"Prevention and Control of Avian Influenza in the U.S."
Avian Influenza Coordinated Agricultural Project
USDA CSREES NRI Integrated Research

Progress Report
University of Maryland, College Park
College Park, MD 20742

The long-term goal of the program entitled "Prevention and Control of Avian Influenza in the US" (AICAP) is to serve as a significant point of reference for the poultry industry and the general public in matters related to the biology, risks associated with and the methods used to prevent and control avian influenza (AI). Our major goal was to develop a collective network research structure that establishes the basis for a better understanding of avian influenza; in terms of the mechanisms that lead to the emergence of avian influenza in poultry, and, more importantly, how to prevent it. Over the course of the past two years, we have established such a structure. Through our research network, several of our groups have engaged in collaborative projects both within the AICAP and outside the network. We have developed a website for our project (http://www.aicap.umd.edu/) which lists current publications, researchers and educational materials from the twenty-two subcontracting institutions under the AICAP research structure. The network of researchers involved in the AICAP project continues to expand, with three new institutions added in the second year, without an increase in funding requested.

Education Efforts

A major emphasis of our project is the development of education programs. Drs. Eric Benson, Robert Alphin, and George Malone at the University of Delaware, in close collaboration with Dr. Nathaniel Tablante at the University of Maryland, have continued to advance and expand their programs to test the environmental stability of avian influenza and practical methods for viral inactivation on poultry premises. A half-day comprehensive classroom training program was developed in the Spring of 2005. Content of the training material included: current status of avian influenza worldwide, human health guidelines for responders to an avian influenza outbreak; requirements, options and procedures for mass depopulation; disposal options, procedures and cost with comprehensive details for in-house windrow composting. Demand for this train-the-trainer program far exceeded the initial projections: a total of 32 sessions were held in 26 different states. Additionally, the Canadian poultry industry requested and supported 5 sessions in four different provinces. A condensed version of this material was also offered at two locations in Brazil (again, supported by the Brazilian government). In the United States, an estimated 1,800 people total attended the 32 sessions. Participants included 45% poultry industry personnel (poultry company health, production, safety and environmental managers), 40% agencies (Department of Agriculture, USDA, emergency management), 10% university professionals and cooperative extension staff and 5% private industry (waste and emergency management firms).

In addition, Dr Eva Wallner-Pendleton at Penn State traveled extensively to host educational trainings, reaching over 1,250 participants in Kansas, South Dakota, Minnesota, Washington, Alabama, South Carolina, Connecticut, Pennsylvania, Tennessee, Texas, Florida and California. Surveys were collected from over 60 live bird market (LBM) producers, the results of which are being tabulated.

Surveillance Efforts

Surveillance of live bird markets (LBM) and wild migratory birds is a critical component of the AICAP project, as the project structure facilitates rapid information sharing between researchers and institutions in different states. Early detection of avian influenza allows for effective culling and quarantine measures, which reduces the possibility of transmission among poultry and to humans. Due to the ever evolving nature of the spread of AI, multiple researchers are working toward effective surveillance and detection throughout the country, utilizing efforts across multiple flyways and in the live bird markets of states across the country.

In order to better understand the procedures of the live bird market producers, Dr. Cardona's team at University of California, Davis delivered a questionnaire to 29 out of 31 custom slaughter markets south of the Tehachapi mountains in California. The questionnaire was delivered in person to each market manager who answered questions on market practices and patterns, depopulations, employee movements and market biosecurity. This survey data is available to researchers in other states, which creates a valuable database of information for both researchers and government institutions. It is essential to determine the flocks that have higher risk for being infected with avian influenza and then educating poultry owners, producers, etc., in mitigating those risks.

In the southeastern part of the United States, Dr. Giambrone's team at Auburn University surveyed for AI in wild ducks in the southern states. In January 2006, 50 samples were taken from hunter-killed ducks from the Bradley, Georgia unit of Lake Eufaula, and 100 samples from hunter killed ducks from a private lake in Lake City, Florida. Of these samples, 5 were HA positive and one was positive for the matrix gene of avian influenza virus using a real-time PCR test.

