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Original Article

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Dual function of the hemagglutinin H5 fused to chicken CD154 in a potential strategy of DIVA against avian influenza disease: preliminary study

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Abstract

In this study we demonstrated that the vaccine candidate against avian influenza virus H5N1 based on the hemagglutinin H5 (HA) fused to the chicken CD154 (HACD) can also be used for differentiating infected from vaccinated animals (DIVA). As the strategy of DIVA requires at least two proteins, we obtained a variant of the nucleoprotein (NP_{49-375}) in *E. coli*. After its purification by IMAC, the competence of the proteins NP_{49-375} and HACD as coating antigens in indirect ELISA assays were tested by using the sera of chickens immunized with the proteins HA and HACD and the reference sera from several avian influenza subtypes. Together with these sera, the sera from different species of birds and the sera of chickens infected with other avian viral diseases were analyzed by competition ELISA assays coated with the proteins NP_{49-375} and HACD. The results showed that the segment CD154 in the chimeric protein HACD did not interfere with the recognition of the molecule HA by its specific antibodies. Also, we observed variable detection levels when the reference sera were analyzed in the ELISA plates coated with the protein NP_{49-375}. Moreover, only the antibodies of the reference serum subtype H5 were detected in the ELISA plates coated with the protein HACD. The competition ELISA assays showed percentages of inhibition of 88-91% for the positives sera and less than 20% for the negative sera. We fixed the cut-off value of these assays at 25%. No antibody detection was observed in the sera from different species of birds or the sera of chickens infected with other avian viral diseases. This study supported the fact that the ELISA assays using the proteins NP_{49-375} and HACD could be valuable tools for avian influenza surveillance and as a strategy of DIVA for counteracting the highly pathogenic avian influenza virus H5N1 outbreaks.

Keywords: Avian influenza virus, CD154, Competition ELISA, DIVA, Hemagglutinin.

Introduction

The twentieth and the early twenty-first centuries have been the scene of several influenza pandemics where avian influenza viruses were outstanding contributors (Zhang, 2012). In the last decade Asia, Europe and Africa were the targets of several outbreaks caused by the highly pathogenic avian influenza virus H5N1. Consequently, millions of birds and hundreds of human lives were lost. Due to the sudden appearance of this virus and its wide distribution, the World Health Organization warned about the emergence of a potential influenza pandemic with devastating consequences (Ligon, 2005). Currently, that threat is no longer a major concern, but the continued presence of the infection in poultry may increase the risk for the emergence of a new H5N1 viral strain able to propagate among humans. Therefore, the protection and control of poultry are crucial. The implementation of vaccination campaigns applying the strategy of DIVA could significantly reduce the avian losses and zoonotic infection of humans with the highly pathogenic avian influenza virus H5N1. Also, the surveillance of this virus could be critical in order to act quickly against the viral outbreaks encoded by the fifth

Few years ago, we developed potential vaccine candidates against the avian influenza virus H5N1 based on the fusion of the protein hemagglutinin H5 from the A/Vietnam/1203/2004 strain and the chicken CD154 molecule (HACD) using different delivery approach (Pose *et al.*, 2011; Ramos *et al.*, 2011). However, an appropriate strategy of DIVA for an accurate application of a probable vaccination policy was not available. As both vaccine candidates contained only one protein of the virus, the strategy of DIVA could only be accomplished by using a different viral protein from that of the vaccine candidates.

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The nucleoprotein (NP) is a polypeptide of 498 amino acids encoded by the fifth single-stranded RNA segment of the eight comprised by the viral genome of avian influenza viruses (Ruigrok and Baudin, 1995). This structural protein is highly conserved among influenza A viruses and confers type specificity (van Wyke *et al.*, 1980). Several studies have demonstrated that the protein NP could be successfully used in ELISA assays for influenza A antibody screening as an alternative technique with rapid, highly specific and sensitive performance compared to other serological methods (Jin *et al.*, 2004; Starick *et al.*, 2006; Wu *et al.*, 2007).

In this study, we provide preliminary data in order to demonstrate that the subunit vaccine candidate HACD can be used to establish a potential strategy of DIVA by adding a segment of the protein NP from the strain H7N1 A/ck/1067/V99 produced in *E. coli*.

Materials and Methods

Isolation of the gene coding the protein NP_{49-375}

We obtained the complementary DNA coding a segment of the protein NP from the total RNA corresponding to the strain A/chicken/Italy/1067/1999 of avian influenza virus, subtype H7N1, using the kit Reverse Transcriptase System (Promega, USA). The access number for the complete sequence of this molecule is AJ584648 in the database of the National Center of Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). The gene coding the protein NP from the aminoacid 49 to the aminoacid 375 (NP_{49-375}) was amplified by PCR using an automatic Master cycler (Eppendorf, USA), the Platinum®*Pfx* DNA polymerase (Invitrogen, USA) and the primers: forward 5´-TATGCTAG**C**AGCGAC TATGAGGGGAGACTG-3´ including the restriction site ***Nhe* I** and reverse 5-TTCGAATTCTTAG GAGT CCATTGTTTCCATGTTC-3´ including the restriction site EcoR I. The PCR reaction was conducted under the following conditions: four minutes at 93°C, followed by 35 cycles of 40 seconds at 93°C, 60 seconds at 55°C and 90 seconds at 68°C. We added a final polymerization step of five minutes at 68°C. The protein NP_{49-375} includes several antigenic epitopes of the native protein NP (Jin *et al.*, 2004).

Cloning and expression of the gene coding the protein NP_{49-375}

The recombinant expression plasmid, named pET-28a-np_{49-375}, was assembled by subcloning the PCR product previously phosphorylated into the plasmid pUC18 (Thermo Scientific, USA) digested with the enzyme *Sma* I, obtaining the plasmid pUC-np_{49-375}. Subsequently, the DNA segment coding the protein NP_{49-375} was removed from the plasmid pUC-np_{49-375} by digestion with the enzymes *Nhe* I and *EcoR* I (Promega, USA) and inserted into the prokaryotic expression vector pET-28a (Invitrogen, USA), previously digested with the same enzymes, to obtain the final construction. The plasmids pUC-np_{49-375} and pET-28a-np_{49-375} were sequenced (Macrogen, South Korea) and checked by a restriction assay using the restriction enzymes *Nhe* I and *EcoR* I to confirm the authenticity of the gene of interest. The *E. coli* strains BL21-CodonPlus® (DE3)-RIL (Stratagene, USA), BL21-CodonPlus® (DE3)-RP (Stratagene, USA) and Rosetta™ (DE3) (Novagen, Germany) were transformed with the plasmid pET-28a-np_{49-375} following the procedures of the instruction manual of BL21-CodonPlus® Competent Cells (Stratagene, USA). We performed the expression induction of the gene coding the protein NP_{49-375} following the instructions of the same manual. The *E. coli* strains were selected due to previous failure in the expression of the gene np_{49-375} using the *E. coli* strain BL-21 (DE3) as host and the existence of several rare codons in the nucleotide sequence of this gene, which could impair the protein translation process (Fig. 1).

