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RESEARCH ARTICLE

Generation of avian influenza (H5N1) virus-like particles from an Egyptian isolate using baculovirus expression system

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Abstract

Influenza virus-like particle (VLP) vaccines are one of the most promising approaches to respond to the emerging threat of pandemic strains, as they possess the potential for higher production capabilities compared to traditional influenza vaccines made in egg-based technology. Highly pathogenic avian influenza (HPAI) H5N1 virus became endemic in Egypt due to defect in the application of AI control strategies and shortage in the vaccine doses. Hence, our study was focused on generation of influenza H5N1 VLP from Egyptian isolate A/Turkey/Egypt/7/2007 (H5N1), using insect cell culture and baculovirus expression system. The generated influenza VLPs were observed photographed by electron microscope and detected by measuring hemagglutination activity, hemadsorbtion activity, SDS-PAGE, and Western plot. This study discuss the creation challenge of identified Egyptian isolate influenza VLPs vaccine production and processing in Baculovirus expression and cell system which could be in large scale is more rapid and easy than other production technologies. It is essential that further immune response results on the Egyptian isolate generated Influenza (VLPs) carried out which is in processing.

INTRODUCTION

H5N1 virus mainly infects birds, highly contagious and can be deadly among birds, especially domestic poultry. Infections of Highly pathogenic avian influenza A (H5N1) virus in birds have been reported since December 2003 in Asia, Africa, and Europe. H5N1 viruses are endemic (ever present) in poultry in at least six countries including Egypt, while sporadic cases in wild birds and poultry outbreaks detected in other countries(WHO/OIE/FAO, 2009).The highly pathogenic avian influenza (HPAI H5N1) virus was detected firstly in Egypt in a common teal (Anas crecca) trapped in a cage by a fisherman at Damietta in December 2005(Saad et al., 2007). On 2006, AI infection caused by the HPAI H5N1 subtype jumped from birds to human in Egypt (Aly et al., 2008). In 2008, Egypt reported to be an endemic are and vital center for the activity of The HPAI H5N1 virus (WHO/OIE/FAO, 2009). From 2003 through December 10, 2013, 15 countries have been officially reported 648 laboratory-confirmed human cases with H5N1 virus infection to WHO, 384 died from these cases with mortality rate (60%). At the current time, there is no active transmission of H5N1 viruses neither any avian influenza A viruses in humans (CDC, 2014). Vaccination remains the most efficient strategy to control the continuous threats of newly emerging influenza viruses. The currently licensed egg-derived influenza vaccines are effective, and protecting up to 90% of vaccine recipients (R. Belshe et al., 2004; Belshe & Gruber, 2001; R. B. Belshe et al., 2004;

Nichol, 2003). Scaling up the production of the egg-derived vaccine is not easily and is limited by the availability of specific pathogen-free (SPF) embryonated chicken eggs (Fedson, 2008). The egg-based influenza vaccine manufacturing process is labor intensive and time consuming and needs long term planning and long annual production cycles (Gerdil, 2003). The evaluation of cell culture-based vaccine production raised up as alternatives to egg dependent production due to those difficulties in production and potential exposure of chicken to avian influenza viruses (Audsley & Tannock, 2008). The World Health Organization (WHO) in 2006 highlighted the need to develop new technologies able to support urgent and large quantities of vaccines in case of pandemics, that's was in the action plan to increase the (WHO, 2006). Using of cell-culture technologies might improve robust vaccine supplies for the manufacture of influenza vaccines (Barrett et al., 2011). In November 2012, the first cell culture-based seasonal influenza vaccine (Flucelvax Novartis) was approved by the Food and Drug Administration (FDA) for adults 18 years of age and older (FDA, 2012). Recently, in mid-January 2013, the first trivalent influenza vaccine was prepared by using recombinant DNA technology and entomovirus (Baculovirus) expression system. Flublok (Protein Science Corporation) was approved as a vaccine for the prevention of seasonal influenza in people 18 through 49 years of age (FDA, 2013), While in animal society there is no influenza virus-like particles (VLPs) approved yet.

