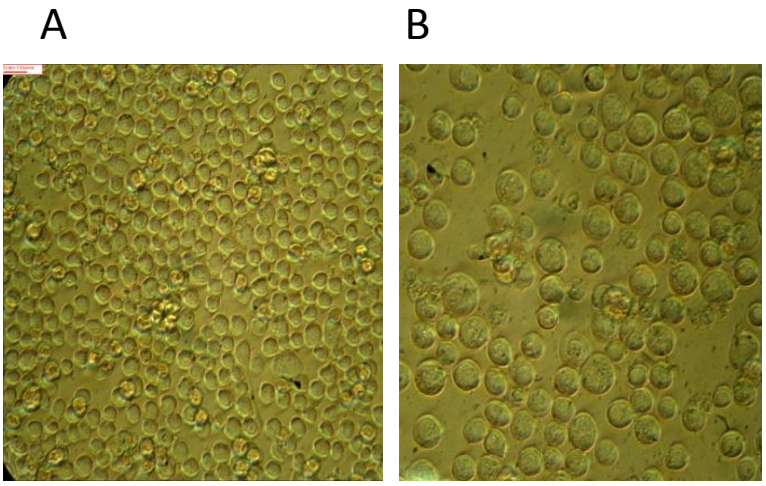


Fig. 3. Inverted microscopy images of Sf9 cells infected with recombinant baculovirus. Normal Sf9 insect cells (**A**); Sf9 insect cells after 72 h of transfection (**B**).

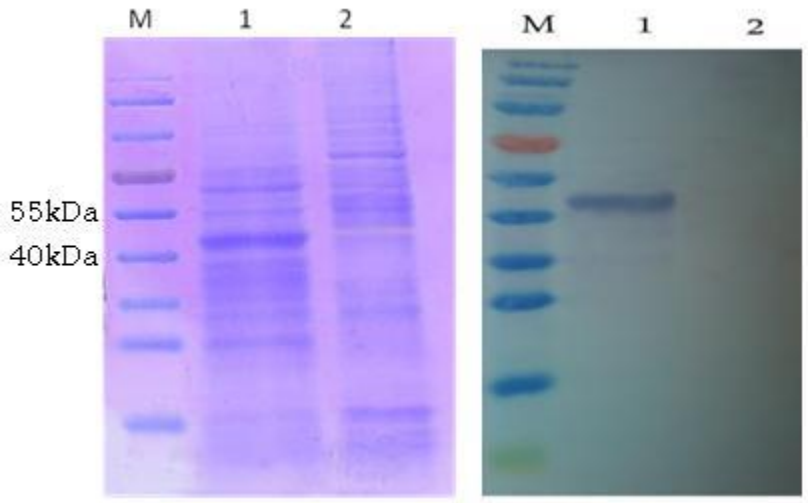


Fig. 4. SDS-PAGE and Western blot analysis of protein samples extracted from Sf9 cells infected by baculovirus recombinants (Lane 1) and uninfected Sf9 cells as control (Lane2). Lane M is protein marker (Fermentas).

recombinant protein was described in 1992 (13). Johnson and colleagues also showed that the neuraminidase protein expressed in this system is functional and estimated that its antigenicity is equal to the native antigen purified from the Influenza virus. It was shown that the NA recombinant antigen could evoke the immune responses without an adjuvant (14). The other growing field of research in influenza vaccinology is generation of Influenza A VLP. To date different systems has been used for VLP construction including recombinant DNA expression vectors (15), recombinant vaccinia viruses (16), and DNA plasmid transfection with T7 RNA polymerase expressing vaccinia virus (17). However, recombinant baculoviruses/ insect cell expression system yields high levels of recombinant proteins and more important, generated VLPs in such systems have been shown that are highly immunogenic able to elicit both neutralizing antibodies and cellular immune responses. Several research groups have considered the NA, as a main surficial glycoprotein, in combination of Influenza A VLPs (18, 19, 20, 21). In this respect, different

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expression strategies have been used to produce influenza proteins complexes in baculoviral expression system (22). While some groups have exploited the use of a single polycistronic baculovirus to generate self-assembling multi-protein complexes, the other researchers used the multiple monocistronic baculoviruses that each one expresses an individual specific envelope protein of influenza A virus. Therefore the recombinant baculovirus containing correct and in-frame sequence of Influenza NA gene under control of polyhedrin promoter constructed in this work can be co-infected with other individual monocistronic baculoviruses carrying HA and M1 proteins to produce influenza VLPs in insect cells. Moreover, the recombinant baculovirus constructed here can be exploited to generate large scale of functional NA protein in insect cells which after purification is applicable in several influenza vaccinology projects.

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