Solubilization and purification of the protein NP_{49-375}

The bacterial culture was collected by centrifugation at 8000 x g for five minutes. It was homogenized in a disruption buffer (5 mM EDTA in PBS 1X). Cell disruption was performed using an IKA®-Labortechnik U200S sonicator (IKA, Germany), set at 70% of amplitude for one cycle. Samples were subjected to intervals of one minute of sonication and one minute of incubation on ice. The procedure was repeated three times. After centrifuging at 10 000 x g for 30 minutes, the pellet was treated with 1 M, 2 M, 4 M and 6 M of guanidine hydrochloride (GuHCl) (Merck, Germany) in PBS 1X during 16 hours at 4ºC.

The protein NP_{49-375} was purified by immobilized ion metal affinity chromatography (IMAC). The solution of 6 M GuHCl containing the solubilized protein NP_{49-375} was adjusted with 5 mM imidazole, and was filtered through a 0.45 µm pore size before applied into a column filled with the chelating matrix, Fractogel®-IDA EMD (Merck, Germany). This matrix was previously loaded with a divalent metal ion solution of 0.1 M $CuSO_4$ (Merck, Germany) and equilibrated with the buffer containing PBS 1X, 6 M GuHCl and 5 mM imidazole, pH 7.5 at a flow rate of 0.2 mL/minutes. After washing with three volumes of the buffer containing PBS 1X, 6 M GuHCl and 20 mM imidazole, pH 7.5, the protein NP_{49-375} was eluted with the same buffer containing 100 mM imidazole. Protein detection was performed by using the chromatography station ÄKTA prime with the ÄKTA prime view software (Pharmacia, Sweden). The eluted fraction was dialyzed in PBS 1X, 2% SDS, pH 7.5. The purity of the protein NP_{49-375} was estimated by densitometric analysis of the SDS-PAGE gels (12.5%) stained with a Coomassie blue R-250 solution at 0.05% using the software TDI's 1D Manager, version 2.0.

Fig. 1. Nucleotide sequence of the gene coding the protein NP_{49-375} highlighting the rare codons.

Immunoenzymatic assays

Indirect ELISA for testing the sera from chickens immunized with the proteins HA and HACD

Polystyrene high binding microtiter plates (Costar, USA) were coated overnight at 4°C with 2.5 µg/mL of the proteins NP_{49-375}, HA or HACD. The last two proteins were produced in mammalian cells by adenoviral transduction (Pose *et al.*, 2011; Ramos *et al.*, 2011). The plates were washed with PBS 1X plus 0.05% of Tween 20 (PBST) and blocked with 1% of bovine serum albumin (BSA) (Sigma, USA) in PBS 1X for two hours at 37°C. We analyzed the sera from chickens immunized with PBS 1X and with the proteins HA and HACD mixed with the adjuvant Montanide 888 (Seppic, France). Also, the sera from non-immunized chickens were tested. The assay was performed with 10 chickens per experimental group. All sera were diluted 1/1000 in PBS 1X plus 0.5% of BSA and two replicates of each serum were added to the coated plates for two hours at 37°C. After washing with PBST, monoclonal antibody anti-IgG (Y) of chicken conjugated to horseradish peroxidase (Sigma, USA) and diluted 1/30 000 in PBS 1X plus 0.5% of BSA was added. After one hour at 37°C, the 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. The reaction was stopped with 3.5% of sulfuric acid and the absorbance measured using a microplate reader model SUNRISE-BASIC TECAN (Tecan, Austria) at 450 nm.

Indirect ELISA for testing the reference sera

Polystyrene high binding microtiter plates (Costar, USA) were coated with either NP_{49-375} or HACD proteins. Washing and blocking steps were performed as described above. The reference chicken sera from H1N1, H2N3, H3N8, H4N8, H5N2, H6N2, H7N1, H8N4, H9N2, H10N1, H12N5 and H13N6 subtypes of avian influenza virus are provided by a reference laboratory from Italy "Istituto Zooprofilattico delle Venezie" OIE Laboratory for AI and NDV. A negative serum was also provided by the same supplier. The reference sera were diluted 1/200 in PBS 1X plus 0.5% of BSA and six replicates of each one were added to coated plates for two hours at 37°C. After washing with PBST, the monoclonal antibody anti-IgG (Y) of chicken conjugated to horseradish peroxidase (Sigma, USA) and diluted 1/30 000 in PBS 1X plus 0.5% of BSA was added. After one hour at 37°C, plates were washed and the absorbance was measured as described above.

Competition ELISA

Polystyrene high binding micro titer plates (Costar, USA) were coated, washed and blocked as described above. For this experiment, the sera from birds of different species, such as: Flamingo (*Phoenicopterus ruber*, n=72), Gamecocks (*Gallus gallus*, n=22), Parakeets (*Melopsittacus undulatus*, n=15), Rosellas (*Platycercus sp*, n=15), Ducks (Anatinae, n=27) and Turkeys (*Meleagris gallopavo*, n=32) were provided by the Laboratory of Avian Investigations and Diagnostic (LIDA), Cuba. These sera were added in duplicate. We also, tested the sera of chickens infected with different avian viral diseases such as: Infectious Bursal Disease, Egg Drop Syndrome, Newcastle Disease Virus and Avian Parvovirus, provided by the National Center for Animal and Plant Health (CENSA), Cuba. In each case these sera were composed by a pool of two infected birds. They were diluted 1/50 in PBS 1X plus 0.5% of BSA and added to the coated plates for two hours at 37°C. Six replicates of the positive and the negative serum were included, as well as the sera of chickens infected with different avian viral diseases. After washing with PBST, the detection antibody anti-HA2 conjugated to horseradish peroxidase (Sancti-Spíritus, Cuba) diluted 1/20 000 was added to the plates coated with the protein HACD and the detection antibody anti-NP5 also conjugated to horseradish peroxidase (Sancti-Spíritus, Cuba) diluted 1/20 000 was added to the plates coated with the protein NP_{49-375}. After one hour at 37°C, the microtiter plates were washed and the absorbance was measured as described above. The reference sera and the sera from chickens immunized

with the proteins HA and HACD were tested by the competition ELISA in the same conditions as described in previous headings. The results were expressed as the percent of inhibition of the detection antibody according to the following calculation: ((ODmax-ODserum)/ODmax)×100 (Starick *et al.*, 2006). The ODmax was the OD obtained from the reaction of the detection antibodies with the target proteins.