The strategy used by Egyptian authorities relied on vaccinating poultry, depopulating infected areas, increasing awareness, and increasing biosecurity levels (Kayali et al., 2013). Several commercial inactivated influenza vaccines were used to control the virus, which mainly based on classical H5 linages (Kayali et al., 2013). These differences play an important role in failure of vaccination strategy in Egypt (Hafez et al., 2010; Kim et al., 2010). So, the availability of safe effective vaccine with the new approaches is a major concern in Egypt.

Influenza VLP does not contain genetic material, but rather are empty particles composed of one or both of the two viral immunogenic proteins of influenza: Hemagglutinin (HA) and Neuraminidase (NA)(Haynes, 2009). Also one of the two influenza matrix proteins, either M1 or M2 may be constructed with influenza VLP (Kang et al., 2009; Pushko et al., 2005; Wu et al., 2010). Currently, influenza VLPs are produced in mammalian, insect or plant cell cultures using a variety of vectors and gene delivery techniques (D'Aoust et al., 2010; Haynes, 2009; Tang et al., 2011).

This study was focused on generation of influenza H5N1 VLPs from Egyptian isolate A/Turkey/Egypt/7/2007 (H5N1), using insect cell culture and baculovirus expression system with assistance of pAcAB4 Baculovirus Transfer Vector; to support emerging of novel production technology and overcoming the limitation of other production technologies in Egypt.

Materials and Methods

Virus, allantoises fluid preparation and cell line system: Highly Egyptian pathogenic avian influenza A/Turkey/Egypt/7/2007 (H5N1) virus was isolated and purified from local surveillance study performed by Influenza Research lab in National Research Center (NRC) in Egypt, and then was used to start this study. Viral suspension was inoculated in 11 day-old specific pathogen free (SPF) embryonated chicken eggs **(**SPF Eggs Production Farm, Agricultural Research Center, Ministry of Agriculture, Egypt) and incubated for 24 h at 37º C, then chilled at 4ºC for 4 h before harvesting the allantoises fluid, clarified, tested for haemagglutination and then stored at -80ºC until use. *Spodoptera frugiperda* (SF9) lyophilized cell line originated from ovaries were grown and maintained in TNM-FH Grace's Insect Medium (Lonza) containing 10% FBS until using for the baculoviral infection.

Amplification of full length HA gene and M1 gene: Viral RNA was extracted from 140 µl of virus suspension using a QIAamp viral RNA mini kit (Qiagen) according to the manufacturer's protocol in a class III bio-safety cabinet. To amplify full length HA gene and M1 gene, primers were designed according to accession numbers CY055191.1 and CY055194.1 respectively **table 1**. Stu1 (AGGCCT) and BamH1 (GGATCC) restriction sites were incorporated in HA and M1 primers respectively. RT-PCR was performed using a QIAGEN One Step RT-PCR Kit with an initial 60-min incubation at50° C; followed by 95° C for 15 min; 40 cycles at 94 °C for 30 s, 55°C for 30 s, and 72° C for 3 min. ; and a final 10 min incubation at 72° C. The resolved amplification products were run on Agaros gel electrophoresis 1% and analyzed by the gel documentation system (UVP, California; USA). The PCR products were purified using QIAquick Gel Extraction Kit according to manufactures' instruction.

Recombinant Baculovirus system: The amplified HA and M1 genes were initially cloned into bacterial pCR2.1-TOPO cloning vector as manufactures' instructions. The clone of HA- pCR2.1-TOPO was digested with Stu1 as described by in instruction of Stu1 enzyme manufacturer (Biolab NEB)**.** The purified HA gene was inserted in the same site of Stu1 digested pACAB4 Baculovirus transfer vector (BD BaculoGold) to be under control of polyhedron promoter, and to have finally the HA-pAcAB4 construct. To verify the construction all purified plasmids were subjected to PCR using H5-155f Lee: 5'-ACA CAT GCY CAR GAC ATA CT-3' and H5-699r Lee 5'-CTY TGR TTY AGT GTT GAT GT-3' primers(Lee, Chang *et al*;2001) . For confirmation of right orientation, the inserts were sequenced in both directions. Sequencing was done using the Big Dye Terminator kit 3.1 (Applied Biosystems) according to manufacturer's instructions, cycle sequencing was performed in a thermolcycler (26 cycles of 95°C, 30 sec; 50 °C, 15 sec; 60°C 4 min); The reaction product was purified by exclusion chromatography (CentriSep columns, Princeton Separations)(Kandil, etal; 2013) the recovered materials were dyed and subjected to ABI prism 310 genetic analyzer. Clone of M1- pCR2.1-TOPO was digested with BamHI to produce M1 gene. The purified M1 gene was cloned into the BamHI digested HA-pAcAB4 construct to be under control of polyhedron promoter and for getting HA-M1-pAcAB4 construct. To verify the construction of the M1 gene into HA-M1-pACAB4, all purified plasmids were subjected to PCR using M30F2/08: 5'-ATGAGYCTTYTAACCGAGGTCGAAACG-3' and M264R3/08: 5'-TGGACAAANCGTCTACGCTGCAG-3'primers .to amplify 244 bp of M1 gene (WHO 2012) For confirmation of right orientation, the inserts were sequenced in both directions as previously mentioned.

