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Construction of Recombinant Baculovirus Expressing HA Protein of Egyptian H5N1 Avian Influenza Virus

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Abstract: Egyptian endemic status of Avian Influenza H5N1 accelerating the urgent need to develop potent H5N1 vaccine among other control measures. In the present study, site directed mutagenesis for the haemagglutinin (HA) gene of a recent Egyptian strain H5N1 AIV (A/chicken/Egypt/VRLCU/2012) was carried out by deletion of multi- basic cleavage site coding sequence using PCR assays. The mutated HA gene was cloned into the baculovirus transfer plasmid (pFastBac TM) to construct the recombinant pFastBac. Purified rpFastBac-HA was transformed into DH10Bac competent cells, transposed with a shuttle vector (Bacmid) to construct the rBacmid-HA which was identified by PCR, restriction digestion and sequencing. Sf-9 cells were transfected with rBacmid-HA and recombinant baculovirus was harvested. In vitro characterization of the expressed HA protein was carried out using hemadsorption assay (HAD), hemagglutination assay (HA), hemagglutination inhibition test (HI), Immunofluorescence assay, SDS- PAGE and western immunoblot. The expressed HA protein was identified in SDS-PAGE with approximately 63 KDa, which further confirmed by western blot assay using reference anti-H5N1 AIV serum. This study reports the successful expression of HA protein of a recently Egyptian H5N1 strain which will provide an effective tool for production of subunit vaccine candidates and diagnostic utilities.

Key words: Endemic · Rbacmid-HA · *Sf-9* · Characterization · Expression · Subunit Vaccine

INTRODUCTION

Highly pathogenic avian influenza virus H5N1 was spread widely and transmitted from domestic poultry to wild birds and mammals posing an increasing threat to humans [1]. The first outbreak of avian influenza H5N1 in Egypt was reported officially in February 2006 and declared endemic in July 2008 [2, 3]. The Egyptian H5N1 viruses continue to mutate and rapidly evolve over time [4, 5]. AI vaccination in endemic areas is becoming an essential tool for prevention and control especially in developing countries due to various economic considerations [6]. Several commercial inactivated H5 vaccines using different H5 virus strains were used during the H5N1 epidemic in Egypt [7]. However, failure of control the continuous H5N1 HPAI outbreaks in Egypt has been occurred [8]. The antigenic diversity of Egyptian H5N1 viruses and improper vaccination coverage may have played a role for vaccination failure in the fleld. As a result of the selective pressure, the HA tends to be mutated frequently resulting in mismatch between the vaccine and the circulating viruses [9]. Current influenza vaccines include; subunit vaccine, DNA vaccine and conventional inactivated vaccines [10-12]. Most avian influenza vaccines used in the field are of the inactivated type, which is propagated in embryonated chicken eggs but cannot provide satisfactory protection against influenza, induce little or no cellular immune response. In addition, egg based influenza vaccine production is dependent on the availability of embryonated eggs, which is difficult to be obtained during the outbreaks of avian diseases and require high level biosafety facility and a stable egg supply for antigen preparation [6]. Also, differentiating infected from vaccinated animals (DIVA) is difficult for conventional egg based vaccines. So, many influenza vaccine studies have been conducted on recombinant influenza virus proteins to overcome these problems [13]. Modern strategies of vaccine production have been developed; subunit vaccine is commonly used depending on a single type of viral protein, so the

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antigenic shift could cause major challenges for immunogenicity when compared with whole virus vaccine [14]. However, preparation of subunit vaccines (recombinant proteins) is simple, safe and widely used especially for the purpose of updating vaccine strains for any epidemiological situation, especially in Egypt [14].

The *Baculoviridae* is a family of arthropod-specifIc viruses. It has been widely used for the expression of recombinant proteins because of the following advantages; protein post-translational modification, a huge viral genome (130 kb) which enable high capacity for multiple genes or a large insert, biosafety as baculovirus naturally does not infect humans and a very high protein yield driven by the strong promoters polyhedrin or p10 [15]. Baculovirus expression system has become the most popular system for production of recombinant proteins which used extensively in the formulation of subunit vaccines [16, 17]. In the present work, we aimed to describe the construction of recombinant baculovirus expressing HA protein of recently Egyptian strain H5N1 AIV.

