National Institute of Allergy and Infectious Diseases
Abstracts: 4th Annual CEIRS Network Meeting

August 3-5, 2010

University of Rochester
Woodcliff Hotel and Spa
199 Woodcliff Drive, Fairport, New York

Table of Contents

Immunology – Activation and Response

Sequential exposure to hemagglutinin from highly pathogenic avian influenza H5N1 does not cause original antigenic sin

Feda N. Masseoud1, Nigel J. Temperton2 and Joshy Jacob1

1Department of Microbiology and Immunology, Emory Vaccine Center, Emory University, Atlanta, Georgia, 2Centre for Medical Molecular Virology, Division of Infection and Immunity, University College London, UK

H5N1 influenza A viruses have continued to impact the global population since its initial outbreak in 1997. It re-emerged in 2003 and since then H5N1 outbreaks have expanded from Asia to Europe, the Middle-East and Africa, raising concerns of a possible pandemic. It is known that sequential exposure to related H1N1 influenza viruses leads to original antigenic sin, a phenomenon in which the immune response is misdirected against the previously-encountered cross-reactive strain rather than the novel protective antigenic determinants in the current strain. It is unknown whether avian H5N1 influenza viruses induce this phenomenon. Here, we determined the extent to which original antigenic sin interferes with immune responses to influenza virus H5 subtype. Briefly, we immunized mice with DNA vaccine encoding full length hemagglutinin (HA) from influenza A/Vietnam/1194/2004 followed a month later, by a subsequent DNA vaccine encoding HA of one of several variant H5N1 strains, ranging in genetic similarity from 100%- 90%. We then determined microneutralization titers directed against the primary strain vs. the variant strain using lenitviruses pseudotyped with H5 HA. We show that antigenic sin did not occur in response to subsequent exposure to H5 HA, irrespective of the antigenic distance/clades of HA molecules. It is possible that the lack of antigenic sin with H5 HA may be a function of its preferential binding to α-2,3 versus α-2,6 sialic acid receptors. Whether H5 viruses will induce original antigenic sin if and when they acquire α-2,6 sialic acid-binding capability remains an open question.

Immune Responses to Experimental and Licensed Vaccines Against Emerging Influenza Viruses

David J. Topham

New York Influenza Center of Excellence, David H. Smith Center for Vaccine Biology and Immunology, Department of Microbiology & Immunology, University of Rochester, Rochester, NY

Evaluation of experimental vaccines against emerging influenza viruses are limited by the inability to measure protection from infection until after the virus has emerged and become a pandemic threat. We therefore sought to determine a more comprehensive immune profile elicited by experimental vaccination in human subjects and in animal models. B cell responses after immunization with a drifted H5 influenza/A/Vietnam/1203/04 vaccine were characterized in the peripheral blood of human subjects primed with experimental recombinant H5 influenza A/Hong Kong/156/97 vaccine. Antibody secreting cells were assayed by ELISPOT against a panel of recombinant hemagglutinin and control proteins. Increased frequencies of H5 HA specific antibody secreting and memory B cells could be observed within 7 days of re-vaccination. Furthermore, these responses were cross-reactive to both H5 HA variants, but not H3 or avian H6 HA strains. In parallel, mice were vaccinated with combinations of recombinant HA proteins derived from drifted A/VN/1203/04 and Indonesia/03/05 viruses, then challenged with live H5/PR8 influenza virus. Mice were protected regardless of which H5 HA sequence they received, and in the absence of neutralizing activity from the serum. Passive transfer of immune serum demonstrated that the protection was mediated by antibody. These observations suggest prior vaccination against H5 influenza HA induces cellular and humoral immune responses that cross-react among drifted variants, without precluding a response to new, or existing HA strains. Finally, we investigated whether pre-existing immunity and age had any effect on cellular and humoral immune responses to monovalent inactivated subunit A/California/04/09 (pH1N1) vaccine. Robust IgG and IgA antibody secreting cells (ASC) were detected in all age groups although responses were somewhat greater in the young adult cohort. Overall, influenza-specific ASC number on day 7 correlated well with the day 28 serum HAI (r=0.52), with a stronger correlation among young adults (r=0.58) than among older adults (r=0.37). These results suggest the immunogenicity of a single dose of unadjuvanted inactivated pH1N1 vaccine is effective in eliciting a clinically relevant, and immunologically robust response in healthy adults.

Encounter with seasonal vaccines and viruses primes memory CD4 T cells capable of recognizing the pandemic H1N1 influenza virus

Katherine A. Richards, Shabnam Alam, Francisco A. Chaves, and Andrea J. Sant

David H. Smith Center for Vaccine Biology and Immunology, AaB Institute of Biomedical Sciences, Department of Microbiology and Immunology, University of Rochester, Rochester, New York 14642

The unexpected emergence of a novel strain of H1N1 influenza in 2009, like the avian H5N1 virus in 1997, generated significant interest in understanding immunological memory to influenza and how previous encounters with viruses and vaccines influence our ability to respond when confronting novel influenza strains. Here we evaluate the anti-influenza CD4 T cell repertoire in normal adults to determine if most individuals have detectable influenza-specific CD4 T cells, and if so, their influenza protein specificity, using cytokine EliSpot assays and synthetic peptides representing the entire translated sequence of 6 major influenza proteins. We also test whether CD4 T cells from individuals not exposed to the pandemic H1N1 strain can respond to this virus. Our studies revealed that most individuals have abundant levels of circulating CD4 T cells that are influenza-reactive and that M1, NP, PB1 and H3 were most consistently the focus of CD4 T cells. When the potential of the CD4 T cell repertoire to recognize naturally generated and displayed peptide epitopes from the novel A/California/04/09 virus was evaluated, we found a strikingly large fraction of influenza-reactive CD4 cells were able to recognize host cells infected with the pandemic strain. In parallel studies in mouse models of influenza virus infection, we have found that previous infection with a seasonal strain of influenza A virus (A/New Caledonia/20/99) primes a memory population of CD4 T cells that promotes a more rapid CD4 T cell and antibody response to challenge with the pandemic A/California/04/09 virus. Collectively, our results indicate that even with novel influenza strains derived from antigenic shift, there can be considerable CD4 T cell reactivity, most likely as a consequence of priming from periodic encounter with seasonal virus strains and vaccines. Current studies focus on effects of vaccination with pandemic vaccines on the specificity and function of CD4 T cell repertoire specific for influenza.

Cytokine diversity in human T cell responses to influenza

Tim R. Mosmann, Iftekhar Naim, Gaurav Sharma, Jonathan A. Rebhahn, James S. Cavenaugh, Jason M. Weaver, Courtney Bishop

Several differentiated effector/memory CD4 T cell subtypes express different cytokine patterns and hence different functions, and as a result are effective against different pathogens. Human circulating anti-Influenza responses are strongly Type 1 biased, including many cells producing IFNgamma and negligible numbers that produce IL-4 or IL-17. However, even within the type 1 cytokine pattern, CD4 T cells can express several combinations of these cytokines. The production of multiple cytokines by individual cells has been linked to the efficacy of anti-pathogen responses, and the patterns may also be influenced by stochastic effects and affinity of the TCR for antigen. To investigate the cytokine patterns expressed by influenza infections and vaccinations, we are using intracellular cytokine staining and flow cytometry. The large number of markers that can now be examined by flow cytometry is a both blessing and a curse – complex patterns can be discerned, but analysis becomes much more difficult with complex (e.g. 16-color) data. To provide more efficient, reproducible and multi-dimensional analysis, we have developed two flow cytometry analysis programs for multi-parameter data, to complement existing programs such as FLOCK (Richard Scheuermann). Gating Assistance for Flow (GAFF) is an automated adaptation of the extensive back-gating approach used by an expert operator. The operator chooses a crude 'seed' population that contains some of the cells of interest, then GAFF performs recursive back-gating to optimize the gates for each of the parameters chosen by the operator. The program is robust - even non-overlapping seed populations converge on the same result, and the gating is similar to, but faster and more consistent than an expert manual operator. Two batch processes accelerate analysis of similar samples from different subjects (gates adjusted) and different stimulations of the same subject (gates locked). SWIFT is a more rigorous clustering program based on Gaussian mixture model fitting, with new innovations to analyze our normal samples (e.g. one million cells, 20 variables) in realistic times. An iterative sampling approach accelerates the Expectation Maximization (EM) algorithm, and the Gaussian components are combined where necessary to accommodate non-Gaussian populations. GAFF rapidly enumerates specific populations of known interest, whereas FLOCK and SWIFT are more suitable for finding novel populations that cannot be easily discerned in conventional 2D gating. These programs are being used to map the detailed cytokine patterns of CD4 anti-influenza responses primed by different histories of antigen exposure.

