Biotechnology Reports 3 (2014) 108–116

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Stable lentiviral transformation of CHO cells for the expression of the hemagglutinin H5 of avian influenza virus in suspension culture

Alaín González Pose[a,*], Nayrobis Oramas Morell[a], Dailenis Abella Matos[a], Elsa Rodríguez Rodríguez[a], Elaine Santana Rodríguez[a], Liliam Rios Cordero[b], María Pilar Rodríguez Moltó[a], Ernesto Manuel González Ramos[a], Anays Álvarez Gutiérrez[a,b], Llilian Gómez Pérez[a], Julia Noda Gómez[b], Armando Vega Redondo[b], Carlos Borroto Nordelo[a]

[a] Animal Biotechnology Department, Center for Genetic Engineering and Biotechnology (CIGB), P.O. Box 6162, Havana 10600, Cuba
[b] Virology Department, National Center for Animal and Plant Health (CENSA), Apdo 10, San José de Las Lajas, Mayabeque 32700, Cuba

ARTICLE INFO

Article history:
Available online 17 May 2014

Keywords:
Hemagglutinin
Lentiviral vector
CHO cells
Suspension culture

ABSTRACT

Avian influenza virus H5N1 has caused extensive damage worldwide among poultry and humans. Effective expression systems are needed for the production of viral proteins required for monitoring this devastating disease. The present study deals with the establishment of a stable expression system for the hemagglutinin H5 (HA^{H5}) of avian influenza virus using CHO cells in suspension culture transduced with a recombinant lentiviral vector. The synthetic gene coding the HA^{H5} protein was inserted in a lentiviral vector with the aim of performing a stable transduction of CHO cells. After the selection of recombinant clones, the one with the highest expression level was adapted to suspension culture and the HA^{H5} protein was purified by immunoaffinity chromatography from the culture supernatant. There were no significant differences when this protein, purified or direct from the culture supernatant of CHO or SiHa cells, was utilized in an immunologic assay using positive and negative sera as reference. It was also demonstrated that the HA^{H5} protein in its purified form is able to bind anti-HA^{H5} antibodies generated with proper and non-proper folded proteins. The results demonstrate that the CHO cell line stably transduced with a lentiviral vector coding the sequence of the HA^{H5} protein and cultured in suspension can be a suitable expression system to obtain this protein for diagnostic purpose in a consistent and reliable manner.

1. Introduction

Since 2003, several outbreaks of the highly pathogenic avian influenza virus (HPAIV) H5N1 have occurred in Southeast Asia. This virus spread across Africa and Europe causing the loss of millions of birds and the death of more than 380 humans until now (http://www.who.int/influenza/human_animal_interface/). As H5N1 virus obviously represents a threat all over the world, several attempts have been made to produce better new generation vaccines and diagnostic systems for counteracting the dissemination of this virus and reinforcing the surveillance [1–4]. The protein hemagglutinin (HA) of influenza viruses has been considered the main antigen during the host immune response against the infection. There are 17 subtypes of avian influenza virus based on the antigenic drift of the HA protein [5]. Thus, the HA protein could be crucial for the detection of these viruses. Because the subtype H5 is one of the avian influenza subtypes that can turn into highly pathogenic viruses, surveillance programs should include diagnostic techniques able to detect this avian influenza subtype. Hence, the HA^{H5} protein could be useful for this purpose. The HA protein has been obtained employing several expression systems, such as bacteria [6], yeasts [7], insect cells using baculovirus vectors [1] and mammalian cells transduced with adenoviral vectors [8]. Moreover, plenty of studies have demonstrated the efficacy of mammalian cells in the expression of heterologous proteins [9]. Among them, Chinese hamster ovary (CHO) is a very well characterized mammalian cell line and is one of the most used expression system for the production of recombinant proteins applied to humans [10]. Therefore, regulatory issues are easier to overcome using this cell line. On the other hand, lentiviral vectors have risen as a promising tool for the stable transformation of mammalian cells. They have several advantages compared to other methodologies

* Corresponding author. Tel.: +53 7 250 4412; fax: +53 7 271 4764.
E-mail address: alain.pose@cigb.edu.cu (A.G. Pose).

http://dx.doi.org/10.1016/j.btre.2014.05.001

utilized for this purpose, such as the stable transformation with calcium phosphate or the use of polycations. Some of these advantages are: (i) the integration in active sites of chromatin, (ii) the transduction of dividing and quiescent cells, (iii) the integration of longer DNA fragments and (iv) the long term expression of the transgene [11]. Therefore, the objective of this study was to generate a stable transformed CHO cell line in suspension culture able to produce the HA protein from the highly pathogenic influenza virus H5N1 (A/Viet-Nam/1203/2004) for diagnostic purpose by transduction with a recombinant lentiviral vector.

2. Materials and methods

2.1. The hemagglutinin gene

The nucleotide sequence of the HA^{H5} protein was obtained from the National Center for Biotechnology Information (NCBI) using the accession number AY818135. The *ha*^{h5} gene was synthesized by GeneArt company (Germany) and encodes amino acids from 1 to 537, which include the native secretion signal of the HA^{H5} protein. It lacks transmembrane region and cytoplasmic tail [2].

2.2. Insertion of the ha gene into the plasmids pAEC-Spt and pLW

The *ha*^{h5} gene was extracted from the vector supplied by GeneArt company with the enzymes *Kpn* I/*EcoR* V and inserted in the mammalian expression plasmid pAEC-Spt [12] previously digested with the same enzymes. The recombinant plasmid was named pAEC-*ha*^{h5}. For the cloning of the *ha* gene into the plasmid pLW [35], it was amplified from the plasmid pAEC-*ha*^{h5} (10 ng of template) by PCR using an automatic Mastercycler (Eppendorf, USA), the *Pfu* DNA polymerase (Promega, USA) and the primers: (forward) 5′-ACTAGTTATTAATAGTAATCAATTACG-3′ and (reverse) 5′-CCAATTATGTCACACCACAG-3′. Three minutes at 93 °C were programmed as the initial step, followed by 35 cycles of 1 min at 93 °C, 1 min at 52 °C and 5 min at 74 °C. A final polymerization step of 5 min at 74 °C was added. The amplified gene was inserted in the plasmid pLW previously digested with the enzyme *EcoR* V. The recombinant plasmid was named pLW-*ha*^{h5}.

2.3. Transfection of the cell line HEK-293 with the plasmid pAEC-*ha*^{h5}

In vitro culture of HEK-293 cell line (ATCC CRL-1573) was carried out in flasks of 25 cm^2 (Greiner Bio-One, Germany) using the culture medium Dulbecco's modified Eagle's medium (DMEM) (Sigma, USA) supplemented with 10% of fetal calf serum (FCS) (PAA, Canada), 0,3 mg/mL of L-glutamine (Sigma, USA), 1 mM of sodium pyruvate (Sigma, USA) and an antibiotic–antimycotic solution 100× (GibcoBRL, USA) at a final concentration of 1×. Cells were incubated at 37 °C, 5% of CO_2 and 95% of relative humidity. One hour before the transfection, the medium of the cell culture at 80% of confluence was changed for fresh medium without FCS. Transfection was performed using the polycation polyethylenimine 25 000 (PEI) (Sigma, USA) at 0,81 mg/mL, pH 7 and the plasmids pAEC-*ha*^{h5} and pEGFP. The last plasmid was generated by including a transcription unit with the enhanced green fluorescent protein (EGFP) gene under the control of the CMV enhanced promoter into the pMOS-Blue backbone. DNA was used at 0,72 μg/cm^2. The ratio pAEC-*ha*^{h5}/pEGFP was 36:1 and the ratio PEI/DNA was 1 μL/1 μg. DNA and PEI were diluted in separate tubes using 5% of glucose until reaching 50 μL each. After samples were vigorously mixed during 10 s and allowed to stand for 5 min, PEI was added to DNA, which were vigorously mixed during 1 min and allowed to stand for 20 min. Subsequently, 900 μL of fresh DMEM was added to the PEI/DNA complex and the mixture of 1 mL was carefully added to the cell culture. Six hours later, FCS was added at a final concentration of 10%. Negative control was performed as above but with the plasmid pAEC-Spt. Transfection was verified after 72 h by observing the production of the EGFP protein in transfected cells at the fluorescence microscope using a magnification of 400×.