MATERIALS AND METHODS

Virus, Plasmids and Cell: Egyptian strain Influenza A virus (A/chicken / Egypt/ VRLCU/2012 (H5N1) with accession number KC625532.1) was used in this study. The Bac-to-Bac® Baculovirus Expression System (Invitrogen, San Diego, CA) was used (Catalog nos. 10359-016). *Spodoptera frugiperda* (*Sf-9*; Invitrogen, San Diego, USA) were grown in monolayer cultures in TNM-FH medium (Gibco BRL, Life Tech, NY).

Site Directed Mutagenesis of Full Length HA Gene of H5N1 AIV: Total RNA was extracted from tracheal homogenate by the guanidium thiocyanate method using TRIZOL reagent (Gibco BRL, Life Tech, NY) according to the manufacturer's instructions.

Site directed mutagenesis of HA gene was carried out by deletion of multi-basic cleavage site coding sequence using PCR. The primer sequences were; HA F 5'- GGA TCC GAA TGG AGA AAA TAG TGC TTC TTC -3', HA-mut-R 5'-GTC CTC GAG TCT CTC GTT GAG GGC TAT TTC TGA GCC CAG TAG C-3', HA-mut-F 5'-CCT CAA CGA GAG ACT CGA GGA CTA TTT GGA GCT ATA-3' and HA R 5'- AAG CTT TTA AAT GCA AGT TCT GCA TTG TAG -3' to amplify 1689 bp.

cDNA synthesis was carried out using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Thermo, UK) according to the manufacturer's instructions. Five µl cDNA product was added to a PCR mixture of 2x ReddyMix® PCR master mix (QIAgen, Germany). The PCR cycling profile for amplification of full length HA gene started with initial denaturation at 95°C for 5 min.; 40 cycles of 94°C for 45 sec.; 55°C for 1 min.; and 72°C for 2 min.; and final extension at 72°C for 10 min. The amplified PCR products (5µl) were loaded into 1.5% agarose gel containing 0.5 μg/ml ethidium bromide for nucleic acid visualization.

Construction of Recombinant Pfastbac- HA (Rpfastbac-HA) Transfer Vector: To construct rpFastBac- HA transfer vector, the PCR products of full length HA were purified from the gel using Montáge DNA gel extraction kit (Millipore, USA), were successfully ligated to the pFastBac™ donor transfer vector (Invitrogen, USA) according to the manufacturers' protocol. Transformation of *E. coli* TOP10 competent cells was carried out using the recombinant pFastBac (rpFastBac-HA). Positive colonies that harbor the rpFastBac-HA were identified by PCR screening assay and sequencing.

Transposition of the Rpfastbac-HA Plasmid into Bacmid DNA: Recombinant plasmids (rpFastBac-HA) were extracted from positive bacterial colonies using QIAprep® Miniprep plasmid extraction kit (QIAgen, Germany), then subjected for transformation into DH10Bac™ *E.coli* cells, which contains a baculovirus shuttle vector (Bacmid) with a mini-*att*Tn7 target site and a helper plasmid. Recombinant bacmids that harbor the target HA gene (rBacmid-HA) were extracted in a highly concentrated pure form using PureLink™ HiPure Mini column (Invitrogen, USA). Correct orientation of rBacmid-HA was carried out using PCR, restriction digestion and sequencing.

Construction of Recombinant Baculovirus: Monolayer cultures of *Sf-9* cells (1 X 10^6 cells per 35 mm dish) were transfected to generate a recombinant baculovirus according to Bac-to-Bac expression system manufacturers' instructions (Invitrogen, USA). Two µg of rBacmid-HA and 8 µl Cellfectin® II (Invitrogen) in unsupplemented Grace`s medium were used for transfection *Sf-9* cells. The transfected cells were incubated at 27°C and once signs of viral infection were started to appear (after 72 hrs), the medium over the cells were harvested, centrifuged at 1000 rpm for 10 min and was stored at 4°C protected from light (P1 viral stock). The P2 stock was prepared after 2 passages of P1 stock and stored at - 80°C until used.