Human Immune Cell Activation by Influenza Virus with Different Receptor Specificities

Irene Ramos, Dabeiba Bernal-Rubio, Natasha Durham, John Steel, Anice Lowen, and Ana Fernandez-Sesma.

Influenza A virus circulates in birds and mammals in a host specific way. This is determined by the binding preference of the hemagglutinin (HA) for the sialic acids (SA) to the underlying sugar chain on the cell surface. Thus, the HA of avian influenza viruses bind preferentially SA bound through α2,3 linkages (SAα2,3), which are abundant in bird intestines, while the human strains HAs bind to α2,6 linked SA (SAα2,6), that are frequent in the human lungs. These receptor restrictions for avian influenza viruses in human lungs may account for the poor ability of avian strains to establish infection in humans. Nevertheless, high pathogenic avian influenza viruses (HPAIV) H5N1 are transmited to humans occasionally, usually resulting in a severe and rapidly progressive pneumonia and subsequent systemic disease, with a fatal outcome rate of approximately 50%. Humans infected by H5N1 HPIAV present unusually high serum concentration of chemokines pro-inflammatory cytokines, which it is thought to contribute to disease severity. Dendritic cells (DCs) are specialized immune cells that sense and respond to pathogens by different mechanisms and become activated by these interactions. One of the features of DC activation is the release of pro-inflammatory cytokines and chemokines, which may account for the hypercytokinemia described in humans infected with H5N1 HPAIV. We generated recombinant influenza viruses bearing the HA and NA from a H5N1 HPAIV in a PR/8/34 backbone with or without mutations (226L, 228S) in the receptor binding domain of the HA protein. Using synthetic sialylglycopolymers (biotin conjugated), we established a solid phase binding assay and a flow cytometry based method to determine the SAα2,3 or SAα2,6 preference of these recombinant influenza viruses. Then, these viruses were tested for their ability to induce DC activation by qRT-PCR and multiplex ELISA. Viruses with the 2,3 linked sialic acid binding site induced higher levels of pro-inflammatory cytokines (IL-6, TNFα) and IFN inducible genes than viruses with SAα2,6 binding site. These data suggest that binding to 2,3αSA on human DCs by H5N1 HPAIV may be sensed differently by these immune cells than 2,6αSA binding viruses and induces an exaggerated pro-inflammatory response in infected individuals, responsible for their enhanced pathogenesis.

Multilevel Modeling of Complex System Dynamics in Immune Responses to Influenza A Virus Infection

Hongyu Miao, Ph.D. , Department of Biostatistics and Computational Biology, School of Medicine and Dentistry, University of Rochester, Rochester, NY

Mathematical modeling has long been introduced into the investigation of a variety of biological problems including immunology research such as immunity and vaccination against influenza A virus (IAV) infection. Due to the complexity of hierarchical interactions with dimensions ranging from nanometers to meters and time scales ranging from nanoseconds to years in immune responses to influenza virus infection, the Center of Biodefense Immune Modeling (CBIM) at University of Rochester has been dedicating to the development of multilevel mathematical models and related statistical and computational techniques to systematically understand immune responses, regulation and vaccination against IAV. For this purpose, we developed mathematical models at genetic, protein, cellular and organ levels, which have led to interesting results regarding predictions of IAV viral dynamics and vaccination strategies against new viral strains. We also developed related biostatistics methods and biocomputing techniques for complex dynamic systems and successfully applied these approaches in understanding IAV immune responses. New insights into data and experiment design benefited from mathematical modeling are also important products of this multidisciplinary research project.

Oseltamivir resistant novel H1N1 viruses lack attenuation in the guinea pig transmission model.

Christopher W. Seibert[1], Randy A Albrecht[1], Silke Stertz[1], Rafael A. Medina[1], Adolfo Garcia-Sastre[1,2,3], and Peter Palese[1,3]

Department of Microbiology[1], Institute of Global Health and Emerging Pathogens[2], Department of Medicine[3], Mount Sinai School of Medicine, New York, New York, USA

Oseltamivir resistance was rare until 2008 when resistant, seasonal H1N1 viruses became prevalent, limiting the use of oseltamivir. In 2009, novel H1N1 viruses arose as the predominant circulating influenza virus strain, and oseltamivir was used extensively during the initial outbreak. More than 250 oseltamivir resistant isolates of novel H1N1 have been reported; all of which contain the classical H274Y mutation traditionally associated with viral attenuation. We show that novel H1N1 viruses with the H274Y mutation are only mildly attenuated in multicycle growth curves and transmit as efficiently as sensitive novel H1N1 viruses. Furthermore, when a 1:1 mixture of wild-type and resistant novel H1N1 viruses is used to inoculate guinea pigs, both wild-type and resistant virus are detectable in the naïve animal nasal washes. Resistant novel H1N1 reassortant viruses containing a seasonal H1N1 NA are not attenuated in a multicycle growth curve, but their transmission efficiency is significantly reduced. These data suggest that the H274Y mutation leading to oseltamivir resistance does not attenuate novel H1N1 viral transmission, while forming a reassortant virus with the resistant seasonal H1N1 virus does attenuate transmission in guinea pigs. The novel H1N1 virus with the oseltamivir resistance mutation H274Y will be further evaluated in the ferret transmission model.

Biological and structural characterization of a host-adapting amino acid in influenza virus

Gabriele Neumann,[1] Shinya Yamada,[2] Masato Hatta,[1] Bart Staker[3,4], Shinji Watanabe,[1] Masaki Imai,[1] Kyoko Shinya,[5] Yuko Sakai-Tagawa,[2] Mutsumi Ito[2], Makoto Ozawa,[1,6] Tokiko Watanabe,[1] Saori Sakabe,[2,7] Chengjun Li,[1] Jin Hyun Kim,[1] Peter J. Myler,[4,8,9] Isabelle Phan,[4,8] Amy Raymond,[3,4] Eric Smith,[3,4] Robin Stacy,[4,8] Chairul A. Nidom,[10,11] Simon M. Lank,[12] Roger W. Wiseman,[12] Benjamin N. Bimber,[12] David H. O'Connor,[12,13] Lance J. Stewart,[3,4]* Yoshihiro Kawaoka[1,2,5,6,7,14]*

[1]Influenza Research Institute, Dept. of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, WI 53711, USA;[2]Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan; [3]Emerald BioStructures, Inc., Bainbridge Island, WA 98110; [4]Seattle Structural Genomics Center for Infectious Disease; [5]Department of Microbiology and Infectious Diseases, Kobe University, Hyogo 650-0017, Japan; [6]Department of Special Pathogens, International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan; [7]ERATO Infection-Induced Host Responses Project, Saitama 332-0012 Japan; [8]Seattle Biomedical Research Institute, Seattle, WA 98109; [9]Departments of Global Health and Medical Education & Biomedical Informatics, University of Washington, Seattle, WA 98195; [10]Faculty of Veterinary Medicine, Tropical Disease Centre, Airlangga University, Surabaya 60115, Indonesia; [11]Collaborating Research Center-Emerging and Reemerging Infectious Diseases, Tropical Disease Centre, Airlangga University, Surabaya 60115, Indonesia; [12]Wisconsin National Primate Research Center, University of Wisconsin-Madison, Madison, WI 53706, USA; [13]Department of Pathology and Laboratory Medicine, University of Wisconsin-Madison, Madison, WI 53706, USA; [14]Creative Research Initiative, Sousei, Hokkaido University, Sapporo 060-0818, Japan.

Two amino acids (lysine at position 627 or asparagine at position 701) in the polymerase subunit PB2 protein are considered critical for the adaptation of avian influenza A viruses to mammals. However, the recently emerged pandemic H1N1 viruses lack these amino acids. Here, we report that a basic amino acid at position 591 of PB2 can compensate for the lack of lysine at position 627 and confers efficient viral replication to pandemic H1N1 viruses in mammals. Moreover, a basic amino acid at position 591 of PB2 substantially increased the lethality of an avian H5N1 virus in mice. We also present the X-ray crystallographic structure of the C-terminus of a pandemic H1N1 virus PB2 protein. Arginine at position 591 fills the cleft found in H5N1 PB2 proteins in this area, resulting in differences in surface shape and charge for H1N1 PB2 proteins. These differences may affect the protein's interaction with viral and/or cellular factors, and hence its ability to support virus replication in mammals.

Changes in pathogenicity of pandemic H1N1 viruses as a result of mutations and reassortment

S. Herfst, S. Chutinimitkul, E.J.A. Schrauwen, B.F. Koel, M. de Graaf, T.M. Bestebroer, A.D.M.E. Osterhaus and R.A.M. Fouchier

Department of Virology and National Influenza Center, Erasmus Medical Center, Rotterdam, The Netherlands

In the first year of the H1N1 swine-origin influenza virus (S-OIV) pandemic, the vast majority of infections were relatively mild. It has been postulated that mutations in the viral genome could result in more virulent viruses, leading to a more severe pandemic. We have tested the effect of several genetic changes on the pathogenicity of S-OIV in animals.