2.4. Assembly of the recombinant lentiviral vector

Transfection of the HEK-293FT cell line (Invitrogen, USA) with the plasmids pLP1, pLP2, pLP/VSVG (Invitrogen, USA), pEGFP and pLW-*ha*^{h5} was carried out in 6 flasks of 175 cm^2 (Greiner Bio-One, Germany) using PEI as explained above. In each flask, DNA was used at 0,411 μg/cm^2. The ratio DNA/pEGFP was 36:1 and the ratio pLW-*ha*^{h5}/each helping plasmid (pLP1, pLP2 and pLP/VSVG) was 2:1. Negative control was performed in the same way but with the plasmid pLW. Six hours after adding the mixture of PEI/DNA to the cell culture, FCS was added until reaching 10%. After 48 h, the supernatant was centrifuged at 1000 × *g*, filtered using a pore size of 0,45 μm and ultracentrifuged at 25 000 × *g* for 1:30 h. The supernatant was removed and lentiviral particles were resuspended in fresh DMEM. Storage was performed in aliquots of 10 μL at −70 °C until use. The recombinant lentiviral vector was named Lv-*ha*^{h5}.

2.5. Transduction of the CHO cell line with the lentiviral vector Lv-*ha*^{h5} and clones isolation

After 24 h of seeding 2×10^3 cells/well of CHO-K1 cells (ATCC CCL-61) in a 96 wells plate (Greiner Bio-One, Germany) with DMEM and 10% of FCS, cells were transduced with 10 μL of the Lv-*ha*^{h5} preparation. Twenty-four hours later, culture medium was replaced by fresh medium. Culture medium was replaced every 24 h until cell recovery. The transduction was repeated 3 times. After transductions, cells were dispersed in plates of 145 mm (Greiner Bio-One, Germany) and cultured until clone expansion in DMEM with 10% of FCS. Clones were named CHO-HA^{H5}. Once they were macroscopically visible, a cellular amplifying process was carried out with the clones of CHO-HA^{H5} randomly selected, until reaching confluent monolayers in 6 well plates (Greiner Bio-One, Germany). Positive clones were selected by taking into account their ability to produce the HA^{H5} protein detected in an ELISA assay described below and by monitoring the insertion of the foreign DNA into the cell genome by PCR. The last procedure was accomplished using an automatic Mastercycler (Eppendorf, USA) and the GoTaq® Green Master Mix (Promega, USA). To amplify a segment of the synthetic *ha*^{h5} gene, the primers: (forward) 5′-ATACCATGGGACTGTGTGACCTGGACGGCG-3′ and (reverse) 5′-GATCTCGAGACACTTGGTGTTACAGTTGCC-3′ were synthesized. Two minutes at 95 °C were programmed as the initial step, followed by 35 cycles of 30 s at 95 °C, 30 s at 66 °C and 1 min at 72 °C. A final polymerization step of 5 min at 72 °C was added. To amplify a segment of the gene corresponding to the cPPT of the lentiviral backbone the primers: (forward) 5′-TGGCTGTGGAA AGATACCTAAAGG-3′ and (reverse) 5′-TCGAATGGATCTGTCTCTGTCTCTC-3′ were synthesized. Two minutes at 95 °C were programmed as the initial step, followed by 35 cycles of 30 s at 95 °C, 30 s at 56 °C and 45 s at 72 °C. A final polymerization step of 5 min at 72 °C was also added. Clones of CHO-HA^{H5} were frozen in liquid nitrogen until use.

2.6. Adapting adherent CHO-HA^{H5} cells to suspension culture

After the CHO-HA^{H5} cells reached 90% of confluence in DMEM and 10% of FCS, a medium change was made. DMEM was gradually substituted by SFM4CHO varying the ratio SFM4CHO/DMEM as follow: 25/75, 50/50, 75/25 and 100/0 every 72 h. Detached cells were recovered by centrifugation at 400 × *g* for 5 min in each

medium change. Suspension cultures were scaled up to spinners of 1 l.

2.7. Purification of the HA^{H5} protein

The immunoaffinity chromatography (IC) purification process of the HA^{H5} protein was the same described by [8] for the HACD protein. Briefly, a Sepharose 4B matrix (Pharmacia, USA) activated with cyanogen bromide and coupled to an anti-HA4 monoclonal antibody (Sancti-Spíritus, Cuba) was used to purify the HA^{H5} protein. Column was equilibrated with EB buffer (1 M NaCl, 20 mM Tris–HCl (pH 7,4) and 3 mM EDTA) at a flow rate of 0,4 mL/min. Solution containing the HA^{H5} protein was concentrated in a 10 kDa Sartocon Slice membrane (Sartorius, Germany), supplemented with Tris–HCl (pH 7,4) and NaCl until final concentrations of 10 mM and 150 mM, respectively and passed through the column. After washing with EB buffer, elution was carried out with 0,1 M of citric acid pH 3. Elution drops containing the HA^{H5} protein were immediately collected in 50 mM Tris–HCl pH 8. Purity was estimated by densitometric analysis of SDS-PAGE gels stained with a Coomassie blue R-250 solution at 0,05% using the software TDI's 1D Manager, version 2.0.

2.8. Immunologic assays

2.8.1. ELISA for the detection and quantification of the HA^{H5} protein

The detection and quantification of the HA^{H5} protein was accomplished as described by [8]. Briefly, polystyrene high binding microtiter plates (Costar, USA) were coated with 2,5 μg/mL of the monoclonal antibody anti-HA2 (Sancti-Spíritus, Cuba) overnight at 4 °C. Plates were washed with PBS plus 0,05% Tween 20 (PBST) and blocked with 1% of bovine serum albumin (BSA) (Sigma, USA) in DMEM medium for 2 h at 37 °C. After washing with PBST, standard curve (for quantification) and culture samples were added for 2 h at 37 °C. Standard curve was performed using values from 100 to 1,56 ng/mL of the HA^{H5} protein purified by IC and culture samples were diluted 1:64 in DMEM plus 0,5% BSA. Plates were washed with PBST and the monoclonal antibody anti-HA3 conjugated to horseradish peroxidase (Sancti-Spíritus, Cuba) diluted 1: 20 000 was added. After 1 h at 37 °C, plates were washed with PBST and visualized with 0,04 M of 3,3′,5,5′-tetramethylbenzidine (Sigma, USA) in dimethyl sulphoxide using hydrogen peroxide as substrate. Reaction was stopped with 3,5% of sulfuric acid and absorbance was measured in a microplate reader model SUNRISE-BASIC TECAN (Austria) at 450 nm. The concentration of the HA^{H5} protein in the supernatant was calculated by extrapolating the optical density (OD) values of samples into the standard curve OD.

2.8.2. ELISA for the detection of anti-HA^{H5} antibodies

Polystyrene high binding microtiter plates (Costar, USA) were coated overnight at 4 °C with 2,5 μg/mL of the HA^{H5} protein obtained from SiHa or CHO cells. The HA^{H5} protein was used in a pure state or directly from the culture supernatant of both cell lines. Blocking and wash were performed as above. Serum samples were diluted from 1/500 to 1/32 000 in DMEM plus 0,5% BSA and added to coated plates for 2 h at 37 °C. The antibody detection in sera of chicken immunized with the HACD protein was performed at a dilution of 1/1000. After washing with PBST, the monoclonal antibody anti-IgG (Y) of chicken conjugated to horseradish peroxidase (Sigma, USA) diluted 1/30 000 in DMEM plus 0,5% BSA was added. After 1 h at 37 °C, plates were washed with PBST. Absorbance measurement was performed as above.

2.9. Statistical analysis

The statistical procedure was done using the statistical software GraphPad Prism v.4.02 (GraphPad, USA). A Kruskal–Wallis test and a Dunn post-test were performed to compare the OD values of the HA^{H5} protein in different clones of CHO-HA^{H5}. The HA^{H5} production by different batches of the clone CHO-HA^{H5} 78 was compared by the ANOVA test and the Tukey post-test. The OD values obtained in the ELISA using positive and negative sera with the HA^{H5} protein purified or in the culture supernatant from different expression systems were compared each time point by the unpaired *t*-test.