At Texas A&M, Drs. Lupiani's team identified three Wildlife Management Areas (WMA) in the state of Texas that have been documented to contain birds primarily from the Central Mississippi migratory flyway with additional species that crossover through merging routes from the Pacific and Atlantic migratory flyways. These three Texas

WMA areas are on the costal region of Texas and approximately 100 miles from a dense poultry production area in Southeast Gonzales, Texas, the site of an H5 HPAI introduction into domestic poultry. Collections occurred two weekends in November 2005 in the Peach Point WMA, Guadalupe Delta WMA, and Mad Island WMA, one weekend in December 2005 in the Peach Point WMA, Guadalupe Delta WMA, and Mad Island WMA, and two weekends in January 2006 at Peach Point WMA, Guadalupe Delta WMA, and Mad Island WMA; one weekend at Matagorda Island WMA. A total of 134 samples have been collected by Dr Lupiani's team. Fifty nine samples were passaged once and eight positive isolates have been obtained, all from teal (blue-wing and green-wing) and mottled ducks. All the positive samples have been confirmed by rRT-PCR and FluDetect test.

Dr. Richard Slemons at Ohio State University continues to monitor avian influenza activity in Ohio and the Eastern Shore in Maryland. In light of the recent LPAI H5N1 outbreaks in both Maryland and Ohio, a database of current and historical samples of wild bird AIV has proven invaluable. In the last 18 months 256 AIV isolates recovered from 6,364 ducks sampled at study sites in Alaska, Missouri, Ohio, Maryland and Delaware have been analyzed. 115 AIV isolates representing 7 HA subtypes, 8 NA subtypes and at least 15 HA-NA combinations were recovered from 3,166 ducks sampled in three of the four North American migratory bird flyways. The integrated format of the AICAP program allowed a rapid dissemination of these results to the researchers, one of the strengths of a collaborative research effort such as this.

Supplemental Surveillance

For the 2006 fiscal year, an additional $1.5 million dollars was allocated by Congress in the area of wild bird surveillance, in particular the surveillance of the Pacific Flyway. Three AICAP institutions received additional funding; notably, the University of Alaska, Fairbanks, under the direction of George Happ, joined the efforts of Dr. Cardona and Dr. Slemons to expand and solidify the database and current sampling areas. In Phase One, supplemental funds were used for collecting an estimated 7700 samples: 4000 from ducks and geese at proven study sites in central and western Alaska in June, July, August and early September, 3000 cloacal samples from ducks, geese and shore birds, and 700 swabs collected from hunter killed birds in both Alaska and Utah. The second phase of the surveillance project involves virus isolation and dissemination of information for positive samples.

Improving diagnostic tools for AI detection

One challenge with avian influenza detection is the difficulty of establishing an effective test to differentiate between vaccinated poultry and infected poultry. Creating a rapid, effective diagnostic test that is cost effective for producers will significantly reduce the current time and expense burden that sample collection entails. As well, the rapid spread of AIV throughout the world makes a rapid diagnostic tool, one capable of detecting a variety of HA and NA subtypes, an increasingly important aspect of AI research.

Dr. Calvin Keeler, at the University of Delaware, continues to develop technology that will significantly improve the speed and reduce the cost associated with traditional sequencing technologies. The cDNA microarray developed by Dr. Keeler has already met a major objective: the ability of one labeling reaction and one slide to identify both HA and NA subtypes. The microarray contains 21 elements representing various avian influenza hemaglutinin (HA) and neuraminidase (NA) subtypes, as well as a pan-influenza probe, based on the matrix (M) gene sequence. These 21 elements are spotted in duplicate (42 spots) creating a "subarray." Eighty percent (80%) of the HA and NA subtypes were correctly identified by the microarray, and all type A influenza were correctly identified. The MAS technology also permits unsurpassed speed: a total synthesis time of less than two hours in the production of high-density custom 24-mer DNA arrays, substantially reducing development time and costs and making customization possible. These arrays can be designed in minutes, and manufactured in less than two hours, resulting in the ability to perform truly iterative experiments in a single day.