Cell line preparation and transfection: Finally, to generate recombinant baculovirus carrying HA & M1 genes of H5N1, SF9 cells were seeded onto 6 well tissue culture plate co-transfected by the Linearized Baculovirus DNA with BaculoGold™ Bright with recombinant transfer vector (HA-M1-PAcB4) according to manufacturer's instructions. Cells were incubated for 4hr at 27ºC, then media was removed and replaced with a fresh TNM-FH medium. Cells were incubated for 4 days at 27ºC, and Cytopathological effects (CPE) were daily observed under inverted Microscope (Olympus ,CKX31) On day 5, cells were harvested and subjected to freezing and thawing three times in order to liberate and detect viral like particle.

Detection of expressed proteins VLPs

HA & M1 expressed proteins were detected by RT-PCR of the purified mRNA from the transfected SF9 cells using RNeasy Mini Kit (QIAGEN) according to the manufacturers instructions. Lysates prepared from control and infected cells were analyzed by SDS-PAGE as previously mentioned by Laemmli (1970). Following electrophoresis, proteinswere detected by Western blotting (Towbin *etal.*, 1979) from the gel to a nitrocellulose sheet (BA85, pore size 0.45 mm; Schleicher and Schuell, Dassel, Germany) at 6 V/cm and 250 mA overnight at 4^{o}C in a transfer buffer. Strip carrying the molecular weight marker was cut and the membranes were cut into individual strips. Strips were washed 3 times with PBS-0.3%T each for 5 min and blocked against non-specific binding at room temperature for one hour in PBS-0.3%T-1%BSA. Strips were washed 3 times again as mentioned above and incubated with diluted first antibody (infected chicken serum; 1: 50) in PBS-0.3%T at room temperature for 2 hrs. After 3 washes, strips were incubated for 2 hrs at room temperature with diluted peroxidase labeled second antibodies (anti-chicken IgG; 1: 500 in PBS-0.3% T, KPL). Developing the strips with DAB, peroxidase specific substrate, allowed visualization of immune complexes on the nitrocellulose membrane. The molecular weights of the immunogenic viral peptides were determined.

Hemagglutination (HA) and Hemadsorption assay:

Hemagglutination of the expressed HA protein from infected Sf-9 cells with recombinant baculovirus was determined from cell laysate using 0.5 % chicken RBCs (INFL, *etal*;2002)A volume of 100µl of 5% chicken red blood cells in PBS was added to each volume of 500µl Infected SF9 cells with recombinant Baculovirus and uninfected control cells, shaken gently for 10 min. Then, a volume of 10 µl of the suspensions was placed on a glass slide and hemadsorption observed under inverted microscope (Olympus ,CKX31) with the40X objective.

Electron Microscope examination:

Morphological observations were carried out with JEM-2100 transmission electron microscope (JEOL, USA) by fixation of Sf9 cells using 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.3 with 3mM CaCl2 for 3 hr. The fixed cells were then washed and loaded in the coated copper grid to examine.

Result and Discussion

Amplification of HA and M1 and cloning into pCR2.1-TOPO (TA cloning vector): Full length HA and M1 genes of A/Turkey/Egypt/7/2007 (H5N1) virus were successfully amplified by RT-PCR using specific designed primers as explained above. Electrophoresis of the amplified PCR products in **1 %** agarose gel stained with ethidium bromide revealed positive bands at molecular size of 1745 bp and 750 bp for HA and M1 genes respectively (fig**.** 1).The specific bands were sliced, eluted and purified using manufacture instruction of QIAquick Gel Extraction Kit(QIAGEN; Cat # 28704), to be used for cloning. The purified HA & M1 genes were sub-cloned using the pCR2.1-TOPO cloning vector (TA cloning vector). Sub-cloning resulted in generation of two vectors pCR2.1-TOPO/HA and pCR2.1-TOPO/M1 (fig.1).