3. Results

3.1. Expression of the synthetic gene coding the HA^{H5} protein and the obtaining of stable clones from CHO cells producing this protein

The cell line HEK-293 was transformed by transient transfection with the plasmid pAEC-ha^{h5} containing the synthetic gene coding the HA^{H5} protein in order to demonstrate its expression (Fig. 1). As the plasmid pAEC-ha^{h5} (Fig. 1A) was co-transfected with a plasmid carrying a transcriptional unit expressing the gene coding the EGFP protein, transfected cells turned fluorescent after the stimulation with ultraviolet light. The fluorescence was homogeneous and intense, indicating a high level of transfected cells (Fig. 1B and C). The production of the HA^{H5} protein in the transfected cells was assessed by SDS-PAGE and western blot using a polyclonal serum (Fig. 1D and E). Several immunoreactive bands were observed in the sample corresponding to the HA^{H5} protein under reducing and non-reducing conditions. The precursor protein HA0 from HPAIV undergoes a proteolytic processing by endogenous proteases generating the subunits HA1 and HA2. Thus, under reducing conditions a partial proteolytic processing corresponding at about 50% of total protein was observed. Three bands were observed in the western blot corresponding to the uncleaved precursor protein $HA^{H5}0$ and the subunits $HA^{H5}1$ and $HA^{H5}2$ with molecular masses of about 75–78 kDa, 55 kDa and 25 kDa, respectively. Under non-reducing conditions, most of the protein was identified as the precursor protein $HA^{H5}0$ and a smear was observed above 200 kDa, which could correspond to multimeric conformations of the HA^{H5} protein. In this assay, the functionality of the genetic construction pAEC-ha^{h5} was demonstrated. Also, the results showed that the HA^{H5} protein is susceptible to proteolytic cleavage by intracellular proteases.

After verifying the correct expression of the synthetic gene coding the HA^{H5} protein, a lentiviral vector was constructed in order to transduce and stably transform the CHO cell line (Fig. 2A). After transduction, six clones of CHO cells carrying the synthetic gene ha^{h5} (CHO-HA^{H5}) and producing high levels of the HA^{H5} protein were selected (Fig. 2B). These CHO-HA^{H5} clones exhibited an OD over 0,50. The clone CHO-HA^{H5} 78 showed the highest production level of the HA^{H5} protein with an OD of 0,78. The OD values of the positive and the negative controls were 0,89 and 0,085, respectively. The HA^{H5} production level of the clone CHO-HA^{H5} 78 was significantly superior to that of the clones CHO-HA^{H5} 12 ($p < 0{,}001$), CHO-HA^{H5} 70 ($p < 0{,}05$) and CHO-HA^{H5} 76 ($p < 0{,}01$). DNA insertion in the genome of CHO-HA^{H5} clones was verified by PCR using specific primers to amplify a DNA fragment of the lentiviral vector (Fig. 2C) and a fragment of the synthetic gene coding the HA^{H5} protein (Fig. 2D). The chromosomal DNA of each CHO-HA^{H5} clone was used as template. The six clones of CHO-HA^{H5} tested showed a DNA band of 500 bp corresponding to a fragment of the genome of the lentiviral vector and a DNA band of 734 bp corresponding to a fragment of the ha^{h5} gene. There was no DNA amplification in the negative controls in which the chromosomal DNA of the wild type CHO cell line was used as template.

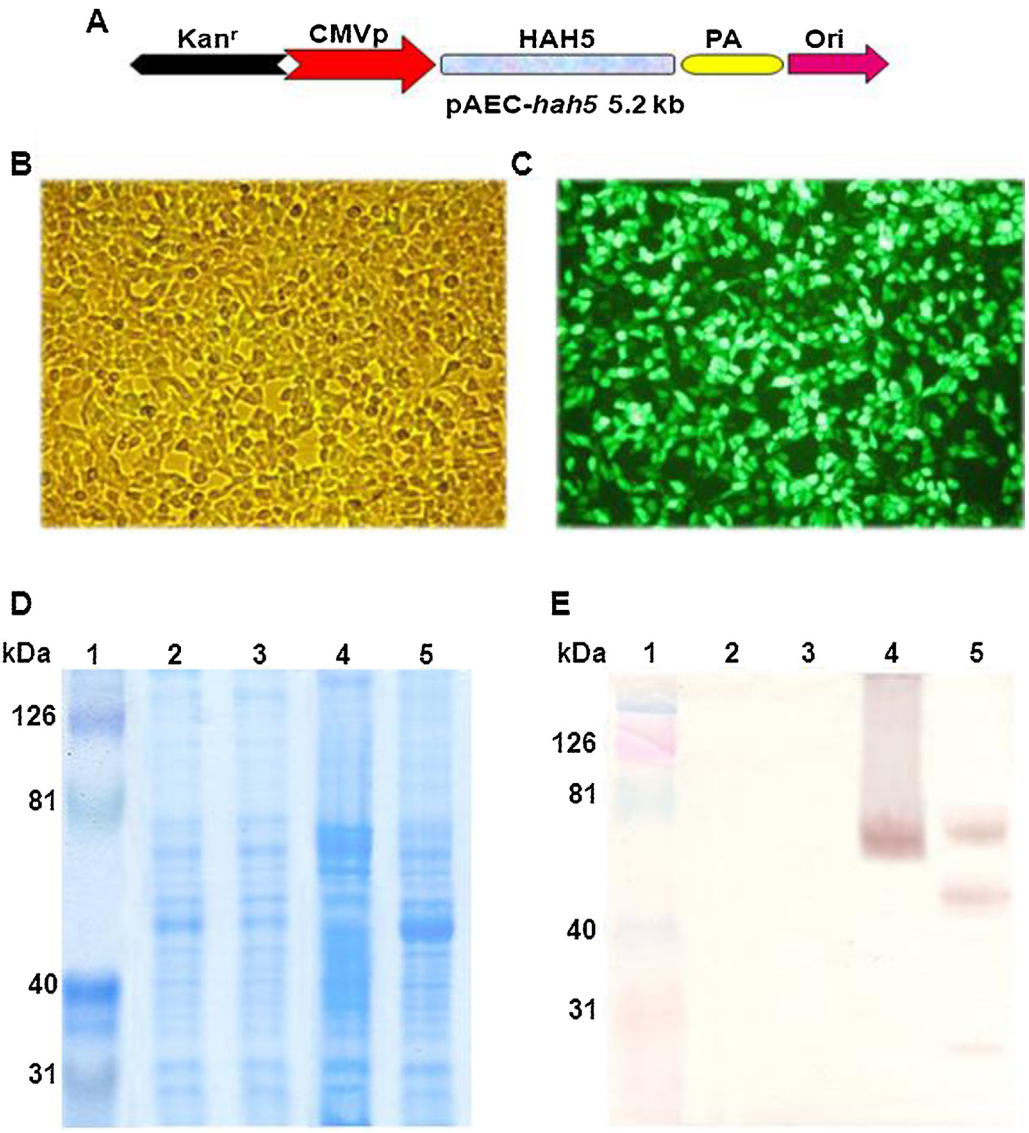

Fig. 1. Expression of the gene coding the HA^{H5} protein in HEK-293 cells. (A) Scheme representing the plasmid pAEC-*ha*^{h5}. Kan^{r}, gene for kanamycin resistance; CMVp, cytomegalovirus major immediate-early promoter/enhancer; HA^{H5}, sequence coding for the extracellular segment of the HA protein from the highly pathogenic strain H5N1 A/Viet Nam 1203/2004; PA, cleavage and polyadenylation sequence; Ori, bacteria's replication origin ColE1. Light field (B) and dark field (C) of HEK-293 cells transduced with the plasmid pAEC-*ha*^{h5} observed to the fluorescence microscope at a magnification of 400×. SDS-PAGE (D) and western blot (E) of trichloroacetic acid-precipitated supernatant of HEK-293 cells transfected with the plasmid pAEC-*ha*^{h5}. (1) Proteins used as molecular weight marker. (2) Supernatant of non-transfected HEK-293 cells under non-reducing conditions. (3) Supernatant of non-transfected HEK-293 cells under reducing conditions. (4) Supernatant of transfected HEK-293 cells under non-reducing conditions. (5) Supernatant of transfected HEK-293 cells under reducing conditions. A hyperimmune chicken antiserum raised against an H5N2 influenza A strain was used for the immunoidentification of the HA^{H5} protein. It was provided by a laboratory of reference from Italy "Istituto Zooprofilattico delle Venezie" OIE Laboratory for AI and NDV. Batch 4/06.

