

Development and production of anti- Avian Influenza Virus (Type A) recombinant nucleoprotein (rNP antigen) specific antibodies.

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INTRODUCTION

The main goal of the present Project was the development and production of anti-AIV recombinant nucleoprotein (NP) specific monoclonal antibodies for appropriate antigen testing in different immunological formats.

As was previously mentioned in the summary of the project Subtask technical report, recent AIV diagnostic data 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 produced in eukaryotic expression systems in vitro has estimated sites for phosphorylation, so the baculovirus expression system of insect cells, which was used in the present work, provides the synthesis of fully processed and folded recombinant proteins. This proposition seemed valuable for generation of “universal” monoclonal antibodies, aimed for the detection of different strains of Avian Flu Virus, considered to be the most changeable (erratic) virus in the family of *Orthomyxoviridae*. Short data concerning the recent advantage in AIV detection by rapid tests is summarized below.

Influenza syndrome is defined by a rapid-onset systemic illness, with patients presenting with fever, chills, cough, myalgias, headache, and sore throat. Differentiation of influenza virus from the other respiratory viruses is of prime importance because the illness caused by influenza virus is associated with higher rates of morbidity and mortality. The recent advent of new treatments for influenza has stimulated the development of rapid diagnostic methods because these treatments have shown clinical benefit only when they are administered within 36 to 48 h of the appearance of symptoms.(1, 4). The "gold standard" for the diagnosis of influenza is tissue culture isolation, which takes from 2 to 14 days. Detection of virus-infected cells in nasopharyngeal secretions by direct or indirect immunofluorescent staining is widely used but is quite technique and technician dependent and its completion still requires 2 h (4,5). Rapid diagnosis of influenza permits the institution of antiviral treatment, helps to control nosocomial transmission of the infection, and contributes to reductions in the cost and the length of the hospital stay. Rapid diagnosis has previously been shown to be cost-effective in a pediatric hospital (29, 43) and useful for controlling influenza epidemics in wild birds and poultry avian flu outbreaks.

At least five different rapid enzyme-linked immunosorbent assay kits are available for the diagnosis of influenza: Directigen Flu A and Directigen Flu A+B (the latter of which is referred to herein as Directigen; Becton Dickinson Diagnostic Systems, Sparks, Md.), QuickVue influenza test (referred to herein as QuickVue; Quidel, San Diego, Calif.), Flu OIA (Bio Star, Inc., Boulder, Colo.) and Zstat (ZymeTx, Inc., Oklahoma City, Okla.). Most of them have been compared with culture or culture and reverse transcription (RT)-PCR, but in some studies RT-PCR has occasionally been used to clarify the results for samples with discrepant results.

Directigen. Directigen is a membrane-based enzyme immunoassay which differentiates between influenza viruses A and B. In brief, 200 µl of each nasopharyngeal aspirate are gently mixed with 8 drops (approximately 120 µl) of extraction buffer in the tube provided with the assay kit. Four drops

(approximately 60 µl) of the specimen extract, then are added to each well of the test device. Subsequently, specific conjugate, washing buffer, and substrate solutions are added within a 10-min period. The results should be read at 5 min, the stop solution should be added, and the test result should be read again. The control dot needed to be visible (unless it was obscured by an intense purple triangle) for a valid test, and if the dot was absent, the result is regarded as indeterminate.

QuickVue. QuickVue is a lateral-flow immunoassay which detects both influenza virus A and influenza virus B but does not differentiate between the two viruses. QuickVue first involves the extraction of influenza virus A and B antigens **by detergents**. The patient specimen is placed in an extraction reagent tube, during which time the virus particles in the specimen are disrupted, **exposing internal viral nucleoproteins**. **After extraction, a test strip is placed in the extraction reagent tube, where nucleoproteins in the specimen react with the lyophilized buffer and mouse monoclonal anti-influenza virus A and anti-influenza virus B antibodies contained in the test strip.** If the extracted specimen contains influenza virus antigens, a pink or red test line along with a blue control line appears on the test strip, indicating a positive result. If influenza virus A or B antigens are not present or are present at very low levels, only a blue control line appears. The test is read after 10 min. The other group have tested the performance of two above-said commercially available rapid test kits for influenza virus detection was compared to that of viral culture by using 356 nasal wash specimens collected during the 2001 to 2002 influenza season. Overall, the two rapid tests were easy to perform and showed comparable sensitivities (70.4 and 72.2%) and specificities (97.7 and 98.3%); for both test kit groups, most of the specimens that yielded false-negative results were found to be growing influenza B virus. The rapid and accurate detection of influenza virus allows hospitals and emergency departments to group or isolate influenza virus-infected patients to reduce the nosocomial spread of infection, reduces the incidence and length of hospital stays, identifies potential epidemic or pandemic strains in a timely manner, **and differentiates influenza virus from other infectious and biological warfare agents such as those that cause anthrax and smallpox, diseases that may begin with flu-like symptoms (1)**. The above mentioned kit was a lateral-flow immunoassay (QuickVue; Quidel, San Diego, Calif.) that detected both influenza A and B viruses but did not differentiate between them. The other test was also mentioned membrane enzyme immunoassay (Directigen Flu A+B; Becton Dickinson Diagnostic Systems, Sparks, Md.) that both detected and differentiated influenza A and B viruses. The rapid test results for both assays were compared to the results of the reference standard of viral culture by using 356 fresh nasal wash specimens. However, in one study, a **rapid neuraminidase detection** assay (ZstatFlu; ZymeTx, Oklahoma City, Okla.) **was shown to be less sensitive for influenza B virus detection.**