Generating Recombinant Baculoviruses: Cloning of HA and M1 genes into pACAB4 transfer vector was carried out as previously described in materials and methods by firstly digest the clones pCR2.1-TOPO/HA and pCR2.1-TOPO/M1 with Stu I restriction enzyme and BamH I restriction enzyme respectively. The purified HA gene were cloned into StuI site of pAcAB4 vector to generate HA-pAcAb4. The resulted PCR amplified fragment was approximately 545bp (fig. 2)**.** As for the purified M1 gene, which was cloned into BamH I site of the HA-pAcAB4 to form the HA-M1-pAcAB4. PCR also was performed to confirm insertion of the M1 gene into pAcAB4 Baculovirus transfer vector. Several selected colonies carried the M1 gene. The resulted amplified fragment was approximately 244bp (fig. 2).The recombinant baculovirus carrying the HA & M1 genes was generated after co-transfecting Sf9 cells and showed a clear CPE which is demonstrated as SF9 cells were detached from tissue culture plates and occlusion bodies were formed in the nuclei of transfected cells fig.(3). The recombinant baculovirus was further propagated and harvested for production of large quantities of VLPs. On contrary, no CPE were observed on uninfected SF9 cells.

Detection of expressed proteins (H5N1 VLPs): Expressed HA & M1 proteins were detected using the RT-PCR of the extracted mRNA from previously infected SF9 cells with recombinant baculovirus. Electrophoresis of the RT-PCR products in agrose gel stained with ethidium bromide, showed positive band identical to the molecular size of HA & M1 genes (Fig. 4). Analyzed results of SDS-PAGE and western plotting, confirms the molecular weights of the immunogenic viral peptides and the specificity of the HA protein in western plot as obviously shown in Fig. (5).

Hem-agglutination (HA) and Hem-adsorption Assay: Recombinant Baculovirus infected Sf9 cells were harvested 5 days post infection, expressed HA protein showed hem-agglutination activity, which revealed 32 HAU/25 µl of infected cells. When RBCs suspension was added to Sf9 infected cells with recombinant baculovirus, RBCs adhered to the cell surface where viral hem-agglutinins are present and didn't wash off (Fig. 6). On contrary, uninfected insect cell didn't show any specific binding of RBCs to Sf9 cells.

EM examination: the morphological appearance of the generated influenza VLP was confirmed through the EM examination (fig. 7).

Discussion:

Obtained results described the formation of influenza VLPs directed only by two viral structural proteins (HA & M1). These VLPs are assembled and released from Sf9 insect cells which co-expressing the two influenza virus proteins as directed by a recombinant baculovirus. The VLPs closely resemble influenza virus in size, particle morphology, and fine structure of the surface spikes according to the observation by TEM. These results are in complete agreement with the results of (Quan, Huang *et al*;2007; Krammer, Schinko *et al*;2010) in which VLP formation was obtained following expression of a similar set of structural proteins. The expressed proteins were detected by RT-PCR of the SF9 infected cellsmRNA as well as with western plot. In addition, the HA activity was detected by HA titration and hem-adsorption assay. In this study we excluded the NA protein in the generated VLPs, as it was reported that the proteins expressed in Sf9 insect cells were devoid of sialylation as a result of the absence of detectable sialyltransferase activities ((Butters, Hughes *et al*. 1981; Hooker, Green *et al*. 1999) and CMP-sialic acids (Stollar, Stollar *et al*;1976; Tomiya, Ailor *et al*;2001) and were N-glycosylated in high mannose type (Kuroda, Geyer *et al*;1990). So that VLP production in insect cells is viable without the aid of NA activity for viral progeny release if HA is incorporated. Also, N-glycans in high mannose type could possibly enhance the HA binding to its

sialylated receptors (Wang, Chen *et al*;2009) and facilitate the uptake of VLPs by Antigen Presenting cells (APCs), so improve the immunogenicity of the VLPs vaccine. The results conclude that, this novel approach for the influenza virus particles assembly has great potential for the design of vaccines against new influenza virus variants in Egypt. That's could extremely support facing several limitation of vaccination in Egypt including; producing safe & effective influenza vaccine either for human or animal use from the Egyptian isolates, and overcoming the limitations of egg-based technology in production of influenza vaccines.