Results

Cloning and expression of the gene coding the protein NP_{49-375} into the E. coli strains

The Figure 2A shows the schematic representation of the cloning steps followed to generate the expression vector coding the protein NP_{49-375}. After obtaining the complementary DNA from the viral RNA of a specific avian influenza viral strain, the gene coding the protein

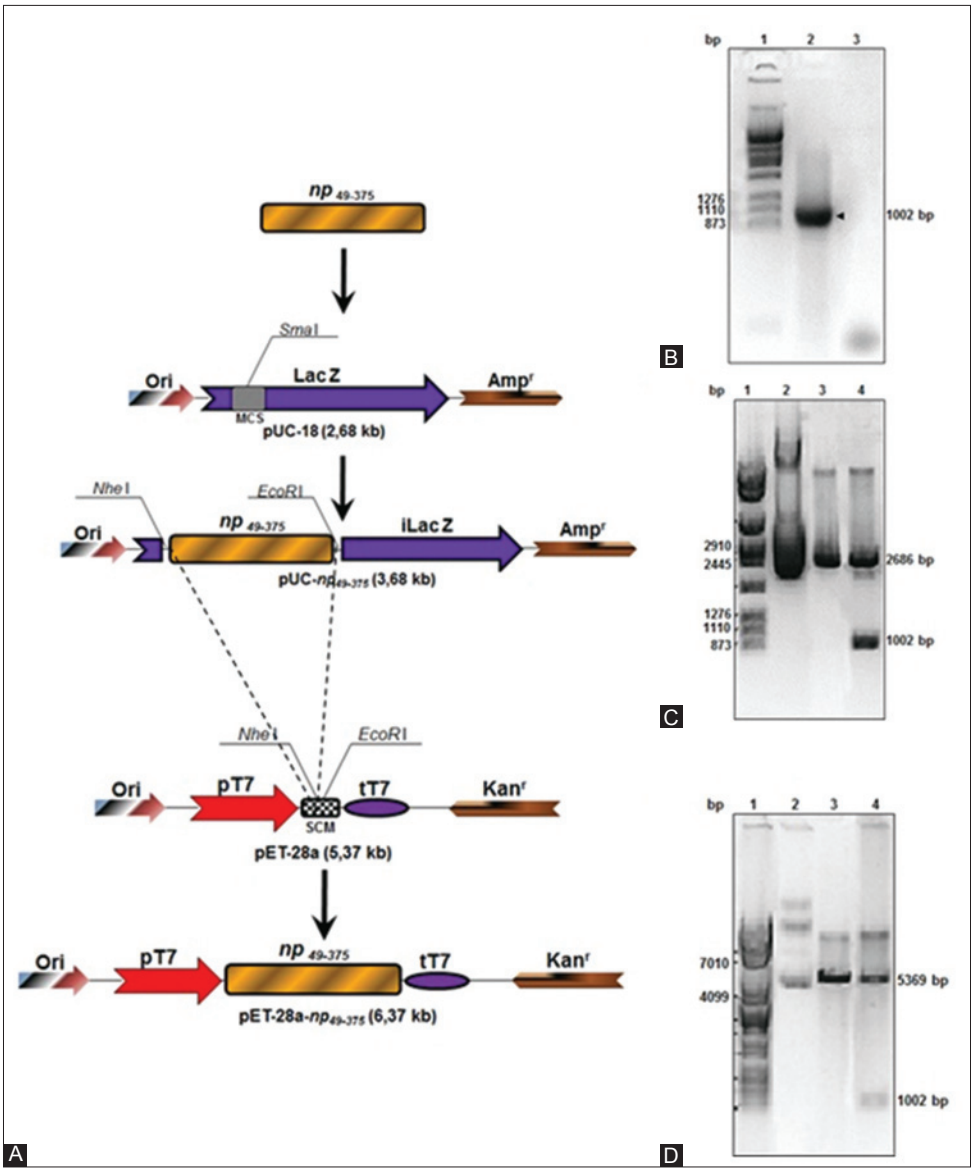

Fig. 2. Isolation of a segment of the gene np and construction of the expression vector. (A) Cloning representation of a gene coding for a segment of the protein NP from avian influenza virus subtype H7N1 comprising the aminoacids 49-375 (np_{49-375}) in the plasmid pUC-18 and in the expression vector pET-28a. (B) Isolation of the gene np_{49-375} by PCR. 1- Molecular weight marker (MWM) (pAdEasy digested with the enzyme Apa I), 2- DNA segment corresponding to the gene np_{49-375}, 3- PCR reaction with primers and without template. (C) Electrophoresis in agarose gel (1%) of the restriction analysis for the plasmid pUC-np_{49-375}. 1- MWM, 2- Plasmid pUC-np_{49-375} undigested, 3- Plasmid pUC18 digested with the enzymes *Nhe* I/*EcoR* I, 4- Plasmid pUC-np_{49-375} digested with the enzymes *Nhe* I/*EcoR* I. (D) Electrophoresis in agarose gel (1%) of the restriction analysis for the plasmid pET-28a-np_{49-375}. 1- MWM, 2- Plasmid pET-28a-np_{49-375} undigested, 3- Plasmid pET-28a digested with the enzymes *Nhe* I/*EcoR* I, 4- Plasmid pET-28a-np_{49-375} digested with the enzymes *Nhe* I/*EcoR* I.