***In Vitro* Characterization of HA Expressed Protein**

***Hemadsorption Assay*:** 0.1 ml of 5% chicken red blood cells in PBS was added to each 0.5 ml insect cells infected with recombinant baculoviruses and uninfected control cells, shaken gently for 10 min. at room temperature. Then, 10 ul of the suspensions were placed on a glass plate and observed under a microscope [18, 19].

Haemagglutination Inhibition (HI) Tests: Haemagglutination activity of the expressed HA protein from infected Sf-9 cells with recombinant baculovirus was determined. Reference H5N1 serum was serially diluted, added to the 96-well (U-shaped bottom plates) 25 ul/well, followed by addition of 25 ul of infected cell (cell pellet) (4 HA units) and incubation for 30 min at room temperature. Finally, 25 ul o f 0.5% chicken red blood cells was added to each well and incubated for 30 min at room temperature. The HI titers were determined as the highest dilutions that were able to completely inhibit agglutination [20, 21].

Immunofluorescence Analysis of Protein Expression: After 96 hrs post infection of insect Sf-9 cells with recombinant baculovirus, the cells were fixed with absolute methanol for 20 minutes at 4°C. Blocking was carried out using 2% bovine serum albumin for 30 min at 37°C, incubation with reference H5N1 AIV chicken antiserum (diluted 1/50 in PBS) for 1 h at 37°C. After three times washing with PBS, 5 minutes each, fluorescein isothiocyanate (FITC) conjugated rabbit anti-chicken IgG antibody (Sigma, USA) was added and incubated for 1 h at 37°C. Following another 3 washing cycles, one drop of mounting buffer (50% glycerol in PBS) was added over the cells before examination under an inverted fluorescence microscope (Olympus IX70) [22].

SDS-PAGE and Western Immunoblot Analysis: Infected *Sf-9* insect cells were harvested after 96hrs by centrifugation at 3000 ×*g* for 10 min and the resulting supernatant and cell pellets treated with two different detergent lysis buffers including either, 1% Triton X-100 or 20% SDS in PBS. Both lysis buffers contain a pepstatin A (protease inhibitor at a concentration of 1ug/ml to inhibit any proteases enzymes) (Sigma, USA), which may degrade the expressed protein [23]. Estimation of molecular weight and binding properties of hemagglutinin were determined by immunoblotting [24] using reference H5N1 AIV chicken antiserum.

RESULTS

Site Directed Mutagenesis of Full Length HA Gene of H5N1 AIV Using PCR Assays: The full length of HA gene of H5N1 AIV was amplified using specific designed primers. Electrophoresis of the amplified PCR products in agarose gel stained with ethidium bromide revealed positive band at the corrected molecular size (1689bp). The obtained band was eluted and purified to be used in the cloning reaction (Fig. 1).

Cloning of the Amplified HA Gene of AIV into Pfastbac^{tm} Transfer Vector: *E.coli* cells that harbor the rpFastBac-HA were grown on LB agar plates containing 50ug/ml ampicillin. Identification of rpFastBac-HA was carried out by colony PCR screening assay and sequencing (data not shown) which revealed that the HA gene was successfully cloned in the transfer vector with correct orientation.

Transposition of the rpFast Bac-HA Plasmid into Bacmid Dna: Insertions of the mini-Tn7 from recombinant pFastBac into the mini-*att*Tn7 attachment site of the bacmid disrupt the expression of the LacZ? peptide, so colonies containing the recombinant bacmid appear white in a background of blue colonies that harbor the unaltered bacmid, white colonies were selected for analysis. Identification of the recombinant bacmid carrying full length HA gene was carried out by PCR screening assay (Fig. 2) and sequencing (data not shown) which revealed the occurrence of successful transposition in correct orientation.