Recently (2006Y) **Standard Diag. Co (Korea)** have produced Influenza A rapid test on the bases of dip-stick format, but the minor data about its reliability and sensitivity is contradictory.

RT-PCR and microarrays. The tests considered as “Rapid tests” needs time and professionally skilled personal. Primers usually targeting the **matrix protein gene** are chosen after the most conserved

regions were identified in GenBank. Again, **low-density oligonucleotide microarrays** with highly multiplexed "signatures" for influenza viruses offer many of the desired characteristics. However, the high mutability of the influenza virus represents a design challenge. In order for an influenza virus microarray to be of utility, it must provide information for a wide range of viral strains and lineages. The design and characterization of an influenza microarray, the FluChip-55 microarray, for the relatively rapid identification of influenza A virus subtypes H1N1, H3N2, and H5N1 were described by Townsend MB et al., (2006). In this work, a small set of sequences was carefully selected to exhibit broad coverage for the influenza A and B viruses currently circulating in the human population as well as the avian A/H5N1 virus that has become enzootic in poultry in Southeast Asia and that has recently spread to Europe. A complete assay involving extraction and amplification of the viral RNA was developed and tested. In a blind study of 72 influenza virus isolates, RNA from a wide range of influenza A and B viruses was amplified, hybridized, labeled with a fluorophore, and imaged. **The entire analysis time was less than 12 h.** The combined results for two assays provided the absolutely correct types and subtypes for an average of 72% of the isolates, the correct type and partially correct subtype information for 13% of the isolates, the correct type only for 10% of the isolates, false-negative signals for 4% of the isolates, and false-positive signals for 1% of the isolates. In the overwhelming majority of cases in which incomplete subtyping was observed, **the failure was due to the nucleic acid amplification step** rather than limitations in the microarray.

Besides fast antigen detection tests, the need in the development of **fast track AIV devices for specific anti-virus antibodies detections (at least for human) is also evident.** Recently, Myers K.P. et al (2007) have examined veterinarians in the United States for evidence of previous avian influenza virus infection. They performed a controlled, cross-sectional seroprevalence study among 42 veterinarians and 66 healthy control subjects using serum samples collected from 2002 through 2004. Serum samples were tested using a **microneutralization assay against 9 influenza A virus strains.** Using multivariable logistic regression modeling, veterinarians exposed to birds demonstrated statistically significant elevated titers against the H5, H6, and H7 avian influenza virus isolates, compared with control subjects. These data suggest that occupational exposure to avian species may increase veterinarians' risk of avian influenza virus infection.

Another approach to differentiate avian influenza virus (AIV)-infected chickens vs. chickens immunized with inactivated avian influenza virus, an enzyme-linked immunosorbent assay (ELISA) was developed by Zhao S. et al., (2005), using a recombinant nonstructural protein (NS1) as the diagnostic antigen, which was cloned from an AIV H9N2 subtype strain isolated during the avian influenza outbreak of 2003-04 and expressed in *Escherichia coli*. Antibodies to the AIV NS1 protein was only detected in the sera of chickens experimentally infected with AIV but not in the sera of chickens immunized with inactivated vaccine. This ELISA is useful for serological diagnosis to distinguish chickens infected with influenza viruses from those immunized with inactivated vaccine. Recently, a microsphere immunoassay (MIA) was developed by Deregt D. et al., (2006) for the detection of serum antibodies to avian influenza virus. **A recombinant influenza A nucleoprotein expressed in baculovirus** was conjugated to

microspheres and incubated with antibodies. High median fluorescent intensities (MFIs) were obtained with a monoclonal antibody and positive chicken sera. Chickens were inoculated with 10 strains of avian influenza virus representing different subtypes, including high and low pathogenic H5 and H7 subtypes. Three hundred and fifty-four samples from experimentally infected chickens and controls were tested with a competitive ELISA (cELISA) and the MIA. MFIs were converted to positive/negative (PN) ratios. The results of both tests, as percentage inhibition and PN ratio, showed a high correlation ($R^2 = 0.77$). From the comparison data, a ratio of $> \text{ or } = 4.5$ was selected as the cut-off value for positivity in the MIA. Using this cut-off value, the sensitivity and specificity of the MIA relative to the cELISA when all discordant experimental samples were retested was 99.3 and 93.1%, respectively. The relative specificity increased to 94.7% when additional negative sera ($n = 68$) were tested. The MIA may be useful for surveillance testing and as a screening test for flocks infected with low pathogenic avian influenza virus and could be expanded for simultaneous detection of antibodies against other avian infectious disease agents.