NP_{49-375} was amplified by PCR. The electrophoresis in agarose gel (0.8%) showed a DNA segment of 1002 base pair corresponding to the size of the gene of interest (Fig. 2B). Next, the gene was subcloned into the plasmid pUC-18 in order to facilitate its manipulation and finally cloned into the expression vector pET-28a obtaining the plasmids pUC-np_{49-375} and pET-28a-np_{49-375}, respectively. To corroborate the authenticity of the gene of interest, the plasmids were submitted to a restriction assay using the enzymes *Nhe* I and *EcoR* I after sequencing. Both plasmids showed the expected pattern of DNA segments (Fig. 2C and D).

The expression of the gene np_{49-375} was performed in three different *E. coli* strains carrying tRNAs for rare codons by transforming them with the plasmid pET-28a-np_{49-375}. The SDS-PAGE and Western blot assays showed that under repressive conditions, the gene of interest was not expressed (Fig. 3A). However, after induction we observed a band of protein at about 37 kDa in the *E. coli* strains BL21-CodonPlus® (DE3)-RIL, BL21-CodonPlus® (DE3)-RP and Rosetta™ (DE3), previously transformed with the plasmid pET-28a-np_{49-375} (Fig. 3B). This protein size corresponded to the one predicted for the protein NP_{49-375}. It was not observed in the induced stage of non-transformed *E. coli* strains. For the final production of the protein NP_{49-375}, the BL21-CodonPlus® (DE3)-RIL strain was selected.

Solubilization and purification of the protein NP_{49-375}

After the BL21-CodonPlus® (DE3)-RIL strain transformed with the plasmid pET-28a-np_{49-375} was grown at a favorable optical density, the bacterial culture was harvested and sonicated. The protein NP_{49-375} was obtained as insoluble inclusion bodies in the lysate (Fig. 4A). It was solubilized using different concentrations of GuHCl (Fig. 4B). The rise of the GuHCl concentration at 1 M, 2 M, 4 M and 6 M increased the solubilization properties of the protein NP_{49-375} and provoked its gradual transition from the lysate to the supernatant.

As the protein NP_{49-375} carries six histidine residues in the carboxyl extreme, it was purified by IMAC. The SDS-PAGE and Western blot assays showed an intense band of protein of 37 kDa approximately with more than 95 % of purity degree estimated by densitometry during the elution step with a buffer containing 100 mM of imidazole (Fig. 4C and D). The protein size was very similar to the one obtained in the gene expression experiments using different *E. coli* strains. A low level of the protein NP_{49-375} was lost in the non-attached material and in the wash steps.

Antibody detection in chickens and reference sera using the proteins NP_{49-375}, HA and HACD in indirect ELISA assays

The sera of chickens immunized with the proteins HA and HACD showed a similar mean optical density

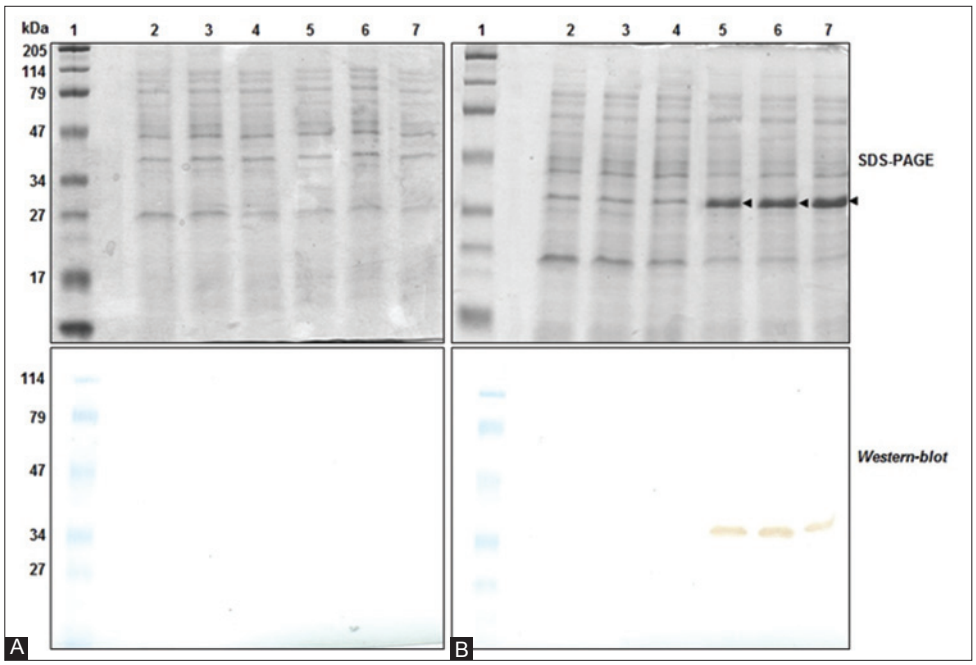

Fig. 3. Expression of the gene coding the protein NP_{49-375} in different *E. coli* strains. SDS-PAGE (12.5%) and Western blot of the samples in the non-induced (A) or induced (B) stages on different *E. coli* strains. 1- MWM (Bio-Rad, USA), 2- Untransformed BL-21-Codon Plus® (DE3)-RIL, 3- Untransformed BL-21-Codon Plus® (DE3)-RP, 4- Untransformed Rosetta™ (DE3), 5- BL-21-Codon Plus® (DE3)-RIL transformed with pET-28a-np_{49-375}, 6- BL-21-Codon Plus® (DE3)-RP transformed with pET-28a-np_{49-375}, 7- Rosetta™ (DE3) transformed with pET-28a-np_{49-375}. Immuno identification was performed with a monoclonal antibody against the six histidine residues (Sigma, USA). Arrow heads indicate the protein NP_{49-375}.