Another strategy is under development with a new researcher added in 2006, Dr Manoj Pastey at Oregon State University. To date, Dr. Pastey has developed 21 Mabs against H5N1 and 25 Mabs against H7N2, while partially characterizing 6 H5N1 Mabs and 5 H7N2 Mabs. The Veterinary Diagnostic Laboratory (VDL) at Oregon State has been designated by the USDA as one of the certified designated diagnostic centers for AI virus, creating a valuable sampling of multiple subtypes of AIV available for the production of Mabs. From Year 2001 to 2005, 358 respiratory samples (178 nasopharyngeal aspirates, 128 throat swabs, 23 throat wash samples, 13 bronchoalveolar lavage samples, 13 sputum samples, and 3 tracheal aspirates) and serum samples were received in the OSU Veterinary Diagnostic Laboratory (VDL) for routine culture of respiratory viruses. In addition, as part of the H5N1 AI surveillance, National Wildlife & Park Services send samples every week to the OSU VDL.

Alternative Vaccine/vaccination strategies against AI

The development of alternative vaccines and vaccination strategies for poultry against AI is of utmost importance both to scientific researchers and to the poultry industry. Even a small scale outbreak of HPAI could have tremendous impact on the economic well-being of the poultry industry. With this in mind, Dr. Haroldo Toro's team at Auburn University conducted experiments where the hemaglutinin (HA) gene of avian influenza (AI) was inserted into a replication-defective adenovirus serotype 5 vector. These experiments show that protective immunity against AI viruses can be elicited in chickens by single-dose in ovo vaccination with a replication competent adenovirus-free human adenovirus vector encoding and avian AI virus H5 (AdH5). The preliminary results show that ocular vaccination with AdH5 induces a specific antibody response against the protein encoded by the H5 transgene. The antibody response detected in chickens vaccinated *in ovo* is of particular importance as this route has been proven to be very efficient for mass immunizations in the poultry industry. Dr Toro’s results also seem to demonstrate the importance of a revaccinating strategy in order to obtain increased antibody responses, important information for successful vaccination strategies.

The integration of AICAP research efforts can be seen most effectively in the research collaboration of Drs. Vikram Vakharia, University of Maryland Biotechnology Institute, and Dr. Maricarmen Garcia at the University of Georgia. Dr. Vakharia has cloned the genes encoding approximately 210 amino acids of the central region of N1, N2, N3 and N7 into a baculovirus transfer vector, while also producing stable recombinant baculoviruses of the N1, N2, N3 and N7 proteins. These recombinant baculoviruses produced by Dr. Vakharia have been utilized by Dr. Garcia at the University of Georgia in an effort to create a DIVA (Differentiating Infected from Vaccinated Animals) serological test. Dr. Garcia’s team has tested sera samples collected from birds vaccinated with H5N9 and H5N2 inactivated vaccines from the North America lineage viruses and challenged with a highly pathogenic H5N1 from the Asian lineage. Three baculovirus clones (AV, INDO, SW) expressing the N1 protein from North American and Asian lineage viruses have been developed. The N1-ELISA with both antigens were specific for the detection of N1 antibodies. Future studies include testing sera samples from H5N2 vaccinated birds will be also tested with the N2-ELISA: this data will be the first evaluation of the ability of the N1-ELISA to differentiate infected from vaccinated animals.

The inclusion of another new investigator, Dr. Chang Won Lee at Ohio State University, adds another dimension of research to the pursuit of a vaccination strategy based on the non-structural protein of the AIV virus. Dr. Lee is working toward the identification of different NS genes, and has found, by cloning and sequencing, 20 different NS genes with unique deletions in different regions. Dr. Lee was also able to biologically purify three different variants that express truncated NS1 proteins. These results, combined with the efforts of Drs. Vakharia and Garcia, yield a more comprehensive picture of the possibility of utilizing alternative elements of the AI virus for rapid and accurate diagnostic strategies.

