

**Development and production of Avian Influenza Virus (Type A) recombinant nucleoprotein (rNP antigen)**

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## INTRODUCTION

Influenza Virus is an etiological infectious agent for a couple of acute respiratory diseases of human, other mammals and birds. At present, three types of flu virus (*Orthomyxoviridae* family) are known: A, B and C, differing in serological characteristics of nucleocapsid (NP) and membrane (M1) proteins (1). Influenza Virus Type A, has 15 estimated virus subtypes, with appropriate amount of hemeagglutinin protein molecules, differing in specific amino acid sequences and/or conformational peptide epitopes. Avian Influenza Virus (AIV) is one of the most dangerous infectious pathogens (List A in O.I.E. and Animal Code, USA classification), affecting wide range of hosts, including mammals, wild birds and poultry, and also is a causative agent of periodic pandemic cases in the world, with the extremely high mortality level (up to 100%) and severe economical losses.

The standard serological diagnostics of AIV or Highly Pathogenic Avian Influenza (HPAI, Fowl plaque), officially regulated by O.I.E. formalities, is based on hemagglutination inhibition (HI) and complement fixation (CF) methods. These technique is evidently laborious (require a continuous source of the appropriate erythrocytes), weakly reliable and ineffective for monitoring or express analysis of large number of samples. The alternative immunological methods based on ELISA, dip-stick and biosensor approaches are much more convenient and reliable, but they need highly purified virus antigens and specific antibodies. In addition, ELISA-like formats of the assay have been used for the detection of influenza virus-specific antibodies and measuring of IgG subtype have been shown to be more sensitive than HI or the CF assay. Again, the demonstration of virus-specific IgA antibodies in poultry serums after AIV infection proved to be of diagnostic value (2).

It is well-known that Flu viruses have 8-segment genome, which provides unique level of variability and adaptability to various environmental conditions and appropriate hosts. AIV, as all viruses with negative-sense RNA genomes encode a single-strand RNA-binding nucleoprotein (NP). The primary function of NP is to encapsidate the virus genome for purposes of RNA transcription, replication and packaging. NP also functions as a key adapter molecule between virus and host cell processes. It is also estimated, that NP is the most conservative protein in AIV proteins tile. As was determined previously, all negative-sense RNA virus genomes encodes three polypeptides, that forms the virus core, and are common to all families: an integral membrane glycoprotein, an RNA-dependent RNA polymerase and a single-strand RNA (ssRNA) binding protein, i.e. nucleoprotein, NP. The recent AIV diagnostic reports confirms, that NP is the most specific virus antigen, regarding immune response to various virus strains, sub-strains and virus geographic isolates (1). This protein may be produced both in

prokaryotic and eukaryotic expression systems in vitro (2, 3). The NP protein has estimated sites for phosphorylation, so the baculovirus expression system of insect cells, which provides the synthesis of fully processed and folded recombinant proteins, seemed to be more advantageous for generation of AIV protein antigen similar to native NP.

**The main goal of the present Project was the development and production of AIV recombinant nucleoprotein (NP) in the baculovirus expression system for appropriate antigen testing in different immunological formats.**

## MATERIAL AND METHODS

**Isolation of viral RNA.** The Influenza Virus strain A/Swine/Hong Kong/9A-1/98 (H9N2), kindly provided by ..... served as a source of single-strand RNA-binding nucleoprotein gene for further development of recombinant constructions and appropriate expression of recNP in E.coli and insect cells. RNA isolation was done using TRIzol (Invitrogen) reagent in accordance with recommended standard methods.

The reverse transcription was done using random primers, i.e. 2 µl of random primers mixture were added to 10 µl of isolated virus RNA and incubated for 10 min at 70°C. After the following incubation at 0°C on ice, the reaction mixture, containing dNTPs (0.5 mM of each nucleotide), RNasin (40 U) and H-MMuV-revertase (200 U), in a standard buffer solution for revertase, was added to the final volume 25 µl with the following incubation for 45 min at 50°C. The reaction was stopped by heating the mixture to 95°C for 3 min. The DNA obtained was used as a template in a NP gene PCR amplification technique.