In summary, results demonstrate that wild-type influenza VLPs of the Egyptian isolate (A/Turkey/Egypt/7/2007 (H5N1)) could be assembled and released from the surface of Sf9 cells following the expression of only two viral proteins. Further studies on the generated influenza VLPs from the Egyptian isolate are recommended to focus on the immunization ability and effectiveness.

Table 1. The designed Primers for amplifying target genes

Primer	Sequence (5' to 3')	Target segment	Size (bp)
FHA-StuI	gat tta AGG CCT AGC AAA AGC AGG GG	HA	1745
RHA-StuI	gat tta AGG CCT AGT AGA AAC AAG GGT GTT TT		
FM1-BamHI	GatttaggatccATGAGTCTTCTAACCGAGGTCG	M1	790
FM1-BamHI	GatttaggatccTCACTTGAATCGCTGCATCTGC		

Fig. 1 Amplification of full length M1 (A) and HA (B) genes using specific designed primers and cloning into pCR2.1-TOPO. Electrophoresis for RT-PCR of A/Turkey/Egypt/7/2007 (H5N1) genome using specific primers showed (A) positive band in the lane 2 at about 790bp for the full length M1 gene, and (B) positive band in the lane 3 at about 1745bp for the full length HA gene. The vector pCR2.1-TOPO used for cloning of above amplified M1 & HA genes generating; (A) pCR2.1-TOPO/M1 clone M1 gene with its specific primer carrying BamHI restriction site, (B) pCR2.1-TOPO/HA clone HA gene with its specific primer carrying StuI restriction site.

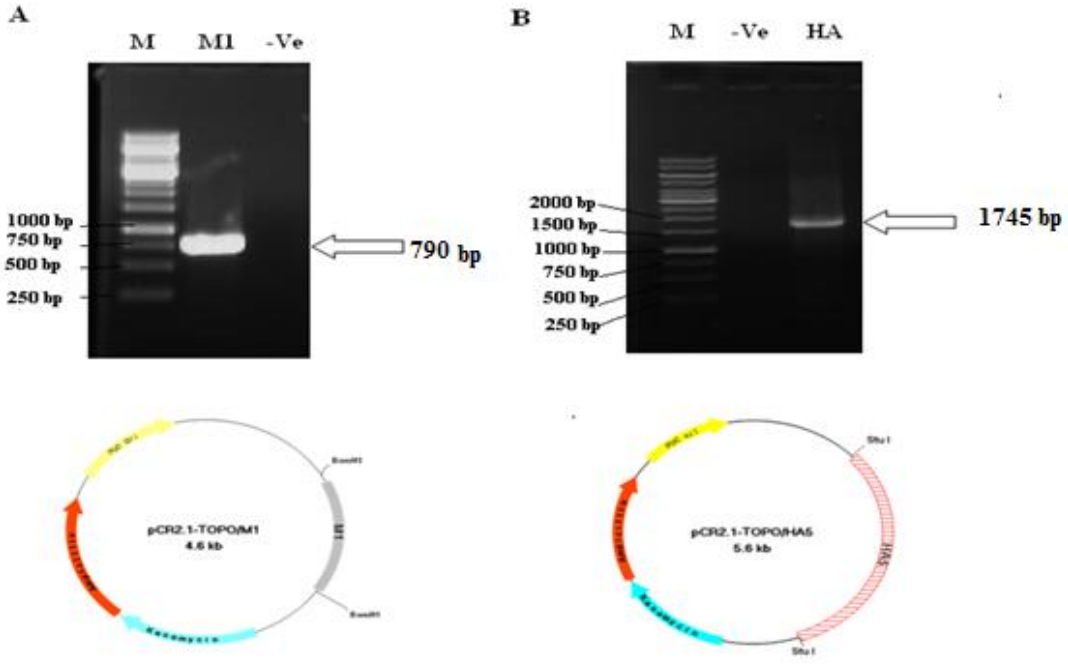


Fig. 2 Colony PCR was performed to confirm insertion of the HA (A) and M1 (B) genes into pAcAB4 Baculovirus transfer vector. Electrophoresis of the colony PCR products for HA-pAcAB4 & HA-M1-pAcAB4 using specific primers for each one, showed positive amplified fragments with approximate size 545bp in lanes 1-8 (A), and 244bp in lanes 2,3,6, & 7 (B). That confirmed insertion of HA gene into HA-pAcAB4 and M1 into HA-M1-pAcAB4 consequently.