3.2. Adaptation of the stable transformed CHO-HA^{H5} cells to suspension culture and purification of the HA^{H5} protein

Due to the clone CHO-HA^{H5} 78 exhibited the highest levels of the HA^{H5} protein measured by ELISA, it was selected for being adapted to suspension culture. The gradual medium change from DMEM plus FCS to SFM4CHO made the cells to detach of the polystyrene surface and successfully adapted to suspended culture with stirring (Fig. 3A and B). The initial inoculum for scaling up the suspension culture to the volume of 1 l in spinners was $2{,}5 \times 10^4$ cells/mL (Fig. 3C). Two days later, cells increased twofold their concentration and began to grow until reaching more than 3×10^5 cells/mL at day 7. The next day of culture cells decreased their concentration to around $2{,}5 \times 10^5$ cells/mL and became stable until day 10. By day 11, the cell concentration abruptly dropped to almost 1×10^5 cells/mL. Cell viability ranged between 100% and 80% from days 1 to 8. At day 9, cell viability began to decrease and by the last day of the experiment there was a 40% of cell viability. The results obtained above led us to maintain the suspension culture until day 10, where cell concentration and viability met acceptable values, hence the production of the HA^{H5} protein could be favored. In this sense, the concentration of the HA^{H5} protein was measured by ELISA (Fig. 4). The average production of the HA^{H5} protein by different batches of the clone CHO-HA^{H5} 78 in suspension culture was approximately 5,1 μg/mL. There were no significant differences among the individual batches analyzed.

The purification process of the HA^{H5} protein obtained in the culture supernatant was carried out by immunoaffinity chromatography (IC) using a monoclonal antibody against the HA^{H5} protein (Fig. 5). The graphic of absorbance *versus* time showed a well-defined peak when the elution buffer was applied to the matrix (Fig. 5A) which could correspond to the elution of the HA^{H5} protein.

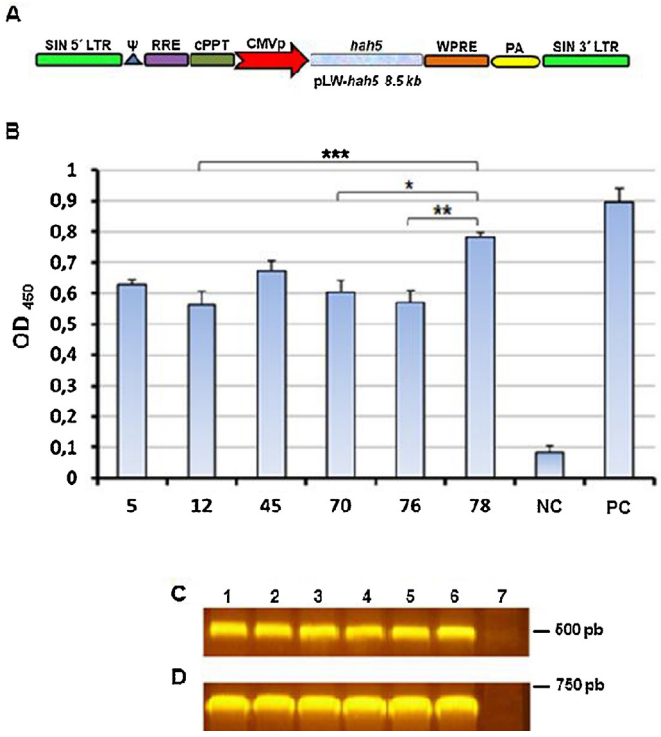

Fig. 2. Transduction of CHO cells with the lentiviral vector Lv-ha^{h5}. (A) Scheme representing the most important characteristics of Lv-ha^{h5}. SIN 5′ LTR, 5′ self-inactivating long terminal repeat; Ψ, packaging signal; RRE, Rev response element; cPPT, central polypurine track; CMVp, cytomegalovirus major immediate-early promoter/enhancer; HA^{H5}, sequence coding for the extracellular segment of the HA protein from the highly pathogenic strain H5N1 A/Viet Nam 1203/2004; WPRE, post-regulatory element derived from woodchuck hepatitis virus; PA, cleavage and polyadenylation sequence; SIN 3′ LTR, 3′ self-inactivating long terminal repeat. (B) ELISA detecting OD_{450} values of CHO-HA^{H5} clones. Data represent the arithmetic mean of nine replicates per CHO-HA^{H5} clone. Negative control (NC): supernatant of untransduced CHO cells. Positive control (PC): Protein HA^{H5} (1 μg/mL) purified by immunoaffinity chromatography. Bars represent the standard deviation. OD values were compared using a Kruskal–Wallis test and a Dunn post-test. $^{*}p < 0{,}05$; $^{**}p < 0{,}01$; $^{***}p < 0{,}001$. (C) PCR bands obtained from CHO-HA^{H5} cells using chromosomal DNA as template and primers specific for a backbone fragment of the lentiviral vector. (D) PCR bands obtained from CHO-HA^{H5} cells using chromosomal DNA as template and primers specific for a fragment of ha^{h5} gene. (1) Clone 5; (2) Clone 12; (3) Clone 45; (4) Clone 70; (5) Clone 76; (6) Clone 78; (7) negative control: untransduced CHO cells.

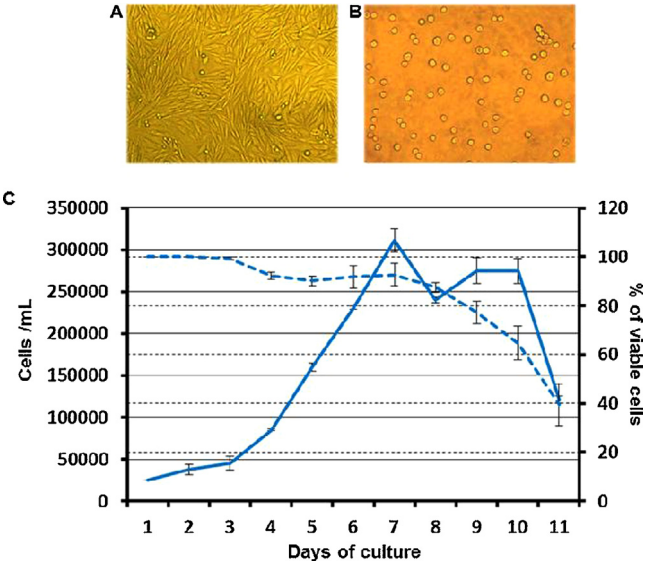

Fig. 3. Kinetic progression of the stable transformed clone CHO-HA^{H5} 78 in suspension culture. (A) Monolayer of adherent CHO cells. (B) CHO cells adapted to suspension culture. Microscopic pictures of cells were observed at a magnification of 400×. (C) Graphic showing cell concentration and cell viability. Data represent the arithmetic mean of three replicates per point. Bars represent the standard deviation.

SDS-PAGE and western blot assays revealed that the peak observed after elution in the graphic of absorbance *versus* time was indeed the elution of the HA^{H5} protein (Fig. 5B and C). The immunoreactive band pattern was the same compared to the observed during the transient transfection of HEK-293 cells. The bands corresponding to the precursor protein $HA^{H5}0$ and the subunits $HA^{H5}1$ and $HA^{H5}2$ were detected. A portion of the HA^{H5} protein was lost in the material not retained to the matrix, which was not observed during the wash of the matrix. The HA^{H5} protein purified by IC was obtained with more than 95% of purity as estimated by a SDS-PAGE densitometric analysis.

