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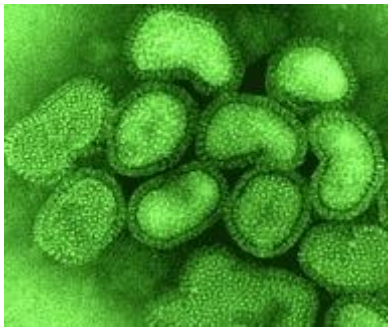
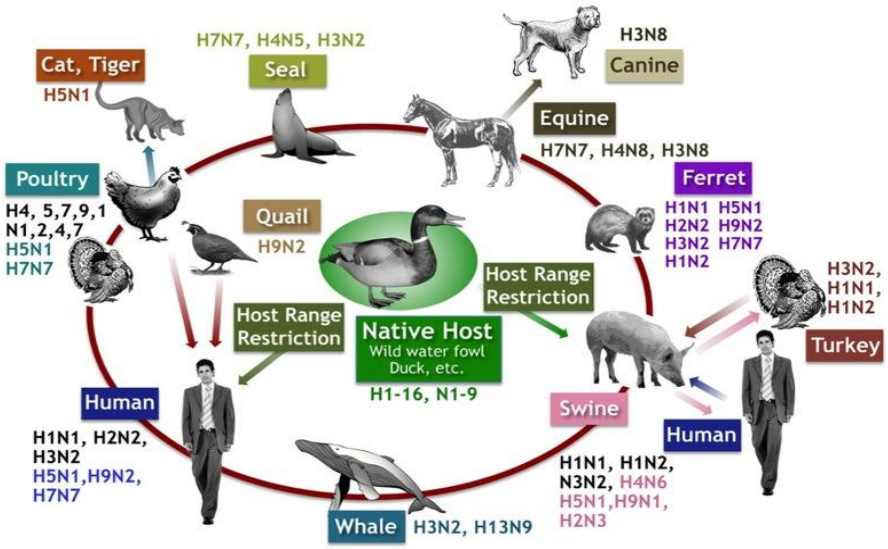
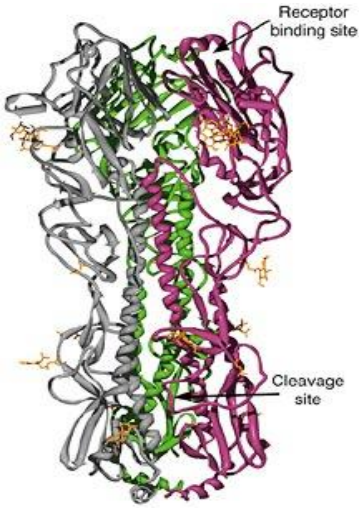
Animal Influenza Viruses Gap Analysis

Workshop Report







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Contents

Animal Influenza Countermeasures Working Group Photo
March 27, 2013

ACRONYMS

APHIS	Animal and Plant Health Inspection Service
ARS	Agricultural Research Service
AI	Avian Influenza
AICWG	Animal Influenza Countermeasures Working Group
AIV	Avian Influenza Viruses
BSL	Bio Safety Level
CDC	Centers for Disease Control and Prevention, HHS
DHS	Department of Homeland Security
ELISA	Enzyme-linked immunosorbent assay
FADDL	Foreign Animal Diseases Diagnostic Laboratory, USDA-APHIS
FAO	Food and Agricultural Organization
GISRS	Global Influenza Surveillance and Response System
GMP	Good manufacturing practices
HHS	Health and Human Services
H1N1pdm09	The 2009 pandemic H1N1 Influenza A virus
HI	Hemagglutination inhibition (HI) assays
HPAI	Highly pathogenic avian influenza
HPAIV	Highly pathogenic avian influenza virus
HSPD-9	Homeland Security Presidential Directive Nine
IAV	Influenza A Virus
Ig	Immunoglobulin
IVPI	Intravenous pathogenicity index
LAIV	Live attenuated influenza vaccine
LPAI	Low pathogenic avian influenza
LPAIV	Low pathogenic avian influenza virus
LPM	Live poultry market
NADC	National Animal Disease Center, USDA-ARS
NAHLN	National Animal Health Laboratory Network
NAPAPI	North American Plan for Animal and Pandemic Influenza
NVSL	National Veterinary Services Laboratories, USDA-APHIS
OIE	World Organisation for Animal Health
OFFLU	OIE FAO network on animal influenza
PCR	Polymerase chain reaction.
PPE	Personal Protective Equipment
PFU/ml	Plaque Forming Unit/milliliter
RT-PCR	Reverse transcriptase-polymerase chain reaction
rRT-PCR	Real-time reverse transcription-polymerase chain reaction
SEPRL	Southeast Poultry Research Laboratory, USDA-ARS
SIV	Swine influenza virus
USDA	United States Department of Agriculture
WHO	World Health Organization of the United Nations
WIV	Whole inactivated virus (WIV) vaccines

EXECUTIVE SUMMARY

The Animal Influenza Viruses Countermeasures Working Group (AICWG) met in Athens, Georgia, March 25-27, 2013, to assess the scientific information and countermeasures available to effectively control and mitigate the impact of an outbreak of an animal influenza virus that is highly pathogenic and/or with zoonotic or pandemic potential. Although all animal influenza viruses were considered, the focus of the workshop was on animal agriculture with emphasis given to swine and poultry production – see workshop agenda in Appendix I. This report provides the state of our knowledge and gaps therein; defines the threats; provides an in-depth analysis of available countermeasures to contain and mitigate the threats; identifies needs for improved countermeasures; and identifies research priorities to fill the gaps in our scientific knowledge, and advance the research and development of new technologies.

GAPS IN SCIENTIFIC INFORMATION

In reviewing the state of our scientific knowledge of animal influenza viruses, the AICWG noted that our fundamental understanding of drivers of virulence, host-range and adaptation process to new host species, and transmission between animals of same or divergent species, and importantly between animals and humans, remains rudimentary.

Virology

There are important gaps in our understanding of influenza virology and studies addressing these gaps are especially critical to support the development of therapeutic and vaccine interventions. Specific gaps in virology include the need to identify the molecular determinants of host specificity, tissue tropism, transmission, and virulence.

Pathogenesis

Gaps exist in our knowledge of many areas of influenza virus pathogenesis. For instance, although the role of the hemagglutinin (HA) protein in the pathogenesis of highly pathogenic avian influenza (HPAI) viruses is well characterized, the role of other determinant(s) contributing to virulence is not fully understood. Many of the gaps in pathogenesis are critical to fully understand virus-host interactions, which are essential before mitigation strategies can be developed. The greatest need is for basic studies investigating the molecular pathogenesis of the virus in different animal species, including differences in binding motifs of hemagglutinin genes and optimization of polymerase complex for adaptation to individual species or families of animals, and the changes needed to move across different animal species or families. These studies should also identify molecular determinates of tissue tropism and relate these to infectivity and pathophysiologic changes. Furthermore, determinates of virulence in addition to the hemagglutinin proteolytic cleavage site need to be determined for waterfowl species and other birds.

The presence of multiple basic cleavage site or long insertion at the cleavage site imparts the biologic trait of high pathogenicity to H5 and H7 avian influenza viruses (AIV) for terrestrial poultry. However, it is unclear why such a change has not occurred naturally in H1-4, H6 and H8-16 AIV and a better understanding is needed to predict the inability or possible occurrence of HPAI viruses among these HA subtypes.

Immunology

Gaps exist in our knowledge of many areas of influenza virus immunology. Although the role of hemagglutinating antibody induced protection from disease is fairly well understood, the role of other contributing factors to immunological protection is less clear, such as non-hemagglutinating antibodies, cell mediated immunity, enhancements of innate immunity (cytokines and interferon) to induce resistance to infection and disease, and induction of antibodies to conserved proteins that may complement protection from hemagglutinating antibodies. Importantly, it is not clear why some birds and mammals are resistant to highly pathogenic avian influenza and others are highly susceptible. Other specific immunology gaps include:

1. Determine the role of unregulated cytokine expression in production of enhanced influenza disease
2. The need to develop adjuvants that result in increased immune responses that are long lived, broadly cross-protective and reduce the number of vaccine boosters, and especially enhance the mucosal immune response for inactivated vaccines.
3. Determine the contribution of host immunogenetics on innate protection of animals by developing transgenic animals to study host-virus interactions
4. Develop and validate tests that are improved immune correlates of protection
5. Identify B- or T-cell epitopes that provide best protection for inactivated traditional and vectored vaccines, and develop systems to predict emerging escape mutants to allow more rapid development of vaccine seed strains
6. Improve vaccine responses *in ovo* or in young animals, particularly when maternal antibody suppresses the vaccine immune response.

Epidemiology

Significant gaps exist in our understanding of the modes and routes of transmission within and between animal species. There are also major gaps in our understanding of how the virus survives in different environments. There is a critical need for research programs that take a systems approach, integrating molecular epidemiology with basic research in predictive biology.

Critical deficiencies in epidemiologic information are dependent on expanding the knowledge base of:

1. Pathogenesis (within different species)
2. Transmission (within and between species) and epidemiology (within the flock/herd). In all cases, strains appropriate to the specific transmission vector, including in some cases to humans, need to be examined.
3. Mathematical modeling and molecular epidemiology

GAPS IN AVAILABLE COUNTERMEASURES

In its assessment of the threats, the AICWG determined that the following countermeasures were important, but several weaknesses were identified.

Vaccines

The group determined that commercially available animal influenza virus vaccines need significant improvements. The pork and poultry industries need highly effective vaccines that can prevent transmission and, in the case of poultry, that can be mass-delivered in water, *in ovo*, sprayed, or feed. A

strategy based on "differentiating infected from vaccinated animals" (DIVA) is also needed in the case of an outbreak with a zoonotic animal influenza virus that has the same hemagglutinin subtype as endemic influenza viruses. There is a need for more coordination between veterinary and human health in vaccine seed strain selection to minimize generation of drift mutants and to maximize vaccine efficacy.

An important goal for improved Influenza A Virus (IAV) vaccines in pigs is to achieve more effective control of influenza infection and spread as a major respiratory pathogen and component of the porcine respiratory disease complex and, in turn, to further minimize the risk of emergence of zoonotic influenza with pandemic potential.

Regardless of the species or the technology used, currently available vaccines all have the same general weaknesses; they have limited cross-protection against antigenic variants within a subtype, even less protection between subtypes, and they cannot be produced quickly enough to keep pace with the ever changing IAV. Solutions include:

1. Evaluation of novel technologies that reduce the time required to produce a vaccine.
2. Development of novel vaccine technologies to produce a broader or universal clinical protection.
3. Development of vaccine platforms that can be used in multiple species.
4. Improvement in the regulatory process for vaccine selection and production.

Diagnostics

The early detection of an animal influenza virus with zoonotic and/or pandemic potential is critical to minimize the spread of disease and reduce the economic impact. New technologies promise more rapid detection and characterization of avian influenza viruses, either in the laboratory or pen-side. One of the major gaps is the lack of companion DIVA diagnostic tests that are validated for routine use.

Influenza is a highly variable virus, which complicates diagnostic tests. Tests for type A influenza viruses are generally reliable, but serological tests require improvement. The key animal influenza diagnostic gap is in subtype identification and in the identification of the subtype specificity of sera. Better serologic tests are needed, both to determine the subtype specificity of antibody (i.e., what subtypes has an animal been infected with) and to characterize the antigenic differences among animal influenza isolates. Characterization of the antibody response and antigenic differences among animal influenza isolates are critical for updating vaccines and evaluating vaccinal protection. Hemagglutination inhibition (HI) assay is the current standard for identifying the subtype specificity of sera and to characterize antigenic differences; however, this is a cumbersome test that lacks precision.

Other areas where improvement would be beneficial are: 1) a rapid molecular test that is not easily affected by genetic mutations; 2) a transport media that stabilizes the virus without refrigeration; and 3) at the very least, efforts to characterize new isolates should be continued to assure that current tests will have optimal sensitivity and specificity.

Surveillance

Gaps in the availability of comprehensive surveillance systems for animal influenza viruses are significant. There is a need for efficient sampling methods, rapid detection of emerging new strains, and identifying influenza viruses with human infection and/or pandemic potential.

Specific gaps in surveillance for animal influenza viruses fall into three broad categories: 1) Using limited resources to provide efficient sampling of animals to confidently characterize circulating endemic influenza viruses, 2) Rapidly detecting emerging new strains, subtypes or incursions between host species to prevent spread and establishment of new lineages, and 3) identifying influenza viruses with human infection or pandemic potential. Specific gaps in surveillance include:

1. Inadequate resources to investigate seropositive flocks identified in the National Poultry Improvement Plan influenza program for virus identification.
2. Lack of permanent sustainable resources to continue development of the USDA SIV Surveillance System as necessary.
3. Lack of understanding of the movement of influenza viruses within the U.S. and across its borders and where to target surveillance to maximize limited resources.
4. Lack of basic studies into the determinants of cross-species transmission and adaptation are critical to enable the identification of genetic signatures that indicate the zoonotic potential of a virus.
5. Need to improve diagnostic tests that target viral properties important for immunity, virulence, and interspecies transmission potential

Drugs/Biotherapeutics

There are no influenza antiviral drugs approved for use in animals. Evidence of drug resistance makes the broad use of these drugs in animals questionable even if less costly generics were available.

Depopulation and Disposal

Depopulation is considered the first line of defense against a foreign animal disease outbreak. In the event of a widespread animal influenza virus outbreak with epizootic and/or pandemic potential in the United States, thousands of pigs and/or millions of poultry could be affected. Current national and international policy requires that affected animals be quarantined, their movement stopped, and the animals humanely euthanized. However, this approach can have devastating effects on the livestock industry, the economy, and the environment. In addition, there are significant risks to public health, animal health, and the environment if these processes are not conducted carefully. Additional work is needed in defining which populations may need to be depopulated, reducing the size of culling zone based on implementation of more rapid diagnostic, modeling of spread and containment and more rapid humane depopulation methods. Consideration for control of influenza viruses that are zoonotic for humans but do not cause disease in animals have to be addressed as part of a One Health approach. The use of public health resources to control and eradicate animal diseases is critical.

Disinfectants

Animal influenza viruses are labile in the environment and inactivated by several inexpensive commercial disinfectants. Additional work is needed to develop methods to collect and dispose of disinfectants after use to minimize negative environmental impact and development of new generation of disinfectants that degrade rapidly in the environment to innocuous materials.

Personal Protective Equipment (PPE)

Negative air pressure respirators are expensive, hot, tight, and uncomfortable for the users. The positive air pressure respirators (PAPRS) are less uncomfortable and cooler because of the blowing air, but the tubes and battery pack are cumbersome in the field. There is a need to improve these products for working under field conditions in hot contaminated environments: 1) lighter weight, 2) cooler, and 3) more

ergonomic in design. In addition, the availability of FDA-approved human drugs and vaccines to protect workers when facing an outbreak with a zoonotic animal influenza virus that can infect people is critical.

COUNTERMEASURES PRIORITIES

The AICWG assessed both commercial products and products known to be in the "pipeline." The value of vaccines and diagnostics in the different animal agricultural production segments were assessed, leading to a prioritized list of segments where vaccines and diagnostics would have the most impact. For the analysis, the working group used a decision model specifically designed for vaccines and diagnostics. The decision model criteria and their respective weight were selected by the working group (see Appendix II). An informal review of other countermeasures was conducted but the decision model was not used to assess methods of euthanasia and other common tools used in an epizootic such as disinfectants and personal protective equipment, as it was felt that an in-depth analysis of these tools had already been done by other working groups. Based on the results of the assessment conducted by the AICWG, the following priorities were identified:

Vaccines

- There is a need to develop and license live-vectored vaccines to include the H7 hemagglutinin subtype and antigenic important variants for H5 subtype avian influenza
- To develop next generation modified live vectored-influenza virus vaccines that can be cost effectively used on poultry after they have been placed into the field
- To explore the use of reverse genetics technology to develop a range of vaccine seed strains with hemagglutinin genes matching likely H5 and H7 threats from around the world with an egg adapted high growth reassortant.
- To explore the use of reverse genetics technology to develop a live-attenuated IAV vaccine for use against new variant SIV strains with pandemic potential.

Diagnostics

- There is a need to improve rapid identification molecular diagnostics of hemagglutinin and neuraminidase subtypes.
- There is a need to develop internal controls to validate the performance of diagnostic test procedures across laboratories.
- More sensitive pen-side tests or commercial test kits that can be used in the field are needed. Even with simultaneous identification of the most important HA and NA subtypes.
- If vaccines are to be used in an eradication campaign, validated companion diagnostic tests will be needed to implement a DIVA (differentiating infected from vaccinated animals) strategy.
- There is a need to develop a reliable test for the HA and NA subtype specificity of avian influenza virus antibody in avian serum.
- There is a need to develop a reliable test for the HA and NA subtype specificity of swine influenza virus antibody in swine serum.
- There is a need to develop a rapid and cost effective full genome sequencing method (e.g., with next generation methods).
- There is a need to develop a viral transport media that can stabilize live influenza virus without refrigeration or maintenance of the cold chain.
- Continue to monitor current molecular diagnostic tests for sensitivity and specificity with novel influenza isolates.

- Continue to evaluate genome sequence from novel influenza viruses for diagnostic needs.
- A rapid and robust molecular test that is not easily affected by genetic mutations.
- Most state and regional diagnostic laboratories already participate in the NAHLN for animal influenza. This program should be maintained to assure that all laboratories meet the minimum diagnostic requirements, pass proficiency testing and are notified of test updates.

Drugs

- None - the use of antiviral drugs to control animal influenza viruses in swine and poultry are not recommended.

Disinfectants

- Commercially available disinfectants are effective but there is a need to develop methods to collect and dispose of disinfectants after use to minimize negative environmental impact
- There is a need to develop new generation disinfectants that degrade rapidly in the environment to innocuous materials..

PPE

- There is a need to improve respirators for working under field conditions in hot contaminated environments.
- There is a need to ensure availability of FDA-approved drugs and vaccines to protect workers.

RESEARCH PRIORITIES

Research priorities were identified that were determined to be critical to address the gaps in our scientific knowledge and importantly, advance the development of countermeasures to effectively control and mitigate an animal influenza virus outbreak with epizootic and/or pandemic potential. Overall, research studies applied to 1) the understanding of viral evolution in animal populations, 2) viral pathogenesis, 3) understanding transmission and epidemiology, and 4) development of improved countermeasures such as vaccines and diagnostics are likely to yield significant improvements in our ability to control influenza virus outbreaks in animals, domestically and internationally.

INTRODUCTION

The threat of new and emerging animal influenza viruses with epizootic and/or pandemic potential is significant. In the last 100 years, the world has experienced four influenza pandemics: 1918, 1957, 1968, and 2009. These pandemics resulted in the death of millions of people worldwide. Since the mid-1990s, the recognition of new or expanding animal influenza viral strains has generated concerns within the public health community that a new pandemic from a domestic animal source was impending. Since November 2003, more than 500 cases of human infection with highly pathogenic avian influenza (HPAI) H5N1 viruses and more than 300 deaths have been reported by more than a dozen countries in Asia, Africa, the Pacific, and Europe. Although public health concerns are justified, HPAI is primarily a poultry disease resulting in up to 90 percent mortality in infected flocks. HPAI viruses also impact international trade by inhibiting exports from an infected country. An outbreak of avian influenza (AI) virus in the United States would devastate our poultry industry and curtail the availability of poultry meat. With more evidence that AI strains continue to breach the species barrier, the cost of an HPAI strain with pandemic potential could be in the billions of dollars. Surprisingly, in 2013, a new "low pathogenic avian influenza (LPAI)" H7N9 Influenza A virus has emerged as a zoonotic threat in China, causing fatal respiratory disease in humans.

Not surprisingly, avian species are not the only source of animal influenza viruses. Since 2005, approximately 370 human cases of variant influenza infections of swine origin have been detected in the United States alone, not including the 2009 pandemic H1N1 (H1N1pdm09). The novel H1N1 influenza virus identified in 2009 in humans caused the first influenza pandemic of the new millennium. Although the H1N1pdm09 virus was highly transmissible in humans, in general it exhibited mild clinical symptoms. The H1N1pdm09 virus currently circulates worldwide in humans as a seasonal strain causing isolated infection. The H1N1pdm09 appeared with a gene segment combination not previously known to circulate in humans or animals prior to its emergence. Six of the gene segments, including the hemagglutinin (HA) gene, were closest in sequence to those of the triple reassortant internal gene (TRIG) influenza viruses that have been isolated from North American pigs since 1997-1998. The remaining two segments of the H1N1pdm09 virus, matrix (M) and neuraminidase (NA) bear the closest similarity with those of Eurasian avian-like swine viruses not previously known to circulate in North America. The H1N1pdm09 virus has since reassorted frequently with other endemic swine IAV worldwide to yield H3N2 viruses with the M segment derived from the 2009 pandemic virus among many other gene combinations. Reassortant H3N2 viruses with the H1N1pdm09 M gene from swine have resulted in more than 300 people infected with variant H3N2 (H3N2v) in the United States since 2011.

The most effective way of controlling zoonotic diseases is at the source (the so called animal host reservoir). In the case of AI, migrating wild birds harbor the virus exhibiting little to no detectable disease; thus the virus is not eradicable from this population for this and many other reasons. However, the source of new genetic variants of IAV infection for people is most likely to be domestic swine and poultry. Therefore, expending resources to control and eradicate outbreaks in these animal populations is the best strategy for safeguarding public health and preventing a potential pandemic.

The Animal Influenza Countermeasures Working Group (AICWG) was charged with the task of conducting an in-depth analysis of the available scientific information and countermeasures to effectively

control, and where feasible, eradicate an emerging animal influenza virus with epizootic and/or pandemic potential. The AICWG assessed both commercial products and experimental products known to be in the "pipeline." The value of vaccines and diagnostics in different animal agricultural production segments were assessed, leading to a prioritized list of segments were vaccines and diagnostics would have the most impact. The group also assessed other countermeasures such as depopulation, disposal, disinfectants, and personal protective equipment (PPE), but did not subject them to an in-depth decision analysis as it was felt that this work had already been done by other working groups. Importantly, to address the gaps in our scientific knowledge and advance the availability of countermeasures, the working group conducted an assessment of research priorities that are likely to have the most impact in preparing for a new emerging animal influenza virus with epizootic and/or pandemic potential.

BACKGROUND INFORMATION

ORGANIZATION OF THE ANIMAL INFLUENZA VIRUSES COUNTERMEASURES WORKING GROUP (AICWG)

An international team of animal and human influenza virus experts from public and private research institutions, including industry, academia, and government, was invited by the Chair of the organizing committee to participate in an animal influenza viruses gap analysis workshop and serve on the Animal Influenza Viruses Countermeasures Working Group (AICWG). The AICWG was charged with making an assessment of specific materials, commercially available and in the research pipeline, which will ensure that the United States has an arsenal of highly efficacious countermeasures to control and mitigate the impact of an outbreak of an animal influenza virus with zoonotic and/or pandemic potential. A total of 56 experts (see Appendix XV, page 133, for the list of workshop participants) accepted to serve on the AICWG. The AICWG was hosted by the University of Georgia and the USDA-ARS Southeast Poultry Research Laboratory in Athens, Georgia, March 25-27, 2013. Sponsors of the workshop included the Global Strategic Alliances for the Coordination of Research on the Major Infectious Diseases of Animals and Zoonoses (STAR-IDAZ, http://www.star-idaz.net) and the UK Biotechnology and Biological Sciences Research Council (BBSRC). Instructions (see Appendix II) and several reference materials were provided by the AICWG Chair prior to the meeting. The AICWG members were tasked by the Chair with assessing the best available countermeasures to rapidly and effectively control and eradicate a new or variant animal influenza virus should an outbreak occur in the United States. When gaps in the information necessary to complete the analysis were identified, AICWG members contacted additional experts directly (see list of ad hoc contributors on page 144).

EXPERT REPORTS

The AICWG used the following reports as background information in evaluating the risks of an animal influenza virus introduction occurring in the United States:

The 2012 North American Plan for Avian and Pandemic Influenza
http://www.spp-psp.gc.ca/eic/site/spp-psp.nsf/vwapj/pandemic-influenza.pdf/$FILE/pandemicinfluenza.pdf

The OIE–FAO Network of Expertise on Animal Influenza (OFFLU)
http://www.offlu.net/fileadmin/home/en/publications/pdf/OFFLU_Research_Priorities_photo.pdf

The FAO Global Animal Disease Information System (EMPRES-i) Genetic Module
http://www.fao.org/docrep/017/i2910e/i2910e.pdf

WHO Pandemic Influenza Preparedness Framework 2013 biennial report

The USDA National Animal Health Emergency Management System (NAHEMS) Guidelines: Vaccination for Contagious Diseases

Strategic research targets to protect American livestock and poultry from biological threat agents. Report from the WMD Counter Measures Working Group -Animal Pathogen Research and Development Subgroup. http://www.ars.usda.gov/research/programs/programs.htm?np_code=103&docid=5815

The Office of Science and Technology Policy (OSTP) Blue Ribbon Panel on the Threat of Biological Terrorism Directed Against Livestock. Conference Proceedings, Washington DC, December 8-9, 2003. http://www.ostp.gov/html/STPI.pdf

SITUATION WORLDWIDE

The threat of a new and emerging animal influenza virus with epizootic and/or pandemic potential in domestic animals is significant. The following section summarizes the animal influenza situation worldwide, the status of our understanding of virology, viral pathogenesis, immunology, epidemiology, and the available tools to effectively control and eradicate new and emerging animal influenza viruses, and current obstacles for controlling an outbreak in the United States.

