

Development of Highly Sensitive Immunoassays and Reagents for Detection of Avian Influenza

DHS Priority Areas Addressed	<input checked="" type="checkbox"/> Prevention <input checked="" type="checkbox"/> Detection <input checked="" type="checkbox"/> Response <input type="checkbox"/> Recovery <input type="checkbox"/> Education/Risk Communication				
Proposal Section Addressed	Sections 5.2.1, and 5.3.1				
Investigators	TAMU: Blanca Lupiani, Sanjay Reddy and John El-Attrache				
Objectives	Deliverables	Progress Toward Deliverables	Percent Complete		
To complete baculovirus expression of avian influenza proteins	Expression and purification of NP protein	rNP protein has been produced using a baculovirus expression system and purified. The quality of the purified protein was evaluated by doing serial dilutions of the protein and testing it against known positive and negative chicken anti-AI sera. The protein was shown to be of high quality and suitable for monoclonal antibody production and for the development of ELISA and Multiplexing diagnostic tests.	100		
	Expression and purification of H6 protein	This project was terminated as decided in the January 2006 IAC at UC Davis and replaced with two new objectives focused on the development of a DIVA vaccine and associated diagnostic test based on the NS1 protein (see below).	Terminated 1/06		
To develop NP monoclonal antibody	Monoclonal NP antibody	Monoclonal antibodies to NP protein have been produced. Several of the monoclonal antibodies work well in ELISA and IFA but not Western blot while others work in all three assays. We are in the process of typing and finalizing the characterization of these monoclonal antibodies.	95		
To develop polyclonal antibodies	Polyclonal H5, H6, and H7 antibodies	Chicken and rabbit anti H6N2 polyclonal sera and chicken anti H5N3 polyclonal serum have been developed. These sera are being used as controls for some of the immunoassays indicated below. However, this project was terminated, as decided in the January 2006 IAC at UC Davis, and replaced with two new objectives focused on the development of a DIVA vaccine and associated diagnostic test based on the NS1 protein (see below).	30 Terminated 1/06		
To develop multiplexing microsphere-based immunoassays for detection and subtyping of AI virus and anti-AI antibodies	Development of an assay for detection of NP antibodies	ELISA tests of serial dilutions of the purified rNP protein, using a reference positive chicken serum (NVSL) indicated that the optimal protein concentration for coating ELISA plates is 0.06 µg/ml. rNP was also evaluated by ELISA test using chicken sera samples of known titer (as determined using Synbiotics AI virus antibody test kit): 20 high, 20 medium, 20 low and 30 negative samples. The titers obtained were in agreement with those obtained with the Synbiotic kit confirming the high quality of the purified rNP protein. NP protein coated ELISA plates were tested for reactivity with reference sera for other 8 avian pathogens (Newcastle diseases virus, infectious bronchitis virus, avian pneumovirus, avian paramyxovirus 2, avian adenovirus, avian reovirus, infectious bursal diseases virus and infectious laryngotracheitis virus), sera from experimentally infected chickens and known negative and positive field sera samples. Our data indicate that our test is comparable to another commercially available and the results obtained present high level of agreement with the gold standard (AGID). Conditions were optimized for coating Luminex fluorescent beads with rNP and rNS1 purified proteins. Both bead sets were used in a duplex reaction with positive and negative serum samples. The results were in agreement with those obtained with the Western blot analysis indicating that the rNP protein is a better choice for the detection of AI infected chickens. NP protein coated fluorescent microbeads were tested for reactivity with reference sera for other 8 avian pathogens (Newcastle diseases virus, infectious	85		