Hurt A.C. et al., (2007), have tested (and compared with cell culture assay) five of the rapid tests (Binax Now Influenza A&B, Directigen EZ Flu A+B, Denka Seiken Quick Ex-Flu, Fujirebio Espline Influenza A&B-N, and Quidel QuickVue Influenza A+B Test). They have demonstrated a similar influenza A sensitivity of between 67-71% and a specificity of 99-100%, however one rapid test (Rockeby Influenza A Antigen Test) had a significantly lower influenza A sensitivity of only 10% (specificity was 100%). For the five kits that detected influenza B antigen, sensitivity was considerably lower than that seen for influenza A (sensitivity for all the kits was 30%), although the number of specimens containing influenza B viruses was low.

Yamazaki M, et al. (2004) evaluated a flow-through immunoassay for rapid detection of influenza A and B viral antigens, RapidTesta FLU AB (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan), by using 507 specimens collected from patients with influenza-like symptoms during the 2002/2003 influenza season in Japan. The specimens consisted of 239 nasal swabs and 268 nasal aspirates; 374 specimens were collected from pediatric patients (under 16 years of age) and 133 from adult patients. RapidTesta FLU AB was compared with cell culture and nested reverse transcription-polymerase chain reaction (RT-PCR). RapidTesta FLU AB had a sensitivity of 83.2% (109/131), a specificity of 98.5% (135/137) and an efficiency of 91.0% (244/268) for influenza A as well as a sensitivity of 82.7% (43/52), a specificity of 97.7% (211/216) and an efficiency of 94.8% (254/268) for influenza B. RapidTesta FLU AB is characterized by high specificity, low false positive rate, and 10-minute detection of influenza virus. These advantages suggest that RapidTesta FLU AB is a useful kit to assist physicians in making a diagnosis of influenza on candidates for antiviral therapy. The specificity of Mabs, used in this test (i.e. AIV antigen) is unknown for us (publication is in Japan language).

The Directigen Flu A assay, designed for rapid in vitro recognition of **influenza A nucleoprotein**, was also used to evaluate this assay for detection of influenza virus in nasal secretions of **naturally infected horses** (Morley PS, et al. 1995). The assay was shown to react with representative strains of influenza virus which cause disease in horses and did not react with nasal secretions from

uninfected horses kept in isolation. In contrast, influenza virus was isolated from only 7% of diseased horses using conventional techniques. Diseased horses which were positive in the Directigen assay had lower pre-exposure influenza antibody concentrations and showed more clinical signs than diseased Directigen-negative horses. This evaluation demonstrates that the Directigen Flu A assay detects influenza virus in nasal secretions of infected horses and is more sensitive than virus isolation.

Among the **avian influenza A virus subtypes, the H5N1 and H9N2 viruses have the potential to cause an influenza pandemic** because they are widely prevalent in **avian species** in Asia and have demonstrated the ability to infect humans (10). Currently, human infections with wild-type (wt) strains of these viruses could occur in the United States in poultry and turkey farm workers and in travelers returning from countries in which avian influenza viruses are prevalent in birds, such as Thailand, Vietnam, China **and now in Russia**. Laboratory-acquired infections could also occur in vaccine researchers working with candidate vaccine viruses, including cold-adapted (ca) viruses (11). Published reports indicate that the Directigen Flu A antigen capture enzyme immunoassay (Becton Dickinson, Sparks, MD) can detect H5N1, H7N2, and H7N3 avian influenza viruses and that a DAKO direct fluorescent antibody reagent (DAKO, Cambridgeshire, United Kingdom) can detect H5N1 avian influenza virus, although these commercial rapid antigen tests are insensitive (13). In these comparative experiments authors cells lines from Diagnostic Hybrids, Inc., Athens, OH, and Bartels **influenza A monoclonal antibody (Trinity Biotech, Wicklow, Ireland)**. The results obtained with the two antigen capture enzyme immunoassays with H5N1 and H9N2 influenza A viruses. show, that neither assay detected virus at 50 TCID₅₀, but H9N2 wt and ca viruses were detected by both assays at 5 x 10⁴ TCID₅₀. H5N1 ca virus was detected at 5 x 10⁴ TCID₅₀ in both assays, but the wt virus was not detected in either assay at this concentration. These data generated the concern that additional strains of wt avian influenza viruses may not be detected by the antigen capture enzyme immunoassays.