Avian Influenza

Since 1959, there have been 35 HPAI epizootics that are reportable to the World Organisation for Animal Health (OIE). H5N1 HPAI is enzootic in six countries: 1) self-declared enzootic (Egypt and Indonesia), 2) continue to report occurrences of outbreaks over multiple years (Vietnam and Bangladesh), or 3) have published data in the literature of continuous reports of infection and molecular evidence of virus continual presence in country (China and east India). H5N1 HPAI is reported from five geographic epicenters: 1) Egypt; 2) Ganges Delta (India, Bhutan, Nepal and Bangladesh); 3) Mekong Delta (south Vietnam and Cambodia); 4) Indonesia; and 5) east to southeast Asia (China, Hong Kong, North Korea, northern to central Vietnam and Myanmar)[1]. Most recently (July 2012- September 2013), 17 countries have reported outbreaks of HPAI in domestic poultry: 12 with H5N1 (Bangladesh, Bhutan, Cambodia, China, Egypt, Hong Kong, India, Indonesia, North Korea, Myanmar, Nepal and Vietnam), two with H5N2 (South Africa and Chinese Taipei), one with H7N3 (Mexico), and two with H7N7 (Australia and Italy). Six subclades of H5N1 HPAI virus have been reported in poultry and wild birds: 1) subclade 2.3.2.1, most frequently reported with wide geographic dispersion including northern and central Vietnam, India, Bangladesh, China, Hong Kong, India, Nepal, and Bhutan); 2) subclade 2.2.1 viruses in Egypt; 3) subclade 7.2 in northern China and Vietnam; 4) subclade 2.1.3.2 and 2.3.2.1 in Indonesia; and 5) subclade 1.1 in southern Vietnam and Cambodia. Human infections were reported with clades 2.3.4.2 (China), 2.2.1 (Egypt), 2.1.3.2 (Indonesia) and 1.1 (Vietnam and Cambodia).

Five HPAI outbreaks have involved subtypes other than H5N1. An outbreak of H5N2 HPAI began in 2011 in South Africa, affecting only ostriches, and continued until resolution in mid-2013[2]. In total, 50 outbreaks have occurred, affecting 57,569 ostriches resulting in 16,402 cases with 4930 birds being destroyed and 47,677 handled via controlled slaughtered. The outbreak was resolved 3 July 2013.

An unrelated outbreak of H5N2 HPAI occurred in Chinese Taipei, being the second such outbreak in Chinese Taipei with first report on 27 February 2012 and resolved 7 August 2012. This involved native chickens on Penchu Islands with 200 deaths and 631 culled chickens. Chinese Taipei has ongoing outbreaks of North American lineage of H5N2 low pathogenicity avian influenza LPAI) virus with first report on 21 October 2008 and most recent 9 September 2013. The H5N2 HPAI virus was derived from the H5N2 LPAI[3].

The H7N3 HPAI epizootic in central Mexico has re-emerged. Initial cases were reported in Jalisco 21 June 2012 with last cases on 12 September 2012, and declaration of freedom 12 December 2012. The epizootic reemerged in Aguascalientes 3 January 2013, with 64 total outbreaks in the states of Jalisco, Aguascalientes, Guanajuato and Puebla. In the resurgence, layers, broiler breeders, backyard poultry and broiler farms were affected with 550,322 deaths, 6,230,022 culled and 284,015 slaughtered birds. The most recent case was 19 August 2013. Two unrelated H7N7 HPAI epizootics have occurred in Italy and Australia. The Australian outbreak began 11 November 2012 in a free-range egg layer farm in New South Wales. The farm experienced 5000 deaths and the remaining 45,000 chickens were culled. The source of the virus was unknown, but farm had a pond with wild ducks. The Italian outbreak occurred in Emilia-Romagna province of Northern Italy, a geographic location of previous HPAI and LPAI outbreaks. The outbreak began on 15 August with the last cases on 4 September 2013. In total, six outbreaks occurred: four in commercial layers, one in turkey flock and one in a backyard free-range layer flock. Deaths numbered 5676 and 946,982 poultry were culled. An outbreak of H7N2 HPAI was reported in Australia on October 26, 2013. The outbreak occurred in New South Wales and involved a single premise of 435,000 free range and cage layer hens aged between 22 and 79 weeks. The outbreak was eradicated by stamping out strategy[4].

For 2014, an outbreak of H5N8 HPAI began in breeder ducks in the Republic of Korea with mostly breeder and meat duck farms affected. In total 18 cases have been diagnosed with 47,000 poultry culled and 70,000 more in process of culling as of January 28, 2014. The outbreak is ongoing at this time.

In addition to HPAI, low pathogenicity avian influenza (LPAI) is reported around the globe. However, the LPAI reporting to OIE is only required for H5 and H7 viruses, and such viruses are only reported consistently by developed countries with adequate surveillance programs. Developing and transition countries have inconsistent surveillance and reporting. However, the outbreaks of H7N9 LPAI in China have garnered global attention. This virus is of avian origin and is responsible for infections in human in large urban areas of China in spring 2013 (first wave)[5]. The original source of the virus from poultry farms is unknown but the live poultry market (LPM) system has served as an amplifier of the virus, especially in wholesale markets in the large cities, with 77% of human cases having known contact with live poultry at a retail live poultry markets[6]. There has been no new human infection in large urban areas where LPM system has been closed. Since late fall 2013, the H7N9 has resurged in China (second wave), still associated in humans with contact to LPM system, but the cases are more geographically distributed suggesting wider infections on poultry farms supplying the LPM.

Swine Influenza

Endemic Seasonal SIV

Swine influenza was first described coincident with the 1918 Spanish flu pandemic, and has been recognized in U.S. swine ever since. Historically, swine influenza was recognized as a seasonal disease

occurring in the fall and winter. With the increased efforts on surveillance, swine influenza virus can be detected throughout the year, although influenza disease continues to primarily have bimodal seasonal peaks. It is the second most diagnosed swine viral respiratory disease behind porcine reproductive and respiratory syndrome (PRRS). Among the co-circulating IAV in the U.S. swine population are at least ten antigenically distinct hemagglutinin (HA) lineages: three classical swine lineages H1α, H1β, H1γ; two lineages derived from human seasonal H1 viruses H1δ1, H1δ2; the H1pdm09; and H3 cluster I-IV viruses.[7,8] The primary implication of these antigenic differences is that controlling infection and transmission via vaccination is less than optimal. Current swine IAV vaccines use multivalent formulations of inactivated, field-sourced virus, each component representing one of the hemagglutinin lineages.[9] These vaccines elicit antibodies with a relatively narrow range of protection that target the hemagglutinin protein, and efficacy is equivocal for drifted strains.

Emerging Variant SIV

The predominant TRIG viruses circulating in North America and Asia evolved by reassortment with Eurasian swine viruses, then emerged in the human population to cause the 2009 H1N1 pandemic. Multiple reverse-zoonotic transmission events of H1N1pdm09 into naïve swine populations occurred around the world. The H1N1pdm09 virus continues to reassort with endemic viruses to yield novel genotypes with unknown potentials for swine or human health. In the United States, variant H3N2 viruses (H3N2v) with M segment and/or additional segments derived from the pandemic viruses and the others derived from swine H3N2 were detected in humans from 2011-13. Based on history following the H1N1pdm09 virus outbreak in humans in the United States, a new or variant human or animal influenza virus with sustained transmission in U.S. swine herds would likely become endemic very rapidly.

ECONOMIC LOSS

Avian Influenza

Economic losses from AI have varied depending on the strain of virus, species of bird infected, number of farms involved, control methods used, and the speed of implementation of control or eradication strategies. Many of the economic losses associated with outbreaks of HPAI are due to consumer avoidance of poultry products and the measures implemented, especially if wide area culling around infected premises is used. In most developed countries, HPAI and LPAI have not been endemic diseases in the commercial poultry industries. Most outbreaks and economic losses have occurred from epidemics of HPAI or LPAI in commercially raised poultry, predominately chickens and turkeys. In some developing countries LPAI is endemic in commercially raised poultry especially viruses of the H9N2 subtype and, in some developed countries, H9N2, H5N2 and H6N1 LPAI has been endemic in backyard and live poultry market (LPM) systems that mainly serve ethnic populations of large metropolitan areas. Since 2003, H5N1 HPAI has become endemic in village poultry, especially domestic ducks in a few countries in Asia and Africa.[10]

Generally, the most accurate reports on losses have come from HPAI eradication programs. Direct losses in HPAI outbreaks have included high morbidity and mortality losses, depopulation and disposal costs, cleaning and disinfection, quarantine and surveillance costs, and indemnities paid for the birds. However, indirect costs such as uncompensated losses to the poultry industry including temporary or permanent loss in poultry exports, income lost by farmers and communities during the production down time, increased

consumer costs from reduced supply of poultry products and losses from decreases in consumer purchases can easily escalate losses by 5-10 folds. The economic costs for eradication of HPAI have varied greatly, but eradication costs have been very high and appear to be proportional to the number of birds that died and were culled. However, in the 1983-84 U.S. H5N2 HPAI epidemic, the projected cost of not implementing an eradication program was $500 million for losses ($1.3 billion in 2012 funds) to poultry farmers and $5.5 billion ($12.2 billion in 2012 funds) in increased customer costs. The HPAI (H7N1) outbreak in the Italy (1999-2000) resulted in 18 million destroyed birds with $100 million in monetary compensation to affected farmers and $500 million in indirect losses. The economic impact of the 2012 AI H7N3 outbreak in Mexico affected a region where approximately 55 percent of the table eggs are produced. More than $22 million in losses were reported by the industry.

Low pathogenicity AI outbreaks have also caused significant economic losses for producers of chickens, turkeys and ducks, especially when accompanied by secondary bacterial or viral pathogens, but accurate documentation of such costs are generally not available.[11] In general, losses have been less than with HPAI outbreaks because infected flocks have typically been eliminated through a controlled marketing program, the mortality rates have been lower, no federal eradication costs were incurred, and national and international trade usually have not been disrupted, although bans are placed on imports by some countries when notifiable LPNAI occurs. Losses from LPAI epidemics include mortality losses, increased condemnations at slaughter, medication against secondary bacterial infections, cleaning and disinfection, delayed placements of new birds, and, for LP H5 and H7 outbreaks, restrictions in trade of poultry and poultry products has become increasingly common. Poorly documented but more costly have been the endemic H9N2 LPAI poultry infections in much of Asia and the Middle East, and H5N2 LPAI poultry infections in Mexico, Central America, and the Caribbean with vaccination programs adding to the cost of production. Since LPAI is usually not dealt with by traditional stamping-out programs, the costs of LPAI are usually unknown. However, when a stamping-out program was undertaken in the Virginia 2002 H7N2 LPAI outbreak, the eradication program had similar costs as previous HPAI outbreaks. In developed countries, stamping-out programs have emerged to be principle control method for H5 and H7 LPAI,[12] although methods used to achieve this vary and include vaccination followed by controlled marketing.[13]

The scale of investment into control of avian influenza following the emergence of H5N1 HPAI in Asia was largely due to concerns that the virus might become readily transmissible between humans and the very high cost of a human pandemic caused by an influenza virus that produced high level mortality in humans.

The economic impact of a new avian influenza outbreak in the United States would be significant. Most poultry premises in the United States are concentrated geographically with allied industries strategically located nearby. One example is Franklin County in the State of Georgia where an outbreak could involve as many as 69 farms and over 7 million birds within a 5-mile radius (Figure 1). Within these areas, truck traffic among farms is common, involving trucks that deliver goods and remove items that could easily facilitate the spread of avian influenza without proper biosecurity measures. HPAI viruses cause the most concern, resulting in up to 90 percent mortality in infected flocks. HPAI viruses also impact international trade by inhibiting exports from an infected country.

Swine Influenza

Economic losses associated with influenza viruses in pigs result from two factors: 1) direct production losses associated with influenza disease in pigs; and 2) potential loss of markets due to decreased domestic consumption and/or exportation associated with public health concerns over pork safety. One hog production company based in the U.S. estimated the direct cost of swine influenza to be $10.31 per market hog,[14] and another estimated their cost to be $3.23 per hog.[15] Although the direct costs may vary between production systems, it is ranked as one of the top three respiratory health challenges causing production losses in the U.S.[16] The U.S. is the world's largest exporter of pork. In 2012, approximately 25% of U.S. pork production was exported to over 100 countries. In 2008, the U.S. exported 20% of U.S pork production valued at $4.9 billion dollars worth of pork. Following the emergence and detection of the H1N1pdm09 pandemic virus, 27 countries banned or threatened to ban U.S. pork and pork products and cost the U.S. pork industry over $5.0 million per day due to the perceived uncertainty of the safety of U.S. pork.

PUBLIC HEALTH

Zoonotic Influenza

Public health remains a strong driver for continued study of influenza viruses in animals, especially in poultry and swine. Ideally, viruses capable of zoonotic transmission with pandemic potential and/or enhanced virulence in humans should be identified while circulating in animals, before they spill over and cause human influenza outbreaks. Currently, the state of knowledge for determining if an animal influenza virus can infect and cause disease in humans is rudimentary. It is therefore difficult to predict the potential for new and emerging animal influenza viruses to become pandemic and infect humans. Nevertheless, it remains imperative that viruses circulating in animals be monitored to allow analyses that identify new threats and support control or eradication interventions.

Dual Use Research Concerns

The 2011 H5N1 studies (Imai[17] and Fouchier[18]) co-funded by the NIH and considered Dual Use Research of Concern (DURC), provided valuable insights into the mechanisms that may allow H5N1, a disease primarily of animals, to adapt to become transmissible between people. In January 2011, 39 influenza researchers around the world declared a voluntary two-month moratorium on research into transmission of H5N1 highly pathogenic avian influenza. This moratorium has extended past the original mandate and in the summer of 2012 the U.S government proposed an indefinite continuation of the moratorium on "gain-of-function studies" with H5N1 viruses. NIH sponsored an international meeting of scientists to discuss the type of research involved and the moratorium on December 17-18, 2012. A number of meeting participants expressed concern that very important research was being stalled by the moratorium.

GAPS IN THE SCIENTIFIC INFORMATION

VIROLOGY

Influenza A virus is a member of the *Orthomyxoviridae* family. Influenza A viruses are segmented, single-stranded, negative-sense viruses with RNA genomes. Two surface glycoproteins, the hemagglutinin (HA) and neuraminidase (NA), are important to the pathogenesis, transmission and life cycle of influenza viruses, are highly variable and targeted by the host immune system. The HA and NA genes may vary due to two types of processes known as antigenic drift and antigenic shift. Antigenic drift results from minor changes in the virus genome due to polymerase errors during replication. Antigenic shift occurs when two or more viruses infect the same cell and exchange the HA or NA gene segments resulting in a reassortant virus, although any of the eight gene segments are subject to reassortment. Human influenza pandemics have been attributed to both types of antigenic changes.

The physical and biological properties of a pathogen are one of three fundamental elements of the epidemiologic triad, which also includes the host and the environment. As such, specific knowledge about IAV contributes to the understanding of disease pathogenesis in the individual host and virus transmission in the population. Understanding these processes is critical for the design of diagnostic and intervention tools aimed at infection control and eradication. Many questions remain across a broad range of aspects of influenza virology. Crucial are issues of pathogenesis, tissue tropism and transmission both among hosts of a particular species and between hosts of different species.

Influenza Viruses

The *Influenzavirus A* virus of the family *Orthomyxoviridae* infects many avian species, swine, humans, dogs, horses, and an array of other terrestrial and aquatic mammals. The virion is enveloped and contains 8 segments of linear, single stranded RNA of negative-sense.

In common with other RNA viruses, the relatively small numbers of proteins expressed are multifunctional. Their functional domains often overlap and interact with other viral and host proteins, making it difficult to obtain holistic views of their function by typical approaches of mutagenesis, heterologous expression and interaction analysis. For example, NS1 is a particularly complex protein, with multiple functions that differ between virus strains and different hosts. Clarifying the complexity of its function will be essential to understanding the range of phenotypes found in influenza viruses in such a broad range of possible hosts.

Avian Influenza Virus

Avian influenza (AI) viruses are classified into 16 hemagglutinin (HA) subtypes (H1-16) and 9 neuraminidase (NA) subtypes (N1-9) based on surface glycoproteins.[19] Avian influenza viruses are further classified as low pathogenicity (LP) or high pathogenicity (HP) AI viruses based on ability to cause severe systemic disease in gallinaceous birds (i.e., chickens, turkeys). Any of the 16 HA subtypes can cause LPAI; however, only the H5 and H7 subtypes have been associated with HPAI, with the vast majority of H5 and H7 viruses being LPAI. HPAI outbreaks in chicken and turkey flocks can result in up to 100% mortality. HPAI is highly contagious among susceptible species. Routes of transmission

include direct and indirect contact with infected birds. Recent outbreaks include the United States, Canada, Europe, Australia, Mexico, the Middle East, Africa, and Asia (www.OIE.int). The classification of HP and LP is a designation based on the intravenous pathogenicity index (IVPI) test used by regulatory authorities. It could be improperly applied if used on other avian species, as the IVPI was designed for use only in gallinaceous species of birds.[20]

LPAI viruses do not cause disease in natural waterfowl host species and although wild birds do not carry HPAI virus, they may become infected from poultry.[19] However, disease in waterfowl is normally mild or absent. A rare exception is some specific isolates of the Asian H5N1 HPAI virus lineage. Isolates that can cause disease and even mortality in Pekin ducks have been collected since about 2003.[21] Age, among other host factors, seems to affect disease presentation and severity.[22] The viral factors that are responsible for virulence in ducks have been investigated, but have yet to be completely elucidated as data are inconsistent.[23-26] The interaction appears to be highly complex and possibly virus strain specific.

Although numerous domestic poultry species can be infected with AI viruses, the natural host reservoir is wild dabbling ducks, particularly Mallards (*Anas platyrhincos*), shore birds and gulls.[27,28] Most AI virus subtypes can be found in dabbling ducks, however two subtypes, H13 and H16 seem to be specific to gulls. This is likely due to the fact that dabbling ducks and gulls do not share habitats, therefore virus exchange does not occur. Limited studies have shown that Mallard ducks and domestic turkeys can be infected with H13 LPAIV, but chickens were not infected under the conditions used.[29] This highlights the fact that among avian species, AI viruses must adapt to a particular host and more importantly that chickens and turkeys are not natural host species for AI viruses. Studies investigating varying bird infectious doses among species and isolates provide additional evidence that not all avian species are equally susceptible to AI viruses.[30] Determinants for virulence in some mammals have been identified, although it is not clear why these viruses are not pathogenic for swine.

Updated unified nomenclature system for the highly pathogenic H5N1 avian influenza viruses

In October 2011, the WHO/OIE/FAO H5N1 Evolution Working Group published an updated unified nomenclature system for highly pathogenic avian influenza A(H5N1) viruses. A unified system facilitates the interpretation of sequence/surveillance data from different laboratories. Since the previous update in 2009, H5N1 viruses have continued to evolve and diversify as they spread and infect animals and humans. The 2011 recommendations were based on detailed analyses and comparisons for nearly 3000 H5N1 virus gene sequences (see http://www.who.int/influenza/gisrs_laboratory/h5n1_nomenclature/en/index.html).

Swine Influenza Virus

Influenza A viruses cause one of the most important respiratory diseases in pigs as well as humans. Repeated outbreaks and rapid spread of genetically and antigenically distinct IAVs represent a considerable challenge for swine production and public health. Although only subtypes of H1N1, H1N2, and H3N2 are endemic in swine around the world, considerable diversity can be found not only in the hemagglutinin (HA) and neuraminidase (NA) genes, but in the other 6 genes as well. This diversity is the result of periodic interspecies transmission from avian- or human-adapted IAV into swine, followed by reassortment and adaptation in the swine host. Regional and intercontinental movement of pigs and/or pig viruses through other intermediates has also resulted in increased global diversity.[31] Human and swine IAV have demonstrated a particular propensity for interspecies

transmission in the past century, leading to regular and sometimes sustained incursions from man to pig and vice versa. The diversity of IAV in swine remains one of the critical challenges in diagnosis and control of this important pathogen to swine health, and in turn contributes to a significant public health risk.

Swine play an important role in the ecology of influenza, and the interplay between human and swine or swine and domestic turkeys are specific influenza niches that exist largely without the interference of IAV from wild waterfowl. Zoonotic transmission of swine adapted IAV to humans has been documented throughout the years since 1918 and generally results in an influenza-like illness similar to human seasonal IAV with little evidence of human-to-human onward transmission. The most dramatic exception to this rule was the H1N1pdm09, a virus with gene segments from two distinct swine IAV lineages, the Eurasian H1N1 and the North American triple reassortant γ-H1 viruses. However, a direct link to an endemic swine IAV has yet to be established, even after more than 4 years of intensified surveillance in swine on a global level. Although the H1N1pdm09 virus likely arose in people in Mexico and spread in pandemic-proportions around the globe, the geographic location and host species in which the pre-pandemic ancestor virus evolved remains a mystery.

The determinants that allow human adapted viruses to cross into swine or swine adapted viruses to cross into humans are unknown. Mammalian adapted viruses tend to have similar predilections for sialic acid moieties in glycan arrays, confusing the concept of differential receptor preferences between these species. Certain subtypes have an apparent higher likelihood for becoming endemic in mammalian hosts (H1N1, H1N2, and H3N2 in pigs and people), but this is not so for horses (H7N7 and H3N8) and dogs (H3N8). Virulence markers detected in avian influenza viruses have not translated to the same effect in mammalian hosts. Population immunity likely has an impact, but also cannot be the sole explanation for interspecies barriers between mammalian hosts of IAV. Some genome constellations of H3N2v (those acquiring the M gene from H1N1pdm09) have been detected in humans more frequently than others, hinting at some selective advantage, but this observation has not been shown to have a definitive effect in humans or pigs.

Standardization of terminology for the variant A(H3N2) virus recently infecting humans

Since July 2011, increasing numbers of human cases of infection with non-seasonal influenza A viruses have been detected in the United States of America (USA), largely represented by H3N2 of swine-lineage. No reports have been received by WHO from other Member States. These viruses have different virological characteristics from current circulating seasonal influenza viruses in humans. In order to improve communications and avoid confusion, the Food and Agriculture Organization of the United Nations (FAO), the World Organisation for Animal Health (OIE) and WHO established a working group of experts to standardize the terminology for animal influenza viruses when they are detected in humans. The joint recommendation was the term "variant" be added to the subtype, such as A(H3N2)v, where "v" stands for "variant".

Canine Influenza Virus

Traditionally, dogs have not been considered a natural host with only limited reports of seroconversion to human H3N2 viruses in the early 1970's, but no reports of natural infections with clinical disease. However, the canine influenza landscape changed dramatically when in January 2004 racing greyhounds with an influenza like illness and fatal pneumonia emerged in Florida. The virus subsequently spread and affected thousands of greyhounds at 20 different tracks in 8 states from June 2004 to June 2006.[32,33]

Molecular analyses of the virus isolates indicated a genetic relatedness to equine influenza H3N8 – in particular, the typical contemporary equine influenza. There appears to be signature amino acid mutations in the canine H3 isolates. There are over 70 million dogs in United States all are considered susceptible if exposed, potentially affecting all breeds and ages. Seven outbreaks were detected in the United States in 2009 with influenza virus being widespread and endemic in many communities. Dogs housed in communal facilities were at greatest risk of exposure and disease (boarding kennels, veterinary clinics, shelters, racing kennels, show dogs). Scientists concluded that a rare interspecies transmission event occurred prior to 2004 where the virus adapted to the canine host and now it is sustained in the canine population. Interspecies transfer of human pH1N1 to dogs has been reported but did not sustain in the dog population. Today there are two subtypes circulating in dogs in the world: H3N8 in the United States and an H3N2 in South Korea. Canine respiratory epithelia contains both α−2,3 and α−2,6 sialic acid receptors – similar to pigs.

Equine Influenza Virus

Equine influenza virus is considered a major disease of horses – particularly for naïve populations. Horses are arguably, the most mobile livestock species on the planet. Equine influenza does not typically kill horses but it does considerable economic harm to equestrian events around the world. Currently, there are only two stable lineages in horses, H7N7 and H3N8. H3N8 is almost worldwide after being first isolated in 1963 with equine epidemics in 1979 and 1989. There was accelerated antigenic drift during the 1980's and 1990's where Eurasian and American lineages emerged. There are three American sublineages. Vaccination in endemic countries is mandatory and subclinical horses can shed the virus. OIE reference laboratories monitor equine influenza antigenic shift and drift (UK, USA, Germany & Ireland), reviewing data annually and publishing recommendations for vaccines as needed in an OIE bulletin. Recommendations have been infrequent with only two recommendations in over the past several years: a recommendation for inclusion of 1995 American & Eurasian strains, and an update of the Eurasian strain in 2004. Of note is that equine influenza transmission to dogs has been recorded for centuries, the recent H3N8 equine influenza virus becoming endemic in dogs being the best documented.

Gaps in our Knowledge of Influenza Virology

There are important gaps in our understanding of influenza virology and studies addressing these gaps are especially critical to support the development of therapeutic and vaccine interventions.

1. Therapeutics and anti-virals. The use of therapeutics and antivirals is often contra-indicated in livestock and poultry because of issues of cost, residues in food products, and importantly, development of resistant strains that could eventually infect people. However, continued research on viral/host protein interactions leading to potential anti-viral drugs will continue to be critical for public health, especially if new pandemic strains emerge that are also resistant to available anti-viral drugs. In addition, anti-virals could play a role in controlling disease outbreaks in animal agriculture in particular situations, e.g., outbreak control (under "vaccination with the intent to slaughter" disease eradication plans) or protecting important genetic stocks, where residues, resistance and cost would not be an issue. The possible application of existing therapeutics/anti-virals (including products rejected for human use) should be evaluated; it would be wise to undertake discovery of new therapeutics/anti-virals relevant to target species.

2. Vaccination. There is an ongoing need for virus studies to support vaccine development. These should explore approaches to improved and sustainable vaccination. Conserved, protective epitopes

need to be identified to support the development of vectored vaccine approaches in particular. In addition, candidate live attenuated influenza viruses (LAIV) should be developed and their safety and stability as vaccines evaluated. Tropism-restricting mutations might be incorporated into the LAIV, or viruses co-modified in the genomic terminal loops and corresponding loop-binding regions of the polymerase might be developed to prevent reassortment with field strains.

Characterization of antigenic structure and evolution is still rudimentary in livestock and poultry (although antigenic cartography is being used experimentally in the analysis of swine isolates to assess matching with vaccines). In poultry, there is a need for antigenic analysis of H5 and H7 viruses to ensure good (neutralizing) matching with potential vaccines. In swine, there is a need for evaluating the risk associated with long-term drift away from human H3N2 strains that could provide a potential source for emergence of new variant viruses for humans. The possibility of developing an approach to vaccine selection that is coordinated with selection of human vaccines to minimize divergence of swine and human vaccines should be considered.