3.3. Establishment of ELISA assays to detect antibodies against the HA^{H5} protein

The production of the HA^{H5} protein in a suspension culture system allowed to obtain enough protein to perform immunodetection assays type ELISA with the aim of detecting antibodies against this protein. When plates were coated with the HA^{H5} protein purified by IC from the supernatant of the clone CHO-HA^{H5} 78, a decrease in the OD values was observed as the sera dilution was increased (Fig. 6A). The comparison between dilutions 1/500 and 1/1000 of positive and negative sera showed the most divergent OD values. The dilution of 1/500 exhibited an average OD value of around 0,93 for the positive serum and around 0,28 for the negative serum. In the dilution 1/1000, the average OD value dropped rapidly to about 0,53 in the positive serum and continued diminishing gradually. The dilution 1/1000 made with the negative serum decreased to an average OD value of about 0,23 and also the decreased pattern was sustained until the last dilution tested. A similar experiment was performed with the HA^{H5} protein directly from the culture supernatant of the clone CHO-HA^{H5} 78 suspension culture (Fig. 6B). The average OD values in the dilutions 1/500 and 1/1000 for the positive serum were 0,81 and 0,51 and for the negative serum were 0,29 and 0,23, respectively. This assay repeated the decreased pattern in the OD values for the next sera dilutions.

In our laboratories, a distinct expression system was already used to successfully produce the HA^{H5} protein, in which the synthetic gene coding this molecule was inserted in an adenoviral vector and used for the transduction of SiHa cells [8]. We had used this expression system for producing several chimeric proteins [13,14]. The HA^{H5} protein obtained by this method was also used to perform ELISA assays directly from the culture supernatant or in its purified form. Although the HA^{H5} protein was produced

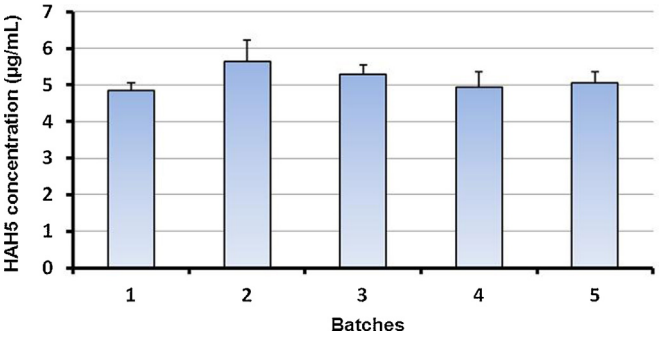

Fig. 4. Quantification of the HA^{H5} protein in the supernatant obtained from different batches of the clone CHO-HA^{H5} 78 in suspension culture. OD values were compared using an ANOVA test and a Tukey post-test. Data represent the arithmetic mean of five replicates per point. Bars represent the standard deviation.

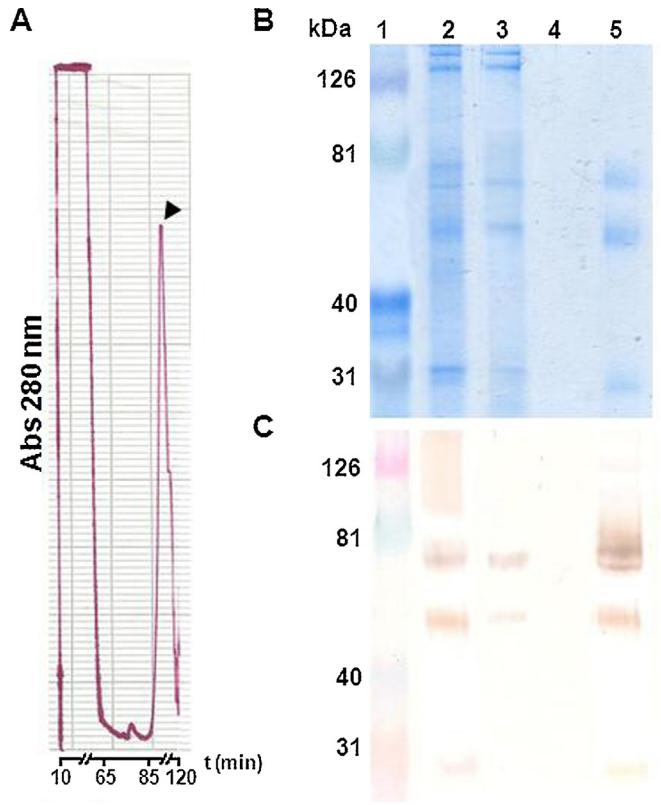

Fig. 5. Purification of the HA^{H5} protein by immunoaffinity chromatography. (A) Graphic of absorbance *versus* time. Arrow head indicates the elution of the HA^{H5} protein. SDS-PAGE (B) and western blot (C) of samples from different stages of the purification process. (1) Proteins used as molecular weight marker; (2) initial sample; (3) non-attached proteins; (4) wash; (5) elution.

in a distinct expression system, the ELISA results using the same conditions as above were very similar. Plates coated with the HA^{H5} protein purified by IC from the supernatant of transduced SiHa cells showed the same decreased pattern in the average OD values when sera dilutions were increased (Fig. 6C). The averages OD of positive and negative sera at dilution 1/500 were 0,91 and 0,29, respectively. In the sera dilutions 1/1000, the average OD for the positive serum was 0,56 and for the negative serum was 0,20. The OD values for the other dilutions continued decreasing. In plates coated with the HA^{H5} protein directly from the culture supernatant of transduced SiHa cells, the average OD for the dilution 1/500 was 0,79 for the positive serum and 0.30 for the negative serum (Fig. 6D). The dilution 1/1000 showed OD values of 0,46 and 0,21 for the positive and the negative serum, respectively. The expected decreased kinetic in OD values for the other sera dilutions was also observed in this assay. The statistical analysis comparing point to point the average OD values of the ELISA assays coating with the HA^{H5} protein from the different expression systems in its purified form or directly from the culture supernatant did not show significant differences.

3.4. Antibody detection in the sera of chickens immunized with the HACD protein having a proper or a non-proper conformation

There is evidence that the renaturation process after the purification of the HA^{H5} protein fused to the chicken CD154 molecule (HACD) by IC could affect the proper conformation of this molecule [8]. Thus, the HA^{H5} proteins purified by IC (HA^{H5}IC) or directly from the culture supernatant of transformed CHO cells (HA^{H5}sC) were used to coat ELISA plates in order to evaluate its capacity to bind antibodies induced by the HACD protein purified by IC (HACD IC) or by size exclusion chromatography (SEC) (Fig. 7). For the positive control, wells coated with HACD purified by SEC (HACD SEC) and the sera of chickens immunized with the same protein were used. In the negative control, measures were carried out coating with the protein HACD SEC and using the sera of chickens immunized with PBS. The ELISA assay coated with the protein HA^{H5}IC showed OD values of 0.61 when the sera of chickens immunized with HACD IC were tested (Fig. 7A), indicating the existence of anti-HA^{H5} antibodies. The proteins HA^{H5}sC and the one obtained in the supernatant of SiHa cells transduced with a recombinant adenoviral vector (HA^{H5}sS) were also able to bind antibodies from the chicken sera used in the previous experiment showing OD values of 0.67 and 0.63, respectively (Fig. 7B). More interestingly, the ELISA assay performed with the protein HA^{H5}IC detected anti-HA^{H5} antibodies in the sera of chicken immunized with the protein HACD SEC, showing an OD value of 0.69 (Fig. 7C). In all cases, positive and negative controls showed OD values around 0.95 and 0.09, respectively.

4. Discussion

After the emergence of the HPAIV H5N1, poultry and human health have been compromised. Also, it has caused a serious economic trouble owing to the obstruction of poultry trade industry worldwide [15]. The Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (OIE) have made huge efforts to organize accurate strategies for circumventing or diminishing the damages caused by the H5N1 virus. Among them, a vaccination program together with biosafety measures which include surveillance, quarantine and sanitation are crucial [16–18]. Establishing analytical methods for differentiating infected from vaccinated animal (DIVA) and surveillance require a strong platform for protein production, which need a robust and reliable expression system able to produce large amount of protein. In this study, an expression system based on the stable transduction of CHO cells with a recombinant lentiviral vector carrying a synthetic gene with the coding sequence of the HA protein from the HPAIV H5N1 was assessed. The generation of genes by chemical synthesis allows the obtaining of the desired genes in a short period of time, avoids manipulation of strains from HPAIV, the codon usage could be rearranged according to the expression system and allows the addition or removal of regulatory sequences that modulate the expression of the gene of interest. The molecule HA derived from the HPAIV H5N1 A/Viet-Nam/1203/2004 was selected for being lethal to chickens, ducks, ferrets and humans [19,20].