Mutations E627K and D701N in the PB2 protein, previously identified as determinants of pathogenicity of avian and pandemic influenza viruses, were introduced in the prototype S-OIV A/Netherlands/602/2009. The mutations did not affect virus replication in the respiratory tracts of mice and ferrets or on pathogenesis, but the viruses were efficiently transmitted via aerosols or respiratory droplets in ferrets. Thus, the impact of key known virulence markers in PB2 in the context of current S-OIVs was surprisingly small.

Introduction of amino acid substitution D222G in the HA of S-OIV – associated with cases of severe disease and fatalities in humans –, also did not result in enhanced pathogenesis in mice and ferrets or changes in transmissibility in ferrets and guinea pigs. However, the virus displayed changes in attachment to human respiratory tissues *in vitro*, in particular we observed increased binding to macrophages and type II pneumocytes in the alveoli and to tracheal and bronchial submucosal glands. Virus attachment studies further indicated that S-OIV with D222G acquired dual receptor specificity for complex α2,3- and α2,6-linked sialic acids. Molecular dynamics modelling of the HA structure provided an explanation for the retention of α2,6 binding. Altered receptor specificity of the virus with D222G thus affected interaction with cells of the human lower respiratory tract, possibly explaining the observed association with enhanced disease in humans.

To study possible changes in antigenic phenotype or receptor binding preference, we introduced a variety of substitutions in the S-OIV HA that were previously identified to be responsible for large antigenic changes in other subtypes or changes in receptor binding specificity. Seven mutations could be distinguished by HAI-assays and antigenic cartography methods, that induced a sufficiently large antigenic change to evade vaccine induced immunity. In addition, 5 of these 7 mutant viruses showed similar or increased replication capacity as compared to wt S-OIV.
To study potential reassortment between S-OIV and seasonal H1N1 or H3N2, an in vitro selection method was used to generate reassortant viruses containing the S-OIV HA. None of the reassortant viruses between seasonal H1N1 and S-OIV caused increased pathogenicity in ferrets. However, S-OIV containing the NA gene of H3N2 virus demonstrated increased replication and more pulmonary lesions in ferrets while maintaining the capacity of transmission via aerosols or respiratory droplets.
These data indicate that some genetic changes indeed may increase the pathogenicity of S-OIV. Surveillance studies on S-OIVs should include detailed characterization of virus phenotypes, guided by genetic signatures of viruses detected in severe cases of disease in humans.

Viral reassortment and transmission after coinfection of pigs with classical H1N1 and triple reassortant H3N2 swine influenza viruses.

Wenjun Ma[1,2,3], Kelly M. Lager[2], Porntippa Lekcharoensuk[3], Bruce H. Janke[3], Alicia Solórzano[4], Richard J. Webby[5], Adolfo García-Sastre[4], and Jürgen A. Richt[1]
[1]Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS, USA; [2]Virus and Prion Diseases of Livestock Research Unit, National Animal Disease Center, USDA, ARS, Ames, IA, USA; [3]Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa, USA; [4]Department of Microbiology, Mount Sinai School of Medicine, New York, NY, USA; [5]Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN, USA.

Since the emergence of the first triple reassortant virus in North American swine in 1998, this lineage of virus has become the predominant virus circulating among North American swine. The internal genes were derived from swine (NP, M, NS), human (PB1), and avian (PA and PB2) influenza viruses forming a constellation of genes that is well conserved. In contrast, the external genes (HA and NA) are less conserved reflecting multiple reassortant events that have produced viruses with different combinations of HA and NA genes. We hypothesize that the maintenance of the avian/human polymerase complex confers a selective advantage to triple reassortant swine influenza viruses. To test this hypothesis, an 8 plasmid reverse genetics system was established for the classical A/Swine/IA/15/1930 (H1N1) (IA/30) virus with a novel restriction enzyme site introduced into each segment, by which each individual gene segment of the IA/30 virus could be differentiated from the corresponding gene from other swine influenza viruses. Pigs were dually infected with the triple reassortant A/Swine/Texas/4199-2/98 (H3N2) (Tx/98) and the classical IA/30 H1N1 viruses and co-housed with a group of sentinel animals. This direct contact group was subsequently moved into contact with a second group of naïve animals. As anticipated, four different subtypes of influenza virus (H1N1, H1N2, H3N1 and H3N2) were identified in bronchioalveolar lavage fluid collected from lungs of experimentally-infected pigs with most of the viruses containing the avian/human polymerase complex from the Tx/98 virus. Surprisingly, only the intact Tx/98 H3N2 virus was transmitted from the infected pigs to two contact groups of animals. These results demonstrate that multiple reassortant events can occur within the lower respiratory tract of the pig; however, only specific gene constellations are able to be maintained and shed from the upper respiratory tract. It is concluded that certain HA and NA gene pairs, in conjunction with the human/avian polymerase complex, may have a competitive advantage over other combinations.

Transmission and pathogenesis of H1 influenza viruses in ferrets

Tompkins, S.M. [1], Smith J.[1], Driskell E.A.[2], Pickens J.A.[1], Nagy, T. [2], Howerth E.W.[1], Stallknecht D.E.[3], and R.A. Tripp
[1]University of Georgia, Department of Infectious Diseases, Athens, GA, USA.
[2]University of Georgia, Department of Pathology, Athens, GA, USA.
[3]University of Georgia, Department of Population Health, Athens, GA, USA.

The 2009 H1N1 pandemic demonstrated the ability of H1 influenza viruses to cross species barriers and infect humans. The 2009 H1 virus had components of avian, swine, and human viruses. In this study, we tested the ability of a panel of H1 influenza viruses of human, swine, and wild aquatic bird origin to infect, cause disease, and in some cases transmit in ferrets. The results showed that in comparison to a contemporary human seasonal H1 influenza strain, A/New Caledonia/20/99, the 2009 pandemic H1 A/California/04/09 replicated to higher titers in the upper respiratory tract despite low inoculation doses, and even with high dose inoculation, CA/04/09 induced limited clinical disease. Seasonal and pandemic human H1 influenza viruses caused similar respiratory pathology. A classical swine H1 influenza virus also readily infected ferrets and replicated to titers similar to the seasonal human H1 influenza virus, but caused limited clinical disease. Most interestingly, one H1 avian influenza virus isolated from a wild aquatic bird readily infected ferrets and replicated in both the lower and upper respiratory tracts causing mild clinical symptoms and limited seroconversion, but mediating robust pathology. Strikingly, this H1 wild bird influenza virus isolate transmitted to naïve contact ferrets without adaptation. These studies highlight the potential for zoonotic influenza to infect humans or other mammals outside of swine, providing opportunities for adaptation or reassortment resulting in novel influenza viruses with pandemic potential.

AIV Infection and the Cytokine Response by Normal Human Bronchial Epithelial Cells

Christine Oshansky-Weilnau, James Barber, Jackelyn Crabtree, and Ralph A. Tripp

University of Georgia Influenza Pathogenesis and Immunology Research Center (IPIRC)

Human respiratory epithelium is the primary target cells for influenza viruses, thus elucidating the viral tropism and host innate immune responses to infection with avian or human influenza virus is important for understanding pathogenesis. In this study, we established primary culture of fully differentiated normal human bronchial epithelial (NHBE) cells and infected these cells with a North American H5 wild bird isolate (A/mute swan/Michigan/451072-2/2006; H5N1), an virus isolated from a live bird market (A/chicken/Pennsylvania/13609/1993; H5N2), a virus isolated from chickens (A/chicken/Texas/167280-4/02; H5N3), or with a virus in the vaccine component of the 2005-2006 season (A/New York/55/2004; H3N2), and evaluated the viral replication kinetics and chemokine responses. We found that avian influenza viruses (AIV) infected NHBE cells independent of α-2,3 sialic acids, and that AIVs differentially induce chemokine expression. Our data show that viruses of chicken origin (H5N2 and H5N3) induce greater IP-10 and RANTES secretion from NHBE cells compared to a wild bird isolate (H5N1) and human H3N2 virus suggesting that these chemokines may be relevant to disease pathogenesis.

Pathogenesis of acute influenza infection in adults and children.

Christine M. Oshansky[1], David Wang[2], Philippa L. Roddam[3], Miguela A. Caniza[2], John DeVincenzo[3], Richard J. Webby[2], and Paul G. Thomas[1].