Construction of Recombinant Baculovirus: After 72 hours for transfection of *Sf-9* cells with rBacmid-HA, the cells stop growing compared with uninfected control cells and few cells were detached from the surface of tissue culture plates. Refractive crystalline structures (occlusion bodies) were found in nuclei of some infected cells (Fig. 3). No CPE was observed in the cell control until the end of incubation period. The supernatant was harvested 72 hours post-transfection for characterization of the recombinant viruses and the cells were incubated for 4 more days to follow up the progress in CPE to confirm the success of transfection.

Hemadsorption Assay (HAD): When RBCs suspension was added to *Sf-9* infected cells with recombinant baculovirus (after 96hrs), some RBCs adhered to the cell

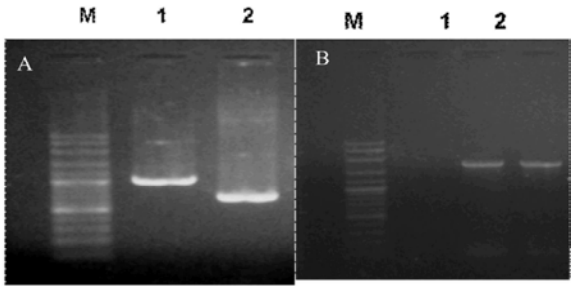


Fig. 1: Ethidium bromide stained agarose gel containing the PCR products of:
A) Lane 1: represents HA1 fragment of HA gene (1020bp). Lane 2: represents HA2 fragment of HA gene (669bp) along with 100 bp plus DNA ladder (M) (Vivantis, Malaysia).
B) Lanes 1 and 2: represent the mutated full length HA gene (1689bp) along with 100 bp plus DNA ladder (M).

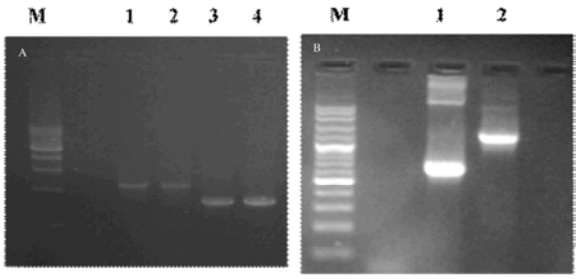
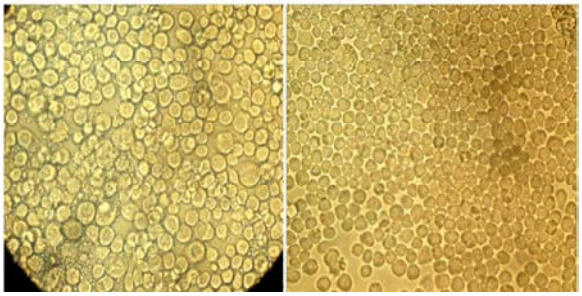


Fig. 2: Ethidium bromide stained agarose gel containing the PCR products of check transformation rpFastBac-HA into DH10Bac™ *E. coli* cells:
A) Lanes 1 and 2: represent the positive amplified product in the tested colonies at 2167 bp. Lanes 3-4: represent the positive amplified product in the tested colonies along with 1 kbp DNA ladder (Lane M).
B) Lane 1: represents the positive amplified product of HA1 fragment of full length HA gene (1020bp) in the tested colonies. Lane 2: represents the positive amplified product of HA2 fragment of HA gene (669bp) in the tested colonies along with 100 bp DNA size marker (lane M) (Vivantis, Malaysia).

surface where viral haemagglutinins are present and do not wash off. On contrary, the uninfected insect cell showed no speciflc binding of RBCs (Fig. 4 A, B).

***Haemagglutination* Inhibition (HI) Test:** Infected *Sf-9* cells with recombinant baculovirus were harvested after



(a) (b)

Fig. 3: Characteristic cytopathic changes observed in the transfected cells (3A) in comparison with normal control uninfected *Sf-9* cells (3B).