Fig. 2

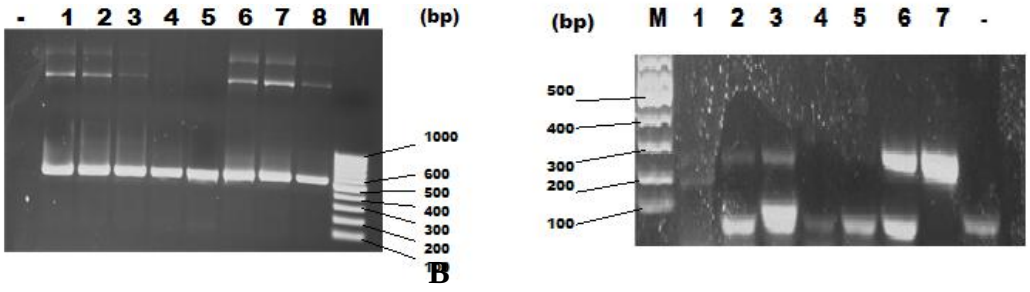


Fig. 3 Characteristic CPE observed in the transfected cells (A) in comparison with normal control uninfected Sf-9 cells (B) after 72 hr. (A) Detached SF9 cells and occlusion bodies formation in the nuclei of transfected cells is a characteristic CPE, that confirm generation the recombinant baculovirus carrying the HA & M1 genes. (B) Normal attached healthy control (uninfected) Sf-9 cells.

Fig. 3

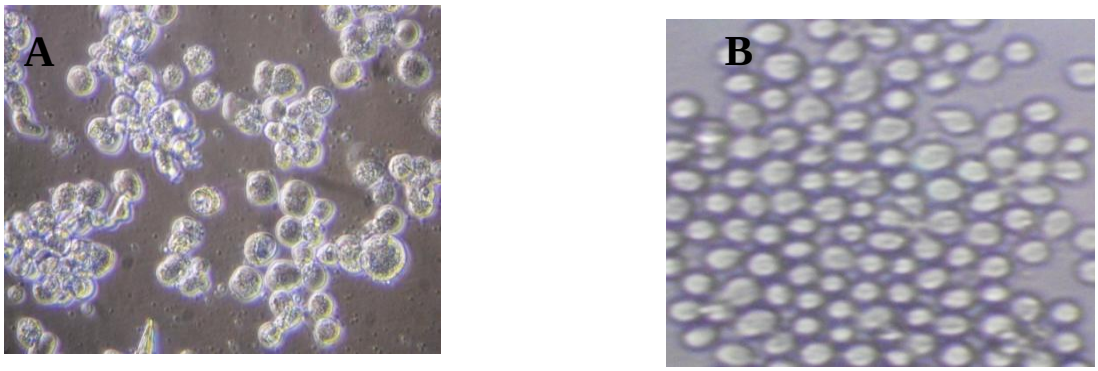


Fig. 4 HA and M1 RT-PCR was used to detect the mRNA expression in infected SF9 cells. Electrophoresis of the RT-PCR confirmed expression of HA in lane 1 at about 545bp (A), and M1 in lane 2 at about 244bp (B) versus negative control of uninfected Sf-9 cells in lane 2 & 1 consequently.