During transient transduction of the cell line HEK-293 with the synthetic gene carrying the nucleotide sequence of the HA^{H5} protein, it was successfully expressed and the HA^{H5} protein was secreted to the culture supernatant. The band pattern observed in the western blot assay was very similar to the one obtained in our previous studies when the same synthetic gene was introduced into an adenoviral platform and expressed in HC11 [2] and SiHa cells [8]. The HA molecule of influenza viruses type A is the most representative molecule of the viral envelope, which is distributed in trimers. Each monomer contains the subunits HA1 and HA2, which are the product of the proteolytic cleavage of the precursor molecule HA0 [21]. This proteolytic cleavage is essential for viral infectivity and it is the most important pathogenicity determinant for avian and human hosts. This cleavage is regulated by the molecule structure and the proteases involved in the viral activation [22]. Low pathogenic avian influenza strains have a monobasic cleavage site susceptible to trypsin-like proteases. Highly pathogenic avian influenza strains have a multibasic cleavage site accessible to subtilysin proteases. They have a wide distribution among several cellular types. For this reason, viral infection spreads to multiple tissues, causing systemic infections and the host death [23]. The *in vitro* expression of the gene coding the HA protein from a low

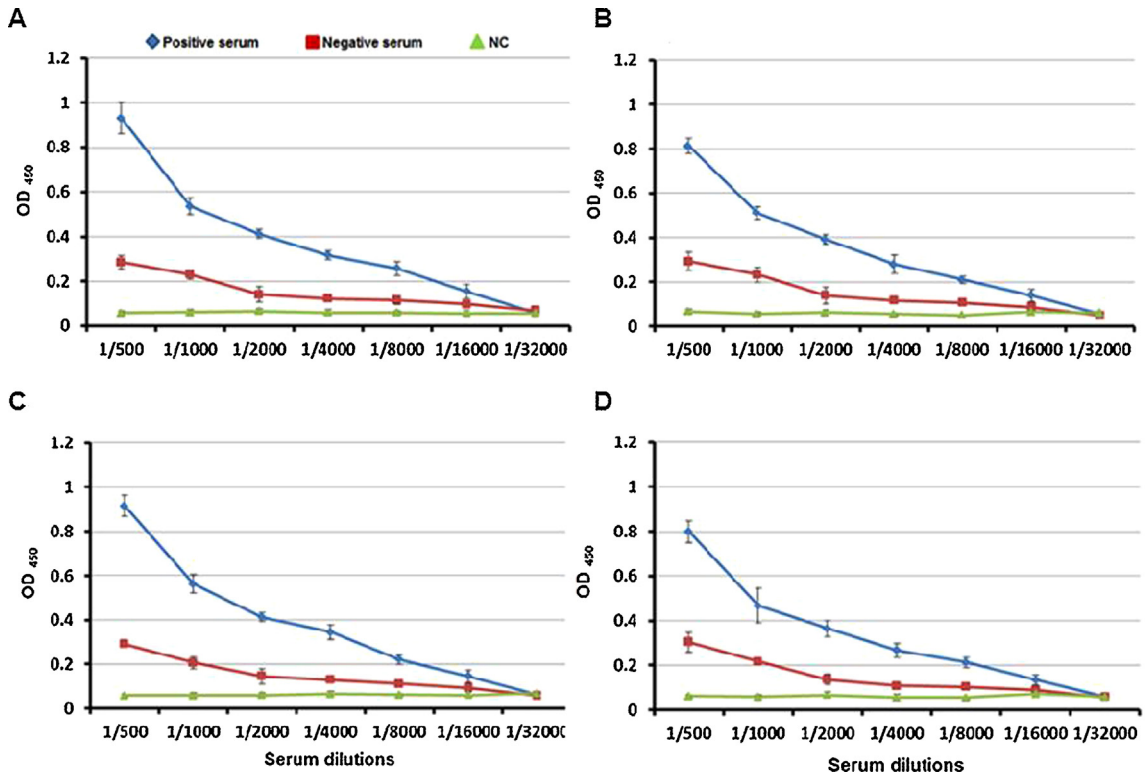

Fig. 6. ELISA assays for detecting anti-HA^{H5} antibodies coating with the HA^{H5} protein produced in different cell lines. Anti-HA^{H5} antibodies detection coating with 2.5 μg/mL of the HA^{H5} protein which was obtained from the supernatant of clone CHO-HA^{H5} 78 in its purified form (A) or cultured in suspension (B). Also, the ELISA plates were coated with the HA^{H5} protein purified from SiHa cells transiently transduced with a recombinant adenoviral vector (C) or directly from the culture supernatant of this expression system (D). Negative and positive reference sera were purchased from a reference laboratory of Italy "Istituto Zooprofilattico delle Venezie" OIE Laboratory for AI and NDV. Batch 4/06 for the positive serum and batch 2/06 for the negative serum. Data were compared each time point by the unpaired *t*-test. NC, negative control. Data represent the arithmetic mean of six replicates per point. Bars represent the standard deviation.

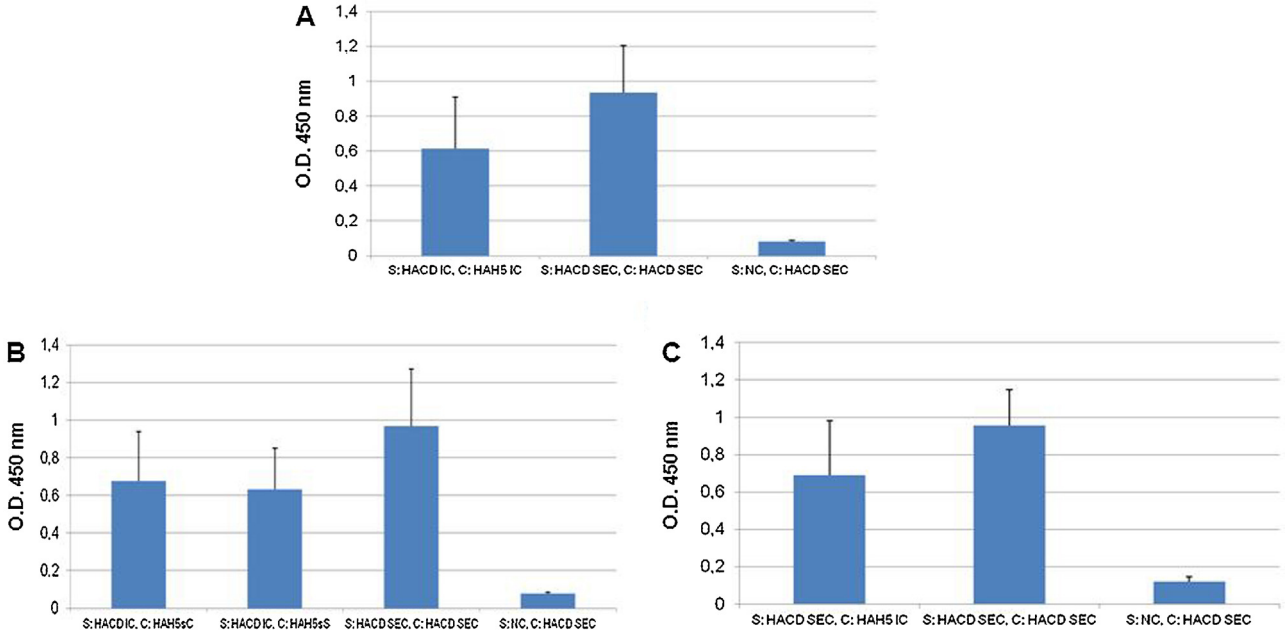

Fig. 7. Antibody detection by ELISA coating with the HA^{H5} protein produced by suspension culture of CHO cells purified by immunoaffinity chromatography or directly from culture supernatant of CHO or SiHa cells. (A) Antibody levels in sera of chickens immunized with the HACD protein purified by IC coating with the HA^{H5} protein obtained in CHO cells also purified by IC. (B) Antibody levels in sera of chickens immunized with the HACD protein purified by IC coating with the HA^{H5} protein from the culture supernatant of CHO (HAsC) or SiHa (HAsS) cells. (C) Antibody levels in sera of chickens immunized with the protein HACD SEC coating with the HA^{H5} protein obtained in CHO cells and purified by IC. As positive controls, sera of chicken immunized with the protein HACD SEC were used. The coating was carried out with the same protein. As negative controls, sera of chicken immunized with PBS were used. The coating was carried out with 2,5 μg/mL of the HA^{H5} protein purified by IC or directly from culture supernatant of both cell lines. Sera of ten immunized chickens per group diluted 1:1000 were used. S, sera of chickens immunized with; C, coating; NC, negative control.