[1]Department of Immunology, [2]Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN, [3]Departments of Pediatrics and Molecular Sciences, University of Tennessee School of Medicine and The Children's Foundation Research Center, Memphis, TN.

A clear understanding of the immune responses in individuals who have been infected with influenza A is critical for the design of effective vaccination and treatment strategies. Here we characterize the immune responses in peripheral blood and nasal wash of children and adults infected with influenza A during the 2009-2010 season. We determine the duration and magnitude of viral shedding in infected patients and ascertain the frequency of infection in close contacts. We describe the kinetics of the systemic and, importantly, site-of-infection immune responses, including cytokines and chemokines, as well as the relationship between these responses and disease severity. Furthermore, we analyze the adaptive immune responses in the peripheral blood and nasal washes to influenza A, identifying cross-reactive T and B cell responses between several strains of viruses.

Funding provided by the NIH/NIAID Center of Excellence for Influenza Research and Surveillance (HHSN266200700005C).

H4N8 subtype avian influenza virus isolated from stints and gulls in Japan causes a severe clinical phenotype in mice

Haruko Ogawa[1], Vuong Nghia Bui[1], Keisuke Saito[2], Yukiko Watanabe[2], Yu Yamamoto[3], Kuniyasu Nakamura[3], Jonathan Runstadler[4], George Happ[4], Kunitoshi Imai[1]

[1]Obihiro University of Agriculture and Veterinary Medicine, Japan; [2]Institute for Raptor Biomedicine, Japan; [3]National Institute of Animal Health, Japan; University of Alaska Fairbanks, AK.

Objective: During AIV surveillance in eastern Hokkaido, Japan, we obtained five H4N8 virus isolates from shorebird samples. While the H4N8 subtype influenza virus is frequently isolated in the US and other countries, the isolation of this subtype has rarely been reported in Asian countries. In Japan, despite extensive surveillance only one other H4N8 isolate has been reported previously. In this study, the newly isolated H4N8 subtype viruses were characterized by virological and genetic methods, and further investigated for pathogenicity in mice in comparison to other H4 subtype viruses.

Materials and Methods: All viral segments of the H4N8 virus were amplified using RT-PCR and sequenced. The obtained sequences were phylogenetically analyzed. For pathogenicity studies, the viral isolates were intranasally inoculated into BALB/c mice ($10^{2.3}$ $TCID_{50}$/head), and the mice were daily monitored for clinical signs of disease. On day 1-7 post inoculation, the mice were euthanized, and tissues (lungs, nasal turbinates, and others) were collected for viral isolation. Tissues were screened using real-time RT-PCR for the matrix segment and inoculated into embryonating chicken eggs for viral isolation. The tissue samples were also analyzed histopathologically to detect viral antigen. The infection study was also performed on A/Dk/Osaka/105 (H4N8), Dk/Shiga/8/04 (H4N6), and Ck/Czechoslovakia/56 (H4N6) for comparison to the current isolates.

Results and Discussion: Phylogenetic analysis of HA and M genes revealed that all 5 isolates of the current study were similar, despite 2 viruses being obtained out of samples from slaty-backed gulls in Yururi island and 3 viruses from rufous-necked stints at Lake Komuke located 125 miles north from Yururi island. These segments are highly related to A/red necked stint/Australia/04 with the homology between 97 and 99%, suggesting a possible transport of the virus from Australia to Japan. The mice inoculated with the H4N8 virus developed marked body weigh reduction and symptoms of severe respiratory diseases with some proceeding to death. The virus was isolated from lungs and nasal turbinates, and viral antigen was detected in the lungs with pneumonia. Interestingly, the mice inoculated with other H4 subtype viruses did not show any symptoms, although virus was detected in the respiratory tissues of these mice at similar titers. We conclude that the H4N8 virus newly isolated from shorebirds in Japan is more virulent for mammalian infection than those previously isolated.

Broad spectrum analysis of influenza A HA subtypes for cleavage-activation and membrane fusion properties

Galloway, S.E.[1], Reed, M.L.[2], Sobolik, J.[1], Russell, C.J.[2], Steinhauer, D.A.[1]

[1] Emory University School of Medicine, Department of Microbiology and Immunology, Atlanta, Georgia, USA
[2] St. Jude Children's Research Hospital, Department of Infectious Diseases, Memphis, Tennessee, USA

Influenza A virus (IAV) is a significant human pathogen whose primary reservoir and enzootic transmission cycle involves aquatic birds. Entry of IAV into host cells relies primarily on the hemagglutinin (HA) surface glycoprotein, which mediates two main events: 1) binding to cell surface receptors containing terminal sialic acid and 2) fusion between the viral and endosomal membranes. The full complement of antigenic surface proteins is represented in wild aquatic birds, of which 16 HA and nine neuraminidase subtypes have been identified to date. HA proteins have been shown to display a range of stability phenotypes with regard to temperature and pH, which, along with the HA cleavage-activation properties, may affect the ability of viruses to adapt or persist in different environments, and may be involved in the ecology, pathology, or interspecies transmission of influenza A viruses. To examine in greater detail the cleavage-activation and membrane fusion properties of HA proteins belonging to all 16 subtypes, we have cloned and characterized avian-origin HA protein representative of all 16 subtypes, as well as four human-origin HA proteins. Our data show that the pH at which HA mediates membrane fusion can vary substantially from subtype to subtype and that cleavage-activation properties of HA may vary to a greater degree that is generally appreciated. Further, we have examined specific mutants in a select group of HA proteins for their effects on the pH of fusion and have found that the effects of a given mutation can vary from one subtype to the next.

Modifications to the hemagglutinin Cleavage Site Control Virulence of a Neurotropic H1N1 Influenza Virus

Gary Whittaker, Cornell University, College of Veterinary Medicine

A key determinant of influenza pathogenesis is mutation in the proteolytic cleavage site of the hemagglutinin (HA). Typically, low pathogenicity forms of influenza are cleaved by trypsin-like proteases, whereas highly pathogenic forms are cleaved by different proteases (e.g. furin). Influenza A/WSN/33 is a well-studied H1N1 strain that is trypsin-independent in vitro and has the ability to replicate in mouse brain. Previous studies have indicated that mutations in the neuraminidase (NA) gene allow the recruitment of an alternate protease (plasminogen/plasmin) for HA activation. In this study we have identified an additional mutation in the P2 position of the WSN HA cleavage site (S328Y) that appears to control virus spread in a plasmin-dependent manner. We reconstructed recombinant WSN viruses containing tyrosine (Y), phenylalanine (F) or serine (S) in the P2 position of the cleavage site. The Y328 and F328 viruses allowed plaque formation in the absence of trypsin, whereas the S328 virus was unable to form plaques under these conditions. In mice, Y328 and F328 viruses were able to efficiently spread following intracranial inoculation; in contrast the S328 virus showed only limited infection of mouse brain. Following intranasal inoculation all viruses could replicate efficiently, but with Y328 and F328 viruses showing a limited growth defect. We also show that wild type HA (Y328) was more efficiently cleaved by plasmin than S328 HA. Our studies form the foundation for a more complete understanding of the molecular determinants of influenza virus pathogenesis and the role of the plasminogen/plasmin system in activating HA.

Cross-talk between polymerase accuracy and replication strategies of influenza A virus

Baek Kim, New York Influenza Center of Excellence, University of Rochester, Rochester, NY

It is widely accepted that the highly error prone replication process of influenza A virus (IAV), together with viral genome assortment, facilitates the efficient evolutionary capacity of IAV. Therefore, it has been logically assumed that the enzyme responsible for viral RNA replication process, influenza virus type A RNA polymerase (IAV Pol), is a highly error-prone polymerase which provides the genomic mutations necessary for viral evolution and host adaptation. Importantly, however, the actual enzyme fidelity of IAV RNA polymerase has never been characterized. Here we established new biochemical assay conditions that enabled us to assess both polymerase activity with physiological NTP pools and enzyme fidelity of IAV Pol. We report that IAV Pol displays highly active RNA-dependent RNA polymerase activity at unbiased physiological NTP substrate concentrations. With this robust enzyme activity, for the first time, we were able to compare the enzyme fidelity of IAV Pol complex with that of bacterial phage T7 RNA polymerase and the reverse transcriptases (RT) of human immunodeficiency virus (HIV-1) and murine leukemia virus (MuLV), which are known to be low and high fidelity enzymes, respectively. We observed that IAV Pol displayed significantly higher fidelity than HIV-1 RT and T7 RNA polymerase and equivalent or higher fidelity than MuLV RT. In addition, the IAV Pol complex showed increased fidelity at lower temperatures. Moreover, upon replacement of Mg(++) with Mn(++), IAV Pol displayed increased polymerase activity, but with significantly reduced processivity, and misincorporation was slightly elevated in the presence of Mn(++). Finally, when the IAV nucleoprotein (NP) was included in the reactions, the IAV Pol complex exhibited enhanced polymerase activity with increased fidelity. Our study indicates that IAV Pol is a high fidelity enzyme. We envision that the high fidelity nature of IAV Pol may be important to counter-balance the multiple rounds of IAV genome amplification per infection cycle, which provides IAV Pol with ample opportunities to generate and amplify genomic founder mutations, and thus achieve optimal viral mutagenesis for its evolution without catastrophic lethal mutagenesis.