**Primers design and synthesis.** For primers search and development the consensus sequence of NP genes from various Influenza virus A strains (NCBI, Genbank) was used. The pair of external and pair of internal primers, containing sites of restriction for Sall and SpeI were developed for the following cloning of the NP gene amplification product into the donor plasmid pFastBacHTc (see Table 1) in appropriate baculovirus expression system (Invitrogen). The nested-PCR technique was used for amplification of the desired cloning NP gene sequence, using High Fidelity PCR enzyme mix (Fermentas) for proper polymerase reaction.

**Table 1. AIV NP gene cloning primers set**

Item	Nucleotide sequence 5'-3'	Use
F-out-NPcom	CAGGGTAGATAATCACTCA	For PCR-1
R-out-NPcom	AGTAGAAACAAGGGTATTT	For PCR-1
FNPBac-Sall	ACTCGTCGACATGGTGTCCCAAGGCACCAAACGAT	
RNPBac-SpeI	GTATACTAGTTTAATTGTCATACTCCTCTGCATTGTCT	

The product of the proposed NP gene amplification was purified using standard DNA extraction kit (Fermentas), treated with appropriate restriction endonucleases and installed in a poly-linker donor plasmid pFastBacHTc, designed for baculovirus expression system. The further development and completion of recombinant baculovirus genome was achieved in accordance with producer's manual (Invitrogene). The recombinant bacmids were used for transfection of *Sporoptera frugipedra* (Sf-21) insect cells monolayer. Thereto, 5-10 µl of purified bacmid were mixed with IPL-41 medium; simultaneously the 10 µl of "CellFECTIN" solution(Invitrogene) was mixed with equal volume of the above-said medium. Both mixtures were combined and incubated 30 min. The cultural fluid was removed from Sf-21 cell monolayer and transfection reagents mixture was added to the cultural flasks. The transfection was carried out for 2-5 hrs at 37°C and after that, the cultural fluid was changed for normal culture growth medium, containing 5% Fetal Calf Serum (FCS, Gibco). The further generation of recombinant baculovirus strains, expressing proposed AIV nucleocapsid proteins, were done in cultivated, transfected insect Sf-21 cells, using IPL-41 growth medium with 5% FCS and 50 u/ml gentamycin.

**Purification of AIV recombinant NP protein.** Recombinant NP was purified by 2 methods.

**Method 1:** metal affinity chromatography under denaturing conditions. The cell pellet (approximately 1,0 gm) was resuspended in 10 ml of lysis buffer 1 (LB1) (100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-base, 8M Urea, pH=8.0) incubated at 25<sup>0</sup>C 10 min and then lysed by sonication (3 times for 1 min). 5 ml 50% slurry of Ni-NTA resin was used for 10 ml of the crude lysate. Mix of lysate and Ni-NTA agarose incubated 1 h at 25<sup>0</sup>C and loaded onto the column. The column washed with 5 volumes of wash buffer (100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-base, 8M Urea, pH=6.3), then with 5 volumes elution buffer 1 (100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-base, 8M Urea, pH=5.9) and finally the bound recNP was eluted by elution buffer 2 (100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-base, 8M Urea, pH=4.5).

**Method 2.** The cell pellet (approximately 1,0 gm) was resuspended in 10 ml ml of lysis buffer 2 (LB2) (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 5 mM Tris-Cl, 10 mM β-mercaptoethanol, pH 7,5), containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were disrupted by sonication for 1 min with cooling using ultrasonic disintegrator. The debris was removed by centrifugation for 10 min at 12,000g. To precipitate nucleic acids, 30 % streptomycin sulfate (1/10 V) was added to the final concentration of 3%. The mixture was kept on ice for 5 min. After centrifugation under the same conditions as above, the supernatant containing recombinant protein was transferred into a fresh tube. Saturated ammonium sulfate solution was gradually added to the supernatant to give a final

30% saturation and incubated for 1 h at 4°C. After centrifugation for 10 min at 12,000g the supernatant was transferred into a fresh tube again and saturated ammonium sulfate solution was added to the supernatant to give a final 40% saturation and incubated for 2 h at 4°C. After centrifugation under the same conditions as above, the pellet was resuspended in 40% ammonium sulfate solution.