3. Identify the molecular determinants of host specificity. Although mutations at the receptor binding site in the HA gene are known to influence the ability of influenza viruses to infect avian versus mammalian hosts, the determinants that promote the transmission and adaptation of influenza A viruses between vertebrate hosts are essentially unknown. To prevent incursions of influenza A virus between man and animals, this void will have to be addressed. This will require basic studies investigating the molecular pathogenesis of the virus in different animal species. Rapid detection of viruses naturally infecting a new host is also critical to begin to identify the virus and host determinants in the natural process of adaptation. This can only happen with partnerships between all influenza sectors conducting surveillance, diagnostics, and research.

4. Identify the molecular determinants of tissue tropism. Where the virus replicates in a host directly affects virus shed, our ability to detect virus, and determine how virus is spread and how much is released into the environment. Beyond the characterization of receptor specificity for different moieties of sialic acid, which is not completely definitive, there is little known about the determinants of tissue specificity within a host.

5. Identify molecular determinants of virulence in avian species. The hemagglutinin proteolytic cleavage site is known to be the major determinant of virulence of influenza for gallinaceous birds, however there is variation among HPAI viruses in the severity of disease (e.g., mean death time) and clinical presentation (e.g., whether there is neurological involvement). The viral mechanisms for these differences are unknown and should be investigated because of their potential impact on virus spread. Additionally, very little is known about the viral factors that are responsible for virulence in other avian species, most critically domestic breeds of ducks, such as Pekins and Muscovys. Although data with other waterfowl species, such as geese and swans (wild and domestic) are limited, the pathogenesis of AI viruses in these species can directly affect virus spread. The viral mechanisms that cause death and disease (e.g., neurological disease) in waterfowl need to be elucidated.

PATHOGENESIS

Avian Influenza Pathogenesis

Avian influenza (AI) viruses infect a wide variety of domestic poultry, captive birds, and free-ranging wild bird species under natural and experimental conditions. Wild aquatic birds are the main reservoirs of AI viruses and such AI viruses are highly host-adapted, replicating in epithelial cells of the gastrointestinal tract, producing asymptomatic infections. Periodically, these AI viruses transmit from wild aquatic to domestic birds producing subclinical infections, or occasionally respiratory disease and drops in egg production. This phenotype of virus is typically termed low pathogenicity or low pathogenic (LP) AI viruses and can be any combination of the 16 hemagglutinin and 9 neuraminidase subtypes. However, a few H5 and H7 LPAI viruses after circulating in domestic poultry have mutated to produce high pathogenicity or highly pathogenic (HP) AI viruses. These HPAI viruses cause severe systemic disease and high mortality in gallinaceous poultry. Historically, with some exceptions, HPAI viruses have not infected wild birds. However, since 2002, the Eurasian-African H5N1 HPAI viruses have caused infections, illness and death in a variety of captive, zoo and wild birds. Several factors affect the complex biology of AI viruses including infectivity, host adaptation, virulence and tissue tropism, and these pathobiological features vary with host species and virus strain.[19,34-37]

Pathotypes

Based on pathogenicity (the ability to produce disease), AI viruses from poultry are classified into two pathotypes: HP and LP. To foster international control of specific AI viruses, the OIE Terrestrial Code lists "notifiable" AI viruses as all HPAI (i.e., HPNAI) viruses and all H5 and H7 LPAI (i.e. LPNAI) viruses.[38] H5 and H7 LPAI viruses became LPNAI in 2006 because these subtypes can mutate to the HP form when allowed to circulate in poultry populations. The OIE Terrestrial Code now lists "notifiable" AI (HPNAI and LPNAI) as follows[38]:

1. HPNAI viruses have an intravenous pathogenicity index (IVPI) in 6-week-old chickens greater than 1.2 or, as an alternative, cause at least 75% mortality in 4-to 8-week-old chickens infected intravenously. H5 and H7 viruses which do not have an IVPI of greater than 1.2 or cause less than 75% mortality in an intravenous lethality test should be sequenced to determine whether multiple basic amino acids are present at the cleavage site of the hemagglutinin molecule (HA0); if the amino acid motif is similar to that observed for other HPNAI isolates, the isolate being tested should be considered as HPNAI viruses;
2. LPNAI viruses are all influenza A viruses of H5 and H7 subtype that are not HPNAI viruses.

In addition, the OIE code creates by default a third category of AI viruses – non-H5 and non-H7 LPAI viruses for which there is no formal requirement to report to OIE, unless they are causing a severe disease, but these viruses may be reportable to national and state/provincial authorities. However, based on pathobiological criteria (disease, lesions and signalment), LPAI viruses are indistinguishable irrespective of the H and N subtype. Although the pathogenicity classification is specific for chickens, similar *in vivo* test results have been obtained for related birds in the order *Galliformes*.[39] However, most AI viruses that are HP for chickens have been LP for domestic ducks except for some strains of H5N1 HPAI virus, which are also highly lethal for young domestic ducks, but not always highly lethal in older ducks.[39,40] Pathogenicity test results are specific for the host used in the test, and being an H5 or H7 subtypes are not predictors of HP; i.e., only a small percentage of H5 and H7 AI viruses have mutated to the HP phenotype. By contrast, all naturally occurring H1-4, H6, and H8-16 AI viruses have been of low virulence (LP) for chickens experimentally when given by the natural route of challenge.

Pathogenicity

Although only two pathotypes of AI viruses can be demonstrated in the laboratory (HP and LP), natural infection by AI viruses results in a wide range of clinical outcomes which are dependent on virus strain, host species, host age, host immunity, and environmental factors.[34] From mortality patterns, clinical signs, and lesions in the field, AI can be categorized into four clinical groups: 1) highly virulent, 2) moderately virulent, 3) mildly virulent, and 4) avirulent.[19] First, the highly virulent clinical group results from infection by HP H5 or H7 AI viruses usually in chickens or closely related gallinaceous birds and is expressed as a severe, highly fatal systemic disease that affects most organ systems. Morbidity and mortality approach 100%. Experimentally, the HPAI viruses alone reproduce the lesions and high mortality rates seen in the field.[41] Second, the moderately virulent clinical group results from infection by LPAI viruses, of any HA or NA subtype, usually with co-infection by secondary pathogens or accompanied by other stress factors.[42,43] The mortality rates vary but range from 5-97% with the highest mortality occurring in young birds, reproductively active hens, or severely stressed birds.[44,45] Lesions usually have been in the respiratory tract, reproductive organs, kidney, or pancreas.[46,47] Third, the mildly virulent clinical group results from infection by LPAI virus producing low mortality and mild respiratory disease or drops in egg production. Mortality is usually less than 5%, and is typically in older birds. Fourth, the avirulent clinical group results from infections by LPAI viruses without any increased mortality or clinical signs. This has been most frequent with infections by LPAI viruses in wild birds of orders *Anseriformes* and *Charadriiformes*.[36] In poultry, this has been seen following the introduction of a poorly host-adapted LPAI virus from wild birds. Such an example would be the first cases of AI in range turkeys following exposure to wild waterfowl AI viruses that resulted in seroconversion detected at slaughter without any previously noted clinical signs.[36]

Avian influenza viral pathogenesis

The HA gene is the primary but not the only determinant of high pathogenicity in chickens; a proper constellation of all eight gene segments is required for the maximal expression of virulence potential.[48] To initiate the infection process in birds, the HA must first bind to α2,3-galactose linkage cell receptors to initiate receptor-mediated endocytosis. In addition, fusion of the viral envelop with endosome wall requires a cleaved HA. This cleavage of the HA protein into the HA1 and HA2 proteins is essential for the virus to be infectious and produce multiple replication cycles. With LPAI viruses, they are released from the host cell with an uncleaved HA protein and are not infectious. The protein can be cleaved by trypsin-like proteases found in restricted anatomical sites, such as respiratory and intestinal epithelial cells, which accounts for the restricted replication and lower virulence. The difference between the cleavage site of LPAI and HPAI viruses is the number of basic amino acids in the HA1 near the cleavage site or an insertion of amino acids near the cleavage site that determines whether trypsin-like proteases or furin-like proteases can cleave the protein. The LPAI viruses generally have only two non-consecutive basic amino acids at the carboxy-terminus of the HA1 that is only cleavable by trypsin-like proteases. In contrast, H5 and H7 HPAI viruses have either multiple basic amino acids or an insertion of amino acids at the carboxy-terminal of the HA1 protein that allows proteolytic cleavage by ubiquitous furin proteases that are present in many cells throughout the body.[49] This increases the cell tropism of the virus leading to virus replication in numerous visceral organs, the nervous system, and the cardiovascular system leading to systemic disease with high mortality.

An additional factor, the presence or absence of a glycosylation site at the amino terminal end of the HA1 protein, has been shown to influence HA cleavage. Changes in a glycosylation site of the neuraminidase (NA) also play a role in the pathogenicity of HPAI viruses in chickens.[50] Additionally, changes in the

polymerase PB2 and PB1-F2, the nucleoprotein (NP), and the non-structural protein (NS) can experimentally alter the pathogenicity of HPAI viruses in chickens.[51-58] In addition to mutations in these genes, the combination of RNP components (NP and polymerases PB1, PB2 and PA), which function as a unit, can effectively attenuate or increase virus virulence in chickens.[24,50,55,59,60]

Pathogenicity of the H5N1 HPAI viruses in ducks appears to be multigenic as increase in virulence has been associated with changes in the HA, NS, NP, PA, PB1 and PB2 genes.[23,26,61-64]

Mechanisms of cellular pathobiology

For LPAI viruses, the nasal cavity is the predominant initial site of virus replication with spread to other parts of respiratory tract and the intestinal tract.[35,37] However, secondary bacterial, fungal or viral infections are usually necessary to produce sufficiently severe respiratory damage to result in illness or death. Rarely, LPAI viruses have spread systemically, causing infection and damage in epithelial-containing tissues of visceral organs such as kidney, pancreas, and oviduct.

With HPAI viruses, infection in chickens is initiated in the nasal epithelium within 16 hours after direct intranasal exposure and, by 24 hours, the nasal epithelium is necrotic with accompanying submucosal inflammation and virus in capillary endothelial cells.[35,37] Inflammatory cells play important roles in the initial replication and dissemination of HPAI viruses, as do virus replication within endothelial cells and spread through the vascular or lymphatic systems. Such viremia, allows dissemination of HPAI virus, and initiates replication in a variety of parenchymal cell types within visceral organs, brain and skin. With the most virulent HPAI viruses, following intranasal exposure, replication may be seen within 24 hours in visceral organs. By 48 hours, the virus titers may be maximal and the lesions severe. However, some HPAI viruses require a longer period of time to produce illness and death. These HPAI viruses produce viremia with lack of or minimal vascular endothelial cell replication but tend to have extensive replication in parenchymal cells of visceral organs. With some HPAI viruses, increased vascular permeability is responsible for edema, hemorrhage and multiple organ failure with associated damaged vascular endothelial cells and accompanying microthrombosis. If the HPAI virus infected chicken survives the peracute phase (days 1-2 after exposure), the virus may disseminate and replicate in multiple critical organs causing single or multi-organ failure and death with involvement of the brain and autonomic nervous system, cardiac myocytes, endocrine tissue (e.g., adrenal gland) and/or pancreas.

Clinical Signs

The pathotype of AI virus (LP or HP) has a major impact on the clinical manifestation of the disease. However, clinical signs of disease are extremely variable and depend on other factors including host species, age, sex, concurrent infections, acquired immunity, and environmental factors.[19]

Most infections by LPAI viruses in wild birds produce no clinical signs. However, in experimental studies in mallard ducks, LPAI virus infections suppressed T-cell function and produced a depression in egg production.[65]

In domestic poultry (chickens and turkeys), clinical signs reflect abnormalities in the respiratory, digestive, urinary, and reproductive organs. The most frequent signs represent infection of the respiratory tract and include mild to severe respiratory signs such as coughing, sneezing, rales, rattles, and excessive lacrimation. In layers and breeders, hens may exhibit increased broodiness and decreased egg production. In addition, domestic poultry will exhibit generalized clinical signs including huddling, ruffled feathers,

listlessness, decreased activity, lethargy, decreased feed and water consumption, and occasionally diarrhea. Emaciation has been reported but is infrequent because AI is an acute, not a chronic disease. In ratites, LPAI viruses produced similar respiratory signs to those in gallinaceous poultry and in some cases green diarrhea or green "urine".[66-68]

In wild and domestic waterfowl, most HPAI viruses replicate to a limited degree and produce few clinical signs because of poor adaptation to non-gallinaceous species. The major exception to this rule are many H5N1 HPAI viruses that can infect and cause clinical disease including neurological signs, depression, anorexia and sudden death.[37] Occasional sporadic, isolated cases of mortality have been reported in wild birds with other HPAI viruses.[69] In domestic chickens, turkeys, and related galliformes, clinical signs reflect virus replication and damage to multiple visceral organs, and cardiovascular and nervous systems. However, clinical manifestations vary depending on the extent of damage to specific organs and tissues.[37] In most cases in chickens and turkeys, the disease is fulminating with some birds being found dead prior to observation of any clinical signs. If the disease is less fulminating and birds survive for 3-7 days, individual birds may exhibit nervous disorders such as tremors of head and neck, inability to stand, torticollis, opisthotonus, and other unusual positions of head and appendages. The poultry houses may be unusually quiet because of decreased activity and reduction in normal vocalizations of the birds. Listlessness is common as are significant declines in feed and water consumption. Precipitous drops in egg production occur in breeders and layers with typical declines including total cessation of egg production within six days. Respiratory signs are less prominent than with LPAI viruses but can include rales, sneezing, and coughing. Other poultry have similar clinical signs but may live longer and have evidence of neurologic disorders such as paresis, paralysis, torticollis, and general behavior aberrations.[70] In ostriches, reduced activity and appetite, listlessness, ruffled feathers, sneezing, hemorrhagic diarrhea and open mouth breathing have been reported.[68,71] In addition, some birds can be uncoordinated, exhibited torticollis, and have paralysis of the wings and tremors of the head and neck.

Morbidity and Mortality

In chickens, turkeys, and related gallinaceous birds, morbidity and mortality rates are as variable as the signs and are dependent on virus pathogenicity and the host as well as age, environmental conditions, and concurrent infections.[72] For the LPAI viruses, high morbidity and low mortality rates are typical. Mortality rates are usually less than 5% unless accompanied by secondary pathogens or if the disease is in young birds. With the HPAI viruses, morbidity and mortality rates are very high (50-89%) and can reach 100% in some flocks. Typically, the virus spreads rapidly among poultry housed on the floor with peak mortality (70-100%) occurring in 3-5 days of first clinical signs, but in poultry housed in cages, the virus spreads slower through the house with peak mortality taking 10-15 days.

H5N1 HPAI virus lineage has caused illness and death a variety of wild aquatic and terrestrial birds. Many H5N1 HPAI viruses have been shown to replicate systemically and produce mortality in ducks.[37,73-77] The age and species of the ducks also influences the outcome of the infection, with younger ducks and some duck species being more likely to show clinical signs after infection.[40,77-80] In ostriches, LP and HPAI viruses usually produce moderate morbidity and low mortality rates but this depends on the strain of virus.

Gross and microscopic lesions

Macroscopically, in LPAI, rhinitis and sinusitis are frequently present, and if accompanied by secondary bacterial infections, swollen infraorbital sinuses and nasal discharge may be present, especially in turkeys.

The tracheal mucosa may be reddened from congestion, with edema and, occasionally hemorrhages and luminal exudates.[19] Occasionally, tracheal exudates form plugs that occlude airways with resulting asphyxiation. When secondary bacterial pathogens are present, fibrinopurulent bronchopneumonia, air sacculitis and coelomitis ("peritonitis") may be present. In hens, the ovaries may regress and mature ova rupture producing free yolk in the coelomic cavity or egg yolk peritonitis. The last few eggs produced may lack pigment, be thin shelled and be misshapen. Rarely, laying hens may have swollen kidneys with accompanying renal failure. Mild enteritis may be present, particularly in turkeys. Microscopically, most frequently, LPAI viruses have produced lymphocytic rhinitis, sinusitis, tracheitis and bronchitis with common demonstration of AI viral antigen in epithelial cells of the upper respiratory tract. On rare occasions, nephrosis and nephritis were present in hens. In experimental studies and natural cases in turkeys during the 1999 Italian H7N1 LPAI outbreak, necrosis in the pancreas was reported.[67]

For HPAI, the frequency of gross lesions varies with species of bird and virus strain, and all lesions are not consistently present in all birds. Generally, HPAI virus infections affect multiple visceral organs, the cardiovascular and nervous systems and the integument, producing necrosis, edema and hemorrhage. In peracute disease, no gross lesions may be seen. In acute disease, birds may have ruffled feathers, and swelling (edema) of the comb, wattles, periorbital and intermandibular areas, upper neck, leg shanks and feet with accompanying subcutaneous hemorrhages, especially of the non-feathered skin. Some virus strains produce edema and hyperemia of the conjunctiva, eyelids, and trachea. The wattles, combs and snoods may contain necrotic foci and hemorrhages, and be cyanotic. Internally, hemorrhages may be present on serosal or mucosal surfaces, and necrotic foci within multiple visceral organs. Unique to the recent Eurasian-African H5N1 HPAI virus lineage and classic fowl plague viruses is the production of necrosis and hemorrhage in Peyer's patches of the small intestine, severe edema and hemorrhage in the lungs, and occasionally, edema of the brain. White foci of necrosis may be present in the heart, and occasionally, liver and kidneys. Urate deposits may accompany the necrosis in kidney. The lungs may be firm from edema and interstitial pneumonia and have congestion and hemorrhages. In young birds, the primary lymphoid organs (cloacal bursa and thymus) may be atrophic, with or without hemorrhage. The spleen may be enlarged with pale necrotic foci or be normal in size.

Microscopic lesions are more frequent than gross lesions in most HPAI cases. Histopathological lesions in birds from experimental studies vary with virus strain and passage history; inoculum dose and route of inoculation; and species, strain and breed of bird host.[19] Most frequently, histological changes consist of necrosis and/or inflammation in multiple organs, most often and severe within the skin (including feather follicles), brain, heart, pancreas, lungs, adrenal glands, and primary and secondary lymphoid organs. In peracute disease, microscopic lesions are lacking in most organs, but occasionally, mild or multifocal necrotic and inflammatory lesion are seen with virus demonstration principally in vascular endothelial cells and cardiac myocytes. In acute disease, visceral organs may have multiple foci of necrosis, and associated inflammation, hemorrhage, and edema. However, necrosis is less prominent and inflammation more prominent in birds that survive longest. AI viral antigen is associated with areas of necrosis and inflammation, but not in apoptotic lymphocytes. Lesions are similar in other gallinaceous species, but since such birds survive longer than chickens or turkeys, inflammation is more common and prominent than necrosis in parenchymal organs.

Swine Influenza Pathogenesis

Pigs infected with IAV show a spectrum of clinical disease, from remaining clinically unaffected to high fevers and severe respiratory signs. The clinical range may be due to prior immunity, properties of the

virus, and many other contributing health and environmental factors. Clinical signs of influenza in pigs are similar to those observed in humans and are manifested as acute respiratory disease characterized by fever, inactivity, decreased food intake, respiratory distress, coughing, sneezing, conjunctivitis and nasal discharge.[81-84] The disease incubation period is between 1 and 3 days with rapid recovery beginning 4 to 7 days after onset. Individual pigs typically begin shedding approximately 24-48 hours after exposure and continue shedding for approximately 5-7 days after exposure.[85] Fevers typically peak 24-48 hours post exposure, and can be a good indicator for actively infected pigs. However, not all infected pigs will consistently demonstrate a febrile response and changes in behavior, such as reduced water or food intake and absence of flight response from humans, may need to be used in addition or instead of monitoring for fevers. Swine influenza is characterized by high morbidity (approaching 100%) and generally low mortality (<1%) rates and may sweep through a naïve herd or more slowly roll through a population with partial or mixed immune status. Macroscopically, swine IAV-infected lungs lesions are described as purple-red, multifocal to coalescing consolidation of predominantly the cranioventral lung lobes. Microscopic changes in the lung consist of necrosis of bronchiolar epithelial cells and sloughing of these cells into airway lumen, which often contains cellular debris, proteinaceous fluid and a few leukocytes. This necrosis is accompanied by peribronchiolar lymphocytic infiltration and interstitial pneumonia of variable severity. During recovery, bronchiolar epithelium becomes proliferative and lymphocytic cuffs become more prominent. Influenza viruses are commonly found in cases of porcine respiratory disease complex (PRDC), acting in concert with other pathogens.[86,87]

Gaps in our Knowledge of Influenza Virus Pathogenesis

Gaps exist in our knowledge of many areas of influenza virus pathogenesis. Although the role of the multi-basic cleavage site (MBCS) in poultry is well understood, the role of other determinants in contributing to virulence in poultry is less clear. It is not clear why HPAI are limited to H5 and H7 subtypes. The basis for the lack of virulence of H5N1 in pigs is unclear, as is its virulence in some species of aquatic birds (e.g. Pekin ducks). Priorities include:

1. There is a need to identify determinants of virulence, innate immune evasion, shedding/transmission, across target species.
2. To identify virus determinants (other than MBCS) of local replication and potential systemic spread in target species.
3. To identify determinants of virus shedding from the respiratory and intestinal tracts (support understanding transmission)
4. To identify the determinants of efficient virus infection at mucosal portals of entry (support transmission studies).
5. To identify virus and host determinants of host range restriction to identify mechanisms by which viruses adapt to new host species, e.g., from birds to mammals, and vice versa, (adaptation should also be studied directly, where necessary or useful, as in ferret transmission studies).
6. To investigate whether increased virulence of H5N1 in ducks is due to enhanced replication or systemic spread, or to altered innate responses (whether increased or decreased).
7. To study immunopathology, particularly dysregulation of the innate responses (or the lack of it) in target species.
8. To explore possible influence of virus on host gene expression by novel mechanisms; e.g., microRNAs
9. To develop appropriate systems for *in vivo* tracking of model viruses in target species.
10. To investigate the influence of co-infecting immunosuppressive viruses (CAV, IBDV) and other respiratory viruses (NDV, PCV2, PRRSV) on influenza transmission and pathogenesis.

Many of the gaps in pathogenesis identified above serve to highlight gaps in the study of the host side of the virus-host interaction. There are, for instance, major gaps in our understanding of what constitutes an optimal acquired immune response for effective suppression of shedding to interrupt transmission. It is also not clear what the optimal balance is for cellular versus humoral, or systemic versus mucosal responses for an effective immune response. There is also a gap in the investigation of potential novel anti-viral mechanisms (including microRNAs), some of which may prove to be species-specific. Concerted and effective analysis of virus:host interactions requires complete and reliable host genome assemblies and annotations, particularly where one species serves as the model template for related species (e.g. chicken for other avian spp.). There is, therefore, a need for critical review of the current status of livestock genome assemblies and annotations, with appropriate remedial action should it be indicated that this is required.

There is often a paucity of reagents for working with livestock species. This is unlikely to be overcome in the short-term but it could be mitigated by promoting effective sharing of reagents and resources for livestock species, such as siRNA and gateway-type innate response gene expression libraries, or monoclonal antibody panels, commissioning the production of such resources where necessary.

IMMUNOLOGY

Avian Influenza Virus Immunology

Because infection of poultry (namely chickens and turkeys) with HPAI may result in death within a matter of days, the adaptive immune response contributes little to protection from disease in unvaccinated birds. In contrast, the innate and adaptive immune response of some wild bird species, including ducks, is able to protect against disease. However, because wild birds may receive exposure to either LPAI or HPAI during their lives they may have established protective immunity to subsequent infection. Thus, the mechanism of immunological protection against influenza, either through innate-immunogenetics or vaccine-induced, emphasizes the need for gaining a thorough understanding of the avian immune system in order to advance more effective control strategies.

Innate Immunity to Avian Influenza Virus

Previous research has demonstrated that AI viruses are sensitive to the antiviral effects of Type I interferon (IFN), in particular IFNα.[88-91] Expression of Type I IFN is strictly regulated by host transcription factors nuclear factor kappa-beta, (NF-κβ) and interferon regulatory factors.[92] Production and detection of IFNα results in host gene transcription of interferon-stimulated response elements (ISRE), including myxovirus resistance gene (Mx), 2',5'-oligoadenylate synthetases (2,5 OAS), and RNA activated protein kinase (PKR), all of which inhibit protein synthesis and/or virus replication.[93] The timing and level of expression of these ISRE in birds, in terms of resistance or susceptibility to Avian Influenza virus infection, remains unknown.

AI viruses have developed various antagonistic mechanisms to overcome the host IFN response, which appears to be the role of the viral NS1 protein. The NS1 protein has not been detected within individual viral particles, but rather is only expressed upon entry into the host cell. Two functional binding domains have been described for NS1, including a RNA-binding domain and an effector domain. The RNA-binding domain is believed to sequester viral RNA from detection by host NOD proteins, including RIG-I, which recognize uncapped 5' triphosphate RNA ends found in RNA genomes.[94]

Detection of viral RNA through RIG-I proteins can result in cytokine and IFN production. The effector domain has been shown to interact with various viral and cellular proteins including, the viral polymerase complex and cellular transcription/ translation factors.[95-97] However, as most of these studies have been done in mammalian systems, it is impossible to extrapolate these results to avian models given the differences in immune systems between the two species. Ducks were recently shown to have a functional retinoic acid-inducible gene 1 (RIG-I), which recognizes foreign nucleotide domains, found in viral genomes of influenza-infected cells.[98] RIG-I is able to mobilize the innate immune response by upregulating transcription factors that produce cytokine and interferon responses. That study has reported that RIG-I is not present in the chicken genome, which suggests a possible mechanism for why chickens and turkeys are more susceptible to disease as compared to ducks. Whether other avian species possess a functional RIG-I or homolog is a critical gap in our knowledge.

A number of recent microarray reports indicate that the early host transcriptome response of chickens to LPAI includes activation of proinflammatory cytokines, IL-1β and IL-6.[99-102] Interestingly, a strain-dependent suppression of antibody response was observed in H9N2 infected chickens, but not H6N2 infected birds.[100] Thus it appears that some LPAI viruses maintain the ability to modulate the immune response following infection.[102] The mechanism of immune modulation remains unknown, although as mentioned above, the NS1 protein appears to interact with interferon regulation. Microarray analysis following HPAI infection has demonstrated a hyperactive/unregulated cytokine response that is believed to contribute to disease pathology. Why this difference exists and how it may explain the difference between a susceptible versus protected bird is a gap in our understanding of host immunity.