Mutations in the Avian-derived PA Gene Contribute to Host Adaptation of the 2009 Pandemic Influenza A Polymerase

Kendra A. Bussey[1], Jonelle Mattiacio[1], Emily A. Desmet[1], Stephen Dewhurst[1], Baek Kim[1], Toru Takimoto[1*]

[1]Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, New York, United States

The recent emergence of the 2009 swine-origin pandemic influenza virus (S-OIV) highlights the importance of influenza virus host adaptation to the emergence of new human pandemic strains. Previous studies have shown that polymerase complexes from avian viruses function poorly in mammalian cells. However, the S-OIV polymerase has two avian-derived polymerase components, PA and PB2, yet it is highly active in mammalian cells. This suggests mutations in these components may contribute to S-OIV adaptation to the mammalian host. We previously reported that the PB2 T271A mutation partially contributes to enhanced activity of the S-OIV polymerase. However, the contribution of the other polymerase components to the high activity of the S-OIV complex is unknown.

We identified the polymerase component responsible for high activity of the S-OIV polymerase in 293T cells by characterizing the activity of A/California/04/2009 (Cal04, H1N1) polymerase complexes with individual components replaced by those of the avian virus, A/chicken/Nanchang/3-120/01 (Nan, H3N2) using a reporter gene assay. Cal04 polymerase complexes containing Nan NP, PB1, or PB2 were still highly active in 293T cells. However, replacement of the Cal04 PA with Nan PA significantly decreased the Cal04 polymerase activity. Conversely, replacement of Nan genes with those of the Cal04 showed that the Cal04 PA greatly increases Nan polymerase activity in mammalian cells. The activity of the Nan complex containing the Cal04 PA is approximately 10-fold higher than the Nan complex containing the Nan PB2 E627K mutation, which has been previously shown to be essential for avian polymerase activity and high pathogenicity in the mammalian host. We also tested polymerase activity in human lung epithelial A549 cells using an adenoviral transduction-based assay, and confirmed that the Cal04 PA contributes to high activity in human cells. Our results indicate PA is a major contributing factor to S-OIV host adaptation. To determine the residues in the S-OIV PA responsible for high polymerase activity and mammalian adaptation, we compared the Nan and Cal04 PA sequences, and identified 20 amino acid differences. We introduced the 20 Nan residues individually to the Cal04 PA, and identified several that decrease Cal04 polymerase activity. We introduced the corresponding Cal04 residues to Nan PA, and found that these mutations enhance Nan polymerase function in mammalian cells. Our results indicate that multiple residues in PA are crucial for the enhanced polymerase activity of S-OIV.

Modifications in the polymerase genes of a swine-like triple reassortant influenza virus to generate live attenuated vaccines against 2009 pandemic H1N1 viruses

Lindomar Pena[1], Amy L. Vincent[2], Jianqiang Ye[1], Janice R. Ciacci-Zanella[2], Matthew Angel[1], Alessio Lorusso[2], Philip C. Gauger[2], Bruce H. Janke[2], Crystal L. Loving[2], Ruben Donis[3], Eduardo O'Neill[3], and Daniel R. Perez[1*]

1. Department of Veterinary Medicine, University of Maryland, College Park and Virginia-Maryland Regional College of Veterinary Medicine, College Park, Maryland, USA.

2. Virus and Prion Diseases of Livestock Research Unit, National Animal Disease Center, USDA-ARS, Ames, IA, USA

3. Molecular Biology and Genetics Branch. Influenza Division. Mailstop G-16. 1600 Clifton Rd. Atlanta, GA, USA

On June 11, 2009 the World Health Organization (WHO) declared that the outbreaks caused by novel swine-origin influenza A (H1N1) virus had reached pandemic proportions. The pandemic H1N1 (H1N1pdm) is the predominant influenza strain in the human population. It has also crossed the species barriers and infected turkeys and swine in several countries. Thus, the development of a vaccine that is effective in multiple animal species is urgently needed. We have previously demonstrated that introduction of temperature-sensitive mutations in the PB2 and PB1 genes of an avian H9N2 combined with the insertion of an HA tag in PB1 resulted in an attenuated (*att*) vaccine backbone for both chickens and mice. Because the new pandemic strain is a triple reassortant (TR) virus, we chose a swine-like TR virus isolate, A/turkey/OH/313053/04 (H3N2) (ty/04), to introduce the double attenuating modifications with the goal of producing live *att* vaccines. This genetically modified backbone had impaired polymerase activity and restricted virus growth at elevated temperatures. *In vivo* characterization of two H1N1 vaccine candidates generated using the ty/04att backbone demonstrated that this vaccine is highly attenuated in mice as indicated by the absence of signs of disease, limited replication and minimum histopathological alterations in the respiratory tract. A single immunization with the ty/04att-based vaccines conferred complete protection against a lethal H1N1pdm infection in mice. More importantly, vaccination of pigs with a ty/04att-H1N1 vaccine candidate resulted in sterilizing immunity upon an aggressive intratracheal challenge with the 2009 H1N1 pandemic virus. Our studies highlight the safety of the ty/04att vaccine platform and its potential as a master donor strain for the generation of live attenuated vaccines for humans and livestock.

Research priorities leading to better therapeutics and diagnostics

Richard W. Compans, Influenza Pathogenesis and Immunology Research Center, Emory University, School of Medicine, Atlanta, GA 30322.

Antiviral agents targeted to the influenza A neuraminidase or M2 protein have been developed more than a decade ago, but the utility of the licensed compounds is limited by the frequent occurrence of resistant viral variants. One approach to improving such antiviral compounds, which has been recently reported, is the development of agents with inhibitory capacity that extends to such resistant variants. The use of combinations of existing or novel agents should also limit the potential for developing resistant variants. Additional new approaches include compounds targeted to other viral proteins including the HA and RNA polymerase, and the therapeutic use of broadly cross-reactive neutralizing antibodies. In addition, a number of host genes are being discovered which play important roles in viral replication, and their proteins represent potential novel targets for antiviral discovery. Such agents also include inhibitors of the host responses to infection which contribute to disease pathogenesis.

Rapid diagnosis of influenza infection has been improved by the availability of new diagnostic tests, particularly involving detection of the viral RNA by PCR-based diagnostics. The recent discovery of anti-HA antibodies which are broadly cross-reactive across influenza A subtypes presents an attractive new approach for developing rapid diagnostic tests.

Priorities for CEIRS Research Post-2009 H1N1 Pandemic: Lessons Learned

Michael T Osterholm PhD, MPH

Director, Center for Infectious Disease Research and Policy
Director, Minnesota Center of Excellence for Influenza Research and Surveillance
Professor, Division of Environmental Health Sciences, School of Public Health
Adjunct Professor, Medical School University of Minnesota
Minneapolis, MN

The 2009 novel H1N1 influenza pandemic has provided the CEIRS network with a "mid-course reality check" regarding our assumptions about a number of aspects of the influenza virus-animal-human interface. Given that our research priorities are based largely on these assumptions, now is the time, as we approach Year 5-7 activities, to determine if our work better prepared us to answer timely questions regarding everything from viral pathogenesis to animal and human infection surveillance. What role did the research and surveillance accomplishments of the CEIRS research network play in improving our international, national and local understanding of and responses to the pandemic? Did our organizational structure facilitate a rapid and directed response that supported policy makers, public health professionals, medical care givers and other influenza researchers in their efforts to respond to the pandemic?

This presentation will provide "one viewpoint" of the performance of the CEIRS network during the past 16 months since the beginning of the pandemic and how that performance facilitated the global response to novel H1N1. Lessons learned will be reviewed and suggestions for how we can capitalize on those lessons for our research over the next 3 years will be detailed.

Where should surveillance be directed, and how to get there; best steps for coordination

Richard Webby
Director, St Jude Center for Influenza Research and Surveillance.