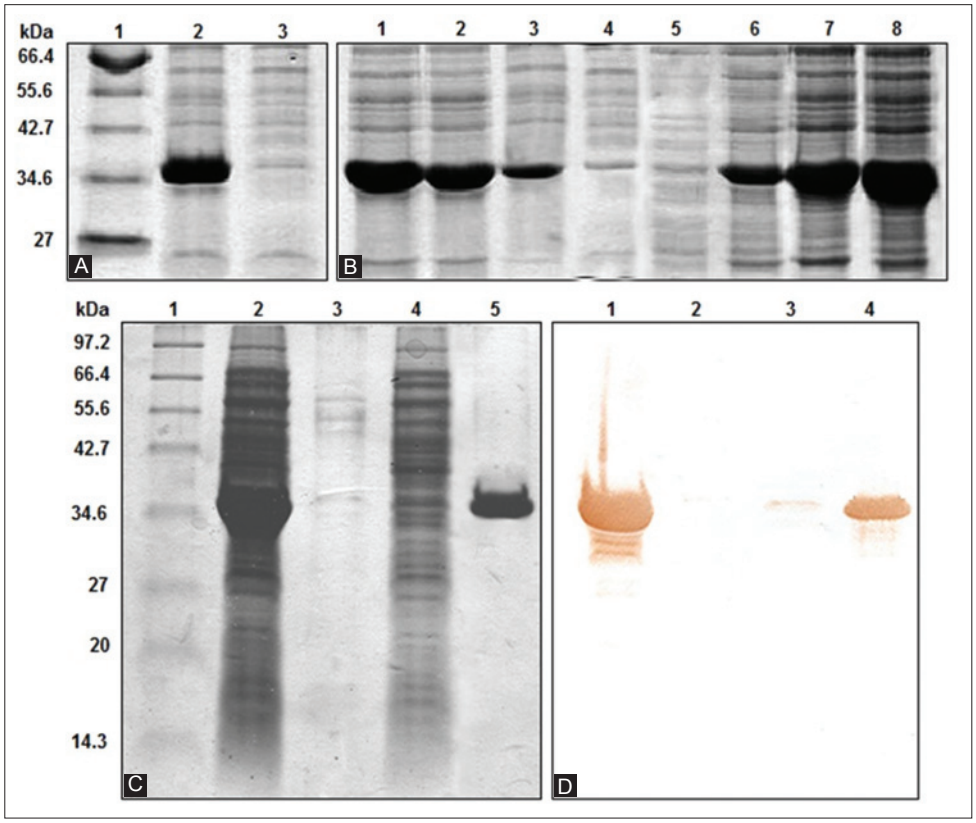

Fig. 4. Solubilization and purification of the protein NP_{49-375}. (A) SDS-PAGE (12.5%) of the different fractions after the rupture. 1- MWM (New England Biolabs, USA). 2- Rupture pellet. 3- Rupture supernatant (B) Solubilization with GuHCl. 1- Pellet GuHCl 1M, 2- Pellet GuHCl 2M, 3- Pellet GuHCl 4M, 4- Pellet GuHCl 6M, 5- Supernatant GuHCl 1M, 6- Supernatant GuHCl 2M, 7- Supernatant GuHCl 4M, 8- Supernatant GuHCl 6M. SDS-PAGE (12.5%) (C) and Western blot (D) of the different stages in the purification process performed by IMAC. 1- MWM (New England Biolabs, USA), 2- Initial sample, 3- Non-attached proteins, 4- Wash 20 mM Imidazole, 5- Elution 100 mM Imidazole.

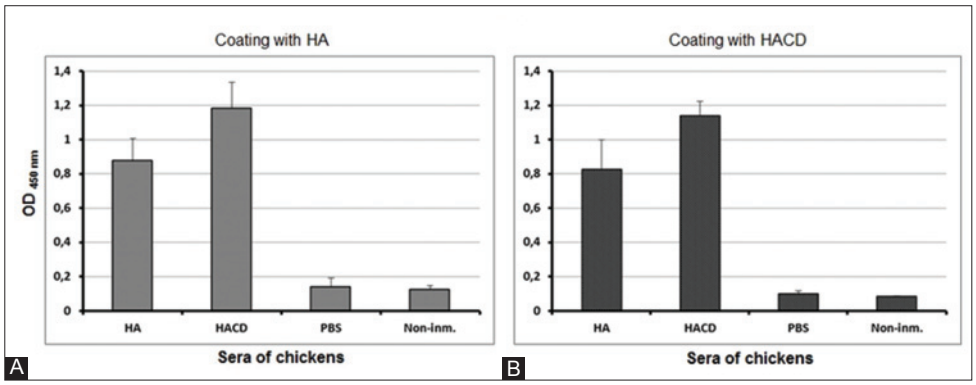

Fig. 5. Indirect ELISA for antibody detection in the sera of commercial chickens immunized with the proteins HA or HACD. Plates were coated with 2.5 µg/mL of the proteins HA (A) or HACD (B). Ten animals per experimental group were used. Duplicate sample per animal were tested. The sera were diluted 1/1000 and the monoclonal antibody anti-IgG (Y) of chicken conjugated to horseradish peroxidase and diluted 1/30 000 was used as detection antibody. Bars represent the standard deviation.

(mOD) when ELISA plates were coated with these proteins (Fig. 5A and B). The mOD was about 0.8 for the sera of chickens immunized with the protein HA and 1.1 for the sera of chickens immunized with the protein HACD. There was no anti-HA antibody detection in the sera from chickens immunized with PBS 1X or in the sera from non-immunized chickens.

The ELISA plates coated with the proteins NP_{49-375} and HACD were used to test the reference sera from different avian influenza subtypes. We observed a

variable pattern of the mean optical densities (mODs) when reference sera were tested in the plate coated with the protein NP_{49-375} (Fig. 6A). Most of the mODs exceeded 0.5. Only the mOD in the wells containing the reference serum corresponding to the H4N8 subtype was below of the previous value. However, they reached a mOD of 0.4, which was more than 2.5-fold compared to the mOD of 0.14 in the wells containing the negative reference serum.

In the ELISA plates coated with the protein HACD the antibody detection of the reference sera was almost irrelevant (Fig. 6B). The mODs behaved similarly to that of the negative reference serum, fluctuating between 0.11 and 0.26. The exception was the high mOD value of 0.97 observed in the wells containing the reference serum H5N2.