Fig. 4

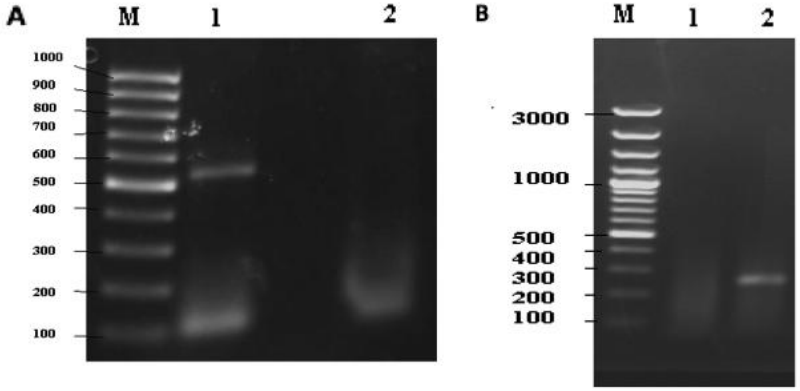


Fig. 5 The SDS-PAGE & western plot of the HA of infected SF9 cells in comparing with control (uninfected cells). Positive Expression of the influenza virus protein HA in infected Sf-9 cells in lane 2 of the SDS-PAGE at about 65-70 KDa (A) was further evaluated with a mixture of anti-HA monoclonal antibodies using Western plot method (B). Contrary the uninfected Sf-9 cells used as controls in lane 1.

Fig. 5

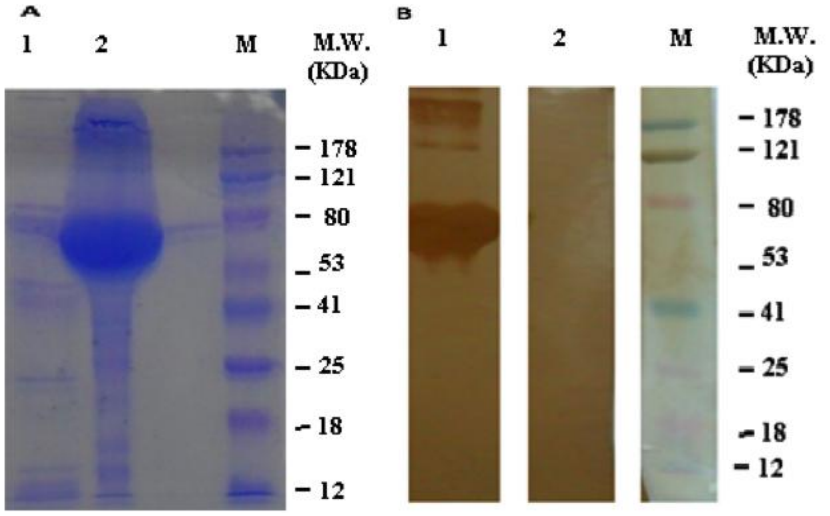


Fig. 6 Hemadsorption & HA activity for developed virus compared with normal control Sf-9 cells. The heamadsorbtion activity was not detected in the normal control Sf-9 cells (A), while the infected cells with recombinant baculovirus adsorbed the RBCs showing remarkable agglutination of the Sf-9 cells (B). The same result is obtained in HA test (C) showing negative button like RBCs that settle down in first well versus positive agglutinated cells in other wells forming a flat sheet of RBCs with titer 32 HAU/25 µl of infected cells.