Three years into the CEIRS contracts it is worthwhile reflecting on the progress made and future directions of the networks surveillance efforts. The initial Broad Agency Announcement stated that the goals of surveillance programs should be "…to provide the Government with information and public health tools and strategies needed to control and lessen the impact of epidemic influenza and the increasing threat of pandemic influenza" and to determine " the prevalence of avian influenza viruses in close contact with humans, understanding how influenza viruses evolve, adapt and transmit." The contribution of the CEIRS network to the Governments response to the H1N1 pandemic clearly showed that we have been successful in the first of these goals. The first three years of surveillance activities have in some respects been targeted towards solidification of collaborations and identification of "hotter spots' for influenza viruses. As we now move towards the utilization of these data to help address the evolution, adaptation, and transmission properties of influenza viruses, the need for inter center collaboration and coordination increases and the time is right for a synergistic cross center approach.

Approaches to surveillance coordination and laboratory quality assurance producing consistent data

Carol Cardona
University of Minnesota
Minnesota Center of Excellence in Influenza Research and Surveillance
Veterinary Sciences, 1971 Commonwealth
St. Paul, MN 55108
(612) 625-5276
ccardona@umn.edu

The need for laboratory quality assurance and proficiency testing in veterinary diagnostics has been driven by the needs of international trade where results are used to verify national claims of compartmentalization, and disease status. Within the CEIRS network, our testing of non-human animals includes a lot of non-regulatory surveillance which should be independent of international trade. Consistent laboratory testing data is still important but is driven by other factors such as the development of collaborative projects between investigators including data sharing policies.

One of the barriers to overcome when collaborating is certainly the variations that one might encounter between field and laboratory practices among different groups involved in animal surveillance. The CEIRS surveillance teams and laboratories can and do consistently isolate influenza viruses, without contamination and artifact. However, to do that, imperfect methods have been adapted to work within the matrix effects encountered in samples collected from the species and environments where we work. This creates a complex system of methods that may not and should not be standardized. However, there is also a need to assure the quality of the data we use and share. In a collaborative project, a step to measure the variance between field and laboratory teams should be included to create a baseline upon which to measure real differences. But, the NIH data sharing policy has brought us to the brink of a new era by creating openness between researchers we know and those we may never meet. Because we don't have that baseline measure, it's important to meet this challenge with a new standard that can assure the quality of what we produce and use, a standard that is reasonable to implement and useful to the centers in their own projects but at the same time, assures a measureable level of quality for data users. At the same time, it is critically important to define the limits and biases of our merged data for all who might be tempted to use it in ways it cannot possibly be stretched to perform.

Natural History of Avian Influenza in Minnesota Ducks; Capturing Subtype Diversity on a Small Temporal and Spatial Scale

Benjamin R. Wilcox, Virginia H. Goekjian, Rebecca L. Poulson, and David E. Stallknecht
Southeastern Cooperative Wildlife Disease Study
Department of Population Health, College of Veterinary Medicine
The University of Georgia, Athens, GA 30602
Phone: (706) 542-1741 email:dstall@uga.edu

Srinand Sreevatsan[a,b], Sagar Goyal[b], Carol Cardona[a,b]
Departments of [a]Veterinary Biomedical Sciences and [b]Veterinary Population Medicine
University of Minnesota
St. Paul, MN 55108

Surveillance for avian influenza viruses (AIV) in wild bird reservoirs is needed to provide data to understand potential transmission risks to domestic animal and human populations and to provide a relevant collection of field isolates to adequately reflect genetic diversity within these reservoir populations. Unfortunately, the acquisition of these data and isolates is a costly endeavor that may be difficult to sustain. For this reason, more efficient approaches to meet these demands are needed. In this study we tested ducks in northwestern Minnesota during 2007 and 2008 in order to better understand the local temporal and spatial variation in both AIV prevalence and subtype diversity. In addition, we further evaluated these data to determine if more targeted species and age-class specific approaches would provide adequate data to capture the temporal variation in prevalence and the subtype diversity present on these sites. During 2007 and 2008, ducks were sampled from July to October corresponding to periods of pre-migration staging and migration. Prevalence was dependent upon species, age, location and time of sampling and peaked during August during both years. Significant subtype diversity was detected and most HA subtypes normally associated with North American ducks (H1-8, H10-H12) were represented each year. These included all of the common HA/NA subtype combinations (H1N1, H2N3, H3N8, H4N6, H5N2, H6N2, H7N3, H8N4, H10N7, H11N9, H12N5) except H9N2. Although mallards (*Anas platyrynchos*) made up 63% of the sampled birds, 80.8% of the 533 AIV isolations originated from this species. Most of the AIV subtype diversity also was represented in this single species during both years. Juvenile mallards were 2.7 times more likely to be infected than adult mallards and all but one of the HA/NA subtype combinations was represented in this cohort. Results from this study indicate that by utilizing published and recent epidemiologic data, surveillance efforts can be targeted to specific times, species, and age classes to more efficiently provide reliable prevalence estimates and capture the AIV subtype diversity present in these wild bird populations.

Establishment and Lineage replacement of H6N2 influenza viruses in domestic ducks in southern China (2000 – 2007)

Yi Guan1, Honglin Chen1, Richard Webby2 and Malik Peiris1
1State Key Laboratory of Emerging Infectious Diseases, The University of Hong Kong, Hong Kong SAR, China and 2Division of Virology, Department of Infectious Disease, St. Jude Children's Research Hospital, Memphis, TN.

Multiple reassortant events between different subtypes of endemic avian influenza viruses have increased the genomic diversity of influenza viruses circulating in poultry in southern China. Gene exchange from the natural gene pool to poultry has contributed to this increase in genetic diversity. However, the role of domestic ducks as an interface between the natural gene pool and terrestrial poultry in the influenza ecosystem has not been well defined. Here we phylogenetically and antigenically analyzed 206 H6 viruses isolated from domestic ducks from 2000 to 2007 in southern China which contains the largest population of domestic ducks in the world. Three distinct H6 lineages were identified. Group 1 contained the majority of isolates with a single internal gene complex and was endemic in domestic ducks in Guangdong from the late 1990's to 2005. Group 2 was derived from reassortment events in which the surface genes of Group 1 viruses were replaced by novel H6 and N2 genes, which appeared in 2004 and gradually replaced the Group 1 viruses and became the predominant H6N2 variant after 2005. Epidemiological and genetic findings also show that the Group 2 viruses started to disseminate from the coastal regions to inland provinces and was also introduced into terrestrial poultry. The Group 3 H6 viruses represent part of an influenza gene pool that undergoes frequent gene exchange with different subtypes. Our study revealed that gene exchanges between viruses from domestic duck and migratory duck occurred throughout the surveillance period. These findings suggest that domestic duck in southern China mediate the interaction of viruses between different gene pools and facilitate the generation of novel influenza variants circulating in poultry.

Understanding the factors that influence low pathogenic avian influenza virus infection in ducks and gulls

Justin D. Brown, Taiana Costa, Camille Lebarbenchon, and David E. Stallknecht

Southeastern Cooperative Wildlife Disease Study
Department of Population Health, College of Veterinary Medicine, The University of Georgia, Athens, Georgia, 30602
Phone: 706-542-1741; email: jubrown1@uga.edu

Wild aquatic birds in the Orders Anseriformes and Charadriiformes compose the reservoir for all subtypes of avian influenza (AI) virus and represent the original source of type A influenza viruses that have occasionally spilled-over and infected poultry or mammals. Over the last 10 years there has been a strong research focus on defining the susceptibility, pathogenesis, and shedding patterns of wild bird species exposed to biologically unique influenza A viruses, including Eurasian-lineage H5N1 highly pathogenic avian influenza (HPAI) and novel H1N1 viruses. Experimental data on these virus strains have proven extremely valuable for supporting field observations and guiding surveillance and response efforts during these epidemics. However, our ability to fully interpret and recognize the novelty of experimental data on these atypical influenza A viruses, particularly H5N1 HPAI virus, is limited by gaps in our understanding on wild bird-origin low pathogenic avian influenza (LPAI) virus infection in wild avian reservoir hosts. To address this, we have conducted a series of experimental infection trials to 1) characterize the pathogenesis, viral shedding patterns and immune response of ducks and gulls exposed to wild bird-origin LPAI viruses, and 2) evaluate the influence that field relevant viral and host factors have on susceptibility and viral shedding in Mallards (*Anas platyrhynchos*). It is our goal that these data provide a strong understanding for what is "normal" with regards to AI virus infection in wild avian reservoirs and serve as a foundation for interpreting "abnormal" viruses or transmission events. To date, through multiple experimental trials, we have evaluated the influence of the following factors on LPAI viral infection in ducks and gulls: viral hemagglutinin subtype, age of host, host species, recent prior exposure to heterologous hemagglutinin subtype, susceptibility to LPAI viruses that have not been passed in eggs or tissue culture, and susceptibility to reassortant LPAI viruses. In addition to improving our understanding on the natural history of AI, these experimental trials provide support for ongoing field studies and yield data that can be used for future ecological modeling of AI virus transmission and maintenance in wild avian populations.