Humoral Immunity to Avian Influenza Virus

Vaccine-induced protective immunity against AI viruses is primarily the result of an antibody response because abrogation of the bird's ability to mount a humoral response, by removing the cloacal bursa, followed by vaccination with AI virus vaccine, eliminated protection against a HPAI virus challenge.[103] Vaccination results in the production of neutralizing IgY (avian IgG equivalent) antibodies against the hemagglutinin protein (HA) that block viral attachment. Such protection is specific to each of the 16 different HA subtypes (H1-16); e.g., H5 vaccines protect against H5 challenge viruses but not against H1-4 or H6-16 AI viruses. Antibodies against the neuraminidase (N1-9) can be complementary in protection by reducing the efficiency of virus release, but are also subtype specific. Also anti-NA antibodies are only partially protective, especially when used as the sole immunogen.[104-108] In addition to the viral coat proteins (HA and NA), infection by AI virus produces an immune response against the internal influenza proteins, such as nucleoprotein, polymerase or matrix protein, but humoral immunity against them does not provide significant protection in poultry.[109,110] Interestingly, the M2 protein is appealing as an immunogen because the ectodomain or exterior portion of M2 (known as M2e) is highly conserved among influenza A viruses and provides an exposed target for immune competent cells.[111-113] M2e-specific antibodies have the potential to generate broad-spectrum immunity across influenza A viruses, and has been demonstrated to be protective in mice.[114,115] Attenuated salmonella vectored vaccines expressing the AI M2e gene have also demonstrated increased protection from disease and viral shedding following LPAI challenge.[116]

Humoral immunity is also affected by rapid mutation of the virus. One of the major biological results of this is rapid antigenic changes and the emergence of immunological escape mutants.[117-119] Practically, this means that vaccine seed strains must be continually updated to maintain adequate efficacy. Currently selection of seed strains relies to some extent on protein sequence identity of the HA between

the vaccine strain and target field virus, however protein sequence and antigenic matches are not always correlated. Antigenic mapping using HI assay data by antigenic cartography has been used to map human seasonal influenza and is used by the World Health Organization (WHO) to help select the seasonal influenza vaccine strains.[119] Application of this tool to poultry AIV vaccines would greatly improve our ability to select efficacious vaccine strains and would illuminate species specific differences in antibody specificity for AIV.

Cell-mediated Immunity to Avian Influenza Virus

Cell-mediated immunity (CMI) is specific immunity mediated by T lymphocytes and has been suggested to be an important factor to the development of protection in vaccinated animals against viral diseases.[120] The subsets of T lymphocytes: CD4+- helper cells and CD8+-cytotoxic cells constitute the principal cells of the CMI response. A number of studies have demonstrated the importance of CD4+ T-cells against influenza.[121-123] Likewise, CD8+ CTLs play a crucial role in controlling infectious virus from the lungs of mice. Many studies have provided evidence that CD8+ CTL directed against viral epitopes conserved among influenza A viruses, including the nucleoprotein (NP), contribute to protection against influenza.[124-126]

In mice and humans, the CTL responses specific for influenza A viruses have been best described, and shown to be broad and multispecific.[127-129] Yet the overall contribution of the cellular aspect to protection remains unknown since it takes 5-7 days for virus-specific CD8+ cells to migrate and localize in lungs following a primary infection, whereas secondary responses by memory T cells have been shown at 3 days.[130] The benefits of a secondary cellular response against influenza A virus have been shown to decrease duration and amount of viral shedding and decrease severity of disease.[131]

There is limited data on CTL responses to influenza viruses in poultry, which requires the availability of inbred chickens. In initial testing, different MHC-haplotypes did not confer resistance to HPAI infection in chickens.[132] In addition, little is known about cross reactive cellular immunity following AI infection in birds. In particular, limited data exists directly examining cross reactive T-cells from birds against homo-and heterosubtypic AI. It has been demonstrated that cross reactive CTLs are produced in chickens following infection.[133] That study demonstrated adaptive transfer of the AIV-specific CD4+ and CD8+ T-cells from H9N2-infected birds protected naïve-birds against lethal H5N1 challenge (A/Chicken/Hong Kong/97), suggesting that cellular immunity alone could protect against HPAI virus challenge. It has also been demonstrated that following natural infection with LPAI, CTL produced against one subtype will react against other subtype.[134] However, because more recent HPAI viruses cause rapid, sudden mortality (mean death times of 2 days), and mobilization of memory T-cells to target organs may take 3 or more days, protection from disease may not be reasonable without vaccination or a rapid memory response.[135]

Recently, a number of T cell epitopes from various proteins of influenza virus have been identified in mice and humans.[136] These studies identified over one-hundred CD4+ T cell and thirty-five CD8+ T cell epitopes within the HA protein alone and some of these epitopes have been shown to induce immune responses and confer protection in challenge studies.[137-140] Functional studies have determined that epitopes derived from nucleoprotein (NP), polymerase acidic (PA) and M proteins of influenza virus demonstrated a strong cytotoxic T cell response.[125,141-143] Thus the contribution of CMI through T cell involvement may be important to disease outcome. With regards to birds, a recent report describes the first identification of a CD4+ and CD8+ specific epitope on the HA protein from H5 avian influenza

in chickens. These epitopes appear to induce cross reactive CTLs against multiple AI subtype.[144] Given the importance of T-cell epitopes to protection, and our lack of knowledge as it pertains to ducks, results of these studies will lay a foundation to better understand the role of CMI and how it relates to disease control and transmission of AI viruses.

Protective Immunity and Avian Influenza Vaccines

Protective immunity may be described as an achieved level of immune-related function that allows a bird to resist disease following exposure to a particular avian influenza virus. More specifically, this term refers to induction of antibodies (humoral immunity) or CTLs (cellular immunity) or a combination of both, following vaccination or natural infection, which results in protection of the host from mortality, and usually morbidity. In the context of AI vaccination, protective immunity should not only protect from disease, but also decrease the magnitude of any infection and the resulting duration and virus titers shed into the environment; thus reducing transmission to susceptible cohorts. In general, inactivated vaccines primarily provide protection via humoral immunity, while live virus or some recombinant vaccines will develop both humoral and cellular immunity that may be superior to either type of immunity produced singularly.

Many different types of experimental AI vaccines have been described and some have been licensed for commercial use.[35,145] Categories of vaccines include the following: inactivated, live, subunit, recombinant-vectors expressing AI virus genes, and DNA vaccines. While many of these vaccines have proven to induce protective immunity in the laboratory under optimal conditions, the final proof of protection and efficacy must be demonstrated under field application. For field use, the overwhelming majority of AI vaccines produced and sold have been oil-emulsion inactivated whole AI virus vaccines delivered via parenteral route (subcutaneous or intramuscular), although recombinant vectored vaccines (fowlpox virus or herpes virus of turkeys) are gaining popularity in use. Because these recombinant vaccines are live, and replicate inside of host cells, they have the ability to induce both humoral and cellular immunity following vaccination of chickens.

Vaccine-induced protective immunity of poultry against AI viruses is primarily the result of an antibody response directed against the hemagglutinin (HA).[135] Antibodies produced against the HA are virus-neutralizing and thus prevent attachment of the virus to host cells or fusion with the host membrane. A minor contribution to immunity is provided against the NA protein, of which there are 9 different NA subtypes.[146] Our current knowledge of protective immunity against AI is derived from experimental HPAI challenge studies,[104-107] in which the NA in a whole AI virus vaccine provided mostly partial protection from mortality. However, immunization with NA protein alone produced only partial protection following 2-3 vaccinations.[108] Because protection is provided through an immune response to the HA, the more efficacious vaccines target the specific phylogenetic lineages of the virus within a HA subtype.[147]

To increase immunogenicity of inactivated AI vaccines, most are blended with oil to form an emulsion. The emulsions are prepared by mixing a water-based antigen phase with an oil phase, normally containing surfactant for stabilization (e.g. sorbitan monoleate and Tween 80) to produce a water-in-oil emulsion. Without other components, oil-based adjuvants stimulate mainly antibody responses, although under some circumstances water-in-oil emulsions may be able to activate CTLs.[148,149] The type of oil used in vaccine production can affect the overall immune response to the vaccine, with non-metabolizable oils (e.g., mineral oil) enhancing antibody responses over biodegradable oils (e.g.,

vegetable).[150,151] Besides having adjuvant properties, oil-emulsion vaccines slowly release antigen over time, resulting in higher immune responses than would be produced by the antigen alone.
In terms of AI vaccination to protect poultry, there are four ideal goals: (i) protection from clinical disease, (ii) increase the threshold of virus dose required for infection, (iii) reduce the amount of virus excretion if the bird is infected, and (iv) the ability to differentiate infected from vaccinated animals. The risk of infection of vaccinated birds with and subsequent excretion of, virulent field virus is usually reduced but not fully prevented. This results in an epidemiological problem in endemic areas where vaccinated birds appear healthy but may well be infected and excrete the field virus without showing clinical signs of disease. Thus, improved AI vaccines are needed in the event of an outbreak of HPAI and to better control commonly circulating H5/H7 LPAI strains that have the ability to mutate to HP. The effectiveness of reduction of virus excretion is linked to both a reduction in titer of the virus excreted and the duration of viral shedding.

Gaps in our Knowledge of Avian Influenza Virus Immunology

Gaps exist in our knowledge of many areas of AI virus immunology. Although the role of antibody induced protection from disease is fairly well understood, the role of other contributing factors to immunological protection is less clear. It is not clear why some birds are resistant to HPAI and others are highly susceptible. The likely genetic basis for this resistance must be identified and applied where possible. Priorities include:

1. To understand the innate immune response of different species including the determination of the timing and expression of cytokines and interferons following influenza virus infection that result in decreased virus shedding from host cells, and results in increased resistance of birds to infection.
2. To determine the mechanisms of influenza virus resistance contributed by the duck RIG-I gene as it relates to enhanced innate immunity and how this can be applied to susceptible avian species.
3. To determine the source and contribution of unregulated cytokine expression following HPAI virus infection, which is believed to contribute to disease pathology. Why differences exist between individual viruses and different hosts, which may explain the difference between a susceptible versus protected bird is a gap in our understanding of host immunity.
4. To determine antibody response profiles against influenza B-cell epitopes to identify those involved with virus neutralization and target receptor binding domains for improved vaccine selection based on virus amino acid sequences recognized by MHC class II molecules.
5. To determine the role and importance of T-cell epitopes to protection following infection, and how cell-mediated immunity contributes to immunity in wild birds versus poultry, in particular how it contributes to disease control and transmission.
6. To determine the immunological response of poultry to recombinant live vaccines and define the humoral and cellular protection induced by their application, to include examination of cross protection.
7. To develop cost effective adjuvants for inactivated or subunit vaccines that improve the host's immune response resulting in longer protection, reduce the number of injections required for protection, and provide for broad cross protection against antigenic variants.
8. To develop adjuvants that work well between species, particularly adjuvants that provide protection for domestic waterfowl species.
9. To determine the immunological mechanism of how maternal immunity suppresses the host immune response after both vaccination and natural infection.

10. To determine the contribution of host immunogenetics on innate protection of birds by developing transgenic animals to study host-virus interactions.

Swine Influenza Immunology

Protective immunity against infection with influenza in many species has been intensively studied and involves innate and adaptive immune responses (for a concise yet comprehensive review see (Doherty et al.[152]). However, specific studies with swine adapted influenza A viruses in the swine host are limited. Innate immunity involves Type 1 interferons produced by infected respiratory epithelial cells, pro-inflammatory cytokines and chemokines, neutrophils, natural killer cells, and phagocytic cells. These early signals clearly play a critical role in the host response and recent advances in the understanding of cellular pathways have shown that pathogen recognition converges in a number of innate responses. These early innate factors can have a limiting effect on influenza infection, but importantly, they signal the adaptive arm of the immune system and stimulate the cells necessary to activate lymphocytes.

The adaptive immune response includes both humoral and cellular immunity in the systemic and mucosal compartments. However, the relationship between protective humoral and cellular immunity is neither simple nor readily predicted.[153] When swine are infected with a virulent influenza virus, complete protective immunity typically develops against re-challenge with homologous virus, i.e., there is little or no detectable virus replication following secondary challenge and there are no lesions associated with challenge.[154,155] Antibodies play a significant role in attenuating/preventing this disease and this provides the basis behind current vaccination efforts. In pigs, the argument for humoral immunity being critical is based on the ability of antibodies to neutralize swine influenza virus,[156] the protective quality of colostrum in young pigs[157] and the protection provided by inactivated vaccines[155,158] that primarily stimulate humoral immune responses. The targets of humoral immunity are primarily the hemagglutinin, neuraminidase and matrix proteins of swine influenza. H1 and H3 HA proteins from human seasonal influenza viruses have been studied to identify the major antibody epitopes and critical amino acids or locations in the 3-dimensional structure of the HA trimer that lead to antigenic drift upon mutation. Although recognition may be similar in swine, studies to compare or confirm the human findings in the swine host have only recently been reported.[159]

Clinical protection against challenge virus is correlated with the hemagglutination inhibition (HI) titer in the serum of an individual animal,[158,160] i.e., a high HI titer provides better protection against challenge than a low HI titer. This information has led to the suggestion that the presence and magnitude of a HI titer could be a predictor of protection, however this is likely only true when the priming HA inducing the HI titer is closely related antigenically to the HA of the challenge virus. Other studies have demonstrated the protective qualities of antibodies at the mucosal level. Pigs immunized with virulent SIV and then challenged with the same virus 42 days later did not have a detectable anamnestic serum antibody response.[154] However, an anamnestic mucosal immune response (rise in IgA and IgG) was detected in the nasal cavity, the site of challenge, indicating that this compartment of the immune system was stimulated. These data support the hypothesis that antibody mediated protection at the mucosal level is important for clearing the respiratory tract of SIV and may not be accurately reflected by systemic antibody levels. Further complicating the reliance on HI titers as the gold standard for a correlate of protection, numerous studies have demonstrated significant protection against infection and clinical disease in the absence of detectable HI titers. Studies with live attenuated influenza vaccines in pigs have shown the HI titers to not always be predictive of protection against challenge.[161,162] Additionally, a recent study with a vectored vaccine given intranasally, induced a mucosal IgA response and provided

complete protection from homologous challenge and partial protection from heterologous challenge, in the absence of detectable serum HI titers.[163]

When the humoral response fails to prevent infection, cell mediated immunity (CMI) is believed to play a dominant role in clearance of the SIV infection; however, evidence for CMI in pigs is primarily associated with natural infection or the use of experimental live attenuated or vectored vaccines rather than commercially approved inactivated vaccines.[163-166] T cells mediating CMI against influenza, can target internal proteins common to heterologous viral strains (as reviewed in Thomas et al.,[167]). Some studies have identified a relatively small number of targeted influenza epitopes recognized by cytotoxic T cells in humans.[168] However, specific target epitopes of CMI in pigs have yet to be identified. Moreover, it is not known whether targeting of such epitopes by vaccines would simply increase the rate of antigenic drift in those regions of the viral genome.

In addition to immune-escaping antigenic drift and shift, vaccine associated enhanced respiratory disease (VAERD)[169-173] and maternal antibody interference with vaccine efficacy[174] are phenomena that diminish the efficacy of killed vaccines in swine. Sows are often immunized with inactivated SIV vaccine with the intent of providing specific antibodies to their pigs following ingestion of colostrum. The maternal antibodies are intended to protect the neonatal pig; however, if still present at the time of immunization, they can also inhibit the pig's immune response, thus leaving these pigs susceptible to SIV at a later date. This is clinically relevant in herds or systems where the strains present in nursery and growing phases of production are antigenically distinct from the strains inducing maternal immunity either by vaccine usage or natural exposure. Previous studies with live attenuated influenza vaccines[162] and a replication defective human Adenovirus 5 vector system indicate these types of experimental vaccines can immunize pigs in the face of passively acquired antibodies;[175] moreover, each of these vaccine platforms avoid the development of VAERD.

Gaps in our Knowledge of Swine Influenza Virus Immunology

Similar to gaps that exist in our knowledge of AI virus immunology, there are many gaps in understanding the SIV immune response. The amino acids involved in antigenic drift have not been well characterized for swine H1 and H3 virus lineages and a direct prediction for loss of antibody protection cannot be reliably made from HA gene sequence alone. There is a positive relationship between SIV humoral immunity and clinical protection that is well described, but the underlying mechanisms of this protection are not well understood. Less is known about the cellular immune response that presumably plays a significant role in clinical protection. Likewise, what promotes or limits cross-protection within a subtype and between subtypes is unknown. Research priorities include:

1. Identification of innate mediators that can be used for attenuating virus for vaccine platforms and/or utilized for modulating the host response to vaccines.
2. Identification of antibody epitopes important for antigenic drift in the swine host and development of models to predict based on HA sequence evolution.
3. Characterize the humoral and cellular immune response to wild-type infection, and compare it to attenuated and inactivated vaccines to identify correlates of protection.
4. Evaluate effect on mucosal immunity of different adjuvants for inactivated vaccines.
5. Identify conserved B- and T-cell epitopes within and between virus subtype to target in new vaccine platforms or improvement of existing vaccines

6. Identify unique B- or T-cell epitopes that are non-overlapping between subtypes to support development of virus and subtype specific diagnosis of previous infection using blood or mucosal specimens.
7. Investigate adjuvants that may result in increased immune responses that are long lived, broadly cross-protective and reduce the number of vaccine boosters.

EPIDEMIOLOGY

The overall distribution of influenza viruses in animals remains complex, with notable changes since the 2009 H1N1 pandemic. H5N1 remains endemic in poultry in several Asian and African countries, causing on-going sporadic zoonotic infections in humans, but without onward human-to-human transmission. Lately, a low pathogenic avian influenza (LPAI) H7N9 virus has emerged as a zoonotic threat in China, causing fatal respiratory disease in humans.

In swine, the previously predominant TRIG viruses circulating in North America since 1998 and subsequently spread to Asia, evolved by reassortment with Eurasian swine viruses and emerged in the human population to cause the 2009 H1N1 pandemic. The 2009 H1N1 pandemic virus reassorted with endemic swine viruses globally, notably yielding H3N2 viruses with the M segment derived from H1N1pdm09 that were transmitted to humans and termed H3N2v. Nearly 350 people have been infected with H3N2v, with children being particularly vulnerable.

Avian Influenza

AI viruses are classified by their pathotype in gallinaceous species as either low pathogenic (LP) or highly pathogenic (HP) based on the standards of the World Animal Health Organization.[20] A HP isolate will kill 75% of intravenously inoculated chickens within 10 days and/or has an amino acid sequence at the HA proteolytic cleavage site (PCS) that is the same as an isolate that has previously been classified as highly pathogenic.[20] An isolate with an intravenous pathogenicity index (IVPI) of $\geq$1.2 may also be considered HP. Historically only H5 and H7 viruses have been naturally HP. Importantly, the OIE definition of LP and HP viruses only applies to AIV in chickens. The term "highly pathogenic" may be used with other species, such as ducks, ferrets or mice, but is then usually used as descriptive of higher virulence.

Wild birds, especially ducks and shorebirds, are reservoirs of LPAI viruses and usually show no signs of disease. Avian influenza is a transboundary animal disease that can spread rapidly from continent to continent. The way in which the virus may spread includes the movements of poultry and poultry products; contaminated objects including clothing, machinery and feed; and in some cases wild birds are implicated in its spread (OFFLU, May 2013).

AIV is a potential zoonotic agent and on rare occasions causes disease in humans. The first report of an HPAI H5N1avian influenza virus being transmitted directly from birds to humans occurred in 1997 in Hong Kong, where 18 people were infected with the virus, 6 fatally. Since 2003, over 641 people in several countries including China, Vietnam, Cambodia, Thailand, Indonesia, and Egypt, have been infected with HPAI H5N1 viruses resulting in over 380 fatalities.[176] The continued reports of H5N1 infections in humans have the public health community concerned about the potential for a worldwide pandemic. However, HPAI H5N1 does not spread efficiently from humans to humans and only a few reports of human to human spread have been documented.

As of 7 October 2013, 135 human cases of influenza A (H7N9) virus infection were reported to WHO.[177] Of these cases, 45 died. Most human A (H7N9) cases have reported contact with poultry or live animal markets. Knowledge about the main virus reservoirs and the extent and distribution of the virus in animals remains limited and, because it causes only subclinical infections in poultry, it is possible that the virus continues to circulate in China and perhaps in neighboring countries. As such, reports of additional human cases and infections in animals would not be unexpected, especially as the Northern Hemisphere autumn approaches. Although four small family clusters have been reported among previous cases, evidence does not support sustained human-to-human transmission of this virus. Continued vigilance is needed within China and neighboring areas to detect infections in animals and humans. WHO advises countries to continue surveillance and other preparedness actions, including ensuring appropriate laboratory capacity. Current information related to avian influenza A (H7N9) can be found at: http://www.who.int/influenza/human_animal_interface/influenza_h7n9/en/index.html.

Although factors influencing successful transmission of influenza viruses from one species to another species are not fully understood, influenza viruses adapted to pigs and humans have been transmitted to birds and vice versa. Opportunities exist for transmission of influenza viruses between pigs, humans, and poultry when they are in close contact. Under such circumstances opportunities also exist for reassortment of genes from these viruses, which may result in a mammalian-adapted influenza virus of public health concern. Although remotely probable, this highly consequential zoonotic threat has heightened public health interest in surveillance for influenza viruses in multiple species (OFFLU, May 2013).

Hosts, epidemiology and distribution

Although AIV has been isolated from hundreds of bird species worldwide, the natural reservoir hosts of AIV are considered to be wild aquatic birds, particularly dabbling ducks, gulls and some shorebird species.[178] Most other wild avian species are not considered to be important reservoirs. AIV has a worldwide distribution in wild birds and most surveillance for AIV in wild birds has been reported from North America and Europe, and is fairly limited for Africa, Oceania and South America. At a molecular level, much work has been reported on the importance of AIV receptor binding specificity and host restriction. Briefly, type A influenza viruses have a molecular preference for either the alpha2-3 or alpha2-6 moiety of sialic acid on the host cell.[179-182] Therefore the species association of influenza is to some degree determined by the moiety of sialic acid expressed in their upper respiratory tracts (e.g., humans express alpha2-6, so human viruses bind alpha2-6 better than alpha2-3.[182] This also determines within host tissue tropism since different tissue will express different sialic acid moieties. Clearly this is not the only viral determinant of host range since influenza can cross-species, but it certainly contributes to effective transmission within a species.

It is generally accepted that the migratory *Anatidae* (biological family of birds that includes ducks, geese, and swans) are the principal source of spillover infection of influenza A viruses in chickens, turkeys and other poultry. The usual pattern is that the viruses circulating in wild birds are LPAI, then a LPAI virus is transferred from the wild birds into chickens, and becomes progressively more pathogenic through successive infection cycles in the spillover hosts (FAO, April 2005). Chickens and turkeys are not considered natural hosts for AIV, and not all duck viruses will easily transmit to chickens and turkeys.[35,178] Also, once an isolate is adapted to gallinaceous birds, an isolate will often not easily infect ducks.[30,183] In addition, chickens and turkeys are not equally susceptible to the same isolates. The 50% minimum infectious dose (MID_{50}) for some isolates of AIV may vary by 2-3 logs between chickens and turkeys.[30]

Also, there is some evidence that turkeys may be generally more susceptible to AIV infection than chickens.[183,184]

Wild birds seemed to play an important role in the dissemination of the HPAI H5N1 throughout Asia and into Africa and Europe.[185,186] Asymptomatically infected wild migratory ducks are suspected of contributing to the spread of HPAI H5N1 viruses from Asia to other parts of the world;[187-189] however, domestic ducks are more likely perpetuating H5N1 HPAI viruses in nature.[189,190] Free-range as well as backyard domestic ducks have been associated with disseminating H5N1 HPAI viruses.[191-193] Domestic ducks are often farmed in open fields, flooded rice paddies, or on ponds or other bodies of water. Duck flocks may also be moved long distances through a region as part of the scavenging process which provides opportunities for further dissemination of H5N1 HPAI viruses.[194] An important difference between H5N1 and other avian influenza viruses is that H5N1 probably evolved to become HPAI in domestic birds and then subsequently was transmitted into wild populations, rather than emerging first in wild populations. It may well therefore behave quite differently in wild birds from other influenza viruses, being more virulent, and perhaps occurring at much lower prevalence (thereby making it hard to detect in wild populations).

In poultry, the incidence of AI varies due to sporadic outbreaks, which are rapidly controlled. However, there are a few regions where LPAIV or HPAIV are considered endemic: H5N2 LPAIV in Mexico; H5N1 HPAIV in parts of Asia and Africa; H9N2 LPAIV in the Middle East and Asia; and H6 viruses in Korea. Further complicating our understanding of the epidemiology of AIV is that some isolates can transmit between birds and mammals. An example is that turkeys are susceptible to swine influenza virus (SIV), and SIV can cause substantial production losses in breeder turkeys. Work with the 2009 swine origin human pandemic H1N1 (pH1N1) has shown that turkeys are not susceptible to respiratory tract infection, but can be infected through the reproductive tract (i.e., by artificial insemination).[195,196] Also in the field, pH1N1 appeared to spread within flocks of breeder turkeys beyond what could be attributed to infection through artificial insemination, so it is not clear how these isolates spread within the flocks.

The immediate source of infection for domestic poultry can seldom be ascertained, but most outbreaks probably start with direct or indirect contact of domestic poultry with water birds. Many of the strains that circulate in wild birds are either non-pathogenic or mildly pathogenic for poultry. However, a virulent strain may emerge either by genetic mutation or by reassortment of less virulent strains. Scientific evidence indicates that the former mechanism occurred in 1983-1987 in the eastern part of The United States of America. Swine appear to be important in the epidemiology of infection of turkeys with swine influenza virus when they are in close proximity. Other mammals do not appear to be involved in the epidemiology of HPAI.