The development of AIV immunological fast tests, based on Mabs generated against concrete HA antigens meets certain difficulties in detecting new serotypes (substrains) of viruses even dominating in environment in the next year. **Horimoto T. et al., (2004)** made an attempt to assess whether the antigenic properties of H5 hemagglutinin (HA) change over time due to antigenic drift. They have produced a panel of monoclonal antibodies (mAbs) against the HA of the index H5N1 human influenza A virus, A/Hong Kong/156/97. By immunizing mice with a plasmid expressing this HA and boosting the initial immunization with cell lysates transfected with the plasmid, a total of six hybridomas producing HA-specific mAbs were established: four to the HA1 subunit with hemadsorption-inhibiting activity and two to the HA2 subunit. **None of the mAbs to HA1 could bind to the HA of a recent human isolate, A/Hong Kong/213/2003, indicating, that there are substantial antigenic differences between the H5N1 human influenza virus isolated in 1997 and that isolated in 2003!** More promising but different result was obtained by Zheng Q.S. et al.,(2005), with interaction of anti-AIV serums with recombinant product of the HA1 gene of H5N1 subtype AIV (15). Western blot analysis proved that the recombinant protein has good reactive ability against H5N1 subtype AIV positive serum (one?). The optional working circumstances for the iHA-ELISA assay (antigenicity concentration: 4 microg/mL; serum dilution: 1:200)

was tried out with chess titration. The positive criterion of this **ELISA assay is OD** (the tested serum) > 0.5 and OD (the tested serum)/OD (the negative serum) > 2.0.

Thus, the experiments achieved by J. T. M. Voeten, et al., (1998) about universal use of recombinant nucleoproteins in ELISA's for detection of virus-specific immunoglobulins IgG and IgA in Influenza Virus A- or B-infected patients, remains conclusive in the selection of AIV antigen for Mabs generation in the present study for evident reasons. Shortly, the above-said team have used the **nucleoprotein genes of influenza virus A/Netherlands/018/94 (H3N2)** and influenza virus B/Harbin/7/94 for cloning into the bacterial expression vector pMalC to yield highly purified recombinant influenza virus A and B nucleoproteins. IgG antibody rises were detected in 88.2% of influenza virus A-infected patients and in 95.8% of influenza virus B-infected patients. On comparison, hemagglutination inhibition assays detected antibody titer rises in 81.3 and 72.7% of patients infected with influenza viruses A and B, respectively. Since the NP is well conserved within the influenza A viruses, the IgG NP ELISA enables the detection of antibodies induced by influenza A viruses of both circulating subtypes (H1N1 and H3N2). Furthermore, this assay does not require the annual adjustment of the viral antigen preparations, in contrast to the HI assay, which measures antibodies against the highly variable hemagglutinin. Huang H. et al., (2007), have produced 6 clones of Mabs against formalin-treated influenza A/CK/Hubei/327/2004 virus. They happened to be specific against HA and have also neutralizing activity to 3 relative Flu virus strains and strong cross-reactivity and neutralizing reaction between virus strains located in different phylogenetic lineages.

In the present report, recombinant NP of influenza viruses A was used for the generation of virus-specific Mabs and development of ELISA systems which can detect virus-specific IgG serum antibodies and virus antigen. These assays may replace the commonly used HI (HA) and CF assays for the serodiagnosis of influenza virus infections and can be performed when respiratory specimens are not available or to confirm results obtained by culture procedures with respiratory specimens also.

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Materials and Methods

Production of recombinant NP. The NP gene of H2N9 avian influenza virus (AIV) A/Swine/Hong Kong/9A-1/98 strain was cloned into pFastBacHTc donor plasmid (Invitrogen) and transformed into DH10Bac competent cells. The recombinant baculovirus stock was prepared by transfecting the recombinant bacmid DNA into Hi-5 insect cell line for protein expression after amplification.

Purification of recombinant NP (rNP). The Hi-5 insect cell line was used for expression of the recombinant NP. After expression, cell culture (2 g) was pelleted, resuspended in 20 ml of LB (50 mM NaH₂PO₄, 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 12000g. PMSF was added to the supernatant to the final concentration of 1 mM. 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 12000g 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 (store in this way).

Preparation of native nucleoprotein (NP). Allantoic fluid containing native NP was obtained by inoculating of influenza viruses (subtype A/H5N1, A/chicken/Kurgan/5/05) in 10-day-old embryonated chicken eggs. Complex NP-M1 proteins was isolated by treatment of viral preparation with 0.1 % nonionic detergent NP-40 and purified by differential sedimentation through 20 to 60 % sucrose gradients at 27000 rev/min 90 min in a SW28 rotor.