		bronchitis virus, avian pneumovirus, avian paramyxovirus 2, avian adenovirus, avian reovirus, infectious bursal diseases virus and infectious laryngotracheitis virus), sera from experimentally infected chickens and known negative and positive field sera samples. Our data indicate that our test is superior to a commercially available ELISA since it yields no false positives and had a higher signal to noise ratio and presents high level of sensitivity and specificity when compared to the gold standard (AGID).	
	Development of an assay for detection of H5, H6, and H7 antibodies	This project was terminated as decided in the January 2006 IAC at UC Davis and replaced with two new objectives focused on the development of a DIVA vaccine and associated diagnostic test based on the NS1 protein (see below).	Terminated 1/06
	Development of an assay for detection of NP, H5, H6, and H7 antigen	The section of this project that involves the development of assays for the detection of H5, H6 and H7 antigen was terminated, as decided in the January 2006 IAC at UC Davis, and replaced with two new objectives focused on the development of a DIVA vaccine and associated diagnostic test based on the NS1 protein (see below). Monoclonal antibodies against NP protein have been developed. These antibodies will be used in the development of an ELISA and Luminex test for the detection of NP protein in infected chickens. The monoclonal antibodies have been characterized and we are in the process of expanding them to use them in the antigen detection tests.	Part of this project Terminated 1/06 50
To develop NS1 mutant viruses with DIVA vaccine potential by reverse genetics	Development and characterization of NS1 mutant viruses with DIVA vaccine potential	Using reverse genetics we have developed a NS1 mutant avian influenza virus, rH5N3/NS1-143, in which stop codons were introduced at amino acid position 143. This mutation results in the synthesis of a 143 amino acid long protein instead of the 230 amino acid long wild type protein. This virus replicated well in 7 day-old but not in 11 day-old embryonating chicken eggs. This virus was able to replicate in chickens but showed reduced pathogenicity. Since the virus had attenuated phenotype in chickens, we evaluated its efficacy as a live attenuated vaccine in 6-week old chickens. The antibody titers, as measured by hemagglutination inhibition assays, reached protective levels one week after vaccination in both vaccine groups (rH5N3/NS1-143 and parental rH5N3). The titers between the two groups did not differ significantly over the next three weeks. Western blot analysis of the serum samples from parental (rH5N3) and rH5N3/NS1-143 vaccinated chickens, using purified rNP and rNS1 proteins indicated that the rH5N3/NS1-143 infected chickens were able to develop a strong immune response to NP but not NS1 proteins. These data suggest that rH5N3/NS1-143 could be used as a vaccine with DIVA properties. However, when chickens were challenged with recombinant wild type virus the level of protection was significantly less compared to protection conferred by the parental (rH5N3) vaccine. Since the protection conferred by the live rH5N3/NS1-143 was significantly less as compared to parental rH5N3 virus as a live vaccine, we evaluated both viruses as killed vaccine preparation. Protective efficacy of killed adjuvant vaccine prepared from rH5N3/NS1-143 virus was compared with a conventional killed vaccine prepared from parental rH5N3. Two doses of vaccines were administered at 3 and 5 weeks of age. The negative control groups received vaccine but no challenge virus, whereas the positive control groups received challenge virus but were not vaccinated. The chickens received challenge virus at 7 weeks of age. Serum samples were collected at weekly intervals to determine antibody titers based on hemagglutination inhibition assays. The antibody titers in both vaccine groups reached protective levels one week after booster vaccine dose. Protection was measured by shedding of challenge virus on the trachea. Real time PCR and virus re-isolation experiments showed that rH5N3/NS1-143 vaccine provided comparable levels of protection to rH5N3 conventional vaccine. The major advantage of rH5N3/NS1-143, as a killed vaccine is the ability to serologically distinguish infected chickens from vaccinated chickens. Work is in progress to evaluate tests developed earlier to distinguish vaccinated from infected chickens.	Project initiated 1/06 95
To develop a NS1 based ELISA test for the differentiation of vaccinated from infected animals	Development of an ELISA test based on baculovirus expressed NS1 protein that can differentiate infected from vaccinated animals	Preliminary data by Western blot analysis suggest that an ELISA test can be developed based on our rNS1 protein and the rH5N3/NS1-143 mutant virus. We are in the process of confirming these results using both ELISA and Luminex tests. With samples from experimentally vaccinated and vaccinated/challenged chickens	Project initiated 1/06 30
To develop a lateral flow immunoassay	Preparation of immunoassay conjugates and optimal selection of lateral flow device	A stock supply of AI polyclonal serum was developed in chickens and further purified to contain IgY component. Concentration of the stock supply IgY was evaluated and	95

(LFIA)	conjugate pad, absorbance pad, and nitrocellulose membranes	reactivity of the purified product was first determined via ELISA for confirmation of stock AI IgY. A selected portion of the AI IgY was then conjugated to colloidal gold and analyzed on the AE98 Fast membrane predetermined in the first quarter to be the fastest and most efficient nitrocellulose membrane.	
	Evaluation of sample preparation and lateral flow immunoassay application	An initial assessment of six sample buffers has been conducted. These buffers included different detergent concentrations (SDS, Triton X-100, and NP40) in addition to different blocking components used to eliminate false positives. Buffers with various salt concentrations were also evaluated. Analysis of all buffer and variable AI antigen combinations were performed in a 96 well plate format. Incubation time was analyzed and the solutions were then applied to the sample pad of the LFI. In approximately 15-20 minutes the test was read. Final data analysis of the appropriate buffer components identified an ideal buffer consisting of 0.1M PBS/0.5% BSA/0.05% Tween/1% EtOH, pH 9.	80
	Sensitivity and specificity determination and data analysis	This project is a year 3 deliverable and has not yet been initiated. Reagents supplied by Drs. Reddy and Lupiani will be utilized to evaluate antigen and serological detection of different subtypes of avian influenza.	10