Understanding AI transmission and pathogenesis

In the second year of the AICAP project, Dr. Jack Gelb’s group at the University of Delaware continued to focus on the pathogenicity of avian influenza infections for broiler chickens. Significantly, broiler chickens were shown by earlier studies conducted by Dr. Gelb to be more severely impacted by LP H7N2 AI infections than turkeys or SPF leghorn chickens based on clinical signs, gross and microscopic lesions. This demonstrates that there are likely genetic differences between meat and egg type poultry responsible for susceptibilities to avian influenza viruses (AIV). The difference in response of different poultry types pointed the way for the next component of Dr Gelb’s research: studies conducted to measure the effects of exposure to CAV, IBD or both viruses. As was expected, viable AIV was not re-isolated from the trachea beyond 7 days post challenge. Of all the birds sampled, viable AIV was isolated from one cloacal swab taken from CAV/IBD exposed bird 14 days post AIV challenge. Compared to birds in the AI challenge control treatment group, birds previously exposed to CAV, IBDV or both viruses generally had lower AI antibody titers determined by HI. However, there were no major differences in the number of positive AI ELISA or agar gel precipitant assays. The information obtained by these studies further helps to tailor economically viable vaccination strategies for poultry producers, based on the type of poultry being produced and the elements that produce a greater risk of AIV outbreak or infection in immunosuppressed chickens.

Dr. Perez's group has focused on the interspecies transmission of AI viruses. The group studied the potential role of quail in the transmission of AI to other animal species. Adaptation studies of a mallard H2N2 viruses were performed in quail. The results of the H2N2 study indicated that quail could provide an environment in which AI from wild birds can adapt and increase their host range. In this case, the quail-adapted virus not only replicated and transmitted efficiently in quail, but also replicated and transmitted very efficiently in chickens, causing depression and mild diarrhea. In contrast, the mallard H2N2 virus was unable to establish a productive infection in chickens. Reverse genetics studies have been performed to determine the minimal changes required for the change in phenotype (Sorrell and Perez, Avian Dis, 2007 in press). Similar studies are being carried out with avian influenza virus subtypes H9N2 of Eurasian origin (Hossain and Perez, submitted) and H5N2 (Ramirez Nieto and Perez, in preparation) and H7N3 of North American origin (Song and Perez, submitted).

Our two years experience with the AICAP

The second year of the AICAP program has demonstrated the value of the coordinated agricultural projects (CAP) created by CSREES/USDA. The addition of several new researchers, the seamless addition of expanded and collaborative surveillance efforts, and the continued cross-communication occurring between AICAP research institutions are markers of success for the second year of the AICAP program. A redesigned website (www.aicap.umd.edu) was launched in 2006, with a secure login area for researchers to share information and updates, along with public access to researcher publications and education materials. In the recently submitted AICAP renewal, problems that had previously delayed the release of funds were surmounted, with funds released to all subcontractors as of the beginning of February. This represents a significant advancement in the unified efforts of all researchers; projects currently underway will continue with no funding interruption and new researchers have a full year to demonstrate scientific progress.

Of invaluable worth to the program is the input of all project participants, our constant interaction with members of the scientific advisory board, executive committee and stakeholders. We want to create the opportunity for education of the general public on different aspects of AI and be a constant source of information for the large and small poultry producer. Thus, the overall goal of the AICAP project remains the same:

1) To continue expanding our wild bird surveillance capacity and characterize the population of AI viruses in wild birds in North America.
2) To establish the molecular basis for avian influenza interspecies transmission and pathogenesis
3) To continue our different education programs and analysis of risk factors in the emergence of AI
4) To continue exploring and validating alternative diagnostic tools and vaccines strategies

For 2007, a total of $1,675,000 was requested and released from CSREES-USDA, including $1,408,628.00 for funding of AICAP research projects, and $88,482.00 for administration of the project by the University of Maryland (including indirect costs charged to subcontracts granted to other institutions). Budget requests submitted from the subcontracting institutions will be revised and finalized based on the recommendations given by the Scientific Advisory Board and the Executive Committee.

In summary, we are certainly excited about the opportunity we have through the CSREES-USDA to build a network research structure aimed at protecting poultry production in the US. We are confident of making steps in the right direction. However, you as a stakeholder are the ultimate judge. Your opinion is highly valuable and necessary to us. We hope to continue to obtain constant input as we strive to make the AICAP project an essential tool in the understanding and eradication of a disease with a tremendous impact in this country's economy and society as a whole.

Sincerely,

Daniel R. Perez, PhD
AICAP Program Director

Cc Richard Slemons, DVM
AICAP Co-Program Director