Once AI is established in domestic poultry, it is a highly contagious disease and wild birds are no longer an essential ingredient for spread (FAO). Infected birds excrete virus in high concentration in their feces and also in nasal and ocular discharges. Once introduced into a flock, the virus is spread from flock to flock by the usual methods involving the movement of infected birds, contaminated equipment, egg flats, feed trucks, and service crews, to mention a few. The disease generally spreads rapidly in a flock by direct contact, but on occasions spread is erratic (FAO). Airborne transmission may occur if birds are in close proximity and with appropriate air movement. Birds are readily infected via instillation of virus into the conjunctival sac, nares, or the trachea. Preliminary field and laboratory evidence indicates that virus can be recovered from the yolk and albumen of eggs laid by hens at the height of the disease. The possibility

of vertical transmission is unresolved; however, it is unlikely infected embryos could survive and hatch (FAO).

Survival of influenza viruses in the environment

Influenza A viruses have exceptionally long survival times outside the living host, when in a favorable environment. When excreted by water birds they can survive in lake water for many months at 17 degrees C, and for even longer at 4 degrees C. Infectivity of this virus for hosts is variable, depending on strain and environmental factors. In less favorable environments, such as in fecal material, the virus survives no more than 24 to 48 hours. Thus, virus survival is a very significant issue for transmission within wild bird populations, where water contamination may continue to provide a source of infection for extended periods, especially in the northern breeding grounds. Under village conditions in Asia, virus survival in the environment can also be important, but its role depends on temperature, the nature of fomites, which may carry virus, and density of birds. Maintenance of infection in wild and domestic bird populations is considerably more important than environmental sources in spreading infection to new locations (FAO, April 2005)

Transmission mechanisms between hosts

A crucial part of replication of influenza A viruses is cleavage of the precursor HA protein in newly formed viral particles, which is an essential step in making the new particles infectious, so that they can infect additional cells. This step is mediated by a host enzyme rather than being achieved by the virus itself. Some strains of avian influenza virus have specific amino acids close to the cleavage site of the precursor HA protein that limit the host enzymes that can split the molecule to those that are found in the respiratory and digestive tracts. These strains are of low pathogenicity in chickens. Viral strains that have different (basic) amino acids at these same sites can be split by enzymes that occur in a much wider range of tissues, and these are highly pathogenic, since they can multiply throughout the body. This increases the transmission potential of the virus. There appear to also be differences between strains in predominant excretion routes arising directly from the genotype of the virus. Most AIV are principally or entirely excreted in feces, and are therefore mainly spread by contamination of food resulting in oral intake, probably causing infection through the oropharyngeal mucosa. This is the natural process in reservoir hosts, and most spillover hosts. Some viruses are also excreted by respiratory aerosol and therefore can much more readily spread from host to host by droplet infection (as in most human influenza viruses). In the outbreak in Hong Kong in early 2002, aerosol transmission may have occurred over moderate distances, but it is difficult to resolve whether such transfers are due to short distance aerosol spread, or to movement of fomites, or similar items, leading to oral exposure. These factors are very important in the epidemiological processes that can potentially lead to pandemic strains, and in the rate and pattern of spread of avian influenza between flocks, and between different species in places such as live bird markets (FAO, April 2005).

Low Pathogenicity Avian Influenza in North American Poultry Populations

Sporadic outbreaks of LPAI occur in the United States in commercial poultry flocks. These are dealt with on a State-by-State basis, usually through biosecurity enhancements, depopulation or controlled marketing of infected flocks, and limited use of inactivated vaccines. Influenza A H7N3 killed virus vaccine was successfully used to help control and eradicate a LPAI H7N2 outbreak in a layer chicken flock in Connecticut in 2003/2004. H7N2 LPAI has been a problem in the live-bird marketing system in the Northeastern United States. Since 1996, six outbreaks of LPAI H7N2 in commercial poultry have been linked to activities associated with the live-bird marketing system.

Reports of avian influenza virus infections in poultry and isolations from wild bird species in Canada, the United States, and Mexico between 2009 and 2011 involved only low pathogenic avian influenza. All three countries reported outbreaks of low pathogenic notifiable avian influenza in poultry during this period.[197] The reports involved outbreaks of H5N2 among commercial turkeys in Canada in 2009 and 2010; outbreaks of H5N3 in turkeys in 2009, H5N2 in chickens in 2010, H7N3 in turkeys in 2011, and H7N9 in chickens, turkeys, geese, and guinea fowl in 2011 in the United States; and multiple outbreaks of H5N2 in chickens in Mexico in 2009, 2010, and 2011. Outbreaks of pandemic H1N1 infections in turkey breeder flocks were reported in Canada in 2009 and in the United States in 2010. Active surveillance of live bird markets in the United States led to the detection of H2, H3, H4, H5, H6, and H10 subtypes. Despite the fact that wild bird surveillance programs underwent contraction during this period in both Canada and the United States, H5 and H7 subtypes were still detected.[197]

High Pathogenicity Avian Influenza in North American Poultry Populations

The last HPAI outbreak in the United States was in Texas in 2004. In June of 2012, an H7N3 highly pathogenic avian influenza (HPAI) virus was identified as the cause of a severe disease outbreak in commercial laying chicken farms in Mexico. The virus had high sequence similarity of greater than 97% to the sequences of wild bird viruses from North America in all eight gene segments.[4] Further molecular characterization of the 2012 H7N3 virus identified virulence determinants associated with virus mutations resulting from recombination with host nucleic acids, incorporating part of the chicken genome into the virus genome, which has only been described in the literature twice. This unique genetic recombination event underscores the need to make sure that poultry are also free of the low pathogenic forms of the virus.

Avian Influenza Virus Outbreaks with Pandemic Potential

HPAI H5N1

Currently, highly pathogenic avian influenza (HPAI) A (H5N1) virus is considered endemic among poultry in six countries (Bangladesh, China, Egypt, India, Indonesia, and Vietnam). This means the virus is commonly found in poultry in those countries. Sporadic outbreaks have occurred among poultry in other countries.

LPAI H7N9

H7N9 virus of avian origin responsible for infections in human in large urban areas of China was reported spring 2013. The original source of the virus from poultry farms is unknown but the live poultry market (LPM) system has served as an amplifier of the virus, especially in wholesale markets in the large cities, with 77% of human cases having known contact with live poultry at a retail live poultry markets. There has been no new human infection in large urban areas where LPM system has been closed. There have been no known human cases on farms or among veterinarians. The virus was shown to be low pathogenic based on intravenous pathogenicity index test in chickens. Furthermore, intranasal inoculation of chickens, domestic ducks, domestic geese, Japanese quail and pigeons with a human H7N9 influenza isolate resulted in infection, but no clinical signs. The high viral shedding from chickens create a likely source of infection for humans. Virus shedding patterns in ducks (with the exception of the Muscovy ducks) and geese were shorter and of lower titer than gallinaceous poultry. Pigeons were difficult to infect unless given high doses of inoculum intranasally and are unlikely to be involved in field spread (NMWR 2013, Pantin-Jackwood et al., unpublished data).

Sporadic human cases and small clusters would not be unexpected in previously affected and possibly neighboring areas/countries of China. The current likelihood of community level spread of this virus is considered low. Continued vigilance is needed within China and neighboring areas to detect infections in animals and humans. WHO advises countries to continue surveillance and other preparedness actions, including ensuring appropriate laboratory capacity. All human infections with non-seasonal influenza viruses such as avian influenza A (H7N9) are reportable to WHO under the IHR (2005) (WHO, 2013b). Influenza A (H7) viruses have been detected in poultry populations in many countries throughout the world. Occasionally, human cases of infection with A (H7) viruses have been detected, mainly in people directly exposed to infected poultry or contaminated environments (OFFLU, May 2013). Generally, these infections cause conjunctivitis or mild influenza like illness; however, in rare cases of human A (H7) infections, notably with A (H7N9), severe and fatal disease was reported. On 14 August 2013, highly pathogenic avian influenza A(H7N7) was reported in poultry in Northern Italy. To date, a total of six outbreaks in poultry have been reported in this region, the last started on 4 September 2013, according to OIE. Three human cases of infection with influenza A (H7N7) virus were identified in men involved in culling operations. All three men developed conjunctivitis, one had also chills and muscle aches. Genetically, these A (H7N7) viruses are similar to low pathogenic viruses circulating in wild birds in Europe and those causing sporadic and limited outbreaks in poultry in Central and Northern Europe.

Swine Influenza

Influenza A Viruses (IAV) Identified in North American Swine Populations

Swine influenza was first recognized in pigs in the Midwestern U.S. in 1918 as a respiratory disease that coincided with the human Spanish flu pandemic. Since then, influenza has become an important disease to the swine industry throughout the world. The first influenza virus was isolated from swine in 1930 by Shope[198-201] and was shown to cause respiratory disease in swine that was similar to human influenza. This classical swine lineage H1N1 virus derived from the 1918 pandemic virus was relatively stable at the genetic and antigenic levels in U.S. swine for nearly 80 years.

The epidemiology of IAV in U.S. pigs dramatically changed after 1998 when triple-reassortant H3N2 viruses containing gene segments from the classical swine virus (NP, M, NS), H3N2 human seasonal influenza virus (PB1, HA, NA), and avian influenza virus (PB2, PA)[202] became successfully established in the pig population.[203] This genome composition of swine IAVs are referred to as the triple-reassortant internal gene (TRIG) cassette.[204] After their emergence, the H3N2 viruses reassorted with classical H1N1 swine IAV acquiring the H1N1 or H1N1 subtypes.[205,206] Reassortant H1 TRIG viruses are enzootic along with the H3N2 viruses in most major swine-producing regions of the U.S. and Canada; since early in the new millennium, the vast majority of the fully characterized swine viruses contain the TRIG cassette, regardless of subtype.[207-210] Outside of North America, genetically related swine viruses that contain the TRIG have been identified in Korea, Vietnam, and China.[211-213] North American TRIG-containing swine viruses can readily infect turkeys, an ability which may play an unidentified role in the epidemiology of IAV in swine and human hosts.[214]

Since 2005, H1N1 and H1N2 viruses with either HA, NA, or both derived from human seasonal IAV have emerged and spread across the U.S. in swine herds.[215] The HAs from the human-like swine H1 viruses are genetically and antigenically distinct from those of classical swine–lineage H1 viruses. However, their TRIG genes are similar to those found in contemporary swine triple-reassortant viruses. To represent the evolution of the currently circulating North American H1 viruses, a cluster classification was established.

Viruses with the HA gene of the classical H1N1 viruses that have circulated in swine since 1918 evolved into the contemporary α-, β-, and γ-clusters; whereas, H1 subtype isolates with HA genes most similar to those of human seasonal H1 viruses circulating in the early 2000s evolved into the δ-cluster.[215] All four HA gene cluster types (α, β, γ, and δ) can be found with NA genes of either the N1 or N2 subtype. The HA genes from the δ-cluster viruses most likely emerged from at least two separate introductions of human seasonal HAs from H1N2 and H1N1 viruses, differentiated phylogenetically into two distinct sub-clusters, δ1 and δ2, respectively.[208] Both sub-clusters have evolved extensively in swine.[216] During investigations of 2008-2010 viruses, the HAs of the δ-cluster were paired with either an N1 or N2 gene of human virus lineage but not with an N1 gene of swine lineage. Prior to 2009, the H1 IAV evolved by drift and reassortment while maintaining the TRIG backbone and giving rise to viruses differing genetically and antigenically, thus having consequences for vaccine and diagnostic test efficacy.[208] Since that time, H1N1pdm09 has become established in the U.S. pig population with subsequent second generation reassortants emerging.

Influenza Viruses Identified in European Swine Populations

Multiple swine IAVs with genetic lineages that are distinct from the North American TRIG viruses evolved in Europe and Asia (reviewed in Van Reeth[9]). Although classical H1N1 swine viruses previously circulated in Europe, Asia, and many other parts of the world for many years, they were eventually replaced by a new lineage in Europe, a wholly avian H1N1 that entered the swine population around 1979. Human-lineage H3N2 viruses descending from the pandemic A/Hong Kong/1/68 (H3N2) human virus and, thus, distinct from the H3N2 IAVs in North America, emerged in Europe in the 1970s. In Europe, these H3N2 viruses reassorted with the avian-like H1N1 swine IAV, from which they acquired the internal gene cassette in the mid-1980s.[217,218] Additionally, a reassortant H1N2 virus emerged in pigs in Great Britain in 1994[219] and subsequently spread to other countries in Western Europe, retaining most of the genotype of the reassortant H3N2 virus but having acquired the HA gene of a human H1N1 virus found in the 1980s. Thus, the three major virus lineages share common internal protein genes, but they have clearly distinguishable HAs. A recent European surveillance study reported the continued circulation of Eurasian avian-like H1N1, human-like H3N2, and human-like H1N2 viruses in swine. All three subtypes were detected in Belgium, Italy, and Spain, but only H1N1 and H1N2 viruses were found in UK and Northwestern France.[220] Since November 2010, structured, coordinated, and harmonized passive surveillance within Europe has detected a 30% incidence of IAV, primarily in pigs with acute respiratory disease, from 14 countries after investigating 3500 herds. The results have revealed the continued circulation of previously identified subtypes and the presence of H1N1pdm09 in at least seven of these countries, although continued circulation of H1N1pdm09 in some countries remains uncertain. Additionally, next-generation reassortants arising from H1N1pdm09 with endemic strains in European pigs have been detected. Additional second-generation reassortants—H1N2 viruses with an avian-like H1 and H1N1 viruses with a human-like H1 derived from the prototype H1N2 viruses—have also been detected,[220-224] but they remain relatively rare.

Influenza Viruses Identified in Asian Swine Populations

In 2010, China and Vietnam produced 476.2 and 27.3 million pigs, respectively, collectively accounting for 53% of global pig production (FAOSTAT-www.faostat.fao.org). The existence of highly dense populations of swine, poultry, and waterfowl in China, Vietnam, Thailand, Bangladesh, and other Asian countries indicates the need to intensify surveillance for IAV in the region. Classical swine H1N1 viruses (first detected in China in 1974 but probably present for many decades before) are enzootic in swine in China and co-circulated with H1N2 viruses that acquired an N2 of contemporary human origin. Human

H3N2 viruses (A/Hong Kong/1/68-like; A/Port Chalmers/1/73-like; A/Sydney/05/97-like) were repeatedly transmitted to pigs and circulated in pigs long after the parent human virus had been replaced in the human population.[225,226] Avian H1N1 viruses were detected in swine in China in 1993. However, these were not descendants of Eurasian avian-like H1N1 viruses and probably represented an independent interspecies transmission from the Asian avian reservoir to swine.[227] European H3N2 and H1N1 viruses were first detected in China in 1999 and 2001, respectively, and North American triple-reassortant viruses were first found in 2002, indicating intercontinental movement of swine viruses, possibly via importation of swine.[228] Co-circulation of different swine influenza lineages was associated with the appearance of reassortants during the intercessory time period between emergence of each new lineage.[228] More recently, H1N1pdm09 and its reassortants have also been detected in swine. Although much of this surveillance has been done in slaughterhouses in Hong Kong, the swine slaughtered in Hong Kong are imported from many provinces in China; therefore, these data probably gives an indication of swine influenza ecology in the wider region. In addition, the H9N2 and H5N1 avian influenza viruses have been sporadically detected in pigs in some Asian countries.[213,229,230]

Classical swine H1N1 virus probably appeared in the Japanese swine population around 1977 (Miwa et al. 1986) and then reassorted with a human seasonal H3N2 virus to emerge in 1980 as an H1N2 virus possessing all the segments from classical swine IAV except the NA gene. [231] Swine IAVs of this genotype have been the predominant isolates from pigs in Japan.[232-234] The results of serologic examination[235] and of virus isolation from swabs taken at slaughterhouses (Saito et al, unpublished data) confirmed that H3N2 viruses of human lineage have occasionally entered Japanese pig populations. After the emergence of the H1N1pdm09 virus in humans, the virus infected pig populations in Japan and reassorted with H1N2 IAVs (Matsuu et al, unpublished data).

H1N1 and H3N2 viruses have been found in swine in Thailand since the early 1980s.[236,237] In general, the H3N2 and H1N1 IAVs circulating in Thai pigs are related to the lineages found in the Eurasian H3N2 and H1N1 viruses and in the classical H1N1 virus. Swine H3N2 viruses from early 2000 to 2007[238] contain human-lineage HA and NA genes, with internal genes from the Eurasian (PB1, PB2, PA and M genes) and classical H1N1 (NP and NS genes) swine lineages. The Thai swine H1N1 viruses (ThH1N1) circulating from 2000 until the emergence of the H1N1pdm09 virus are unique reassortants of classical and Eurasian swine lineage. These H1N1 viruses could be grouped into classical-HA and Eurasian-NA swine lineages, with internal genes being either all Eurasian swine (7+1) or Eurasian swine with the classical swine NS gene (6+2).[238] The NA genes of ThH1N1 and H1N1pdm09 have less than 90% nucleotide similarity, indicating that although the NA genes originated from the same Eurasian H1N1 ancestor, they have evolved separately. The third subtype, H1N2, was first isolated from pigs in 2005[239] and contained combinations of genes from the endemic human-like H3N2 and ThH1N1 viruses that were circulating in swine herds.[238] Reassortant H1N1 containing seven genes of H1N1pdm09 and the NA gene of endemic ThH1N1 virus were detected repeatedly in a commercial swine herd with IAV-associated respiratory disease,[240] suggesting an increasing genetic diversity among future circulating IAV in Thai pigs.

In Vietnam, reassortant H3N2 viruses with the North American TRIG were detected in swine.[211] The H3 and N2 genes were acquired by reassortment with a human seasonal virus circulating in humans around 2004-2006. The H3 and N2 genes were very similar to those of H3N2-pdm09 swine reassortant viruses isolated in China.[241] In South Korea, the classical swine H1N1, H1N2 and H3N2 IAV of the North American triple-reassortant lineage co-circulate[242] with an additional human-lineage H3N2 virus that is distinct from the lineages in Vietnam and China.[212] Serologic screening of commercial pigs in Malaysia

has detected H3N2 and H1N1 in 41.4% of the farms surveyed,[243] but information about the distribution and gene flow of swine IAV in most other countries in the Asia-Pacific region is unavailable.

Influenza A Viruses Identified in South American Swine Populations

Few published reports about virus isolations or sequences are available to document the presence of IAV in swine in many countries in Central and South America. Argentina has recently reported the presence of distinct human-lineage viruses of the H1N1 and H3N2 subtypes.[244,245] The Argentinean viruses are distinguishable from similar subtypes in North America and represent independent human-to-swine transmission events. In late 2008, a wholly human H3N2 was isolated from pigs with clinical signs of respiratory disease and fever typical of influenza. Experimentally reproduced infections showed that the virus was transmitted efficiently between pigs and that the inoculated pigs had characteristic lesions of influenza, suggesting that this virus was completely adapted to swine and could be maintained in the swine population.[245]

In 2009 and 2010, Argentina reported the isolation of reassortant viruses with internal genes from H1N1pdm09 and surface genes (HA and NA) from human-like (North American δ2-like) H1 swine IAV. Therefore, some indirect evidence of circulation of this δ2 H1 cluster in Argentina exists.[245] In 2011, another reassortant virus was isolated, with surface genes from the wholly human H3N2 virus first isolated in 2008 and internal genes from the H1N1pdm09 virus. The clinical signs observed in all these cases were typical for influenza (e.g., fever, dyspnea, coughing, and sneezing). It is postulated that absence of vaccines and the characteristics of pig production in Argentina (i.e., presence of all ages of pigs from neonatal to adult at one site) may have contributed to the emergence of these new reassortants.

In Brazil, few reports of influenza virus infection in pigs existed before 2010. Recently, coinciding with the H1N1pdm09 in humans, numerous outbreaks of acute respiratory infection in pigs of different age groups were reported in Brazil, and the H1N1pdm09 virus was identified as being the cause.[246] In addition, a new H1N2 IAV identified in a recent study contains H1 and N2 genes of human seasonal origin (similar to North American Swine δ cluster) and internal genes (M, NP, PB1, PB2 and PA) from H1N1pdm09. ELISA testing (IDEXX Influenza MultiS-Screen ELISA) of sera collected from 106 commercial farms from July 2009 to December 2011 had a 60% positivity rate (1889 positive/3150 total sera). Furthermore, analyses of sera collected from pigs in Brazil revealed that HI antibodies against H1N1pdm09 were not detected in pigs in Brazil prior to 2009.[247] A separate study, which involved serologic detection, virus isolation, genomic sequencing, and study of the dynamics of infection, detected IAV in pigs in six Brazilian states: It was concluded that the IAV was circulating in at least 64.7% of the farms studied. The HA gene of the isolates was very similar to H1N1pdm09. Samples collected from those farms before 2009 showed IAV infection but not H1N1pdm09 infection (Ciacci-Zanella, unpublished). The population of wild *Suidae*, including both captive wild boars reared under intensive management and free-range feral pigs of the Pantanal Region in Brazil, was also evaluated. IAVs were detected in 11 of 60 lungs with macroscopic pneumonia lesions by using quantitative PCR. The M gene sequence was 98-99% identical to that of H1N1pdm09 (Ciacci-Zanella, unpublished). Of the 141 feral pigs tested, IAV antibodies were found in 105 (74.5%).

Equine Influenza

Influenza A virus infection of equids has been reported world-wide with the exception of a small number of island countries including New Zealand and Iceland. Equine influenza (EI) is endemic in Europe and America. Other parts of the world such as Japan, South Africa, India and Hong Kong suffer occasional incursions but the disease is not endemic. Although the mortality rate associated with equine

influenza virus (EIV) infection is very low it is considered the most important respiratory virus of horses because it is highly contagious and has the potential to cause significant economic loss due to the disruption of major equestrian events. The virus is spread by the respiratory route, by personnel, vehicles contaminated with virus, and by fomites. Large outbreaks are often associated with high density stabling, the congregation of horses at equestrian events and their dispersal over a wide geographic area after the event (OFFLU), May 2013). EI can be controlled by vaccination. However, subclinically infected vaccinated horses can shed virus, and antigenic drift of the viruses impacts on vaccine efficacy (OFFLU), May 2013).

Only two stable subtypes of EI have been reported in horses, H7N7 and H3N8 (OFFLU, May 2013). The first reported outbreak of equine respiratory disease to be confirmed as equine influenza occurred in 1956 in Eastern Europe. The virus isolated was characterized as H7N7. Subsequently H7N7 viruses were identified as the cause of outbreaks in Europe, Asia and the United States. Although H7N7 viruses co-circulated with H3N8 viruses in horses for many years, it is generally accepted that these viruses have not been active for a long period and may be extinct. The first isolation of a H3N8 virus took place in Florida in 1963. Since then H3N8 influenza viruses have been responsible for epizootics in all continents. Antigenic drift occurs less frequently in equine influenza viruses than in human viruses but the H3N8 subtype has evolved into two distinct lineages designated the "American-like" lineage and the "European-like" lineage based on the initial geographical distribution of viruses. Three American sub-lineages subsequently emerged the Argentina, Kentucky and Florida. The Florida sub-lineage has more recently diverged into two Clades: Clade 1 includes the viruses responsible for the epizootics in South Africa, Australia and Japan respectively, and Clade 2 includes viruses that have been circulating in Europe since 2003.

Influenza virus reassortants originating from avian, human and/or swine viruses have not been identified in horses and to-date the epidemiology of EI appears to be somewhat less complex than that of swine or avian viruses. The current EI viruses are believed to be of avian ancestry and more recent transmission of avian viruses to horses and donkeys has been recorded. During 2004-2006 swine influenza surveillance in central China 2 equine H3N8 influenza viruses were isolated from pigs. Despite the successful experimental infection of human volunteers with EIV and the occasional identification of seropositive persons with occupational exposure there is currently little evidence of zoonotic infection of people with EI More recently avian H5N1 has been associated with respiratory disease in donkeys in Egypt.

Canine and Feline Influenza

Canine influenza virus (CIV) belongs to the genus *Influenza-virus A* of the family *Orthomyxoviridae*. An equine-origin H3N8 influenza virus was first isolated from racing dogs affected with acute respiratory disease in the United States in 2004.[32] Subsequent outbreaks were reported, and the infection spread rapidly across the United States. EI viruses have the potential to cross species barriers and have been associated with outbreaks of respiratory disease in dogs (primarily but not exclusively, greyhounds and dogs in shelters) in North America, quarry hounds in England and dogs on premises with horses affected by EI in Australia. Interspecies transmission of EIV to dogs maintained in the same stable as experimentally infected horses was demonstrated but there is to-date no evidence of EI transmission from dogs to horses (OFFLU, May 2013).

Between May 2006 and March 2010, sporadic cases of a different subtype of CIV, namely, H3N2, were identified from sick dogs at animal clinics in China.[248] In 2007, CIV H3N2 also caused an outbreak of contagious canine respiratory disease in South Korea.[249] This virus appears to be entirely of avian origin and the first low pathogenic avian influenza (LPAI) virus reported to cause respiratory disease in dogs. CIV H3N2 infection results in clinical outcomes ranging from mild respiratory illness to death.

Recently, isolation of H9N2 influenza virus from dogs was reported in southern China. Genetic analysis of an isolate revealed that it was a novel genotype closely related to avian H9N2 virus. Epidemiologic studies demonstrated that this new H9N2-subtype virus was the causative agent of disease in canines.[250]

Due to frequent interactions with humans and other animals, domestic cats and dogs are uniquely positioned to serve as an intermediate host for influenza virus infection. For many years, however, canines and felids were thought to be naturally resistant to influenza virus infection. As a result, their potential role in the interspecies transmission of influenza viruses has been largely overlooked. Previous reports have shown that dogs can be infected by equine H3N8, canine H3N2, H5N2, avian highly pathogenic H5N1, and pandemic H1N1 influenza viruses.[249,251-253] Furthermore, cats are susceptible to canine H3N2, avian H5N1, H1N9, H6N4 and pandemic H1N1 influenza virus.[254,255] The susceptibility of domestic cats and dogs to H9N2 avian influenza virus was evaluated by intranasally or orally inoculating animals with an H9N2 influenza virus. H9N2 AIV can efficiently infect cats and dogs via the upper respiratory tract. H9N2 AIV transmission between cats and dogs is inefficient.[256]

Zoonotic Influenza

The first report of multiple cases of an avian influenza virus being transmitted directly from birds to humans occurred in 1997 in Hong Kong, where 18 people were infected with a HPAI H5N1 virus, 6 fatally. Since 1997, over a hundred people in Thailand, Vietnam, Cambodia, and Indonesia have been infected with the HPAI H5N1 strain of AI resulting in over 50 fatalities. The continued reports of H5N1 infections in humans has the public health community concerned about the potential for a worldwide pandemic.