The immunogenicity of the purified recombinant protein was tested by indirect ELISA and Western blotting with reference chicken sera, specific to 15 influenza virus A subtypes, kindly provided by “AffiniTech”Co, USA.

#### **Indirect ELISA with reference serum.**

For AFV ELISA development, the recNP, purified as described above for Method 1, using metal affinity chromatography, was diluted in 50 mM carbonate buffer, pH = 9.2 to the final concentration 1 – 5 µg/ml and was adsorbed to immunoplates “Greiner Bioone”, Germany.

The recNP antigen, purified according to Method 2, was dialysed against buffer, containing 8 M urea, 10 mM Tris-HCl, 10 mM β-mercaptoethanol, pH 7,5, than we have diluted the antigen in the same buffer, **but with pH 9.5**, to the final concentration 2 – 10 µg/ml, added equal volume of 50 mM carbonate buffer, pH = 9.5 and adsorbed to immunoplates, consequently.

Antigens were adsorbed at +4<sup>0</sup> C, overnight in Greiner ELISA plate (Cat.# 762070). Plates were washed 5 times with PBST (PBS containing 0.1% Tween 20) and 100 µl of chicken anti-NP positive or negative serum (1/100 v/v) were added in PBS, containing 0.5% BSA, and were incubated 1h at 37<sup>0</sup>C. Plates were washed 5 times with PBST, 100 µl of anti-chicken IgG HRP conjugate (Sigma) 1/1000 (v/v) was added and incubated 1h at 37<sup>0</sup>C. After washing, 100 µl H<sub>2</sub>O<sub>2</sub>, TMB solution (Sigma) was added. After 20 min, the color development was stopped by adding 50 µl 1M H<sub>2</sub>SO<sub>4</sub>; the extinction values were then measured at 450 nm in a Multiscan EX spectrophotometer (Thermo).

#### **SDS-PAGE and immunoblotting.**

The protein profile of antigens was examined by SDS-PAGE and immunoblotting. A 3.5% stacking gel and a 12% separating acrylamide gel were used in a minigel apparatus (Bio-Rad Laboratories). Samples from fraction were diluted 1/1 in Laemmli sample buffer and heated in a boiling-water bath for 3 min before being loaded onto the gel. The proteins were separated at a constant voltage of 200 V. Molecular markers in the range 14.2 to 96 kD, obtained from Sigma. Proteins, were stained with CBB G-250.

Electrophoretic semi-dry transfer of the separated proteins to Immobilon PVDV (Millipore) was performed with Multiphor II apparatus (LKB) at a constant current of 200 mA for 1 h. Membranes were blocked with PBST containing 3% “Top-block” (Juro) and incubated with dilution of chicken anti-NP positive or negative serum in PBST containing 0.5% BSA for 1 h at 37<sup>0</sup>C.

After three washes in PBST the membranes were incubated with anti-chicken IgG-HRP for 1 h at 37<sup>0</sup>C. The blots were stained with diaminobenzidine plus 4-cloro-1-naphtol.