Establishment of a strategy of DIVA by competition ELISA

Competition ELISA assays using the reference sera and the sera of chickens immunized with the proteins HA and HACD were employed to perform the strategy of DIVA. The competition ELISA assays also involved the sera from different species of birds including flamingo, gamecocks, parakeets, rosellas, ducks and turkeys, which were already tested as negative by the hemagglutination inhibition assay using antigens of the subtypes H5, H7 and H9. Likewise, we evaluated the sera of chickens infected with Infectious Bursal Disease, Egg Drop Syndrome, Newcastle Disease Virus and Avian Parvovirus. As expected, the pattern for the percent of inhibition detected in the reference sera when the ELISA plates were coated with the protein NP_{49-375} was consistent with the mODs observed in the indirect ELISA coated with the same protein (Fig. 7A). There were variable values ranged from 34.8 to 88.3. No antibodies against the protein NP_{49-375} were detected in the sera from chickens immunized with the proteins HA and HACD. The percent of inhibition was around 6.5. In the plates coated with the protein HACD the pattern of the percent of inhibition for the reference sera was also much related to the mODs observed in the indirect ELISA. The values ranged from 4.8 to 14.6, except for the serum H5N2 and the sera from the chickens immunized with the proteins HA and HACD. They showed percentages of inhibition of 86.2, 88.0 and 89.6 respectively. In the sera from birds of different species it seemed there were no antibodies against the proteins NP_{49-375} or HACD (Fig. 7B). The percentages of inhibition for the plates coated with the protein NP_{49-375} ranged from 7.2 to 15.5 and for the plates coated with the protein HACD the values ranged from 13.5 to 18.9. These results were similar to those observed with the sera of chickens infected with other avian viral diseases (Fig. 7C). The values ranged from 4.3 to 10.1 in the plates coated with the protein NP_{49-375} and from 14.0 to 17.4 in the plates coated with the protein HACD. None of the sera which were expected not to have antibodies against both proteins exceeded the 20% of inhibition, but there were values very close to this percent of inhibition in the ELISA plates coated with the two assayed proteins. Therefore, the cut-off value of each competition ELISA was determined at 25%.

Discussion

Effective methods of control and prevention are necessary to counteract the highly pathogenic avian influenza virus H5N1 due to the threat it represents. To achieve these tasks, it is crucial to establish proper surveillance and vaccination plans. Once an avian influenza outbreak starts, vaccinated birds should be more resistant to the infection with a higher threshold for an infective viral charge to establish. Also, clinical signs tend to disappear and viral shedding must be significantly reduced. Although conventional vaccines against avian influenza viruses are protective against the clinical signs and diminish the viral excretion to the environment, they interfere with serological

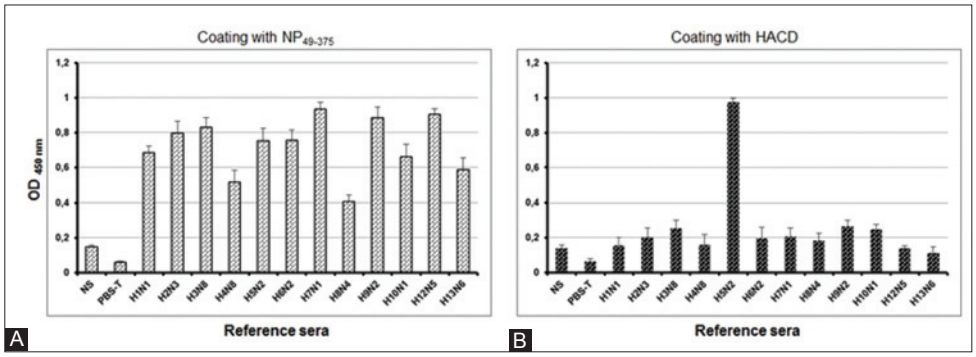

Fig. 6. Indirect ELISA coating with 2.5 µg/mL of the proteins NP_{49-375} (A) or HACD (B) for measuring the antibody levels in reference sera from different subtypes of the avian influenza virus provided by a laboratory of reference from Italy. The sera were diluted 1/200 and the monoclonal antibody anti-IgG (Y) of chicken conjugated to horseradish peroxidase and diluted 1/30 000 was used as detection antibody. Six replicates per reference serum were tested. Bars represent the standard deviation. NS: Reference negative serum.