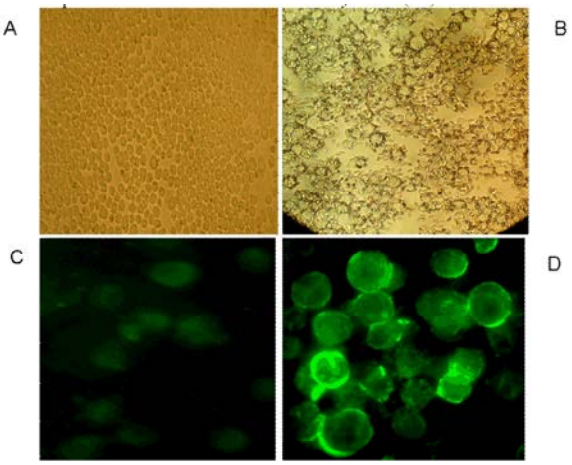
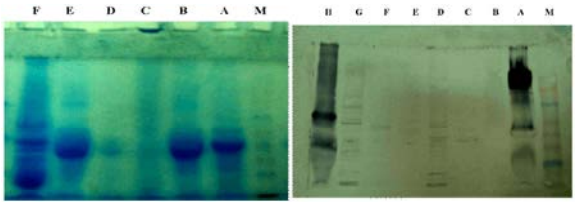


Fig. 4: 4 A and B: represent *Sf-9* infected cells with recombinant baculovirus expressing HA protein with Hemadsorption activity compared with normal control cells.
4C and D: Indirect Immunofluorescence assay of insect cells *Sf-9* infected with recombinant baculovirus expressing HA protein of H5 AIV revealed diffuse and peripheral fluorescent aggregation especially in the cytoplasm of infected cells in comparison with uninfected control cells.

96 hours incubation. Haemagglutination activity of the expressed HA protein was inhibited when reference H5N1 AIV antiserum was used (data not shown).

Indirect Fluorescent Antibody Technique (IFAT): Infected *Sf-9* cells with recombinant baculovirus produce high fluorescent intensity in the form of diffuse and peripheral fluorescent appearance especially in the cytoplasm whereas no fluorescence appearance was seen in control cells (Fig. 4 C, D).



(1) (2)

Fig. 5: 1) SDS-PAGE analysis of recombinant hemagglutinin (HA) protein. Lane M: Prestained low range prestained standards protein marker (Vivantis, Malaysia). Lanes A, B, E: HA expressed in *Sf-9* cells 96h after infection with recombinant baculovirus. Lane C: Normal *Sf-9* cells (control). Lane D: Supernatant of infected cells with recombinant baculovirus. Lane F: Positive control H5N1 AIV.

2) Western immunoblot analysis of recombinant hemagglutinin (HA) protein. Lane M: Prestained low range standards protein marker. Lane A: Positive control H5N1 AIV. Lane B: Normal *Sf-9* cells (control). Lanes C, E, F: Cell lysate of Sf-9 cells infected with recombinant baculovirus. Lanes D, G: Reducing condition for cell pellet of *Sf-9* cells infected with recombinant baculovirus. Lane H: Non-reducing condition for cell pellet of *Sf-9* cells infected with recombinant baculovirus.

SDS-PAGE: *Sf-9* infected cells with recombinant baculovirus after 96 hrs incubation revealed the expected molecular weight (approximately 63 KDa) when stained with Coomasie blue stain (Fig. 5; 1).

Western Immunoblotting Assay: Western blot analysis for *Sf-9* infected cells protein using reference H5N1 AIV antiserum with recombinant baculovirus revealed a distinct band (approximately 63 KDa) at the expected molecular weight of HA (Fig. 5; 2).

DISCUSSION

In the present study, site directed mutagenesis for the HA gene of recently Egyptian H5N1 AI (A/chicken/ Egypt/VRLCU/2012) strain was carried out by deletion of multiple basic amino acids coding sequence using PCR assays. Haemagglutinin protein is the receptor-binding, membrane fusion glycoprotein of influenza virus and the target for neutralizing antibodies [25, 26]. Successful transfection process was proved by sequential development of typical signs of baculovirus infection in the transfected insect *Sf-9* cells in compared with the normal cell control. CPE of transfection may delay until the 4th or the 5th day post-transfection. The culture medium collected 72 hours post-transfection is considered the initial source of recombinant baculoviruses which contains active and viable viruses (P1 stock). P2 stock was prepared by propagation of P1 stock in *Sf-9* cells. In vitro expression of the recombinant protein, expression of HA protein was carried out by inoculation of recombinant baculovirus into *Sf-9* cells. A rapid and sensitive assay is needed to monitor protein expression and to choose the right harvest time because the HA gene is regulated by the polyhedrin promoter, which is a late stage promoter in baculovirus infection to prevent degradation of the expressed protein by a variety of proteases released during lysis of *Sf-9* infected cells [27, 28].