Gaps in our Knowledge of the Epidemiology of Influenza Viruses

Gaps exist in our understanding of the mode(s) and route(s) of transmission within and between species. There are also gaps in our understanding of how the virus might survive in the environment. Many of these gaps and research needs have been previously recognized (OFFLU Agenda for Influenza Research Priorities in Animal Species; STAR-IDAZ Global Network for Animal Disease Research; DISCONTOOLS, Avian Influenza: http://www.discontools.eu/Diseases/Detail/37). Informed by on-

going systematic analysis of field viruses, particularly those that display new features (whether sequence, pathogenicity, antigenicity, transmission, species jumps), there is a need to investigate:

1. Pathogenesis (within different species)
2. Transmission (within and between species) and epidemiology (within the flock/herd). In all cases, strains appropriate to the specific transmission vector, including in some cases to human, need to be examined.
3. Surveillance
4. Mathematical modeling and molecular epidemiology

Priorities include:

Pathogenesis

Host susceptibility
Gaps:

- Role of different species in the genesis, persistence and spread of the virus
- Defined symptoms or surrogate markers of infection
- Incubation period in vaccinated animals
- Genetics of reservoir and susceptible species
- Lack of knowledge on resistance mechanisms in different animal species
- Effect of immunosuppression has on the duration of carriage of influenza viruses in partially immune animals
- Effect of severe stress on susceptibility to infection

Research needs**:**

1. Although quail has already been recognized as an important avian species associated with interspecies transmission, there is a lack of knowledge with regards to the entity, function and consequence of mutations that emerge during replication in this species.
2. Role of turkeys in IAV epidemiology (it has been shown to be a likely bridge species in which a virus from the wild reservoir can adapt to other domestic species).
3. Develop a better understanding of farming systems of domestic ducks and farmed wild waterfowl species and their interactions with free ranging waterfowl with implications for virus control including interaction with other reservoir and potential bridging hosts (including wild birds).
4. Establish infection dynamics in domestic ducks i.e. how they get infected, transmission from environmental sources, role of maternal antibodies and any effect from previous exposure to other AI viruses.
5. Conduct a review of the genetic information available for AI reservoirs and poultry and generate data on the genomics characteristics that confer resistance to avian influenza viruses in some species.
6. Conduct studies to determine if some village chicken breeds contain genes that confer AI resistance.
7. Explore the impact of variable host susceptibility on avian influenza virus persistence in different ecosystems (i.e., domestic ducks in South East Asia).
8. Provide the poultry community with improved predictive tools for how viruses are likely to increase in virulence over time

9. Develop a better understanding of the differences in adaptive and innate immune responses in different avian species and the relationship to susceptibility to AIV infection and responses to vaccination.
10. Develop a better understanding of duration of immunity in ducks and the possibility of re-infection of partially-immune ducks or stressed immune ducks if re-exposed to the virus.
11. Develop improved and more predictive immunological tools to study correlates of humoral, mucosal, cellular and innate immunity on clinical protection from disease after infection.
12. Effect of immune status on intra-host virus population evolution.
13. Improved transmission models that are more applicable to the complexities of virus in the field.
14. It is difficult to consider all the variables and thus, to figure out what should be studied. Models needed to understand data. Are new transmission models needed? Is there a need for challenge studies in larger populations?
15. Unknown field situation—lack of linkages between field scientists and bench scientists.
16. Do bench scientists use field observations to generate hypotheses? Are better data links needed? Computational models? Trained collectors? Changes in the design of field studies?
17. Are there instances where viral transmission between different populations can be blocked? What was done to prevent transmission? Is there a way to systematically collect this information? Most field data are passively collected and not part of a larger study design. Disease outbreaks are great opportunities to validate laboratory findings.
18. There is a need to study the extent of antigenic variants and heterosubtypic protection between influenza viruses in pigs, and the immune mechanisms involved.
19. Study the extent of cross-protection among influenza viruses of swine, humans and birds, and the immune mechanisms involved.

Viral factors

Gaps:

- Rate of genetic changes and triggers influencing these rates, and their effect on epidemiology, host specificity and pathogenesis.

Research needs**:**

1. Review/clarify the role of mutations at receptor binding sites on replication and pathogenesis, especially which mutations are important in changing host specificity.
2. Determine host-virus factors that influence infection outcome in different poultry hosts.
3. Study the viral and host factors that contribute to the successful transmission of SIVs to other species, and identifying markers of swine influenza viruses with human pandemic potential.
4. Undertake thorough characterization of SIV isolates from humans, birds or other species, to identify potential factors involved in interspecies transmission of SIVs.

Transmission

Source of viral infection

What is the source of virus in a specific case? How can future introductions be prevented? What tools/approaches are needed to determine this?

Gaps**:**

- Pathways for interaction between wild birds and poultry
- Mode of environmental transmissions (virus in surface water, sediment, etc.)

- Exposure to AI viruses in live animal markets and in vaccinated flocks
- Understanding of marketing systems and biosecurity protocols

Research needs**:**

1. Develop appropriate systems for measuring shedding of model viruses in target species, allowing quantification of levels of released virus required for transmission (so that measurement of virus released might serve as a correlate of transmission) and helping to assess transmission from asymptomatic animals.
2. Develop a better understanding of ecological factors and risk pathways for interaction between wild birds and poultry
3. Determine risk of transmission of virus in markets or in flocks of vaccinated poultry
4. Better ways to study transmission at the field level.
5. Predictive biology, prevalence studies
6. Develop standarized procedures and tools for conducting transmission studies in experimental settings (interhosts, intrahosts, aereosol, etc)
7. Conduct studies on the population dynamics of SIVs; e.g., to address issues of persistence of virus at the farm level and the major routes of virus spread between farms

Virus properties involved in transmission

Gaps also exist in our understanding of virus evolution and population dynamics. There continues to be insufficient surveillance of equine, swine and avian populations for influenza viruses worldwide to have a clear picture of what is circulating in the field. Although surveillance in the United States is probably the most extensive and transparent for animal agriculture around the world, some influenza viruses like equine or canine influenza are poorly sampled and enhanced swine influenza surveillance is contingent on soft funds. The detection and isolation of virus should lead to characterization of representative viruses at the genetic and phenotypic level and should include complete genome sequencing. With the availability of large amounts of sequence data, it is necessary to have appropriate capacity for downstream phylogenetic and bioinformatics analysis (enhanced to include valuable field metadata), but this remains a major gap of knowledge and capacity. Whether there are constraints to evolution imposed upon the viruses is a question important to the assessment of threat levels. Are there limits to reassortment, what are they, and how do they work? What are the relative pressures imposed by virus structure, innate responses, cellular interactions and transmission (and the influence of hosts and production systems upon those pressures)?

Gaps**:**

- Data on virus survival properties is missing for several subtypes including those with zoonotic implications
- Rate and selection pressure of mutation at the cleavage site. Is it true? And if so, why? Are HPAIV usually generated in gallinaceous poultry? Why has HPAI H5N2 not re-emerged in Mexico despite circulation of LP virus for many years, but the related genetic lineage in Taiwan has mutated to HP? Why have LP H7N3 virus in Italy never become HP despite years of circulation in an epidemiological situation that is similar (e.g., geographic area, poultry involved, etc) to the one where HP H7N1 emerged?
- Tissue tropism of LPAIV; some strains replicate beyond respiratory and intestinal tissues (e.g., kidney, adrenal glands, etc.)
- Survival of virus on feathers

- Mechanisms of adaptation of LPAI to poultry; what predisposes a LPAIV virus to replicate in gallinaceous poultry?
- Transmission rates of LPAIV and HPAIV. Virus shedding titers and kinetics of shedding; relation with transmission rates
- Genetic and phenotypic factors that influence improved survival at various temperatures and relative humidity

Research needs**:**

1. Determine virus survival characteristics, persistence, and underlying factors in a range of settings relevant to animal production.
2. Establish the mode of transmission and mechanisms of persistence of LPAI compared with HPAI, identifying virus-host factors that influence virus transmissibility.
3. Prediction of which wild bird LPAI will infect poultry
4. Prediction of which H5/H7 LPAI can become HPAI, and develop a risk scale

Other factors related to transmission

Gaps:

- What role does environment play in transmission? Alternatively, can environment be altered to prevent or reduce transmission? (Engineering controls). Role of humidity, temperature, housing conditions, dust, litter, ventilation, transport conditions, population density, animal turnover, contact rates with infected materials, water source and status
- Links to other populations: fomite linkages, air space connections, production links, biological vectors, biosecurity
- Epidemiological factors more likely to lead to cross species transmission
- Role of aerosol or neighborhood transmission and relative contributions of various routes of transmission.
- Other modes of transmission/infection. Conjunctiva transmission?
- Is there a carrier states in certain species (pheasants) or in immunocompromised birds
- Uncontrolled and illegal trading activities with live poultry and all kinds of poultry products. Spill-over into wild bird population and (secondary) spread with migratory species possible
- Role of psittacines as a possible nidus of infection and spread through illegal movement of birds as related to the Mexican H5N2 outbreak
- Mode and frequency of contacts between wild birds and poultry (transboundary potential)
- Seasonal cycle (seasonality). Possible link to bird migration patterns (LPAIV). HPAIV: Annual shifts in incidence in endemic regions (SE Asia, Egypt) linked to cooler/more humid times of the year or to increased poultry production and trading movements during nation-wide celebrations/holidays (e.g. Tet/Vietnam, Ramadan/Egypt, Indonesia).

Research needs:

1. Develop environmental and fomite systems (both in vivo and in vitro) for evaluating the transmission of model viruses within and between species of interest. Systems to be investigated would include controlled airflow devices, applied to animals and to tissue explants (such as airway epithelium cultures)
2. Develop appropriate systems for detecting and measuring survival of model viruses in the environment, to measure survival on fomites and surfaces, including e.g. clay particles, for short and long distance spread and to investigate whether any virus determinants are involved in survival

in the environment (recent work shows the importance of the activation pH of HA in host adaptation; it is conceivable it might also affect environmental survival).
3. Identify risk factors in poultry production that favor transmission and spread of avian influenza to poultry.
4. Develop a standardized avian transmission model to better assess the inter-species transmission potential of AI viruses, especially from wild birds to poultry.
5. Identify virus-host correlates of virus transmissibility both within and between host species.
6. Develop tools for trade control.
7. Study how population density at farm, region, and market levels can affect transmission of influenza virus.

Avian/human interface

Gaps:

- Reservoirs and source of transmission of influenza viruses capable of infecting humans remains unknown.

Research needs:

1. Conduct closer monitoring and virological investigations of people in contact with poultry, especially those who present with influenza-like illness.
2. Develop better ways to test for human exposure. How do we detect exposure to reassortants with human/swine viruses?
3. Look for avian /human virus reassortants at the human/animal interfaces. Historical evidence suggests movement of a single gene or small subset of genes is the likely source of genes leading to a pandemic virus. .
4. Conduct closer monitoring and virological investigations of people in contact with pigs, especially those who present with influenza-like illness.
5. Investigate the possible role of humans in influenza virus infection of pigs and virus introduction into farms.

Surveillance

Gaps:

- Define trigger points for investigations in different types of flocks including vaccinated flocks
- Knowledge on surveillance systems applied country by country. Tools to weight the sensitivity of surveillance system and to assess the impact of disease control programs
- Systems for rapid incorporation of antigenic variants to vaccines (only China has done so in a timely manner).
- Lack of sufficient level and consistency of surveillance, lack of adequate compensation and lack of education of farmers, traders and veterinarians
- Structure and nature of poultry sector (usually rapid uncontrolled growth)
- Data gaps regarding outbreaks of non-OIE notifiable animal influenza viruses
- Data gaps exist worldwide regarding reportable outbreaks that are not reported to OIE or that are not reported in a timely fashion.

Research needs:

1. Conduct a review of the surveillance systems applied at a global level for AI.
2. Develop innovative tools to compare and weight efficacy of different surveillance systems.
3. Develop innovative tools to improve assessment of the impact of disease control programs.

4. Develop quantitative tools for the evaluation of surveillance systems in both animal and human health.
5. Novel approaches of disease reporting in poultry should be identified. Transparency should be not confined to notifiable influenza subtypes (H5 and H7).
6. Identification and characterization should be promoted also for non pathogenic subtypes and in particular for all avian influenza subtypes demonstrating increased potential to infect mammals (H1, H2, H3, H7, H5, H6, H9, and H10) or unusual propensity to undergo genetic reassortments (H6) with zoonotic subtypes viruses.
7. A good way to test policy and regulation before implementation to understand what unintended consequences might be involved.
8. Improved sharing of viruses between laboratories and between countries.

Mathematical modeling and molecular epidemiology

Gaps**:**

- Precise epidemiological data in public sequence databases- Availability of information on temporal, demographic and geographic processes of viral spread.
- Intra-host and outbreak dynamics of mutation occurrence

Research needs**:**

1. Install systematic surveillance systems that collect representative molecular data and the metadata involved. This will allow the use of modern phylogenetic/evolutionary techniques which integrates molecular data and phenotypic data (such as time of sampling and sampling location; antigenic data) to elucidate temporal, demographic and geographic processes of viral spread.
2. Promote whole genome sequence analysis to trace the evolution and the spread of novel viral gene constellations (eg. Reassortant strains).
3. Use of modern evolutionary methods to evaluate the effect of distinct hosts on virus evolution (eg. Domestic against Wild reservoir) and selective pressure. Increase accuracy related to the species or 'host status' (domestic or wild) in public sequence databases as it affects our ability to effectively study host shifts and their consequences on viral evolution.
4. Link phylogeny with socio-economic information, poultry trade data to better understand spread and movement of viruses within and across countries and continents.
5. Develop standards for data collection and placement on GIS maps/GPS databases for locations where isolates from wild birds and poultry are collected to enhance traditional and molecular epidemiologic investigations.
6. Risk factor modeling of the spatio-temporal pattern of avian influenza need to consider further variables such as socio-economic factors, poultry trade factors and factors related to wild bird distribution.
7. Develop computer simulation modeling methods to study multi-strain interaction (e.g., H9N2 and H5N1) and possible outcomes of multiple strain circulation. Speed of spatial spread during an outbreak
8. Apply sequencing tools recently developed (Next generation sequencing) to better elucidate the intrahost and outbreak genetic diversity and how this can affect evolutionary and intra and interspecies transmission dynamics.

GAPS IN AVAILABLE COUNTERMEASURES

VACCINES

The rapid development of candidate influenza vaccines and associated reference reagents is increasingly becoming an important part of preparing for a disease outbreak caused by a new and emerging animal influenza virus with epizootic and/or pandemic potential. However, developing influenza vaccines for new and emerging strains presents significant challenges as many of these viruses are considered transboundary animal diseases and is a limited market for these vaccines in the United States.[257,258]

Avian Influenza Vaccines

Effective vaccination programs for avian influenza can lead to a robust immune response that can reduce clinical disease symptoms, reduce the amount of virus that birds excrete if infected, and increase the resistance of the bird to being infected. All these factors can help break the transmission chain that can end an epidemic.[118] However vaccination if improperly applied, either by ineffective application, insufficient coverage, or through poor antigenic matching of vaccines to field strains, may contribute to the persistence of infection and disease in the region.[259,260]

Protection from vaccination is primarily through antibodies targeted to the hemagglutinin protein, which is the surface glycoprotein that initiates viral infection through attachment to the host cell and facilitates release of the viral genome after entry into the cell.[261] Antibody particularly to regions around the receptor binding site can interfere with viral attachment and infection. Antibodies to the neuraminidase protein and matrix protein may also provide some protection when present in high concentrations in the host, but current vaccine platforms do not induce high enough levels for practical value.[108,262] Antibodies to internal proteins, although valuable for diagnostic purposes, provide no additional protection to infection. Both innate immunity and cell mediated immunity can contribute directly to protection from clinical disease and it mediates the overall intensity and duration of the immune response. For cell-mediated immunity, it requires either previous infection or vaccination with a live vaccine to stimulate protective immunity. Currently no live attenuated influenza viruses are commercially available or are likely to be available in the near future. However, several viral vectored vaccines that express avian influenza antigens are commercially available.[263,264] These vaccines produce both an antibody mediated as well as a cell mediated immune response that provides additional protection over just the antibody response. The strengths and weaknesses of viral vectored vaccines are described below (see Countermeasures Assessment, Page 73). A strong cellular immune response, although contributing to effective protection from clinical disease has not been shown to provide long lasting immunity or broad protection for avian influenza. It is also much more difficult to quantify the cellular immune response, which makes prediction of effective levels of protection harder to achieve.

One of the biggest problems of vaccination for avian influenza is the antigenic variability that is present in the wild bird reservoir and the rapid antigenic drift that is present when the virus enters the poultry population. Currently 16 antigenically distinct hemagglutinin subtypes are present in the wild bird reservoir.[261] By definition antibody to one subtype protects only for viruses of the same subtype and not for any of the other 15 hemagglutinin subtypes. This antigenic variability makes it impractical to provide

protection for all influenza viruses. Although almost all hemagglutinin subtypes found in wild birds have also been found in poultry, the list of subtypes that has caused persistent and widespread outbreaks is a much smaller number, but even this reduced number makes preventive vaccination an unrealistic target. With few exceptions in poultry, vaccination has been targeted to specific outbreak viruses; i.e., vaccination is targeted to a single subtype that is currently circulating in the field. However, an equally difficult problem is that influenza viruses have a high mutation rate that can result in a rapid change in the antigenic properties of the hemagglutinin protein and can decrease the protective effect of vaccination.[265,266] If the host has high levels of antibody that closely matches the challenge strain, then the virus is quickly neutralized and virus replication is greatly decreased. However, as antigenic drift occurs because of the high mutation rate, variant viruses can be produced that are neutralized less effectively by the same levels of antibody, and these variant viruses will replicate at higher levels and have increased opportunities to spread to naïve flocks.[259] The hemagglutinin protein has multiple protective antigenic sites, and therefore antigenic drift is generally thought to occur in a stepwise fashion as variant viruses gradually change so that the original antibody becomes slowly less protective allowing increasing levels of virus replication and shedding in vaccinated birds.[267] Eventually, enough antigenic drift can occur so that the vaccine, even though targeted to the same hemagglutinin subtype, is completely ineffective. The same principle is seen with human influenza viruses, which has resulted in a worldwide surveillance program through the WHO that allows prediction of the most likely circulating antigenic variants, with subsequent changes to vaccine seed strains as often as twice a year, although historically vaccine seed strains are changed only every 2 years for each subtype. However, for a variety of reasons, avian influenza vaccines are only rarely updated.

Swine Influenza Vaccines

There is a recognized need for improved vaccines over our current commercially approved vaccines to better serve the need of the swine industry. This entails the use of novel technologies that will involve new regulatory hurdles and likely face economic constraints. Current influenza vaccines are primarily used in adult sows to protect the gestating sow and her suckling piglets or during the grow/finish phase of production to decrease IAV disease, lung lesions and transmission. Vaccinating piglets may be desired in some clinical situations, but the presence of maternal antibodies interferes with vaccine efficacy of inactivated vaccines. Although passive maternally derived antibody may reduce clinical illness, it often is not effective in preventing replication and transmission, particularly with antigenically drifted viruses, allowing weaned pigs to infect downstream nursery and/or finishing sites. Whole inactivated virus (WIV) with adjuvant is the only preparation currently readily available commercially for use in swine. Protection induced by intramuscular administration of fully licensed or autogenous WIV rely on systemic immune responses for correlates of immunity, primarily measured by hemagglutination inhibition (HI) assays with serum antibodies. The production of "off-the-shelf" commercially available efficacious WIV vaccines has proven difficult due to the number of valences required to minimize the antigenic distance between vaccine strains and circulating IAV strains in swine. Current commercially licensed WIV preparations used in swine include two or more representatives of H1 and H3 cluster-types mixed with oil based adjuvants, but WIV have consistently been shown to provide only partial protection against heterologous IAV infection and shedding. Formulating and updating effective WIV vaccines is further challenged by the difficulty in updating vaccine seed viruses faster than the rate of evolution, the time needed to approve and license WIV products, maternal antibody interference, and the lack of an adequate mucosal and cell-mediated immune response. Immune pressure appears to impose positive selection at putative antibody epitopes, contributing to the observed evolution and subsequent antigenic diversity.

Apart from sub-optimal protection provided by WIV, they may be associated with vaccine-associated enhanced respiratory disease (VAERD) under certain criteria. This phenomenon is characterized by severe respiratory disease that results when there is sufficient antigenic drift between the vaccine and challenge strains in spite of having the same hemagglutinin subtype. Alternative IAV vaccine platforms and methods of delivery are needed to improve protection from heterologous infection without the risk of VAERD. Live attenuated influenza virus (LAIV) vaccines administered by the mucosal route mimics natural infection have the potential for broader cross-protective immunity at the respiratory mucosa and appear to have less risk for VAERD.

The antigenic diversity of IAV in swine has made it difficult to produce multivalent inactivated vaccines that fit all production needs. Additionally, public health needs may demand vaccines that perform above the current veterinary label claims. There is a recognized need for improved vaccines over current commercially approved vaccines to better serve the need of the swine industry. Experimental vaccines must meet new regulatory hurdles that are currently unclear, as these vaccines may not fit current paradigms. The National Environmental Policy Act (NEPA) is a United States environmental law that established a U.S. national policy promoting the enhancement of the environment and also established the President's Council on Environmental Quality (CEQ). NEPA set up procedural requirements for all federal government agencies to prepare environmental assessments and environmental impact statements that contain statements regarding the environmental effects of proposed federal agency actions. This includes the approval of veterinary vaccines involving genetically modified organisms. The USDA Animal Plant Inspection Service (APHIS) first established procedures in 1991 that set forth the principles and practices to follow to comply with the NEPA.[268-270] These procedures are now being updated to take into consideration the genetic engineering technology available today for manufacture of next generation vaccines. APHIS is responsible for regulating the introduction (importation, interstate movement, and environmental release) of genetically engineered organisms. To achieve this APHIS has developed implementing rules for NEPA for environmental assessment for field-testing of genetically engineered organisms. The next generation of influenza vaccines are virtually all based on genetic engineering methods for the development, rapid updating of relevant antigens and manufacture. As such, the Center for Veterinary Biologics-APHIS is seeking a categorical exclusion for genetically engineered vaccines that have been proven safe and efficacious to enable rapid updating of the protective antigens contained in these vaccines. If these hurdles are overcome, the cost of goods for producing the vaccines would become a consideration in widespread adoption in the swine industry.

Human Influenza Vaccines

There is a recognized need for human influenza vaccines for first responders in the case of an outbreak with a variant animal influenza virus with epizootic and/or pandemic potential is critical.

Global influenza vaccine production capacity

Since its launch in 2006, the WHO Global Action Plan for Influenza Vaccines (GAP) has proved to be an effective catalyst for a significant expansion in influenza vaccine manufacturing. Seasonal vaccine production has increased from 350 million doses in 2006, to close to 1 billion doses per year by the end of 2010. By 2015, enough seasonal influenza vaccine will be produced to immunize two billion people. Given the close association between seasonal and pandemic influenza immunization, the increased capacity of seasonal influenza directly improves global pandemic preparedness.

GAP also has facilitated a significant expansion in the manufacturing capacity of influenza vaccines in both developing and developed countries. As of 1 October 2012, fourteen developing-country manufacturers had received WHO seed grants and technology-transfer support, including the newly added companies from Kazakhstan, South Africa and China. Of these manufacturers, five currently have licensed vaccines on the market (India, Indonesia, Romania, Republic of Korea and Thailand) and the remaining nine are in late stage development. Financial support was provided by the U.S. Department of Health and Human Services, the Government of Japan, the Asian Development Bank, the Government of Canada and the UK Government.

Summary of Vaccine Gaps

Regardless of the species or the technology used, currently available vaccines all have the same general weaknesses; they have limited cross-protection against antigenic variants within a subtype, even less protection between subtypes, and they cannot be produced quickly enough to keep pace with the ever changing Influenza A virus. Gaps in vaccine research include:

1. Evaluation of novel technologies that reduce the time required to produce a vaccine.
2. Development of novel vaccine technologies to produce a broader or universal clinical protection.
3. Development of vaccine platforms that can be used in multiple species.
4. Improvement in the regulatory process for vaccine selection and production.

DIAGNOSTICS

Obstacles for Detecting Animal Influenza Viruses

Current tests are overall accurate and reliable, although new technology can always improve sensitivity and specificity. Because of the highly variable nature of influenza viruses, continued monitoring of test for their ability to detect variants is necessary. The use of both a screening test and a confirmatory test is optimal in most cases.

Some other obstacles are process based, for example sample collection in a medium that does not require the cold chain would be beneficial for virus isolation, which is frequently used to confirm the results of molecular tests (which do not require viable virus). More importantly, influenza virus isolates are necessary to characterize the biology and genetics of new isolates.

Surveillance programs should be optimized for each animal species, however limited resources will constrain some ability to collect and characterize surveillance samples. Each animal and production system has a unique structure and needs and there are biological variations among influenza viruses in poultry, swine and horse that need to be taken into account. The largest obstacle to implementing the best surveillance programs is resources and funding.

Once an outbreak is detected a more targeted approach can be adapted and the diagnostic methods can be refined to the needs to the situation.

Summary of Diagnostic Gaps

Influenza is a highly variable virus, which complicates diagnostic tests. Tests for type A influenza viruses are generally reliable, but serological test require improvement. The key animal influenza diagnostic gap

is in subtype identification and in the identification of the subtype specificity of sera. Better serologic tests are needed, both to determine the subtype specificity of antibody (i.e., what subtypes has an animal been infected with) and to characterize the antigenic differences among animal influenza isolates. Characterization of the antibody response and antigenic differences among animal influenza isolates are critical for updating vaccines and evaluating vaccinal protection. Hemagglutination inhibition (HI) assay is the current standard for identifying the subtype specificity of sera and to characterize antigenic differences. This is a cumbersome test that lacks precision.

Other areas where improvement would be beneficial are: 1) a rapid molecular test that is not easily affected by genetic mutations; 2) a transport media that stabilizes the virus without refrigeration; and 3) at the very least, efforts to characterize new isolates should be continued to assure that current tests will have optimal sensitivity and specificity.