pathogenic avian influenza strain requires the addition of trypsin for the proteolytic cleavage to occur. However, the HA protein from a highly pathogenic avian influenza strain does not need the addition of any external protease to be cleaved, the endogenous proteases of the cell line that secrete the HA protein are able to cleave it [24]. Our studies showed spontaneous proteolytic cleavages of the HA^{H5} protein, which demonstrate that this molecule came from a highly pathogenic avian influenza strain. Nevertheless, only part of the HA^{H5} molecule was cleaved. Western blot shows a segment of protein without cleavage corresponding to the precursor protein $HA^{H5}0$, suggesting an incomplete processing of this protein.

The stable production of the HA^{H5} protein in CHO cells transduced with a recombinant lentiviral vector could become a suitable alternative for controlling and monitoring avian influenza disease. This system could produce proteins not only for diagnostic purposes but also as vaccine candidates and constitute another valid approach to counteract the spreading of HPAIV H5N1. Avian influenza viruses infect eukaryotic cells. Thus, the environment in which their proteins are produced provides complex post-translation modifications to the molecules. Specifically, HA protein is a highly glycosylated molecule. The type and pattern of glycosylation are important features for the HA protein to perform its biological function [25]. In humans, influenza virus subtype H1N1, the number of potential N-glycosylation sites of the HA protein are highly controlled. Five potential N-glycosylation sites in the globular head of each HA monomer are selected, but only up to three are used [26–28]. Also, there seems to be a relationship between antigenic variation and the number and position of N-glycosylation site which can regulate the avidity and specificity of the union of the HA protein to its receptor, the influenza strain virulence and the evasion to antibodies recognition [25]. Prokaryotes and inferior eukaryotes expression systems are able to glycosylate [29,30]. However, the glycosylation phenomenon in the traditional prokaryotic expression system *Escherichia coli* is very rare [31]. Inferior eukaryotes, like yeasts, are able to perform N-glycosylation, but the hyper-mannosylated glycans attached to the polypeptide chain are significantly different from those of mammalian cells [32]. Although there are some strategies in bacteria and yeast to efficiently obtain the HA molecule as a vaccine candidate able to confer protection in mice [6,7], mammalian cells are the closest alternative to produce a soluble HA protein with post-translational modifications similar to the native one, thus preserving the original properties of this molecule. In fact, we have already obtained the HA^{H5} protein in mammalian cell culture able to induce high levels of HIA in chickens [8]. Also, the protein bands obtained for the HA^{H5} protein in SDS-PAGE under reducing condition corresponds to a glycosylated version of this protein, since we have already demonstrated that the deglycosylation of the HA^{H5} protein with the enzyme PNGase-F provides a lower band pattern [8].

In the last decade, mammalian cell culture has become the most demanded expression system to obtain complex recombinant proteins in response to their increasing need for structural and functional studies and for field experiments. There are several cell lines used for this purpose, such as HEK-293, BHK, NSO, among others. However, CHO cells have been so far the most utilized [33,34]. Currently, the majority of recombinant proteins intended to biopharmaceutical industry is produced in this cell line because it has several advantages with respect to the other cell lines: (i) its safety is thoroughly demonstrated, so it is easy to overcome regulatory issues in order to gain the consent of supervisory institutions; (ii) low productivity can be improved by gene amplification systems available for CHO cells and (iii) the change of culture conditions from adherent serum-dependent to serum-free suspension culture can be easily achieved for this cells. This is a desired feature for scaling up the production system and to reduce the costs [10]. All these characteristics of CHO cells make them a suitable expression system to produce antigens of the HPAIV H5N1 in a safe way and with higher quality.

The comparison of OD values in the immunodetection assay type ELISA using the HA^{H5} protein purified by IC produced in the CHO cells stable transformed with a recombinant lentiviral vector or by transient transduction of SiHa cells with a recombinant adenoviral vector showed no significant differences. The OD values observed during the antigen–antibody interaction of the positive reference serum with the HA^{H5} protein purified or directly from the culture supernatant produced in different expression systems were very similar, as well as the OD values detected when the negative reference serum was assayed. Despite the differences in the viral vector and the expression system used, it seems that the HA^{H5} protein did not suffer dramatic post-translational changes during its production and posterior secretion able to alter its recognition by antibodies. Thus, the use of the HA^{H5} protein directly from the culture supernatant for the recognition of anti-HA^{H5} antibodies could lower the costs in a large scale process because of the exclusion of the purification stage.

On the other hand, the fact that the HA^{H5} protein purified by IC have shown a similar antibody levels compared with the unpurified variant when the sera of chickens immunized with the HACD protein purified by IC was assayed is a very interesting result. There are evidences that the renaturation after the acidic elution during the purification by IC of the HACD protein make it inefficient to induce HIA, while the same protein purified by SEC is able to induce such type of antibodies [8]. This suggests that HA^{H5} molecule purified by IC could undergo conformational changes upon renaturation. Regardless of the failure in inducing hemagglutinating antibodies, the HACD protein purified by IC is able to trigger a humoral immune response detected by ELISA containing antibodies able to recognize both the HA^{H5} protein purified by IC or directly from the culture supernatant. Also, the antibodies induced by the HACD protein purified by SEC bind the HA^{H5} protein purified by IC. Therefore, the protein HA^{H5}, although purified by a method that can affect its conformation, preserves epitopes able to bind antibodies induced by a protein with a conformation very close to the native HA. It suggests there are other antibodies than HIA which are induced during the immune response against the HA protein that, although incapable of neutralizing the molecule, can be detected in ELISA assays using the HA protein purified by IC. Hence, this protein can be useful in diagnostic by detecting H5 subtype of avian influenza virus.

5. Conclusions

There is no doubt that avian influenza caused by HPAIV H5N1 is one of the viral diseases which currently could put in danger poultry and all mankind with the sudden appearance of a new strain able to cross species from birds to human and rapidly propagate among them. Consequently, there are a lot of research projects directed to basic investigations for controlling and making better surveillance methods to eradicate this disease. The present study focused on the establishment of a stable CHO cell line producing the HA protein from HPAIV H5N1 with the main objective of detecting antibodies against this protein and indeed results demonstrated that a reference positive serum succeeded in recognizing epitopes on the HA^{H5} protein obtained in CHO cells. The production of the HA^{H5} protein from a HPAIV is only the beginning from what could represent a safe and consistent system of producing antigens from avian influenza viruses, not only for diagnostic reinforcing the surveillance, but also for mass producing vaccine candidates against these viruses. Further experiments must be performed in order to enhance the stability, the viability and the concentration

of CHO cells in suspension culture. Also the production levels of the HA^{H5} protein and the cell line characterization must be improved. However, it is undoubtedly a more secure, rapid and less expensive method compared to diagnostic methods or conventional vaccines which utilize the natural or the pseudotyped viral particles.

References

[1] Y.J. Lin, M.C. Deng, S.H. Wu, Y.L. Chen, H.C. Cheng, C.Y. Chang, M.S. Lee, M.S. Chien, C.C. Huang, Baculovirus-derived hemagglutinin vaccine protects chickens from lethal homologous virus H5N1 challenge, J. Vet. Med. Sci. 70 (2008) 1147–1152.

[2] O.S. Ramos, A.G. Pose, S. Gómez-Puerta, J.N. Gómez, A.V. Redondo, J.C. Benites, L.S. Amarán, N.S. Parra, J.R. Toledo Alonso, Avian CD154 enhances humoral and cellular immune responses induced by an adenovirus vector-based vaccine in chickens, Comp. Immunol. Microbiol. Infect. Dis. 34 (2011) 259–265.