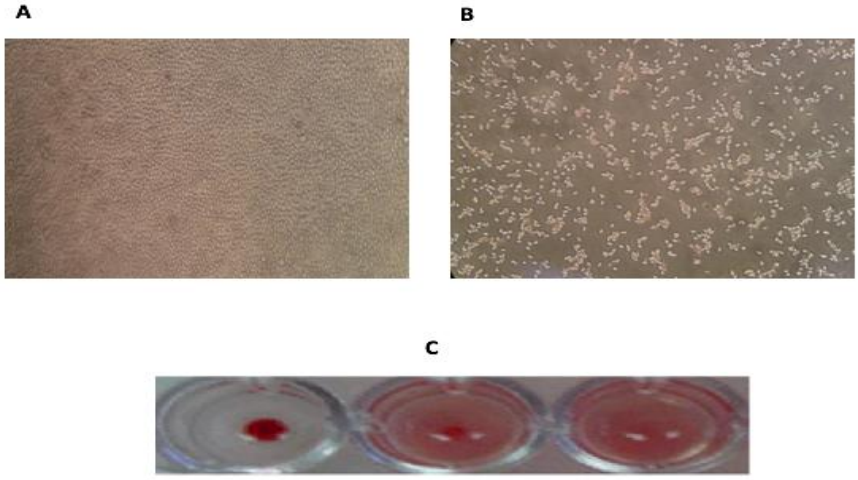
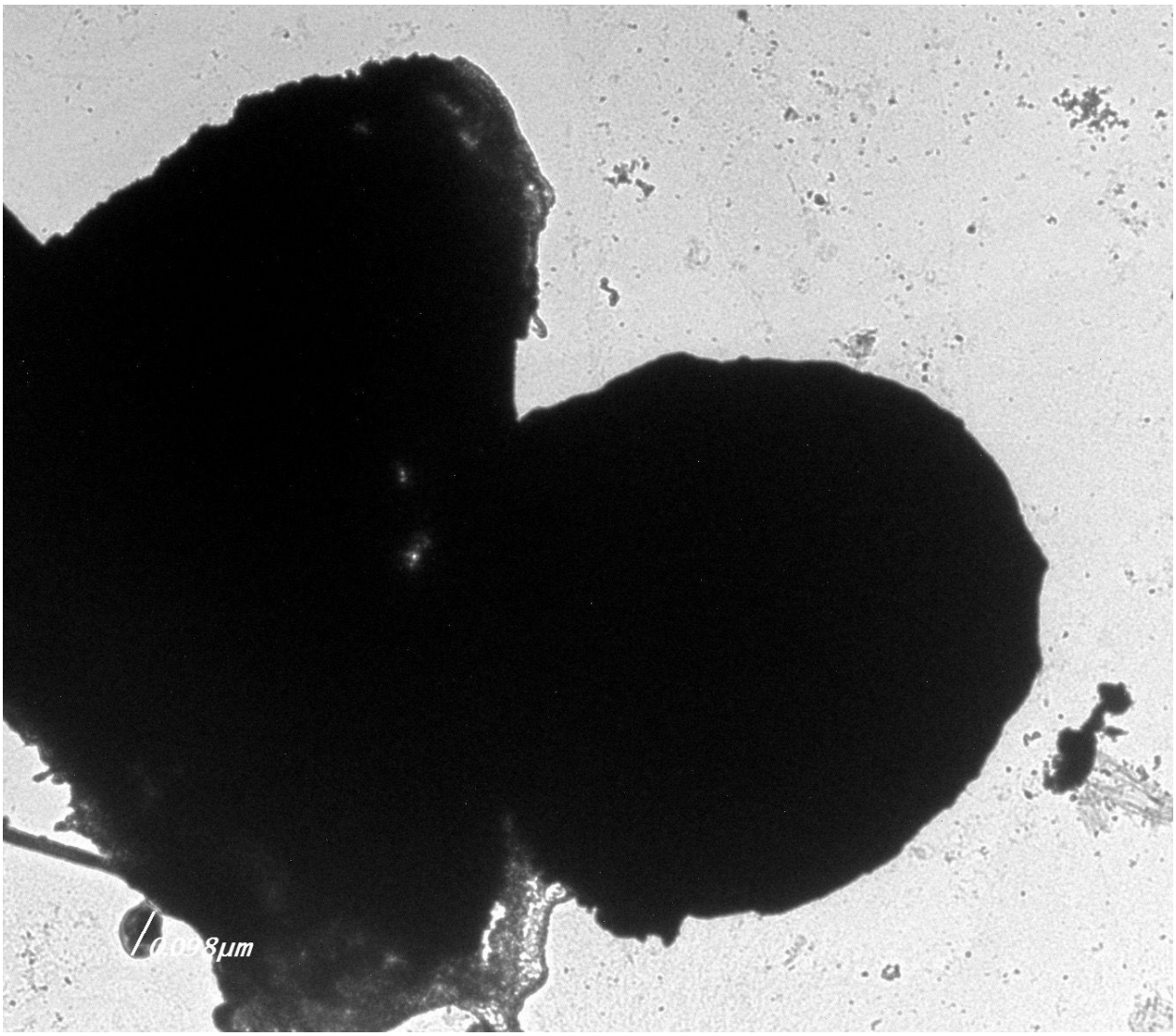


Fig.7 Electron microscope image of the generated Influenza VLPs. The image clearly illustrated the budding of the influenza VLP from the co-infected Sf9 cells, with the characteristicmorphology as influenza virus surface spikes, and with diameter 0.098 µm like size of influenza virus particle that range from 0.08 µm up to 0.12 µm.

Fig. 7



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