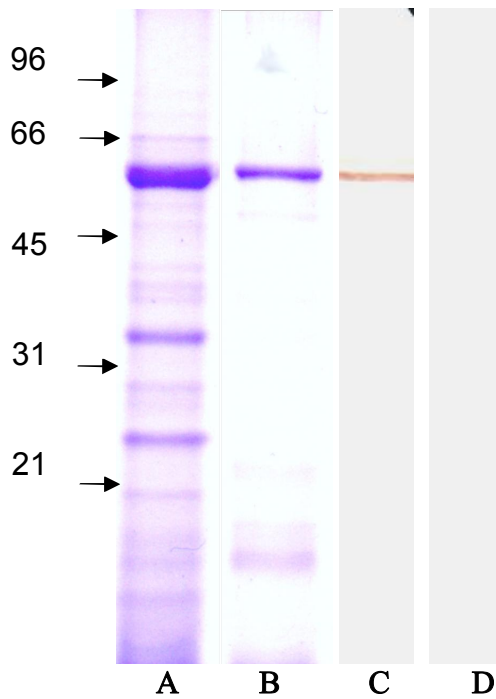
## RESULTS

After the expression of desired NP gene product in baculovirus system of insect cells Sf 21, the major protein band (> 50% of the total sample protein) was found in the virus-containing lysate by SDS-PAGE electrophoresis, with a molecular mass about 56 kDa (Fig. 1A). Further experiments were focused on optimization of recNP purification, stabilization and storage of the NP antigen. It was shown, that metal affinity chromatography allows to obtain recNP with a high degree of purity (95-98%), but with a relatively low yield, not exceeding 100 – 250 µg of protein per g of producing Sf 21 insect cells.

In addition, it was estimated, that during the storage of the purified recNP in the final elution buffer (see above) its concentration in the solution was significantly decreased, as a result of the non-specific adsorption of this extremely highly hydrophobic protein on the glass surfaces of the tubes. That is why we have used the alternative methods of rNP purification using step fractionation with streptomycin sulfate and ammonium sulfate. This approach let us to obtain highly purified recNP preparation with a final yield 1.5 – 2.0 mg of protein per gram of producing insect cells, with a stable initial antigenic (immunogenic) activity in 40% ammonium sulfate during storage at 0<sup>0</sup> – 4<sup>0</sup>C.

The purification degree of isolated recNP was determined by SDS-PAGE. The bands of total proteins were visualized by staining with CBB G-250 (Fig. 1, line A and B). These data obtained by SDS-PAGE indicated, that the purity of NP antigen was more than 95%. The Fig 1(lines A and B), shows the recombinant NP preparation protein patterns after metal affinity chromatography isolation. The similar data was obtained for rNP, purified by stepwise precipitation (data not shown).

The specific immunoreactivity, i.e. identity of the antigen was determined and confirmed by immunoblotting with chicken reference monospecific anti-NP positive or negative sera (Fig. 1, lines C and D).



**Fig. 1. Determination of the purity and immunospecificity of the recNP by SDS-PAGE and Western blot analysis.**

SDS-PAGE protein profiles of cell lysate (A) and purified recNP (B). After electrophoresis, purified recNP was transferred to PVDF membrane. Strips were incubated with reference anti-NP positive(C) or negative (D) chicken serum. The strips were incubated with anti- chicken IgG-HRP and stained with mixture of diaminobenzidine and 4-chloro-1-naphtol. The values on the left are molecular mass protein markers.

The results obtained from Western immunoblot shows, that the NP-positive serum recognized bands corresponding in size to appropriate rec-NP gene product. The identity of the recNP was further confirmed in indirect ELISA.

The activity of recombinant proteins was tested by indirect ELISA with reference chicken anti-NP positive or negative serum (Fig. 2)