During the last two decades, a wide variety of expression systems have been developed for *in vitro* production of recombinant proteins [29]. Baculovirus expression system is a popular eukaryotic system used for producing large quantities of recombinant proteins (up to 600 mg/liter) in insect host cells which are soluble and biologically active [30- 32]. In addition, baculoviruses are known to infect a narrow host range, which requires little or even no safety considerations either during preparation of the recombinant proteins or during their use in practice [31].

Vaccinations with recombinant baculovirus have been previously demonstrated to induce strong humoral and cell-mediated immunity against various antigens in animal models [32]. In vitro secretion of inflammatory cytokines can be mediated by baculovirus wild-type alone and confer protection from lethal virus infection in mice inducing innate immune responses through toll-like receptor dependent and independent pathways [25]. The immunogenic properties of baculovirus were the ability to stimulate cell-mediated and humoral immune responses against the encoded antigens [25, 33]. Development of recombinant HA influenza vaccines using the baculovirus-insect cell expression system has been tested in Phase I and Phase II human clinical trials that demonstrated safety, immunogenicity and efficacy [34].

Hemadsorption assay ensured the optimal HA production and determined the right harvest time for expression which was after 96 hrs in this study beside other harvest parameters such as cell's morphology and viability confirming that the HA expressed protein is properly folded and biologically active [28]. HA protein expressed on the surface of the infected cells causing

hemadsorption of red blood cells, like the natural protein. The antigenicity of the expressed HA protein was confirmed by Haemagglutination inhibition (HI) test [20, 21]. Previous studies reported that recombinant HA protein of H5N1 AIV from the baculovirus system possesses the same biological activity, antigenicity and immunogenicity like the natural protein [14]. Complete protection of SPF chickens challenged with genetic and antigenic Egyptian H5N1 HPAI viruses of clade 2.2.1 has been achieved, when vaccinated with single-shot scheme of a baculovirus expressed H5 protein vaccine [14].

Reactivity of the expressed HA protein with reference H5N1 AIV antiserum was also conflrmed by indirect immunoflorescence assay which appear in the form of diffuse and peripheral fluorescent aggregation especially in the cytoplasm of infected cells. Recombinant protein was migrated on SDS-PAGE gel and had approximate molecular weight 63 kDa. Western blot assay under reducing and non-reducing conditions was confirming the specificity of the developing a distinct band of the expressed HA protein at the expected molecular weight. Non specific bands of low molecular weight were observed could be explained by the action of 2-mercaptoethanol in case of reducing conditions beside cellular proteases on the expressed HA protein.

Finally, the expressed protein may be utilized for advanced structural, functional and immunological studies. Also, it can be used for development of a subunit vaccine candidate for control of the H5N1-induced disease and to overcome the drawbacks of conventional vaccines. This type of subunit vaccines may be included as an additional tool for the prevention and control of HPAI H5N1 in Egypt beside other control measures.

CONCLUSION

Cloning for full length HA gene was successfully carried out after site directed mutagenesis by deletion of cleavage site. Recombinant pFastBac-HA was transformed into DH10Bac for transposition to construct recombinant bacmid which was the precursor of recombinant baculovirus. Transfection and expression for recombinant baculovirus was conducted followed by in vitro characterization for the expressed HA protein by different assays including HA, HI, HAD, IFAT, SDS-PAGE and Western immunoblot which prove the successful expression of HA protein. Expressed HA protein was tested in vitro for antigenicity and immunogenicity which indicate that the expressed protein was acting like the natural protein.

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