SURVEILLANCE

Poultry

Surveillance in poultry is routinely conducted in the U.S. The National Poultry Improvement Plan has a program to certify poultry flocks as avian influenza free or monitored. The U.S. poultry industry generally eradicates avian influenza virus when it is detected. Nearly 100% of chicken flocks are tested for AIV prior to movement to slaughter. Long lived birds such as turkeys, breeders, and egg layers are periodically tested for avian influenza virus. This testing is mostly for antibody. Detection of AIV or AIV antibody in poultry has major trade implications therefore the industry works to keep U.S. poultry free of AIV.

Small holder poultry, back yard flocks, live bird markets and specialty birds, such as upland game birds are less stringently monitored. The programs for theses birds are administered by the states, although most states work closely together to harmonize programs (e.g., the live bird market testing standards). Most testing of small holder and non-commercial poultry is passive, where birds that are exhibiting clinical disease consistent with AIV infection are submitted to a veterinary diagnostic lab and tested for AIV as a differential diagnostic test at no cost to the owner (federally funded). The amount of testing conducted by the states in these types of operations is related to the availability of federal funds.

All poultry testing is initially conducted at local or regional state laboratories, then positive or suspect samples are submitted to the USDA National Veterinary Services Laboratory (NVSL), Ames, Iowa, for official confirmation. Only the NVSL can officially diagnose reportable AIV.

Swine

USDA SIV Surveillance System

Swine influenza caused by H1N1 was historically characterized as a seasonal disease, primarily in weaned pigs with waning maternal immunity. Today, clinical disease still peaks during times of the year associated with dramatic fluctuations in temperature and decreased ventilation, and was recently shown to have a primary peak in November-December and a secondary spike in March-April. However, contemporary influenza illness and diagnosis can be found at any time of the year, in nearly all age groups of pigs, even suckling pigs from sows with high titers of influenza specific serum antibodies. This is likely

due to the increasing numbers of antigenic cluster types as well as the variability in passive transfer and populations with mixed levels and specificity of immunity.

The USDA SIV Surveillance System was initiated in 2009 and roughly 2000 swine influenza virus (SIV) isolates have entered into the system to date. Currently, three gene segments (HA, NA, and M) are being sequenced routinely by participating National Animal Health Laboratory Network (NAHLN, APHIS) laboratories and the sequences submitted to the GenBank database. Approximately 1700 isolates have the three gene sequences deposited (October 2013).

From the sequence analysis, novel viruses may be selected for associated *in vitro* and *in vivo* study of SIV isolates, including pathogenesis and transmission, antigenic characterization, or vaccine protection experiments. Additionally, influenza A virus isolates may be identified through public health investigations of zoonotic transmission events or from the animal health sector for unusual phenotypes or evasion of vaccine immunity. Importantly, the USDA SIV Surveillance System virus isolate repository at the National Veterinary Services Laboratories (NVSL) provides candidate vaccine viruses and reference reagents for epizootic and pandemic preparedness upon request.

SIV Surveillance Gaps

There is not a systematic approach for analyzing and reporting summarized results of the sequencing efforts on a single gene or whole virus genome level or an approach for evaluating the antigenic consequences of the genetic diversity. This is a gap in providing a useful output from the surveillance system for determination of significant virus evolution and identification of viruses of interest.

Human

Global Influenza Surveillance and Response System (GISRS)

The GISRS is an international network of influenza laboratories under the coordination of the WHO. The WHO GISRS laboratories work collectively to: 1) monitor the evolution of influenza viruses and provide risk assessment and recommendations in areas including laboratory diagnostics, vaccines and antivirals; and 2) serve as a global alert mechanism for the emergence of influenza viruses with pandemic potential.

GISRS is comprised of four complementary categories of laboratories:
- 138 National Influenza Centres (NICs) in 108 countries
- 6 WHO Collaborating Centres (WHO CCs)
- 4 WHO Essential Regulatory Laboratories (WHO ERLs)
- 12 WHO H5 Reference Laboratories (WHO H5 Ref Labs)

During May 2011–May 2012, WHO CCs performed detailed analyses to characterize a total of 205 isolates of influenza A(H5N1) from 7 different countries. The analysis of H5N1 or other influenza viruses with human pandemic potential represents only a small portion of the more than 1.1 million total specimens that GISRS laboratories processed during that period. Diagnosing influenza requires special reagents that are updated regularly. During the report period, NICs and other influenza laboratories in 130 different countries, areas or territories had access to, without charge, reagents developed by the United States (CDC, Atlanta GA) WHO CC to test for seasonal and other influenza viruses, including those with human pandemic potential.

During May 2011–May 2012, GISRS developed and made available the following candidate vaccine viruses and reference reagents for pandemic preparedness:

- A(H5N1): 4 new candidate vaccine viruses and 3 new reference reagents
- A(H9N2): 1 new candidate vaccine virus
- A(H3N2)v: 3 new candidate vaccine viruses

GISRS Gaps/Improvement Needs

- Efficient and timely transport of specimens or virus isolates may be hampered by logistical challenges in some countries that can delay diagnosis, virus characterization and risk assessment.
- Countries do not always have financial resources necessary to ship virus samples from NICs or other national laboratories to WHO CCs and other reference laboratories for advanced analysis and characterization.
- The GISRS need to be strengthened through efforts to build capacity at country level, which is integral to robust pandemic preparedness and response.
- The 2009 H1N1 pandemic revealed multiple gaps in global influenza surveillance capacity. To address these gaps, WHO embarked on a multistep process to revise global influenza surveillance guidelines; this process formally commenced with the *Global Consultation on Influenza Surveillance Standards* held in Geneva in March 2011. An interim document, *WHO Interim Global Epidemiological Surveillance Standards for Influenza (July 2012),* has been posted for review at: http://www.who.int/influenza/resources/documents/INFSURVMANUAL.pdf.

Summary of Surveillance Gaps for Animal Influenza Viruses

Gaps in surveillance for animal influenza viruses fall into three broad categories: 1) Using limited resources to provide efficient sampling of animals to confidently characterize circulating endemic influenza viruses, 2) Rapidly detecting emerging new strains, subtypes or incursions between host species to prevent spread and establishment of new lineages, and 3) identifying influenza viruses with human infection or pandemic potential. Specific gaps in surveillance include:

1. Resources to investigate seropositive flocks identified in the National Poultry Improvement Plan influenza program for virus identification.
2. Permanent sustainable resources to continue development of the USDA SIV Surveillance System as necessary.
3. Understanding the movement of influenza viruses within the U.S. and across its borders and where to target surveillance to maximize limited resources.
4. Basic studies into the determinants of cross-species transmission and adaptation are critical to enable the identification of genetic signatures that indicate the zoonotic potential of a virus.
5. Improved diagnostic tests that target viral properties important for immunity, virulence, and interspecies transmission potential.

DRUGS

The effectiveness of this countermeasure is medium at best. There are no AI antiviral drugs approved for use in animals. Tamiflu (oseltamivir phosphate) and Relenza (zanamir) are both approved for human uses and are very effective against AI virus. They are however prohibitively expensive. There are no residue data for food animals. Symmetrel and Symadine (amantadine), and Flumadine (rimantidine), are also approved for human use and are also effective against AI virus but drug resistance is common with over 50

percent of AI strains developing resistant to these drugs. The issue with drug resistance makes the use of these drugs in livestock and poultry questionable even if less costly generics were available.

DEPOPULATION AND DISPOSAL

Although every effort is made to safeguard the welfare and lives of animals, depopulation is often considered the first line of defense against a foreign animal disease outbreak. In the event of a widespread animal influenza virus outbreak with epizootic and/or pandemic potential in the United States, thousands of pigs and/or millions of poultry could be affected. Current national and international policy on notifiable animal diseases may require that affected animals be quarantined, their movement stopped, and the animals humanely euthanized. HPAI is an OIE notifiable disease and the U.S. has developed state and national response plans in accordance OIE standards. IAV in swine that are defined as "emerging" may reach the criteria to become reportable to OIE, but the response to these outbreaks is not defined internationally or at the national level in the USA. When outbreaks require such measures, large numbers of animal carcasses must be safely and quickly disposed of without causing environmental harm, allowing the decontaminated facilities to resume livestock production as soon as reasonably possible. However, as has been seen recently during outbreaks in the UK, Japan, and South Korea, this approach can have devastating effects on the livestock industry, the economy, and the environment, and can be unsustainable for the country involved. Specifically, these processes carry the potential for significant risks to public health, animal health, and the environment if not conducted carefully. For example, human responders might be exposed to a zoonotic animal influenza virus, and healthy domestic animals, and the public may be exposed if improper decontamination and disposal occurs. Viruses may contaminate ground water, surface water, soil, or the air, if there are large numbers of shedding animals or if they are improperly disposed. Hazardous materials used or generated during the response could also contaminate the environment if disposal is improperly performed, leading to additional environmental liabilities and significant cleanup costs.

DISINFECTANTS

Animal influenza viruses are labile and inactivated by several inexpensive effective commercial disinfectants. EPA-approved disinfectants commercially available include quaternary ammonia, phenolics, alkylating agents, sodium hypochlorite, and iodine. All of these compounds are cost effective and available in the United States.

PERSONAL PROTECTIVE EQUIPMENT (PPE)

PPE is necessary to safely work in influenza virus contaminated environments, but it is expensive, hot, and uncomfortable for the users. There is a need to improve these products for working under field conditions in contaminated environments. The most important PPE when working with a zoonotic influenza virus that is virulent for humans is a respirator, either a disposable particulate respirator or a powered air purifying respirator (PAPR) unit. "CDC Guidelines and Recommendations - Interim Guidance for Protection of Persons involved in U.S Avian Influenza Outbreak Disease Control and Eradication Activities" state that disposable particulate respirators are the minimum level of respiratory protection that

should be worn. Workers must be fit tested to the particulate respirator model; thus, workers who cannot wear a disposable particulate respirator should wear a loose-fitting PAPR unit with high efficiency filters. The poultry industry does not generally store PAPR units and they would not be readily available in the case of an outbreak. APHIS currently stockpiles respirators that would be available to a limited number of workers involved in an AI eradication campaign.

Workers disposing of AIV infected carcasses or conducting post mortems should wear chemical resistant aprons and long sleeved gloves. It is also standard practice to wear "Standard Outfit" protective clothing, consisting of one piece disposable coverall, disposable shoe covers, disposable hair covering, chemical resistant gloves, eye protection, face masks/face shields, and other foot protection if needed (boots, steel toe shoes).

The availability of FDA-approved human drugs and vaccines to protect workers when facing an outbreak with an AI virus that can infect people will also be critical.

OBSTACLES TO PREVENTION AND CONTROL

AVIAN INFLUENZA

The poultry industry is complex and the control of diseases in the various poultry segments brings forth different challenges. The control of HPAI in short-lived broilers, produced in the billions in the United States, compared to longer-lived layers and breeders requires different strategies. An AI outbreak in these segments may demand different interventions, depending on the inherent attributes of the available countermeasures. For instance, a vaccine that requires individual animal inoculations and two doses will be prohibitive in the broiler segment. On the other hand, a vaccine that allows mass vaccination through the drinking water and prevents transmission would have a significant role in containing an AI outbreak. Thus, assumptions and the criteria used to compare countermeasures are critical for a valid analysis.

The following were determined to be significant obstacles to effective prevention and control of AI:

1. AI viruses continue to evolve resulting in new strains that have the ability to cross species barriers and become virulent in man and animals. We do not have the predictive tools necessary to effectively mitigate the impact of new and emerging strains. We need access to validated rapid diagnostic tests to rapidly respond to new disease outbreaks.
2. Vaccines specifically designed for the control and eradication of AI in intensive poultry product systems have not been developed. We need vaccines that are efficacious across different avian species, provide mucosal immunity, prevent viral shedding, have mass delivery applications, and the ability to differentiate infected birds from vaccinated birds.
3. Studies have shown that the use of antiviral drugs to control AI virus infections in poultry may contribute to the rapid emergence of drug resistant strains, making their use in animals questionable, especially when it reduces the efficacy of these drugs in people. Antiviral drugs are currently not recommended for production veterinary medicine.
4. Implementation of strict biosecurity measures are logistically difficult, expensive and unreliable, making their use difficult to justify to producers during disease-free periods.
5. The euthanasia of millions of birds and subsequent carcass disposal is difficult logistically and the economic, or social costs are often unacceptably high.
6. Proper safeguards must be in place to provide adequate protection for individuals working in infected premises during a zoonotic AI outbreak.

SWINE INFLUENZA

Endemic Influenza

The U.S swine industry is also large and complex. Although some pigs may be raised in a backyard setting, the vast majority of U.S. pork is raised in intensive production systems that have unique challenges for control of endemic influenza virus. Five basic production systems can be found in the U.S: 1) farrow-to-finish farms that involve all stages of production, from breeding through finishing to market weights of about 265 pounds; 2) farrow-to-nursery farms that involve breeding through marketing 40- to 60-pound

feeder pigs to grow-finish farms; 3) farrow-to-wean farms that involve breeding through marketing 10- to 15-pound weaned pigs to nursery-grow-finish farms; 4) wean-to-finish farms that involve purchasing weaned pigs and finishing them to market weights; and 5) finishing farms that buy 40- to 60-pound feeder pigs and finish them to market weight. Additionally, niches such as the show pig and wet-market industries may present unique obstacles to control and prevention of IAV infection compared to commercial pork production since they have considerably more human-animal interface with naïve members of the public and less defined systems of operation. A 2009 NAHMS bulletin reported in a 2006 survey that 75% of swine herds that did not use influenza vaccine were seropositive for H1 or H3 or both subtypes.

New and Emerging Strains

Over the last 15 years the ecology of SIV in North America has been dynamic with the emergence of antigenic SIV variants through species jumps of human and avian viruses into pigs that led to novel reassortant viruses. With improved surveillance efforts in the swine population globally, it is clear that human seasonal influenza viruses have been the single most important contribution to the increase in genetic diversity in endemic swine influenza viruses worldwide (Nelson et al, 2012). Subsequent to the incursions of human seasonal viruses into swine populations, the surface glycoproteins drift antigenically and eventually the human population becomes susceptible again as the human population immunity wanes. This cycle is likely to continue, making swine influenza a credible threat to human health and vice versa. Although direct transmission of avian IAV to swine has occurred less frequently than that of human seasonal strains, these events have been documented and remain a potential threat to the swine population.

Influenza viruses in swine have evolved rapidly in recent years, resulting in new antigenic variants that have the ability to spread regionally and sometimes globally to establish new endemic lineages. The high incidence of SIV in the U.S. swine herd combined with the frequent transmission of human influenza viruses into swine indicates eradication of SIV with current technology it is not feasible, leaving prevention and control as the only option.

The following are significant obstacles to effective prevention and control of IAV in swine:

1. Production practices or virus-host interactions that drive rapid antigenic evolution of endemic swine IAV are not known.
2. Factors involved in influenza spread within and between production systems are not well understood.
3. The predictive tools, rapid identification, and appropriate response activities necessary to effectively mitigate the impact of new and emerging strains are not available or well defined.
4. The determinants of anthroponotic transmission of human seasonal strains to swine are unknown.
5. The determinants of zoonotic transmission of swine IAV to humans are unknown.
6. Sensitive “pen-side” diagnostic tests to rapidly detect and respond to new disease outbreaks are not available.
7. Effective and affordable vaccines for the control and eradication of IAV in intensive pork production systems are not uniformly available. Vaccines that are efficacious across different age groups in different production settings that provide robust and broad immunity to prevent disease and viral shedding are needed.
8. Rapid (<6 months) availability of vaccines with updated strain changes to prevent epidemics with a novel emerging strain is not supported by the current manufacturing and regulatory practices.

9. Implementation of biosecurity measures and movement restrictions to control IAV may be logistically difficult, expensive, and unrewarding, making their use difficult to justify to producers for an endemic disease.

ANALYSIS

The following captures assumptions made by the AICWG in assessing potential countermeasures to enhance our ability to contain and eradicate an outbreak of an animal influenza virus with zoonotic and pandemic potential in the United States.

AVIAN INFLUENZA

The AICWG was charged with the task of conducting an in-depth analysis of available countermeasures. Since the AI strain, the location, or the poultry segment affected cannot be predicted, the group agreed to certain assumptions prior to conducting its analysis. First, the group agreed that the outbreak should be the worst case scenario: HPAI virus strain that can infect people with high morbidity. Second, the AI outbreak would occur in two separate locations simultaneously in a poultry concentrated area. Third, the group defined the attributes of a vaccine or diagnostic test needed to quickly contain and eradicate an outbreak. For the analysis, the AICWG used a decision model specifically designed for these countermeasures. The decision model criteria and their respective weight were agreed to by the group prior to conducting the analysis.

Situation

Countermeasures assessed for worst case scenario: outbreak with highly pathogenic avian influenza strain that can infect people with high morbidity.

Target Populations at risk

Countermeasures assessed for target flocks in priority order:

1. Valuable genetic poultry stock
2. Commercial turkeys
3. Long-lived poultry, such as parent breeders
4. Layers
5. Backyard birds
6. Rare captive birds
7. Commercial ducks
8. High risk situations; e.g., ring vaccination around HPAI infected birds
9. Broilers/meat production poultry

Scope of Outbreak

Countermeasures assessed for two outbreaks occurring simultaneously at two different locations each with a target population of 7 million birds or less.

Vaccine Administration

1. Commercial poultry firms can logistically vaccinate 1 million birds in 20 days using commercially available killed vaccines.

2. Federal and state vaccination crews can vaccinate 1 million birds in 4 weeks using commercially available killed vaccines.
3. If mass vaccine available (water/spray delivered) the entire target population could be vaccinated.

Diagnosis

Pen-side tests are available for type A influenza detection. These tests are considered only presumptive tests and all test samples must be sent to NVSL and NAHLN avian influenza-certified laboratories for confirmatory testing. The available pen-side tests are regulated for use at the state level, and may not be approved for use in all states.

SWINE INFLUENZA

The assumptions made in assessing potential countermeasures for swine influenza are complex since it is endemic in the United States and in most countries where swine production occurs. Notwithstanding, novel swine influenza virus strains that may have zoonotic potential are constantly emerging and posing significant concerns. Accordingly, the analysis of countermeasures for both "seasonal" endemic swine influenza and new and emerging variant viral strains were included in the assessment of countermeasures, recognizing that the needs and gaps may be significantly different. The AICWG group agreed to the following assumptions prior to conducting its analysis of countermeasures for variant swine influenza viruses.

Situation

Countermeasures assessed for worst-case scenario: outbreak with a highly virulent swine influenza virus strain that can infect people with high morbidity.

Target Populations at risk

Countermeasures assessed for specific segments in priority order:

1. Sow breeding operations
2. Pig production
3. Valuable genetic stock

Scope of Outbreak

Countermeasures assessed for two outbreaks occurring simultaneously at two different geographical locations where the majority of swine production occurs in the United States: Iowa and North Carolina.

Vaccine Assumptions

1. Highly efficacious: prevent pneumonia, clinical disease and shedding; multivalent with cross protection within subtypes; quick onset of immunity; 6 mo duration of immunity;
2. Safe to workers and use in pregnant animals
3. Manufacturing method yields high number of doses
4. Mass vaccination compatible
5. Rapid speed of production and scale-up
6. Reasonable cost
7. Short withdrawal period for food consumption (all currently 21 d)
8. Single dose

9. Regulatory issues: approval time & pathway

Vaccine Administration

1. Commercial swine operation can logistically vaccinate 1 million pigs in 20 days.
2. Federal and state vaccination crews can vaccinate 1 million pigs in 4 weeks.

DECISION MODEL

The AICWG used the quantitative Kemper-Trego (KT) decision model to assess available countermeasures. Instructions for using the model were provided to the working group prior to the meeting of March 25, 2013 (see Appendix I). The model was modified by the working group for the purpose of assessing animal influenza vaccines and diagnostics (See Appendices III-XI).

CRITERIA

The AICWG selected core criteria to enable the comparison of countermeasures using a pertinent and valid analysis, as follows:

Avian Influenza Vaccines

- Efficacy
- Cross-protection within haemagglutinin subtypes
- < 1 week onset of immunity
- No maternal antibody interference
- Two year shelf life
- Safe vaccine
- No high containment required
- DIVA compatible
- Rapid scale-up (> 10 million doses)
- Reasonable cost
- Short withdrawal period
- Feasibility of registration
- Add new antigens
- Accelerated delivery

Swine Influenza Vaccines

- Efficacy
- Cross-protection within haemagglutinin subtypes
- < 1 week onset of immunity
- No maternal antibody interference
- Two year shelf life
- Safe vaccine
- No high containment required
- DIVA compatible
- Rapid scale-up (> 10 million doses)
- Reasonable cost
- Short withdrawal period
- Feasibility of registration
- Add new antigens

- Accelerated delivery

Diagnostics

- Sensitivity
- Specificity
- Validation to purpose
- Speed of scale-up
- Throughput
- Pen-side test
- Rapid result
- Need for a confirmatory test
- Need for serological test to show recovery (absence of circulating virus)
- DIVA compatible
- Easy to perform
- Cost to implement

Weight

Each criterion was weighted to allow a quantitative comparison of the impact of the selected interventions (see Appendix II).

Product profile

To ensure a consistent and meaningful assessment, the desired product profile (i.e., the benchmark) that would enable the control and eradication of an animal influenza virus outbreak was identified for each countermeasure:

Ideal Avian Influenza Vaccine Profile

1. Highly efficacious: prevents transmission in all major target animal species; efficacy in young animals
2. Cross-protection (cross-protection within haemagglutining subtypes)
3. Cross-serotype protection (cross-protection against all 7 serotypes
4. One dose with >1 year duration of immunity
5. One week or less onset of immunity
6. No maternal antibody interference
7. Two year shelf life
8. Safe vaccine: non-abortegenic; all species; pure vaccine
9. No reversion-to-virulence
10. No high containment required for manufacturing (eliminate need to grow live virus)
11. DIVA compatible
12. Rapid speed of production and scale-up
13. Reasonable cost
14. Short withdrawal period for food consumption (21 days or less)
15. Feasibility of registration (environmental release of a recombinant)
16. Ability to rapidly incorporate emerging viral antigens

Ideal Swine Influenza Vaccine Profile in order of weight

Adult animals (sows)

1. Highly efficacious in preventing pneumonia, disease and shedding, one dose with >6 months duration of immunity, and one week or less onset of immunity
2. Cross-protection (cross-protection within HA subtypes)
3. Safe vaccine: to workers and in pregnant animals
4. Prevents transmission.
5. No maternal antibody interference
6. Rapid speed of production and scale-up
7. Number of doses and cost of goods
8. Combination vaccine compatible with other viruses or bacterins
9. DIVA compatible
10. Short withdrawal period for food consumption (21 days or less)

Ideal Diagnostic Test Profile

1. Direct tests (e.g., antigen, nucleic acid) for control and eradication
2. Indirect tests for post-control monitoring/detection sub-clinical cattle and wildlife
3. Rapid test
4. >95% specificity
5. >95% sensitivity
6. Pen-side test
7. DIVA Compatible
8. Field validated
9. Easy to perform/easily train NAHLN's personnel
10. Scalable
11. Reasonable cost
12. Detect all influenza virus strains

VALUES

The values assigned by the AICWG for each of the interventions reflect the collective best judgment of AICWG members (see Appendices III-XI)

COUNTERMEASURES ASSESSMENT

The protection of animals against animal influenza viruses has been a concern of livestock and poultry producers for decades. Animal influenza viruses are extremely contagious and have complex epidemiological profiles that include several animal species, and therefore require an integrated approach for control and eradication. Paramount is the availability of effective vaccines and diagnostics.

VACCINES

Effective immunological prophylaxis for the control of influenza virus is probably one of the most complex problem facing animal health authorities worldwide. The following sections provide specific information on the history and breakthroughs in avian and swine influenza vaccine development and a detailed analysis of available commercial and experimental vaccines.

History of Avian Influenza Vaccine Development

The history of AI vaccines has its origins back to the late 1920s, when chickens infected with fowl plague virus (now known as HPAI virus) recovered from the disease and were resistant to re-exposure.[271,272] Initially, experimental vaccines against fowl plague were based on the experiences of Pasteur with rabies, which used spinal cords from infected animals as vaccines to protect against virulent rabies virus. Although many AI vaccine failures were observed, either from a lack of immune response, inefficient viral inactivation, or mismatched subtypes, eventually, efficacious AI vaccines were produced that protected birds from disease. However, by that point, the policy of "stamping-out" HPAI virus infections of poultry to control spread of disease had gained support throughout Europe, and vaccines were generally not used as part of any control strategy against AI.

More recently, vaccines have been developed and approved for use against LPAI virus infections of poultry. Beginning after the mid-1960s when the economic impact of LPAI virus infections in poultry was realized, control strategies were implemented based on economic need. Early field management strategies included controlled exposure of pullets to LPAI viruses to produce immunity prior to egg lay. In the United states beginning in 1979, AI vaccines were primarily used to prevent production losses in turkeys and egg-laying chickens (breeders and table-egg production). In the past decade, H1N1 and H3N2 swine influenza virus infections of turkeys have resulted in significant decrease in egg production and quality [273,274] (E. Gonder, personal communications). However, because these are low pathogenicity isolates, limited conditional-use inactivated vaccines have been used in such turkey flocks as a management strategy as has been done since the first LPAI vaccine, a H4 and H6, used in 1979 in the United States[135] Finally, following an outbreak H7N2 LPAI in an isolated commercial chicken facility containing laying hens in Connecticut, agreements between state, federal and industry representatives provided the use of an inactivated LPAI vaccine as part of a comprehensive strategy as an alternative to immediate depopulation.[275] As a part of the control strategy, vaccinated flocks were intensively monitored for virus shedding through dead bird testing, and serological surveillance using non-vaccinated sentinels and a neuraminidase (NA)-based DIVA (differentiation of infected from vaccinated animals) approach to detect infections in vaccinated birds. Taken together, vaccination can now be considered as a valuable

component in comprehensive AI control strategies. While situational or local outbreaks of HPAI may always require stamping-out in non-endemic areas, in the face of an epizootic event, vaccines can be formulated and used based on field isolates recovered.