[3] D.L. Suarez, A. Das, E. Ellis, Review of rapid molecular diagnostic tools for avian influenza virus, Avian Dis. 51 (2007) 201–208.

[4] R. Wu, S. Hu, Y. Xiao, Z. Li, D. Shi, D. Bi, Development of indirect enzyme-linked immunosorbent assay with nucleoprotein as antigen for detection and quantification of antibodies against avian influenza virus, Vet. Res. Commun. 31 (2007) 631–641.

[5] S. Tong, Y. Li, P. Rivailler, C. Conrardy, D.A.A. Castillo, L.M. Chen, S. Recuenco, J.A. Ellison, C.T. Davis, I.A. York, A distinct lineage of influenza A virus from bats, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 4269–4274.

[6] A. Jegerlehner, F. Zabel, A. Langer, K. Dietmeier, G.T. Jennings, P. Saudan, M.F. Bachmann, Bacterially produced recombinant influenza vaccines based on virus-like particles, PLOS ONE 8 (2013) e78947.

[7] X. Saelens, P. Vanlandschoot, W. Martinet, M. Maras, S. Neirynck, R. Contreras, W. Fiers, W. Min Jou, Protection of mice against a lethal influenza virus challenge after immunization with yeast-derived secreted influenza virus hemagglutinin, Eur. J. Biochem. 260 (1999) 166–175.

[8] A.G. Pose, J.N. Gómez, A.V. Sánchez, A.V. Redondo, E.R. Rodríguez, R.M. Seguí, E.M.G. Ramos, M.P.R. Moltó, E.S. Rodríguez, L.R. Cordero, Subunit influenza vaccine candidate based on CD154 fused to HAH5 increases the antibody titers and cellular immune response in chickens, Vet. Microbiol. 152 (2011) 328–337.

[9] J. Zhu, Mammalian cell protein expression for biopharmaceutical production, Biotechnol. Adv. 30 (2012) 1158–1170.

[10] J.Y. Kim, Y.G. Kim, G.M. Lee, CHO cells in biotechnology for production of recombinant proteins: current state and further potential, Appl. Microbiol. Biotechnol. 93 (2012) 917–930.

[11] S. Ansorge, O. Henry, A. Kamen, Recent progress in lentiviral vector mass production, Biochem. Eng. J. 48 (2010) 362–377.

[12] A.M. Herrera, E.G. Rodríguez, T. Hernández, B. Sández, C.A. Duarte, A family of compact plasmid vectors for DNA immunization in humans, Biochem. Biophys. Res. Commun. 279 (2000) 548–551.

[13] O. Sánchez, J.R. Toledo, M.P. Rodríguez, F.O. Castro, Adenoviral vector mediates high expression levels of human growth hormone in the milk of mice and goats, J. Biotechnol. 114 (2004) 89–97.

[14] J.R. Toledo, O. Sánchez, R.M. Seguí, G. García, M. Montañez, P.A. Zamora, M.P. Rodríguez, J.A. Cremata, High expression level of recombinant human erythropoietin in the milk of non-transgenic goats, J. Biotechnol. 123 (2006) 225–235.

[15] S.L. Layton, D.R. Kapczynski, S. Higgins, J. Higgins, A.D. Wolfenden, K.A. Liljebjelke, W.G. Bottje, D. Swayne, L.R. Berghman, Y.M. Kwon, B.M. Hargis, K. Cole, Vaccination of chickens with recombinant Salmonella expressing M2e and CD154 epitopes increases protection and decreases viral shedding after low pathogenic avian influenza challenge, Poult. Sci. 88 (2009) 2244–2252.

[16] V.J. Lee, D.C. Lye, A. Wilder-Smith, Combination strategies for pandemic influenza response – a systematic review of mathematical modeling studies, BMC Med. 7 (2009) 76–84.

[17] M. Peyre, G. Fusheng, S. Desvaux, F. Roger, Avian influenza vaccines: a practical review in relation to their application in the field with a focus on the Asian experience, Epidemiol. Infect. 137 (2009) 1–21.

[18] A. Schuchat, B.P. Bell, S.C. Redd, The science behind preparing and responding to pandemic influenza: the lessons and limits of science, Clin. Infect. Dis. 52 (2011) S8–S12.

[19] R.O. Donis, Evolution of H5N1 avian influenza viruses in Asia, Emerg. Infect. Dis. 11 (2005) 1515–1521.

[20] W. Gao, A.C. Soloff, X. Lu, A. Montecalvo, D.C. Nguyen, Y. Matsuoka, P.D. Robbins, D.E. Swayne, R.O. Donis, J.M. Katz, Protection of mice and poultry from lethal H5N1 avian influenza virus through adenovirus-based immunization, J. Virol. 80 (2006) 1959–1964.

[21] C.S. Kim, R.F. Epand, E. Leikina, R.M. Epand, L.V. Chernomordik, The final conformation of the complete ectodomain of the HA2 subunit of influenza hemagglutinin can by itself drive low pH-dependent fusion, J. Biol. Chem. 286 (2011) 13226–13234.

[22] O.P. Zhirnov, M.R. Ikizler, P.F. Wright, Cleavage of influenza a virus hemagglutinin in human respiratory epithelium is cell associated and sensitive to exogenous antiproteases, J. Virol. 76 (2002) 8682–8689.

[23] S. Bertram, I. Glowacka, I. Steffen, A. Kühl, S. Pöhlmann, Novel insights into proteolytic cleavage of influenza virus hemagglutinin, Rev. Med. Virol. 20 (2010) 298–310.

[24] J. Stevens, O. Blixt, T.M. Tumpey, J.K. Taubenberger, J.C. Paulson, I.A. Wilson, Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus, Science 312 (2006) 404–410.

[25] J.I. Kim, M.S. Park, N-linked glycosylation in the hemagglutinin of influenza a viruses, Yonsei Med. J. 53 (2012) 886–893.

[26] S. Sun, Q. Wang, F. Zhao, W. Chen, Z. Li, Glycosylation site alteration in the evolution of influenza A (H1N1) viruses, PLoS ONE 6 (2011) e22844.

[27] S. Sun, Q. Wang, F. Zhao, W. Chen, Z. Li, Prediction of biological functions on glycosylation site migrations in human influenza H1N1 viruses, PLOS ONE 7 (2012) e32119.

[28] C.J. Wei, J.C. Boyington, K. Dai, K.V. Houser, M.B. Pearce, W.P. Kong, Z. Yang, T.M. Tumpey, J.C. Nabel, Cross-neutralization of 1918 and 2009 influenza viruses: role of glycans in viral evolution and vaccine design, Sci. Transl. Med. 2 (2010) 24ra21.

[29] F. Schwarz, M. Aebi, Mechanisms and principles of N-linked protein glycosylation, Curr. Opin. Struct. Biol. 21 (2011) 576–582.

[30] E. Weerapana, B. Imperiali, Asparagine-linked protein glycosylation: from eukaryotic to prokaryotic systems, Glycobiology 16 (2006) 91R–101R.

[31] O. Sherlock, U. Dobrindt, J.B. Jensen, R.M. Vejborg, P. Klemm, Glycosylation of the self-recognizing *Escherichia coli* Ag43 autotransporter protein, J. Bacteriol. 188 (2006) 1798–1807.

[32] S.R. Hamilton, T.U. Gerngross, Glycosylation engineering in yeast: the advent of fully humanized yeast, Curr. Opin. Biotechnol. 18 (2007) 387–392.

[33] B. Gaillet, R. Gilbert, S. Broussau, A. Pilotte, F. Malenfant, A. Mullick, A. Garnier, B. Massie, High-level recombinant protein production in CHO cells using lentiviral vectors and the cumate gene-switch, Biotechnol. Bioeng. 106 (2010) 203–215.

[34] P.M. O'Callaghan, D.C. James, Systems biotechnology of mammalian cell factories, Brief Funct. Genomics Proteomics 7 (2008) 95–110.

[35] J.R. Toledo, Y. Prieto, N. Oramas, O. Sánchez, Polyethylenimine-based transfection method as a simple and effective way to produce recombinant lentiviral vectors, Appl. Biochem. Biotechnol. 157 (2009) 538–544.