Production of anti-NP monoclonal antibodies (Mabs). Six-week-old female BALB/c mice were inoculated subcutaneously with 40 µg native NP dissolved in PBS with 0,5 M urea and emulsified in Freund's complete adjuvant (1:1, v/v). Two booster inoculations of protein in Freund's incomplete adjuvant were administered at 14-day intervals. Two week after the last immunization the blood was obtained for testing of antibody titers by indirect ELISA, using NP as a screening antigen. Three days before cell fusion, the mice were immunized intravenously with recNP without adjuvant.

Spleens were removed, cell suspensions were fused with myeloma cell line SP2/0-Ag14, and hybrids were selected in hypoxantine-aminopterin-thymidine medium. Supernatant tissue culture fluids were screened by ELISA, and positive (against NP) cell lines were cloned by limiting dilution. The isotype of MAbs was determined by ISO-2 Antibody Isotyping Kit (Sigma).

Preparation of precipitated NP for use in indirect ELISA. Suspension of precipitated NP was centrifuged at 10000 g 5 min. The pellet was dissolved in buffer containing 4 M Urea, 10 mM Tris-Cl, 10 mM β-mercaptoethanol (pH 9,5). The protein solution was transferred to dialysis tubing and dialyzed versus two changes of 0.1 M sodium carbonate buffer (pH 9.6) overnight.

Indirect ELISA with monoclonal antibodies. 100 µl of NP antigen (10µg/ml) were adsorbed in 50 mM carbonate buffer, pH = 9.2 at +4⁰ C, overnight in Greiner ELISA plate (Cat. # 762070). Plates were blocked 30 min at 37⁰C with PBS containing 3% “Top-block” (Juro), washed 5 times with PBST (PBS containing 0.1% Tween 20) and 100 µl MAb (cultural fluid, ascites fluid or purified Mab’s) were added in PBS containing 0.5% BSA and incubated 1h at 37⁰C. Plates were washed 5 times with PBST, 100 µl of anti-mouse IgG HRP conjugate (Sigma) 1/1000 (v/v) was added and incubated 1h at 37⁰C. After washing, 100 µl H₂O₂- TMB solution was added. After 20 min, the color development was stopped by adding 50 µl 1M H₂SO₄; the extinction values were then measured at 450 nm in an Multiscan EX spectrophotometer (Thermo).

Sandwich ELISA. 100 µl IgG Mab (NP3, 10 µg /ml) were immobilized in 50 mM carbonate buffer, pH = 9.2 at +4⁰ C, 24 h in Greiner ELISA plate (Cat.#762070). Plates were blocked 30 min at 37⁰C with PBS containing 3% “Top-block” (Juro), washed 5 times with PBST, added 100 µl antigen (recNP contained cell lysates, native NP, (or from poultry pathogenic material) in PBST containing 0.5% BSA + 2.0M Urea and incubated 1.0 h at 37⁰C. After washing 100 µl *NPS-HRP conjugate in PBS containing 0.5% BSA was added and incubated 1.0 h at 37⁰C. Plates were washed 5 times with PBST and 100 µl H₂O₂- TMB solution was added. After 20 min, the color development was stopped by adding 50 µl 1M H₂SO₄; the extinction values were then measured at 450 nm in an Multiscan EX spectrophotometer (Thermo). * NPS – clone of anti NP generated Mabs, selected for sandwich ELISA procedure.

SDS-PAGE and immunoblotting. The protein profile of antigens and specificity of Mabs were 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 were diluted in Laemmli sample buffer with 2-mercaptoethanol and heated in a boiling-water bath for 5 min before being loaded onto the gel. The proteins were separated at a constant voltage of 200 V.

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 Mab in PBST containing 0.5% BSA for 1 h at 37⁰C.

After three washes in PBST the membranes were incubated with anti-mouse IgG-HRP for 1 h at 37⁰C. The blots were stained with diaminobenzidine plus 4-cloro-1-naphtol.

RESULTS

1. Characterization of anti-NP monoclonal antibodies (Mabs).

In the developed indirect ELISA, based on “native” NP as an antigen, 6 hybridoma clones were selected (i.e. monoclonal antibodies from culture fluid with maximal signal to noise ratio (A450>2.5). After appropriate subcloning, 6 stable hybridoma cell lines were used in the further study. NP-specific Mabs were produced in preparative amounts, partially purified by ammonium sulfate stepwise precipitation and dialyzed against PBS.

Using Mouse monoclonal antibody Isotyping Reagents (Sigma, ISO-2), the subtypes of immunoglobulins were determined. The results of each NP-specific Mabs isotyping assay are presented in Table 1.

Table 1. Characterization of anti-NP Mabs immunoglobulins subtypes.

Clone	Isotype	Specific reactivity in indirect ELISA
NPS	IgG1	+
NP1	IgM	+
NP2	IgG1	+
NP3	IgG1	+
NP4	IgG1	+
NP5	IgG1	+

Comparative immunoblotting analysis of the selected Mabs reactivity with recombinant and “native” virus NP showed that two clones, i.e. NPS and NP3 specifically interact with both types of antigens (see Fig.1).