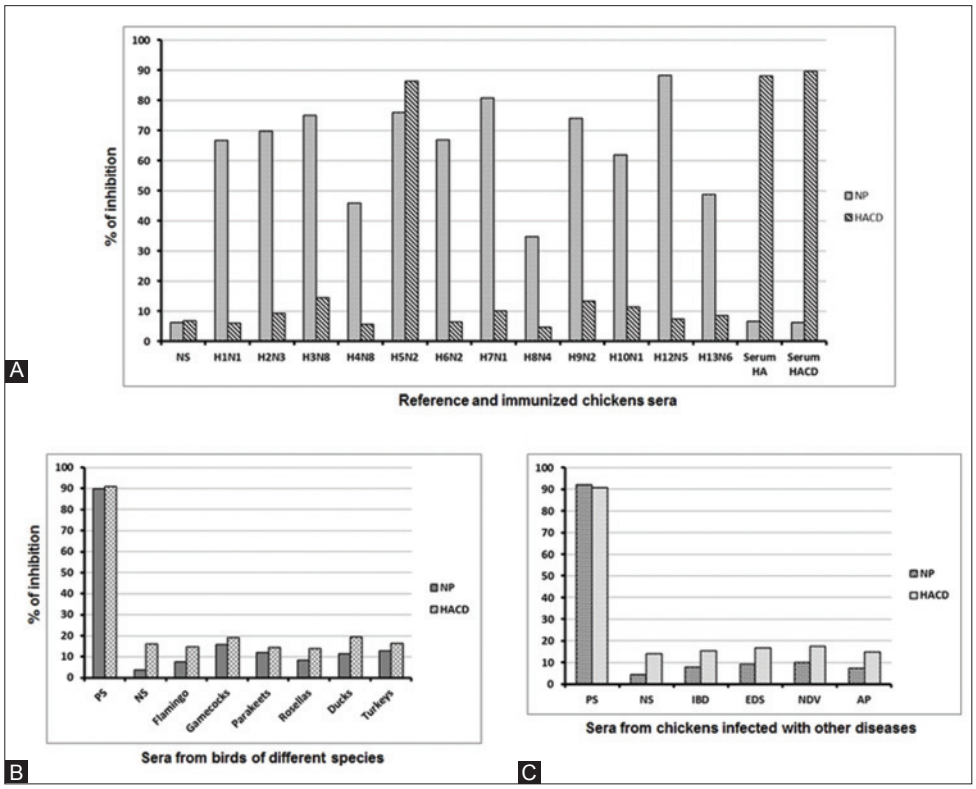

Fig. 7. Establishment of competition ELISA assays for a potential strategy of DIVA directed to the avian influenza virus H5N1. (A) Competition ELISA assays coated with 2.5 µg/mL of the proteins NP_{49-375} and HACD. The reference sera from different avian influenza virus subtypes diluted 1/200 and the sera from chickens immunized with the proteins HA and HACD diluted 1/1000 were tested. Duplicated samples from the sera of ten immunized chickens per experimental group and six replicates of each reference serum were evaluated. (B) Antibody detection in duplicated samples of the sera from birds of different species. Plates coated with 2.5 µg/mL of the protein NP_{49-375} used the monoclonal antibody anti-NP5 diluted 1/20 000 as detection antibody. Plates coated with 2.5 µg/mL of the protein HACD used the monoclonal antibody anti-HA2 diluted 1/20 000 as detection antibody (C). Antibody detection in the sera from chickens infected with distinct avian viral diseases. Plates were coated as above and the same detection antibodies were used. Sera were diluted 1/50. Six replicates per serum from infected chickens were used. Results were expressed as the percent of inhibition. PS: Reference H5N2 serum. NS: Reference negative serum. IBD: Infectious Bursal Disease. EDS: Egg Drop Syndrome. NDV: Newcastle Disease Virus. AP: Avian Parvovirus.

surveillance because the antibodies induced by these vaccines cannot be distinguished from those induced by the live virus infection (Lee *et al.*, 2011). The stamping out policy is often preferred for controlling avian influenza disease, especially when the birds or their products are destined to the market. Obviously, high economical losses are generated because of the elimination of the infected birds together with the vaccinated and non-infected ones. Subunit vaccines can overcome this issue. As they are generally composed by one or two viral proteins, the vaccinated birds can be differentiated from those infected by using in the serological assays a viral molecule distinct from that included in the subunit vaccine. Several studies have obtained successful results applying the strategy of DIVA for the control of avian influenza using inactivated or reverse genetic vaccines (Capua *et al.*, 2003a,b; Lee *et al.*, 2004). In this study, we presented a preliminary evidence of a successful strategy of DIVA by using the avian influenza subunit vaccine candidate HACD (Pose *et al.*, 2011) in conjunction with a segment of the protein NP from avian influenza virus.

The protein NP from the avian influenza viruses have been obtained in different expression systems and thoroughly used to detect antibodies against these viruses (Jin *et al.*, 2004; Starick *et al.*, 2006; Wu *et al.*, 2007). In this study, *E. coli* was selected as the host because all of the known benefits this expression system offers (Baneyx, 1999). Also, some studies have demonstrated the effectiveness of the protein NP in recognizing antibodies raised against avian influenza viruses upon its production in *E. coli* (Jin *et al.*, 2004; Wu *et al.*, 2007). Our initial attempts to produce the protein NP_{49-375} in the *E. coli* strain BL-21 (DE3) were unsuccessful. This phenomenon was already observed for the full length protein NP in the same *E. coli* strain (Jin *et al.*, 2004). It is known that codon usage from distinct hosts is an essential feature for the expression

of heterologous genes (Wakagi *et al.*, 1998; Novoa and de Pouplana, 2012). The gene np_{49-375} used in this study contained 39 rare codons for *E. coli* (31 coding Arginine, four coding Isoleusine, two coding Leucine and two coding Proline), some of them in double or triple repeats. These rare codons represent the 12% of the molecule. For this reason, the *E. coli* strains BL21-CodonPlus® (DE3)-RIL, BL21-CodonPlus® (DE3)-RP and Rosetta™ (DE3) were used to express this gene. These strains supply tRNAs for the rare codons previously mentioned. The expression of the gene np_{49-375} was successfully achieved in the three *E. coli* strains selected, which suggests that codon usage is critical for the expression of this specific gene in prokaryotes. Although densitometric analysis did not reveal significant differences in the expression of the gene np_{49-375} on the three *E. coli* strains, we selected the strain BL21-CodonPlus® (DE3)-RIL for the final production of the protein NP_{49-375} because it comprises most of the tRNAs for the rare codons found in the gene np_{49-375} and also because of our experience working with this *E. coli* strain in the laboratory.

After being purified and solubilized, we used the protein NP_{49-375} as antigen in the ELISA assays. For the same purpose, we also used the proteins HA and HACD, previously obtained in mammalian cell culture by adenoviral transduction (Pose *et al.*, 2011; Ramos *et al.*, 2011). The antibody responses of chickens immunized with the proteins HA and HACD were compared by indirect ELISA coated with both proteins. The similar antibody detection observed in the plates coated with the proteins HA and HACD was an outstanding result, which suggested that most of the immune response was against the HA molecule. Moreover, it indicated that the fusion of the CD154 to the HA molecule did not interfere with the antibodies to recognize the HA epitopes. It was also observed that the antibody detection in the sera of birds immunized with the protein HACD was higher than the sera of birds immunized with the protein HA. It was an expected result due to the effect of molecular adjuvant that the CD154 molecule must exert, particularly in the enhancement of the humoral and cellular immune responses (Elzey *et al.*, 2011). Therefore, the protein HACD not only can be used as vaccine candidate but also as coating antigen in the ELISA assays to carry out the strategy of DIVA. This could lower the cost of a future vaccination campaign against the avian influenza virus H5N1 because only two proteins would have to be produced: the protein HACD for the vaccination program and to be used as antigen for differentiating vaccinated birds from the infected ones together with the protein NP_{49-375}.

The high recognition level of the protein NP_{49-375} by the antibodies in all the reference sera by competition ELISAs showed the high sensitivity of this assay. Although the detection of antibodies was variable depending on the avian influenza subtype of each reference serum, this issue reinforces the utility of this protein for the detection of influenza A viruses due to its conservation degree among them (van Wyke *et al.*, 1980). The competition ELISA assays also demonstrated to be highly specific. It seemed that neither NP_{49-375} nor HACD proteins attached antibodies when the sera of chickens infected with other avian viral diseases were assayed. Also, the antibody detection levels were irrelevant when we tested negative sera from different species of birds. Additionally, the protein HACD did not show cross reaction with the sera of other avian influenza subtypes, but we observed high antibody levels in the sera of chickens immunized with the same protein, as expected. Interestingly, the protein HACD attached antibodies from the reference serum of the H5 subtype. Despite the hemagglutinin has a highly mutagenesis rate due to the *antigenic drift* of avian influenza viruses (Sugita *et al.*, 1991), it is known that partial antibody cross-reaction can occur among these proteins owed to the presence of conserved epitopes in distinct avian influenza viral strains (Lee *et al.*, 2008; Chiu *et al.*, 2013).