Gaining Insight into the Natural History of Influenza A Viruses and the Ecology and Epidemiology of Influenza A Virus Infections in Waterfowl Using the Mississippi Migratory Bird Flyway

Richard Slemons, Jacqueline Nolting, Tony Fries, The Ohio State University, Department of Veterinary Preventive Medicine

This project, initiated in September 2008, includes two main components: 1) evaluating the ecological aspects of skewed distributions of influenza A virus infections in wild ducks at established study sites and 2) gaining insight into the natural history of influenza A viruses and factors driving the dynamics of influenza A virus infections in waterfowl along the Mississippi Migratory Bird Flyway (MS Flyway). A progress report for the first component is a poster presentation at this meeting and authored Mr. Tony Fries (*Ecology and epidemiology of influenza A infections: using genetic, elemental, and isotopic biomarkers to determine the origins of wild, migratory mallards accessioned into type A influenza virus surveillance investigations*). The progress report for the latter component of this project is the subject of this oral presentation.

Results were obtained primarily from mutually beneficial, collaborative relationships established with various combinations of Federal and state agencies, universities, private organizations and private individuals in WI, IL, and MS. Additional data was obtained from pilot investigations conducted in NE, IA, and IN. Also, compatible data for the MS Flyway is available from ongoing USDA APHIS NIFA AI CAP-2 projects being conducted in MO and OH. Data from these USDA efforts are not included in this report. In 2008-2009 and 2009-2010, 15 and 25 study sites were used, respectively. Three study sites used in 2008-2009 and two sites used in 2009-2010 were dropped as they did not meet selection criteria. In 2008-2009, 850 waterfowl were sampled and meta-data collected at 15 sites in four states. The 35 influenza A virus isolates recovered represented 10 HA and 9 NA subtypes and 16 HA-NA combinations. In 2009-2010 field activities were increased to include a total of 1833 waterfowl sampled and meta-data collected at 25 sites in six states. The 88 influenza A virus isolates recovered represent 8 HA and 6 NA subtypes and 15 HA-NA subtypes. Subtyping has not been completed on all isolates so more antigenic diversity is expected. All subtyping has been done by our collaborators at the USDA APHIS National Veterinary Service Laboratory, Ames, IA who are responsible for detecting and reporting avian-origin influenza A viruses of concern to U.S. agriculture. A total of 27 of the 35 2008-09 isolates were submitted to the J. Craig Venter Institute for full-length genomic sequencing. The eight samples not submitted contained mixtures of influenza A and/or Newcastle disease viruses. The sequencing on the 27 of the 2008-09 isolates is near completion and the data will be released into GenBank as a batch as soon as the sequencing of all isolates has been completed. Sequencing of the 88 2002-10 isolates at JCVI is scheduled for this coming year. As expected, simple data sorting of the current database has shown marked differences in results by sites, by dates during the same year at the same site, and by years. The influence of concurrent environmental events on these variations is unknown. These results clearly demonstrate the importance of a database which includes sequential sampling at specific sites each year, the longitudinal and latitudinal spread of viruses during waterfowl migration, and ecological data over several years. Our meta-data are currently undergoing validation in Microsoft Excel and MySQL in preparation for more comprehensive data analysis efforts.

In the coming year, preliminary descriptive, analytical, and genomic analyses will be initiated on the viral and meta-data accumulated over the last two years. Simultaneously efforts will be initiated for more rigorous and comprehensive geospatial and bioinformatics analyses of the database at the end of year three. In the field, approximately seven new study sites will be evaluated for potential contributions to the project. These new sites will be used to fill in gaps within the MS Flyway and to contribute to building the MCEIRS Central Flyway research

network in collaboration with MCEIRS researchers working in North and South Dakota. The Central Flyway is also a very important waterfowl flyway in North America and inclusion into the MCEIRS influenza A virus surveillance network will be a valuable addition.

Epidemiology and household transmission of pandemic and seasonal influenza A in 2009

Malik Peiris, The Department of Microbiology, The University of Hong Kong

There are few data on the comparative epidemiology and virology of the pandemic H1N1 2009 and seasonal influenza viruses. Furthermore, many epidemiological parameters, even those that are used as the basis for complex mathematical models informing public health decisions on influenza, are indirect estimates. We used infrastructure set up for conducting community based-studies on influenza virus transmission within families to compare the epidemiology and virology of pandemic H1N1 and seasonal influenza viruses in Hong Kong. The families of 99 index cases who tested positive for influenza A virus by rapid diagnostic tests were followed clinically and virologically with three home visits within 7 days. Quantitative viral load was assayed using both molecular and infectious-virus titration methods. A subset of individuals was also followed up serologically. Secondary attack rates among household contacts of index cases was similar for pandemic H1N1 (8%; CI 3-14) and seasonal (9%; CI 5-15) influenza. The pattern of viral shedding and scores of clinical illness was also similar in these two groups. In the subset of patients for whom serological data was available, 36% of patients with seroconversion had no overt clinical illness. In contrast to data observed in animal models, the pandemic H1N1 virus is broadly similar to seasonal influenza in terms of viral shedding, clinical illness and transmissibility in a household setting. Experimental infection of ex vivo and in-vitro cultures of human respiratory epithelium also supports a similar contention.

Estimation of the age-stratified infection-attack rates and severity of the pandemic H1N1 (pdmH1N1) has proved challenging. Hong Kong, a geographically compact Asian city with a homogenously mixing population of 6.8 million, and with a public-funded centralized system of hospital care provided an opportunity for reliably estimating these parameters. Using large scale sero-epidemiological surveys combined with virologically confirmed data on hospitalization, intensive care admissions and mortality, we estimated the age specific infection attack rates and rates of intensive care utilization and death caused by pdmH1N1 infection. While children had very high infection attack rates, the severity of the disease in those infected was dramatically higher in older adults. However, even in older adults, the estimates of severe illness and death was not higher than that estimated for seasonal influenza.

Swine influenza A viruses (H1) from long-term systematic surveillance (1998-2010) in Hong Kong

D Vijaykrishna[1,2], Gavin JD Smith[1,2], Yi Guan[1], and Malik Peiris[1]
[1]State Key Laboratory of Emerging Infectious Diseases & Department of Microbiology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong Special Administrative Region, China; [2]Duke-NUS Graduate Medical School, Singapore.

The emergence of the 2009 pandemic from viruses that have been circulating in swine for more than 10 years highlights the huge gap in our understanding of the evolutionary dynamics of swine influenza. Here we investigate the prevalence, serological and genomic data generated from systematic surveillance for influenza A viruses conducted in a large abattoir in Hong Kong over the last 12 years. As 80% of the swine tested originate for neighboring province of China, this data represents the diversity of a larger region with the highest demand for and the largest livestock holding population of pork in the world.

During this period 573 H1N1 or H1N2 viruses were identified and isolated. Phylogenetic analyses of the hemagglutinin genes of all 573 viruses shows that three major lineages of swine H1 influenza viruses have been circulating in pigs during the last 11 years as identified through our surveillance. Of these, 321 were classic swine (CS), 188 European 'avian-like' (EA) and 52 American triple reassortant H1N2 viruses (TRIG). Ten viruses isolated since October 2009 were derived from the pandemic H1N1 lineage, whereas only two viruses were from the human seasonal H1N1 lineage. Phylogenetic analyses of the neuraminidase (NA) genes showed that among these 431 were H1N1 viruses, whereas the remaining 142 were H1N2 viruses. An additional 61 swine viruses isolated between 1977-1979 and 32 viruses isolated between 1993-1994 were all CS except two isolates that had HA gene and NA genes which were derived from the natural gene pool of wild aquatic birds.

Since 1998, H1N1 and H1N2 viruses have continued to circulate in swine in China with a clearly demonstrated seasonal pattern of virus isolation peaking in the winter months from October to March. From August 1998 until December 2002 the majority of isolates were CS HA lineage. From February 2001 and July 2002 EA lineages viruses and TRIG lineage viruses were detected sporadically, however, from early 2003 to late 2005 there was a distinct shift in the genetic composition of the viruses being isolated as all three major swine virus lineages co-circulated and were frequently isolated. Directly following this period, EA viruses became dominant although CS and TRIG were still occasionally isolated throughout the surveillance period. Interestingly, it was around the time when EA viruses became predominant that total isolation rates reduced significantly in comparison to previous years of surveillance. This suggests that virus fitness of CS and TRIG viruses is higher than EA in swine.

This study illustrates the high degree of genetic diversity of influenza in pigs. Not only are dynamic changes in the dominant HA lineages observed, but the number of reassortant viruses circulating is also seen to vary. These results highlights the importance of pig populations in the emergence of influenza viruses in humans and confirms the importance of long-term surveillance in this host, particularly if attention is paid to gene flow from pigs to high risk individuals such as agricultural workers.