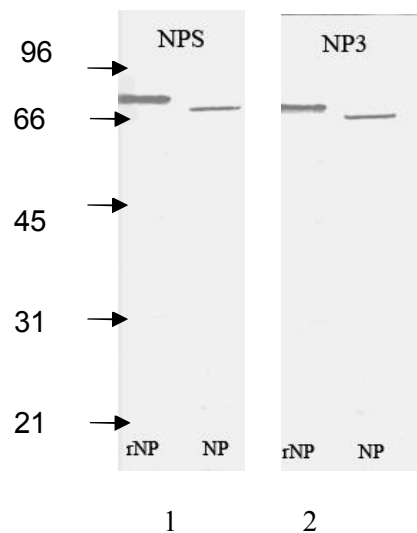


Fig.1. Immunological identification of rNP and virus NP by Western Blot with generated Mabs.

The proteins (rNP – recombinant NP, NP – native NP) were boiled in SDS-sample buffer and separated by SDS-PAGE. After electrophoresis proteins was transferred to PVDF membrane. Strips were incubated with different Mabs (1 – NPS, 2 – NP3). Further, strips were incubated with anti-mouse IgG-HRP and stained with mixture of diaminobenzidine and 4-cloro-1-naphtol.

As can be seen from Fig. 1, the both protein bands, corresponding to purified preparations of recombinant (rNP) and native NP are stained NPS and NP3 Mabs with evident specificity. Small differences in molecular mass of recombinant and “native” NP is conditioned by additional segment in *np* gene construction installed in expression vector.

At the next stage, the Mabs from each clone were conjugated with horse radish peroxidase (HRP) and used as a secondary antibodies in a sandwich ELISA format for detection of recombinant NP as follows. Purified Mabs (10 mkg/ml in 0.1 carbonate buffer) were adsorbed on immunoplates during 18 hrs at 4 °C. Different amounts of recNP in **PBST, containing 0.5% BSA + 2.0 M Urea** were added to the wells and incubated 1.0 hr at 37°C. After standard washing Mabs-HPR conjugates from each clone were added. The optimal dilutions of the above said conjugates were obtained in a previous experiment (data not shown). In our hands, the most sensitive variant for the detection of recombinant NP was a system, where Mab NP3 were used as a capturing antibody and NPS-HRP conjugate Mabs were used as a detecting antibody (see Fig. 2).

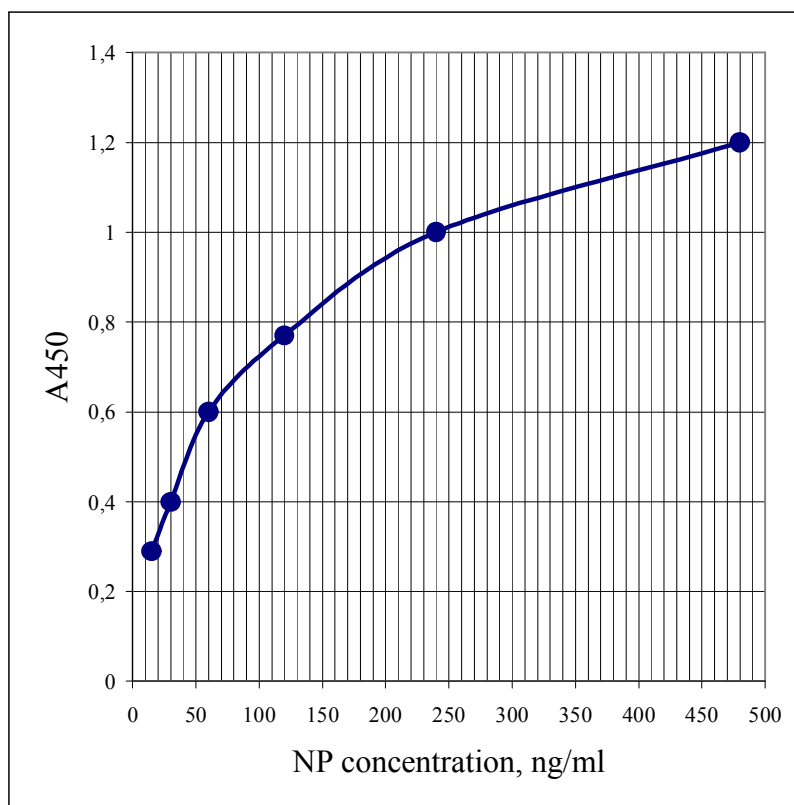


Fig.2. Detection of recNP in a sandwich ELISA format.
NP3 – capturing Mabs; NPS-HRP conjugate – detecting Mabs.