This study showed percentages of inhibition below 20% for the negative sera, while the percentages of inhibition for the positive sera were around 88-91%, which reinforces the idea that the test is highly specific and sensitive for the avian influenza disease. Our results are consistent with those obtained by Starick *et al*. (2006), where a competition ELISA based in the protein NP was highly specific and sensitive showing comparable percentages of inhibition for positive and negative sera.

In conclusion, this study presents initial evidence for a potential strategy of DIVA to succeed using the competition ELISA assays coated with a segment of the protein NP from the strain A/chicken/Italy/1067/1999 of avian influenza virus and the protein HACD, which would be the same protein used as vaccine candidate in case of an outbreak of avian influenza virus H5N1 to occur. Although further experiments should be conducted to optimize the methodology, it could be advantageous from the economic point of view. As the protein HACD can play a dual function, there is no need to produce an additional protein for detecting antibodies against the H5N1 virus. Another possible application for this competition ELISA, is in the influenza virus monitoring program due to the broad capacity of the protein NP_{49-375} to detect antibodies induced by influenza A viruses.

Conflict of interest

The authors declare that there is no conflict of interest.

Reference

Baneyx, F. 1999. Recombinant protein expression in Escherichia coli. Curr. Opin. Biotech.10, 411-421.

Capua, I., Cattoli, G. and Marangon, S. 2003a. DIVA--a vaccination strategy enabling the detection of field exposure to avian influenza. Dev. Biol. 119, 229-233.

Capua, I., Terregino, C., Cattoli, G., Mutinelli, F. and Rodriguez, J.F. 2003b. Development of a DIVA (Differentiating Infected from Vaccinated Animals) strategy using a vaccine containing a heterologous neuraminidase for the control of avian influenza. Avian Pathol. 32, 47-55.

Chiu, C., Wrammert, J., Li, G.M., McCausland, M., Wilson, P.C. and Ahmed, R. 2013. Cross-reactive humoral responses to influenza and their implications for a universal vaccine. Ann. NY. Acad. Sci. 1283, 13-21.

Elzey, B.D., Ratliff, T.L., Sowa, J.M. and Crist, S.A. 2011. Platelet CD40L at the interace of adaptive immunity. Thromb. Res. 127, 180-183.

Jin, M., Wang, G., Zhang, R., Zhao, S., Li, H., Tan, Y. and Chen, H. 2004. Development of enzyme-linked immunosorbent assay with nucleoprotein as antigen for detection of antibodies to avian influenza virus. Avian Dis. 48, 870-878.

Lee, C.-W., Senne, D.A. and Suarez, D.L. 2004. Generation of reassortant influenza vaccines by reverse genetics that allows utilization of a DIVA (Differentiating Infected from Vaccinated Animals) strategy for the control of avian influenza. Vaccine 22, 3175-3181.

Lee, D.-H., Park, J.-K., Lee, Y.-N., Song, J.-M., Kang, S.-M., Lee, J.-B., Park, S.-Y., Choi, I.-S. and Song, C.-S. 2011. H9N2 avian influenza virus-like particle vaccine provides protective immunity and a strategy for the differentiation of infected from vaccinated animals. Vaccine 29, 4003-4007.

Lee, L.Y.-H., Do Lien Anh Ha, C.S., de Jong, M.D., Chau, N.V.V., Schumacher, R., Peng, Y.C., McMichael, A.J., Farrar, J.J., Smith, G.L. and Townsend, A.R.M. 2008. Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals. J. Clin. Invest. 118, 3478-3490.

Ligon, B.L. 2005. Avian influenza virus H5N1: a review of its history and information regarding its potential to cause the next pandemic. Semin. Ped. Infect. Dis. 16, 326-335.

Novoa, E.M. and de Pouplana, L.R. 2012. Speeding with control: codon usage, tRNAs, and ribosomes. Trends Genet. 28, 574-581.

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

Ramos, O.S., Pose, A.G., Gómez-Puerta, S., Gomez, J.N., Redondo, A.V., Benites, J.C.Á., Amarán, L.S., Parra, N.C. and Toledo Alonso, J.R. 2011. Avian CD154 enhances humoral and cellular immune responses induced by an adenovirus vector-based vaccine in chickens. Comp. Immunol. Microb. 34, 259-265.

Ruigrok, R.W.H. and Baudin, F., 1995. Structure of influenza virus ribonucleoprotein particles. II. Purified RNA-free influenza virus ribonucleoprotein forms structures that are indistinguishable from the intact influenza virus ribonucleoprotein particles. J. Gen. Virol. 76, 1009-1014.

Starick, E., Werner, O., Schirrmeier, H., Köllner, B., Riebe, R. and Mundt, E. 2006. Establishment of a competitive ELISA (cELISA) system for the detection of influenza A virus nucleoprotein antibodies and its application to field sera from different species. J. Vet. Med. B53, 370-375.

Sugita, S., Yoshioka, Y., Itamura, S., Kanegae, Y., Oguchi, K., Gojobori, T., Nerome, K. and Oya, A. 1991. Molecular evolution of hemagglutinin genes of H1N1 swine and human influenza A viruses. J. Mol. Evol. 32, 16-23.

van Wyke, K.L., Hinshaw, V.S., Bean, W.J. and Webster, R.G. 1980. Antigenic variation of influenza A virus nucleoprotein detected with monoclonal antibodies. J. Virol. 35, 24-30.

Wakagi, T., Oshima, T., Imamura, H. and Matsuzawa, H. 1998. Cloning of the gene for inorganic pyrophosphatase from a thermoacidophilic archaeon, Sulfolobus sp. strain 7, and overproduction of the enzyme by coexpression of tRNA for arginine rare codon. Biosci. Biotech. Bioch. 62, 2408-2414.

Wu, R., Hu, S., Xiao, Y., Li, Z., Shi, D. and Bi, D. 2007. 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, 631-641.

Zhang, J. 2012. Advances and future challenges in recombinant adenoviral vectored H5N1 influenza vaccines. Viruses 4, 2711-2735.