Highlight for Research Briefs

- Recombinant NP and NS1 proteins of avian influenza virus has been produced in baculovirus.
- Monoclonal antibodies to rNP protein have been produced.
- ELISA and Luminex tests have been developed for the detection of chicken antibodies to NP protein. Both assays present high specificity and sensitivity when compared to commercially available ELISA and AGID tests.
- A recombinant avian influenza virus with truncation in the NS1 protein, rH5N3/NS1-143, has been generated.
- Live attenuated vaccine prepared from rH5N3/NS1-143 showed attenuated phenotype in chickens but induced a good antibody response. Challenge experiments showed that it did not confer comparable levels of protection compared to rH5N3 vaccines as measured by histopathology and virus shedding.
- Killed adjuvant vaccine prepared from rH5N3/NS1-143 provided comparable levels of protection compared to conventional killed vaccines.
- Chicken immune response to this recombinant virus suggests it has potential as a vaccine with DIVA properties.
- Preliminary data suggest that a DIVA diagnostic test can be developed with the baculovirus expressed rNS1 protein and the rH5N3/NS1-143 mutant virus.

Interpretive Summary

Early detection is essential for the control of avian influenza virus infection in poultry. Using molecular techniques we have produce large amounts of the highly conserved NP protein, rNP, of an avian influenza virus using a baculovirus expression system. This protein was shown to be recognized by antibodies produced in AI virus infected chickens. We have developed EISA and Luminex based tests that allow the detection of chicken antibodies to influenza virus. In addition we have developed and characterized a series of monoclonal antibodies to NP that will allow the development of test for the detection of AI virus in infected chickens. These tests and reagents will aid in an early detection and response to AI virus infection in commercial poultry.

Vaccination of commercial poultry with inactivated AI vaccines has been shown effective in controlling the spread of AI. However currently available vaccines do not allow differentiation of infected from vaccinated animals (DIVA). Using reverse genetics we have generated a mutant AI virus in which the NS1 protein is truncated, rH5N3/NS1-143. The live rH5N3/NS1-143 virus showed attenuated phenotype in chickens, induced good antibody responses but the protection conferred by this live virus was significantly lower compared to the parental H5N3 virus. However, the killed

vaccine preparation of rH5N3/NS1-143 showed high levels of protection as measured by virus shedding and histopathology. Since this recombinant vaccine induces minimal responses to the NS1 protein, the combination of a vaccine produced with this recombinant NS1 mutant virus and the baculovirus expressed rNS1 protein can be used for the development of a vaccine and diagnostic test with DIVA properties. Killed adjuvant vaccine prepared from mutant AI virus in which the NS1 protein is truncated, rH5N3/NS1-143 was able to protect chickens from virulent AI challenge. The level of protection was comparable to conventional killed vaccine. The ability to use DIVA diagnostic tests to distinguish vaccinated from naturally infected chickens makes this vaccine superior to the currently available vaccines.

Technology Transition Plan for Vaccine Project:

There is a big market potential worldwide for avian influenza vaccines with DIVA strategy. Steps toward product development and technology transition will include:

1. The avian influenza vaccines will be developed and tested for induction of protective neutralizing antibodies in chickens and turkeys at TAMU.
2. Invention disclosures will be filed to protect intellectual property.
3. Results will be discussed with major commercial vaccine manufacturers, including Fort Dodge, Intervet and Merial.
4. The benefits of the avian influenza vaccine will be discussed with USDA/APHIS leadership representing end users, personnel from DHS representing biosecurity users, the USDA/APHIS/Center for Veterinary Biologics representing the regulatory authority for animal health, and presented at the annual meeting of the United States Animal Health Association where producers, marketing, academic and federal officials convene to recommend implementation of new vaccines into poultry production systems.
5. Encourage interested commercial manufacturers and vendors, e.g. Intervet, Fort Dodge, and Merial among others, to license the vaccine and assume responsibility for product development and federal approval for distribution to state and federal officials.

Technology Transition Plan for Diagnostic Project:

Steps toward product development and technology transition for the final NP, NS1 and H5 diagnostic test will include:

1. Basic research on the development of reagents and diagnostic test for avian influenza infections will be carried out at Texas A&M University and UC Davis.
2. Invention disclosures will be filed to protect intellectual property.
3. A dossier of finalized GLP-like protocols, and manufacturing concepts will be packaged for delivery to major commercial manufacturers and vendors of biologics, including Synbiotics and Idexx.
4. The concept for the benefits of the NP and NS1 diagnostic tests will be discussed with USDA/APHIS leadership representing end users, personnel from DHS representing biosecurity users, the USDA/APHIS/Center for Veterinary Biologics at Ames, IA representing the regulatory authority for animal health, and presented at the annual meeting of the United States Animal Health Association where producers, marketing, academic and federal officials convene to recommend implementation of new technology into livestock production systems.
5. Encourage interested commercial manufacturers and vendors, e.g. Synbiotics and Idexx among others, to license the integrated array based multi-select agent platform and assume responsibility for product development and federal approval for distribution to state and federal officials for detection and diagnosis of select agents of livestock and zoonoses.