The titration curve shows that in the range of 10-30 ng/ml recNP can be reliably detected in ELISA though the sensitivity limit might be much higher due to the further optimization of the system including new detergents for antigen sampling buffer. It is known, that Influenza A nucleoprotein tends to form in vivo different types of NP-NP-associates, including termo and proteinase-resistant oligo- and multimers (Semenova N.P. et al., 2007) and in some cases (pre-heating of pathogenic virus-containing samples at 100 °C, etc.) conformational Mabs did not recognize any immunoreactive NP structures in cell or virus lysates (Prokudina-Kantorovich et al., 1996; 2001). These phenomena indicates, that in the case of virus RNA-binding proteins, the development of sampling buffer for dip-stick format may be a serious independent research study (Portela A. and Digard P., 2002). In the present work, the selected pair of

Mabs proved to be rather sensitive in detection of recNP in standard sandwich ELISA format and additional validation of generated Mabs efficiency in anti-AIV serum IgGs competitive ELISA is in progress.

2. Validation of anti-rNP sandwich ELISA for detection of Avian Flu Virus in biological material.

The sensitivity and specificity of the developed ELISA test-system was further studied using virus detection of different AIV strains and geographic isolates in cell cultures and real pathogenic materials from birds and laboratory animals. The preparation of paramyxovirus (Newcastle Disease virus, strain La Sota) was used in criss-cross specificity studies, as a relative negative control virus antigen. The results of the ELISA-Flu experiments are presented in Table 2.

Table 2. Determination of Avian Influenza Virus subtypes by sandwich ELISA, based on Mabs NP3 and NPS.

№	Influenza A Viruses (etalon strains)	Antigenic formula	Titer in ELISA	ID ₅₀ /0,2 ml.
1	A/USSR/90/77	H1N1	1/27	10 ⁵
2	A/Aichi/68	H3N2	1/27	10 ⁸
3	A/turkey/Massach/3740/65	H6N2	1/27	10 ^{7,75}
4	A/duck/Kazach/5187	H7N2	1/2	10 ⁵
5	A/turkey/Wisconsin/66	H9N2	1/81	10 ^{7,75}
6	A/ch/Germany/"N"/49	H10N3	1/81	10 ^{8,5}
7	A/gull/Astrachan/13/76	H16N6	1/16	10 ⁵
8	***A/chicken/Kurgan/5/05	H5N1	1/81	10 ⁵
9	A/duck/Altai/1285/91	H5N2	1/64	10 ⁵
10	A/duck/Primoric/2621/01	H5N2	1/32	10 ⁷
11	A/duck/Primoric/2621/01	H5N2	1/32	10 ⁷
12	A/duck/Primoric/2632/01	H5N3	1/32	10 ⁹
13	A/duck/Primoric/2632/01	H5N3	1/32	10 ⁷
14	A/duck/Primoric/2632/01	H5N3	1/8	10 ⁵
15	A/duck/Primoric/2633/01	H5N3	1/32	10 ⁷
16	A/duck/Primoric/2634/01	H5N3	1/32	10 ⁷
17	A/duck/Primoric/2634/01	H5N3	1/16	10 ⁸
18	A/duck/Primoric/2635/01	H5N3	1/16	10 ⁷
19	A/duck/Primoric/2635/01	H5N3	1/2	10 ⁵
20	** Newcastle disease virus, strain La Sota		negative	negative

As can be seen from Table 2, the specificity of the developed ELISA test-system is sufficient for detection of all tested AIV strains and field isolates* with a variable hemagglutinin/neuraminidase (HA/NA) antigen repertoire. The sensitivity of the method allow to detect influenza A virus strains in a range of dilution of original virus preparation varying - 10³ - 10⁵. Newcastle disease virus preparation showed negative criss-cross reactivity in the above said sandwich ELISA format.

*The practice of virus-containing field sample collection of animal and wild bird tissues in some cases meets evident difficulties in sample storage, and re-isolation of viruses from such biomaterials is essential procedure for monitoring of the flu outbreaks in RF. In the present work the detection of AIV was done also with wild birds cloacal swabs samples, collected in 2002. After original (initial) AIV

isolation, the influenza virus-positive samples were stored at -45°C more than 5 years. The sandwich ELISA developed in this work proved to be more sensitive in comparison with standard HA testing in parallel reactions (Table. 3), and showed positive results in all cases of previously estimated AIV strain isolates samples.

Table 3. Detection of AIV subtypes by sandwich ELISA.

№	AIV Antigenic formula	Titer in ELISA	Titer in Hemagglutination (HA)
1	H5N1	16	6
2	H3N8	8	6
3	H4N8	2	2
4	H10N4	2	2

This promising result indicates, that new immunological formats, based on the recommended pair of Mabs, can be used for AIV detection at least in virus culture preparations and also for AIV outbreaks monitoring in birds and animals species.