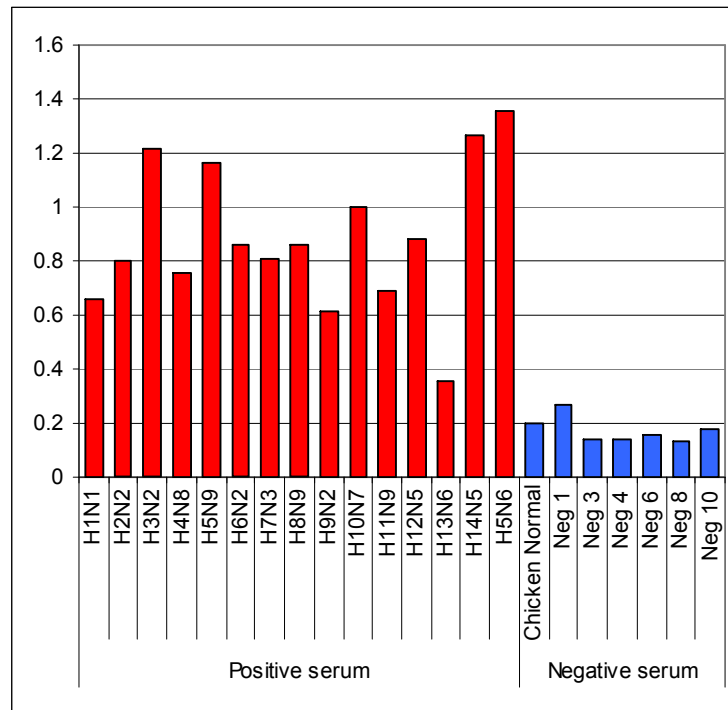


Fig.2. Detection recNP by indirect ELISA with reference chicken sera.

Plates were coated with rNP, incubated with chicken monospecific sera, raised against influenza virus A subtypes, and stained with anti-chicken IgG HRP conjugate. The ordinate –is an optical density (450 nm).

Thus, the evidence was obtained that recNP, as an antigen, at least in indirect ELISA, allow to discriminate AIV positive and negative poultry sera and to detect IgG antibodies specific to 15 subtypes of influenza virus A.

The specificity of rNP antigen was validated in cross-reactivity studies with sera, received from poultry species, vaccinated against Newcastle Disease, Infectious bronchitis and Gamboro Disease (infectious bursal disease). The further cross-reactivity experiments validating the specificity of influenza A recNP against other influenza viruses isolates (Type B. etc.) are in progress.

### CONCLUSION

The main result of Task duration period - is the development in the baculovirus expression system the appropriate NP gene construction and production in eukaryotic insect cells of the folded AIV recombinant nucleocapsid protein (rNP), consequently purified practically to homogeneous state. The immunoreactivity and specificity of the proposed antigen was confirmed in Western blot и indirect ELISA analysis with reference chicken anti-NP positive or negative sera and numerous poultry sera, obtained from birds, affected with AIV, including H5N1 serotype. The final rNP product may be used as an antigen for ELISA and other immunological formats testing of anti-AIV specific IgG at least for all known 15 subtype virus variants.

## REFERENCES

1. Portela A., Digard P. The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication. *J. Gen. Virol.* (2002), 83, 723-734.
2. Voeten J.T.M., Groen G., van Alphen D., Claas E.C.J., de Osterhaus R., Rimmelzwaan G.F. Use of recombinant nucleoproteins in enzyme-linked immunosorbent assays for detection of virus-specific immunoglobulin A (IgA) and IgG antibodies in influenza virus A- or B-infected patients. *J Clin Microbiol.* (1998), 12, 3527-31.
3. Rota P.A., Black R.A., De B.K., Harmon M.W., Kendal A.P. Expression of influenza A and B virus nucleoprotein antigens in baculovirus. *J. Gen. Virol.* (1990), 71, 1545-1554
4. de Jong M.D. and Hien T.T. Avian influenza A (H5N1). *J. Clin. Virol.*, (2006), 35, 2-13.

### **Milestones Completed**

The rNP protein in appropriate amount and appropriate experimental protocols were delivered to New Horizons Diagnostics Corp in October 2006 for further joint investigations.

The immunochemical characterization of anti-rNP Mabs, developed from 4 stable hybridoma clones is in progress. The preliminary data obtained in WB, ELISA shows evidence, that Mabs, generated against natural AIV H5N1 subtype virus preparation, specifically interact with AIV H5N1 isolate.