Pandemic H1N1/2009 in Thai swine farms, 2009-2010

Pravina Kitikoon, Donruethai Sreta, Siriporn Tantawet, Suparlark Nuntawan Na Ayudhya, Aunyaratana Thontiravong, Manoosak Wongphatcharachai, Jiradej Lapkuntod, Napawan Bunpapong, Ranida Tuanudom, Sanipa Suradhat, Linda Vimolkept, Yong Poovorawan, Jiroj Sasipreeyajun, Roongroje Thanawongnuwech and Alongkorn Amonsin

Chulalongkorn University & Minnesota Center of Excellence for Influenza Research and Surveillance (MCEIRS)

The emergence and rapid spread of pandemic H1N1/2009 influenza virus (pH1N1) throughout the world caused major health problems in humans and ripple economic impacts to the swine industry. Shortly after its appearance, reverse zoonoses of pH1N1 from human to swine were identified around the world. The clinical characteristics of pH1N1 infection in pigs are indistinguishable from infection caused by contemporary circulating swine influenza viruses (SIVs). However, the possibility of further re-assortment between pH1N1 (containing a human, avian and swine triple reassortant internal gene (TRIG) cassette) and other influenza A viruses (IAVs) in pigs is a major concern and calls for continuing molecular SIV surveillance around the world. Subsequent to the emergence of pH1N1 in April 2009, the first confirmed human case in Thailand occurred in May 2009. The first official pH1N1 report in Thai pigs was issued on December 17, 2009, from a university research swine farm. In this study, we describe the outcome of a pH1N1 monitoring program in 14 commercial swine farms facing outbreaks of respiratory disease in different regions in Thailand from November 2009 to April 2010. During each monitoring visit, at least 20 nasal swab samples were collected from pigs with respiratory clinical signs and/or pigs in contact with clinically positive pigs. The findings demonstrated two pH1N1 positive farms in the western and eastern regions of Thailand. One farm had a prolonged (~ 3 months) pH1N1 co-infection with Thai H1N1-Clb SIV without evidence of further gene reassortment. Interestingly, a novel reassorted H1N1 virus (rH1N1) was detected in a separate medium-sized swine farm in the central region of Thailand. The rH1N1 virus consisted of the pH1N1 backbone with a Thai H1N1-like NA gene. Both pH1N1 and rH1N1 viruses were detected from clinically affected pigs (fever, coughing and swollen eyes) but with various degrees of clinical signs. This finding substantiates the role of pigs in generating new IAVs. In addition, close attention should be paid to SIV isolates containing the TRIG cassette to reduce potential economic losses in the swine production system and prevent possible human influenza pandemics in the future.

Pandemic H1N1/2009 surveillance in Thailand

Yong Poovorawan, S. Kanchana, W. Vuthitanachot, Alongkorn Amonsin, Thaweesak Chieochansin, P. Vichaiwattana, K. Praianantathavorn and Apiradee Theamboonlers

Chulalongkorn University & Minnesota Center of Excellence for Influenza Research and Surveillance (MCEIRS)

Human pandemic 2009 influenza A (pH1N1) virus quickly became the predominant circulating strain in Thailand, and prevalence of infection was highest in children and young adults. From May 2009 to June 2010, we studied the epidemiology of pH1N1 by real-time RT-PCR using virus detection in 4,067 nasopharyngeal or throat swabs. We collected specimens from out-patients or in-patients who experienced respiratory tract diseases in Chumphae provincial hospital, Chumphae district, Khon-Kaen province, Northeast Thailand (n=904); Toungsong provincial hospital, Toungsong district, Nakorn-Srithamraj province, South Thailand (n=798); and Bangkok International Hospital Network, Bangkok, Central Thailand (n=2,365). The results showed that 143 of 904 (15.2%), 66 of 798 (8.3%) and 873 of 2,365 (36.9%) samples from Nakorn-Srithamraj, Khon-Kean and Bangkok were positive for pH1N1, respectively. The results from our study suggested that three waves of pandemic influenza (pH1N1) outbreaks (1st wave Jun-Aug 2009; 2nd wave Jan-Mar 2010; and 3rd wave starting Jun 2010) had occurred. The usefulness of a targeted, longitudinal surveillance system to provide a comparable assessment of influenza activity over time was clearly demonstrated. In conclusion, epidemiological data from virological surveillance, as demonstrated in our study, provide crucial information for outbreak prevention, outbreak control and human pandemic influenza (pH1N1) vaccination planning in Thailand.

Avian influenza in Egypt 2006-2010: Research activities

Ali, M.A.[1], Webster, R.G.[2], Webby, R.J.[2], Kayali, G.[2]
[1] Infectious Diseases and Immunology Group, Virology Laboratory, the Center of Excellence for Advanced Sciences, National Research Centre, Egypt and [2]Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN.

Avian influenza (AI) virus (H5N1) emerged in Egypt in Feb. 2006 and had infected numerous poultry farms in more than 18 governorates. The virus rapidly crossed the host barrier to infect humans and recorded about 18 infected cases at the end of 2006 with 10 deaths and by year 2010 the human cases raised to 109 with 34 deaths. The strategy of control and prevention of the disease was not adequate and slightly haphazardly. As a result the virus became endemic in Egypt without active surveillance or control.

The virology laboratory at the National Research Centre in Egypt took the responsibility of researching the AI viruses in Egypt and to participate in the control and prevention of the disease through a very valuable cooperation with the Virology Division at St. Jude Children's Research Hospital in Memphis via a subcontract funded by the NIAID. The subcontract involves a research agenda that comprised four individual, but very much intertwined, activities: 1) comparative H5 vaccine efficacy in poultry, 2) molecular determinants of H5N1 virus virulence and transmission, 3) surveillance at the human-animal interface, and 4) training of the junior scientists.

The first activity has been conducted on 6 types of avian influenza imported vaccines available in the Egyptian market and compared with an Egyptian vaccine. The results revealed that the homologous H5N1 vaccine prepared from an Egyptian isolate is much better than the imported vaccines. Virus-host relationship was also studied on the level of susceptibility and receptor incidence and distribution in cell culture and pulmonary organs of domestic poultry. The results showed that the two configurations of sialic acid receptors of human and animal influenza are distributed on the upper and lower respiratory tracts with variable densities. Animal surveillance for AI viruses was another activity in which more than 5000 swab samples were collected from poultry in farms, markets, and houses in six Egyptian governorates. The swabs were tested for influenza A viruses and the positive samples were subtyped. H5N1 was the dominant influenza A subtype. In the last activity different constructs are being prepared to be used as alternative vaccines of individual HA segment and multivalent vaccines with different segments of AI subtypes.

A human study has been just started and will be conducted for three years with follow up on 750 poultry exposed Egyptians and 250 unexposed individuals. All paper work and permissions were taken and the team work received the recommended certificates related to human studies.

Active swine influenza surveillance in the United States of America

Mariette Ducatez[1], Evelyn Stigger-Rosser[1], Marie Gramer[2], James Lowe[3], Daniel Darnell[1], Cesar Corzo[2], Kevin Juleen[2], and Richard Webby[1]
[1] Department of Infectious Diseases, St Jude Children's Research Hospital, Memphis TN; [2]University of Minnesota, St Paul, MN; [3]Carthage Veterinary Service, Albers, IL.

In March 2009, a novel H1N1 influenza virus of the H1N1 subtype emerged and spread in humans. This new influenza virus contained a unique combination of genetic elements from Eurasian and North American swine viruses. To date, the direct ancestor of the human pandemic 2009 strain has not been identified in swine demonstrating a failing of detection systems. In response, and to get an accurate measure of the amount of influenza activity, we implemented an active surveillance program in US swine that started in June 2009. Sampling sites were selected to be representative of both the US swine population and the most common risk factors (comingling of multiple sources in the same region and proximity to poultry) for novel Influenza viruses to emerge. Five geographically distinct regions of intensive swine production have thus been identified: Northwest Iowa, Central Iowa, South East Illinois, North Central Indiana, and Eastern Indiana / Western Ohio. These regions share a high geographic density of pigs, both a local production and an importation of growing pigs, as well as a high numbers of intensive domestic poultry production. In total, 18000 pig nasal swabs should be randomly collected throughout the study year, and screened for the presence of influenza virus. Ten months into the program a number of samples have been analyzed by real-time RT-PCR and virus isolation. 5% of the samples were positive for influenza A, demonstrating a high viral burden in this population. We have also observed differences in viral epidemiology with seasonal differences in the ratios of viral subtypes seen. The H1N1 pandemic strain has also been identified in a couple of herds since last October.