3. Detection of Avian Influenza Virus, subtype H5N1, in pathogenic tissues of infected animals.

Highly Pathogenic Avian Influenza (HPAI, Fowl plague) virus with determined subtype - H5N1, was isolated from broiler's pathogenic material, received from AIV poultry outbreak in 2005Y. The genom sequence of this virus strain was estimated and etalon virus preparation, named as - ***A/chicken/Kurgan/5/05 was established.

At the next stage of the present work the efficiency and sensitivity of the developed AIV/NP ELISA was verified with tissues samples from different organs of laboratory mice, experimentally infected with A/chicken/Kurgan/5/05, H5N1 virus subtype.

White laboratory mice (18-19 grams/ wt) were infected by intranasal injection of allantoic fluid, containing 10^5 EID₅₀ of selected H5N1 etalon virus subtype. After 4 days of the challenge, the tissues from different organs of animals were taken for further histopathology investigation and AIV antigen immunoassay. For sandwich ELISA tests, the 10% (*w/v) tissue suspension was prepared in standard PBS. The presence of AIV in the tissues of challenged animals was confirmed by consequent positive re-infection of 9-10-day chicken embryos with the appropriate virus-containing tissues suspension prepared from infected mice.

Table 4. Detection of AIV/H5N1 in organs of infected animals by sandwich ELISA.

№	Organs	AIV Antigenic formula	Titer in ELISA	Titer in HA	EID ₅₀
1	Lungs (mouse № 1)	H5N1	128	24	10^4
2	Lungs (mouse № 2)	H5N1	64	8	10^4
3	Liver (mouse №1)	H5N1	16	6	10^4
4	Spleen (mouse №1)	H5N1	8	4	10^4
5	Trachea (mouse №1)	H5N1	2	+-	10^4

As can be seen from Table 4, the maximal content of virus was determined both by ELISA and HA reaction in lungs, and minimal virus content was found in trachea of the challenged laboratory animals. These data was confirmed by histopathology investigations of appropriate tissues. The evident clinical signs of periapical niduses of pneumonia in lungs, strong hyperemia and size changes in liver and spleen were estimated.

In re-infected chicken embryo tissues AIV virus was detected by ELISA in all experimental cases (data not shown).

4. Fast AIV/NP Dip-Stick format assay.

In the frames of the development we have done preliminary initiative research for estimation of the NPS and NP3 Mabs immunoreactivity and “sandwich” efficiency in lateral flow chromatographic strip format. IgGs purified from both types of Mab clones were conjugated with colloidal gold and used as a detecting or capturing antibody in immunostrip version of the assay. The completion of strip device was kindly provided by biotechnology laboratory of Shortly, at present, the only one variant of the strip construction showed AIV/NP positive results: NP3 Mab were used as a capturing antibody plotted onto membrane support and NPS antibodies were conjugated with gold particles and used as a floating detecting antibody (Fig.3). It should be pinpointed that *sampling buffer* for the native NP (from A/H5N1-containing preparations) which provide and assure the evident and reliable positive “red line” result in this format, was purchased from commercial FeLV-kit produced by European Veterinary Laboratory (EVL, Netherland).

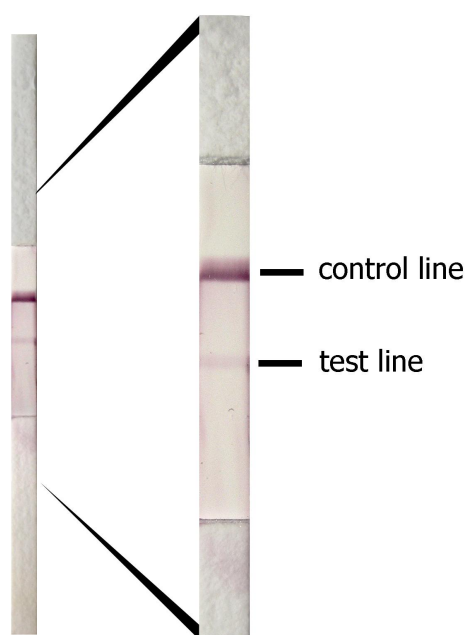


Fig.3. Detection of native NP (A/H5N1) in a Fast Dip-Stick format assay.

At present moment the search for appropriate detergent-containing sampling buffer system for optimization of AIV dip-stick analysis is under our intensive research.

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Milestones Completed

Completed. The anti-rNP protein specific Mabs in appropriate amounts and appropriate product certificates were delivered to New Horizons Diagnostics Corp. in April 2007 for further joint investigations.

Completed. The immunochemical characterization of anti-rNP Mabs, developed from 4 stable hybridoma clones and data obtained in WB, indirect and sandwich ELISA gave evidence that Mabs, generated against natural AIV subtype, specifically interact with different AIV strains and field isolates.

On schedule. -stick immunological format is under our joint research for AIV antigen detection in real field samples from wild birds and poultry.