Until the recent H5N1 outbreak in Southeast Asia and Egypt, vaccination against AI had not been widely used worldwide. A multivalent inactivated AI vaccine containing H5N2, H6N2, H10N2 along with Newcastle disease virus (NDV) was reported to have been used in Italy in 1980 to control multiple subtypes of LPAI virus.[276] In addition, ring vaccination with inactivated vaccines against H6N2 and H9N2 strains enzootic in Italian turkeys have been applied in breeder birds.[277]

An inactivated H5N2 vaccine was used in Mexico as a result of the widespread HPAI outbreaks caused by H5N2 virus that began in December 1994.[278] Between 1995 and 1997, 847 million doses of vaccine were licensed for use. Inactivated H7N3 vaccine was also used extensively in Pakistan following the widespread HPAI outbreaks in 1995.[279]

Following the outbreak of H5N1 HPAI in 1996 in China, an inactivated, oil-emulsified vaccine was developed using an H5N2 low pathogenic virus, A/turkey/England/N-28/73. The vaccine was first approved in August of 2003. In total, 2.5 billion doses of H5N2 inactivated vaccine were used to control the initial outbreak, however because of mismatched antigens, the vaccine was not ideal [280]. More recently, a plasmid-based reverse genetics approach was employed to create vaccines that matched the HA from field isolates to the vaccine [281]. Using the internal genes from A/Puerto Rico/8/34 (PR8) virus, and the HA and NA genes from recent H5N1 viruses including GS/GD/1/96 (Re-1), A/bar-headed goose/Qinghai/3/2005 (Re-3) and A/duck/Anhui/1/2006 (Re-5), new viruses were constructed and utilized as inactivated vaccines. Safety concerns were met by replacing the multiple basic amino acid motif found in the cleavage site of the HA protein of high pathogenicity in H5 avian influenza viruses (-RRRKKR-) and replaced with those of low pathogenicity (-RETR-). The Re-1 vaccine was approved for use in the field in 2004, and over 20 billion doses applied in China, Vietnam, Mongolia and Egypt.

New technologies utilizing live vaccines including, fowl poxvirus recombinants expressing H7,[282] and herpes virus of turkeys expressing H5,[283] have recently been approved for commercial use.

Avian Influenza Vaccines

Numerous studies have shown that vaccines can be effective at protecting poultry from both low pathogenic and highly pathogenic avian influenza. An antigenically closely matched vaccine to the challenge strain, when properly administered and producing high antibody levels, will prevent or greatly reduce clinical disease signs, prevent or greatly reduce the amount of virus being shed into the environment, and make birds more resistant to infection. The increased resistance to infection and reduction in viral shedding can be valuable tools in breaking the transmission chain allowing vaccination programs to be effective control tools during outbreaks. However, recent experience has shown that when an avian influenza outbreak is already widespread, vaccination by itself is not a good control tool. Vaccination as part of an eradication program has to be integrated in to a larger control strategy where quarantines, animal movement controls, increased biosecurity, education, and animal euthanasia of infected animals is used.[260]

Worldwide there are a number of different vaccine technologies used for avian influenza including traditional killed vaccines, reverse genetics killed vaccines and viral vectored vaccines expressing different influenza hemagglutinin proteins including a fowlpox, turkey herpesvirus, Newcastle disease virus, and a duck enteritis virus. Even with this wide range of tools, the use of vaccination has not been as effective as experimental studies would predict. Several different reasons have been identified as likely contributing to this suboptimal protection but the primary culprit is antigenic drift of the hemagglutinin protein.

The influenza viral genome encodes 10 or more viral proteins, but only three proteins have been shown to elicit neutralizing antibody: the hemagglutinin, neuraminidasen, and the matrix 2 (M2) proteins. Numerous studies have shown that antibodies to the hemagglutinin protein are the most important, and all the commercially available vaccines produce antibodies, many exclusively, to the hemagglutinin protein. Unfortunately the avian influenza hemagglutinin gene has extreme genetic variation that has resulted in many antigenic variants of the virus. Avian influenza virus has 16 defined hemagglutinin subtypes that by definition means that antibody to an isolate from one subtype should provide at least partial protection for other viruses of the same subtype, and that same antibody will not protect against other avian influenza subtypes. For example, H5 antibody will at least partially protect for all H5 viruses, but it will not protect against viruses with the H1, H2, H3, etc, subtypes. Therefore, using current vaccine technology, it would require a minimum of 16 different hemagglutinin proteins to be expressed to control for all avian influenza viruses. This approach is currently not practical, but in domestic poultry certain subtypes are much more important and prevalent than other subtypes. The H5 and H7 subtypes are the most important, because viruses of this subtype are and can mutate into the highly pathogenic form of the virus, which by definition causes high mortality in infected chickens. In addition to the H5 and H7 subtypes, the H9, H6, H1, and H3 subtypes are either endemic in poultry (H6 and H9) or routinely jump species to infect poultry (H1 and H3). Even though the list of problematic influenza subtypes can be reduced to a smaller number, with few exceptions, vaccination for avian influenza has always been done in the face of an outbreak and is rarely done as a prophylactic measure. One of the principal reasons for not using prophylactic vaccination is cost of both the vaccine and the administration of the vaccine. It is currently just impractical to vaccinate for all avian influenza subtypes.

Although the hemagglutinin subtype is used as a convenient way to classify an avian influenza virus, there is actually a high amount of genetic and antigenic diversity within a subtype. The most extreme example of antigenic diversity is with human and swine influenza. In 2009, a pandemic virus emerged in humans through a complex series of reassortants that resulted in a virus with a swine-like H1 hemagglutinin gene that replicated well in humans, and this virus was not neutralized by antibodies to the human seasonal H1 virus. So although the seasonal influenza and pandemic influenza virus were classified as H1 influenza subtypes, they were different enough that infection or vaccination from one did not protect against the other. Antigenic drift in human influenza is a well characterized phenomenon. Because of this antigenic drift the World Health Organization has developed a worldwide network of laboratories to monitor how the virus is changing and they recommend new seed strains for vaccine use when the antigenic variation reaches a certain point. This typically means that at least one of the three viruses in a seasonal influenza vaccine is changed each year (H1N1, H3N2, type B). Human influenza, because of the rapid movement of people on a daily basis all around the world, is maintained as a single worldwide population, with a single primary lineage for H1N1, H3N2 and Type B influenza, which allows global cooperation and cost sharing in vaccine selection. For avian influenza virus, the same process of antigenic drift occurs, but in many ways it is more complicated to monitor. Unlike humans, influenza infected poultry are not routinely monitored around the world. Sanitary measures and diagnostic testing are designed to block the

movement of virus and infected birds between countries and within a country. This has resulted in multiple antigenic variants evolving in different countries, and even within a country, that has varying levels of cross protection. The biggest and most costly poultry disease outbreak in modern times is the Asian lineage HPAI outbreak that was first reported in China in 1996. The virus persisted within the borders of China and in 2003 it rapidly spread to several neighboring countries. The virus spread widely in wild birds in 2005, and ultimately over 60 countries reported outbreaks of this lineage of virus. Most countries have been successful at eliminating the virus, but the virus has remained endemic in at least 6 different countries. Although the initial source of virus in 2003 was similar for the affected countries, if the virus became endemic in a country and the virus was isolated in the country, it developed a unique antigenic character different from virus in other countries. This antigenic variation resulted in vaccines that provided good protection early in an outbreak, but eventually lost efficacy. The best protection for vaccination is when the vaccine strain is antigenically closely related to the field strain. As a vaccine diverges from the field strain, the more virus replication and shedding occurs that perpetuates a virus remaining endemic in the population rather than contributing to its eradication. Natural antigenic variation in the wild bird reservoir and antigenic drift that occurs in poultry remains as the largest hurdle to effective vaccination.

Summary of Obstacles to Vaccinating against Avian Influenza Viruses

Although vaccine technologies are now available to match the antigenic drift that occurs in the field, many different issues are present that currently prevent the development of the ideal poultry vaccine. Some of these factors include:

1. Effective transparent surveillance of avian influenza in poultry is not being done in many countries. Only a small number of countries perform enough surveillance of their poultry populations to have a reasonable idea of what influenza subtypes are circulating, and then have transparent reporting of the results. These countries include the United States, Canada, Australia, many European countries and a few others. Most countries are either not performing adequate surveillance or they are not reporting the results transparently for economic reasons. **Without adequate worldwide surveillance and investments in predictive biology research to determine the basic characteristics of avian influenza viruses, it is impossible to adequately respond to new outbreaks for effective disease control, including the proper selection of or development of new vaccines.**
2. Vaccine development and licensing has been primarily a private enterprise, and vaccine companies only develop products that have a reasonable return on investment. Avian influenza vaccines must consider both subtype antigenic variation and within subtype antigenic variation. Vaccines are most effective when the vaccine is antigenically closely matched to the field strain. At the current time it is not cost effective for vaccine companies to make targeted vaccines for every potential market for a number of different reasons. One factor is regulatory because currently every variant vaccine has been considered as a "new" vaccine that requires separate licensure. The cost of licensing a new vaccine remains a high cost for the company, and therefore companies have only licensed a limited number of vaccines. This is perhaps most apparent for the live viral vectored vaccines, which require additional regulatory scrutiny because they are genetically modified organisms (GMOs) that are given as live virus that have the potential for spread to non-target species. Of the two licensed viral vectored vaccines in the United States (fowlpox-AI and HVT-AI), both are licensed only for the H5 subtype with a single hemagglutinin gene available for either vector. The two additional vaccines (NDV and DEV) licensed in other counties are also only

available for the H5 subtype, although there are more than one H5 hemagglutinin gene available. Therefore, we don't have viral vectored vaccines for any avian influenza subset besides H5, and even within the H5 subtype we have only a limited number of antigenic variants. The newer technologies, because of intellectual property issues, also requires a higher cost for development and use of the technology, which again raises the cost of the vaccine. Finally, vaccine companies are reluctant to license vaccines without a defined market. In the United States (and many other countries) we don't have endemic avian influenza, and therefore we don't routinely vaccinate poultry for avian influenza. Therefore, there is not a market for avian influenza vaccines, and no incentive for vaccine companies to license products in the United States. One of the primary reasons the fowlpox and HVT vectored vaccines were licensed in the United States was to use our scientific based regulatory system to provide assurance of the data that could be used for application in other countries. In other words, by licensing in the United States it would be easier to license the vaccine in other countries. However not all vaccine companies use this approach, and therefore not all types of avian influenza vaccines will be available in this country. **Vaccine companies currently do not have the economic incentive to develop and license vaccines, particularly next generation vaccines, for all important subtypes or for important antigenic variants in the United States.**

3. The worldwide poultry industry is a hugely diverse agriculture system that deals with a wide range of scale of production and numerous different species of birds. The bird species includes gallinaceous birds, like chickens, turkeys, and quail, waterfowl species including pekin duck, Muscovy duck, and different types of geese, and other birds ranging from pigeons to ostriches. For different avian influenza viruses, the ecology of the virus and the type of host it infects can be completely different. The important consideration is that different species are involved in the maintenance of a virus in different countries, and therefore vaccines, if they are to be used for control of an outbreak, must be effective in all these different bird species. The viral vectored vaccines, in general, are host specific and can't easily be used in other species. For example, the fowlpox-AI vaccine is designed for use in chickens, and it requires a 10 times higher dose to get a similar response in ducks.[263] Even the traditional killed adjuvanted vaccines are not equally efficacious in all avian species, as the adjuvants used have generally been optimized for chickens. For example, in a comparison study that included chicken, pekin ducks, and Muscovy ducks using the same killed vaccine, the chickens responded with the highest antibody level, pekin ducks were intermediate, and the Muscovy ducks had the poorest immune response.[79] However, little research is performed to understand how different types of vaccines work in different species, and although vaccination may be used in certain circumstances it may not result in an effective immune response. **Vaccination programs have to consider the different hosts involved in the ecology of the outbreak, and vaccines or vaccination schedules must be optimized for a variety of bird species for effective control.**
4. One of the biggest hurdles in using vaccination for the control of an outbreak is the difficulty in vaccinating birds already placed in the field. For example, egg laying poultry complexes in the United States may have over a million hens in cages, and it is logistically extremely difficult to vaccinate these birds with the existing vaccines. The killed adjuvanted vaccines require each bird be injected separately. The viral vectored vaccines are generally administered in the hatchery, and it would also require individual handling of the birds for vaccination of older birds in the field. For vectored vaccines like the fowlpox-AI vaccine where the research has been done, if the hens were exposed to fowlpox through either natural exposure or by vaccination, they will not even immunologically respond to the vectored vaccine.[284] Currently there are no good options for

vaccinating most poultry for avian influenza once they have been placed in the field. **Therefore, one of the biggest needs for avian influenza is to have a vaccine that can provide a robust immune response to birds in the field using a product that can be administered by a mass administration approach, such as aerosol, water, or feed.**

5. International trade of poultry and poultry products is an important part of the U.S. agricultural system, and the U.S. poultry industry tests millions of diagnostic samples yearly, mostly serologic testing to assure that U.S. poultry are free of avian influenza. If vaccination was used prophylactically or as a response to an outbreak, a huge concern would be that our trading partners would refuse our exports because of concern over avian influenza infection. Multiple different systems have been described that would allow the differentiation of infected from vaccinated animals, commonly called a DIVA system, to provide trade partners assurance that a product is safe. In particular the subunit vaccines, where only the hemagglutinin protein is expressed, are well suited for use in a DIVA strategy. However, relatively little field data and statistical analysis have been done with these subunit vaccines to show that they can be used as part of a DIVA strategy that will assure trading partners that it is a viable approach.[285] **Additional research is needed to provide the data to allow the DIVA system to be accepted by trading partners internationally to facilitate trade of vaccinated poultry.**
6. An ideal vaccine for avian influenza would have many attributes that our current vaccines do not have. Some of the factors would include the ability to get an effective immune response in the face of maternal antibody to avian influenza, the development of a protective immune response with a single dose of vaccine, the ability of a vaccine to have a short or no withdrawl time after vaccination before a bird is marketed, a rapid onset of a protective immune response, broad subtypic or even heterosubtypic immunity, a vaccine that can be used in wide range of avian species, and finally a vaccine that is inexpensive to produce and to administer. All of these attributes are unlikely to be found in a single vaccine, but our current and emerging vaccine technology is likely to make important incremental advances that will improve our vaccine technology to be more effective. **Additional research is needed in many aspects of vaccines, including the use of new vectored vaccines, to produce products that will be more effective at controlling and ideally eliminating avian influenza form our poultry populations**.

Assessment of Commercial AI Vaccines (See Appendix III)

Avian influenza viruses causing disease outbreaks worldwide have different characteristics that vary from country to country, and even within a country. In addition, each country regulates vaccines differently and vaccine availability is extremely variable. Because many developing countries do not have the resources to independently regulate every type of vaccine, it has become a common practice for vaccine companies to license vaccines in countries with a strong and respected regulatory structure, and use the approvals from those countries to support applications in other countries. For this reason several viral vectored vaccines have been licensed in the United States, but have never been used. The only type of vaccine used in the United States is the killed adjuvanted vaccines using a LPAI virus as the seed strain.

Conventional killed AI adjuvanted vaccines

Vaccination for influenza has been widely practiced in humans and animals for over 60 years. The traditional approach for the production of these vaccines for poultry is to identify a low pathogenic seed strain that grows well in embryonating chicken eggs, grow the virus to high titer, harvest the allantoic fluid, inactivate the virus, and use the inactivated antigen with an adjuvant for administration to the bird.

Vaccines for other species have been similarly produced with the exception that human influenza vaccines are also purified to reduce the amount of egg proteins in the vaccine and concentrate the levels of hemagglutinin and neuraminidase protein in the vaccine. Human vaccines also contain more influenza antigen in part because they typically don't include an adjuvant. The other major difference in human vaccines is that the seed strains used are reassortant viruses that contain the hemagglutinin and neuraminidase genes from current circulating strains, but the internal genes are from the virus strain A/Puerto Rico/8/1934 H1N1 (PR8) (Taylor et al., 1943) because of its high growth potential in eggs. Poultry vaccines, because of cost concerns, are cruder products and they use potent adjuvants as an antigen sparing approach to further reduce cost.

Avian influenza virus has 16 defined hemagglutinin subtypes that by definition means that antibody to an isolate from one subtype should provide at least partial protection for other viruses of the same subtype, and that same antibody will not protect against other avian influenza subtypes. For example, H5 antibody will at least partially protect for all H5 viruses, but it will not protect for H1, H2, H3, etc. The only subtypes of influenza known to have highly pathogenic isolates are H5 and H7, although most viruses of these subtypes are low pathogenic in standard chicken pathotyping studies. The H5 and H7 low pathogenic avian influenza (LPAI) can occasionally mutate to the highly pathogenic form by a relatively small number of amino acid changes in the hemagglutinin gene. The LPAI viruses can provide protective antibody for highly pathogenic avian influenza (HPAI) viruses, so the changes that makes a virus highly pathogenic is separate from the neutralizing epitopes that antibodies attach too. Because of biosecurity concerns and later because of human biosafety concerns, LPAI viruses were used as the seed strains for vaccines for both LPAI and HPAI viruses. Historically for both LPAI and HPAI outbreaks where vaccination has been used, control leading to eradication of that virus strain has been achieved in a relatively short period of time. In this situation, the field virus does not have enough antigenic drift to affect vaccination. However, in the last 20 years there have been several different influenza outbreaks where vaccination was used but the virus was not controlled.[259] In these cases, the field virus has drifted such that the vaccines commonly used do not provide any meaningful protection. The most problematic case has been the Asian lineage H5N1 HPAI outbreak that is endemic in at least 6 Asian countries and Egypt. Because of the antigenic drift in this lineage, none of the available LPAI seed strains are antigenically close to the variant H5N1 viruses. Several countries, including Russia, Kazakhstan, and Indonesia, decided to use the HPAI viruses as seed strains. The Russian vaccine was produced in BSL3 production facilities. However, the Indonesian vaccine was produced in BSL-2 facilities. The production in these facilities creates a potential for spread in the community or for worker safety, but no laboratory incidents have been reported from vaccine manufacture in Indonesia. However, it is not recommended for BSL2 facilities to be used to produce highly pathogenic avian influenza viruses. There is no production capacity to make vaccine in the United States using a HPAI seed strain.

Reverse genetics killed AI vaccines

This recent technology has allowed the production of vaccines for both HPAI viruses as well as LPAI and greatly increase safety and biosecurity. Reverse genetics technology provides a method of cloning all eight influenza gene segments in a DNA plasmids in such a way that it can be transfected into cells to rescue a live influenza virus.[281,286] The power of this technology is twofold. First, with the influenza genes being in bacterial plasmids, it allows the manipulation of the genes to change individual amino acids. For HPAI, the hemagglutinin cleavage site is changed from having a multiple basic amino acids to having a sequence similar to other LPAI virus. This allows the change of a HPAI virus into a LPAI virus without affecting the antigenicity of the virus. The second factor is that it allows the creation of unique

reassortant viruses. Genes from different influenza viruses can be recombined, allowing a hypothesis-driven approach to determine the biological characteristics of viruses that could evolve from the reassortment of different gene segments (i.e., genetic shift). For vaccine work, a technique similar to what is used for human influenza vaccines can be achieved such that the internal genes from a high growth virus (PR8) can be combined with the modified HA and NA genes of relevant circulating strains to produce a LPAI virus that consistently grows to high titers, making a vaccine more economical to produce.[287] This powerful technology is routinely used for human influenza vaccines, but it has been used sparingly in veterinary medicine because of intellectual property issues. The reverse genetics technology has been patented, and the additional cost of producing a reverse genetics vaccine has dissuaded most vaccine manufacturers from attempting to incorporate the technology. A single company has licensed a reverse genetics vaccine in the United States, and the vaccine has seen some use internationally. However, the higher cost of the vaccine appears to have priced the vaccine out of the market for the poultry industry, where vaccines command a low price because of high number of vaccine doses required to vaccine the billions of birds produced in our modern poultry production system.

Fowlpox virus vectored AI vaccines

The fowlpox virus (FPV) is a large double stranded DNA virus in the *Avipox* genus in the *Poxviridae* family. There are many unique poxviruses in birds that have varying abilities to infect and cause disease in domestic poultry, but the term fowlpox virus is usually used for pox viruses of chickens. The FPV can cause serious economic losses to the poultry industry, and a attenuated live vaccine was developed over 70 years ago.[288] Because of the large DNA virus, FPV was one of the first viruses considered for use as a platform to express foreign genes. The first report of FPV being used to express the avian hemagglutinin protein was in 1988.[289] The FPV was an attenuated vaccine virus and the hemagglutinin gene from the H5 subtype A/turkey/Ireland/1378/1983 was inserted in a non-essential gene of the vector virus. The recombinant virus (FPV-AI), although it only expresses the hemagglutinin protein, has been shown to provide protection to a wide range of highly pathogenic H5 viruses.[146] However, the FPV-AI vaccines, like conventional inactivated vaccines, provide no protection for other hemagglutinin subtypes, including the H7 subtype, and it will not even protect against all variant subtypes of H5 avian influenza. The FPV-AI vaccine with the A/turkey/Ireland/1378/1983 insert was licensed for emergency use in the United States in 1988, and it remains the only FPV vectored AI vaccine licensed in the United States.[284] This vaccine was shown to provide protection for the H5N2 highly pathogenic virus in Mexico, and was used as a control tool in Mexico starting in 1998 with billions of doses being used.[290,291] Multiple investigators have developed different FPV recombinant vaccines expressing different hemagglutinin inserts and in some cases in conjunction with the neuraminidase gene. Most of the vectored vaccines have not been licensed for commercial use with the exception of a virus in China that expressed both the HA and NA genes from A/goose/Guangdong/1/1996 (H5N1), one of the earliest isolates from the Asian H5N1 HPAI lineage that is found in multiple countries in Asia and Africa.[292] This vaccine was used commercially in 2006, with 615 million doses reported to have been used, but it is unclear if the vaccine has been used after 2006 based on published reports of amount of vaccine used.[280]

The fowlpox vectored vaccine, because of species specificity, replicates preferentially in chickens with lower replication levels in other species. The immune response to the avian influenza insert correlates with the replication of the vector, and therefore the vaccine will not work equally in all birds species. Studies in domestic waterfowl have shown that a dose 10 times higher than is required for chickens is needed to get a protective immune response.[293] One of the primary benefits of the FPV-AI vaccine is that it can be used in day old chicks, which provides a cost efficient vaccine program in the hatchery. Because

so few influenza HA genes are licensed using the FPV vector, it has had less use over time because of the increasing antigenic variation found in the field. One alternative application that has been proposed is the prime-boost response in chickens with maternal antibodies. The FPV vaccine can replicate in the presence of maternal antibody to both the vector and insert, and although the AI part of the vaccine response is suppressed, the use of the live vaccine can still prime the immune response, which provides an improved response to vaccination with killed avian influenza vaccines at a later time.[294,295]

Turkey Herpesvirus vectored AI vaccines

Another vectored vaccine has been developed and commercially licensed in the United States using the vaccine strain turkey herpesevirus that expresses the A/Swan/Hungary/4999/2006 (H5) hemagglutinin gene (rHVT-AI).[283] The turkey herpesvirus is also a large double stranded DNA virus commonly used as a vaccine for Marek's disease in day old chickens and has also been used for *in ovo* vaccination. The vaccine is highly cell associated and it can replicate in the presence of maternal antibody.[283] The virus does have a limited host range and likely can only be used for vaccination of chickens and turkeys. The rHVT-AI vaccine has the insert from the Asian H5N1 highly pathogenic lineage, and its efficacy against a range of H5 subtypes is unclear, although protection has been demonstrated with H5N1 HPAI viruses from Egypt and Indonesia.[264,296] The rHVT-AI has been tested in the field in Egypt and looks to be a valuable tool for control of the H5N1 Asian lineage directly or through a prime-boost response. However, few published reports, particularly of field trials, are available to understand the full value of these vaccines in the field. However, these vaccines are likely to be hampered in effectiveness by the availability of only a single hemagglutinin gene insert and the limited host range of the vector.

Newcastle vectored AI vaccine

The Newcastle disease virus (NDV) vectored vaccine has been developed into a vector for avian influenza genes, and it has been licensed and commercialized in both China and Mexico.[280,297] Newcastle disease is a small single stranded RNA virus that is similar to avian influenza in host range and pathogenesis. Several laboratories have successfully inserted the hemagglutinin of different subtypes into NDV allowing the expression and protection for both viruses. The potential benefits of the NDV-AI vaccine is that it can potentially be administered by aerosol or water allowing cost effective administration in birds after they have been placed in the field. The NDV-AI vaccine should produce both humoral and mucosal immunity, which for NDV alone provides superior protection over just killed vaccines.[298] The major concern about the NDV-AI vaccine is the presence of both maternal antibody and active immunity to the NDV vector because of widespread use of vaccination in almost all commercial chickens. So although experimental studies in specific pathogen free birds show good protection with NDV-AI vaccines, the field experience in China, based on declining usage, suggests this may be a serious impediment to its use.[280,295,299-302] In Mexico, a La Sota NDV vaccine virus was modified to express the A/chicken/Mexico/435/2005/DCV (H5N2) hemagglutinin gene and field application began in July of 2008.[297] The same product was also developed as an inactivated vaccine for protection against NDV and AIV.[303] Although these products have been available for over 5 years, the efficacy and use of these vaccines in the field have not been reported. In China, several different NDV-AI vaccines were produced including using the hemagglutinin gene from A/Bar-headed goose/Qinghai/3/2005 (H5N1), A/goose/Guangdong/1/1996 (H5N1), and A/duck/Anhui/1/2006.[280,302] The first NDV vaccine was introduced in 2006 with over 2.6 billion doses being used, but less than half of the amount was used in subsequent years.[280] It has also been proposed that the NDV-AI vaccine can provide improved protection when administered with a killed vaccine in a prime-boost vaccination scheme or in combination with the fowlpox vectored vaccine.[304,305] Although the NDV-AI vaccine has been available for a number of years, the efficacy of this type of vaccine in the field

has not been published, and the relatively small number of hemagglutinin inserts available appears to limit its applicability.

Duck enteritis vectored AI vaccine

The three other described vectored vaccines for avian influenza virus were developed primarily for use in chickens. Although the vaccines could potentially be used for other species, it typically required larger doses or multiple vaccinations to get good protection.[293,305] However, domestic waterfowl, including Pekin ducks, Muscovy ducks, and domestic geese play an important role in the ecology of the Asian H5N1 HPAI lineage of virus in several countries in Asia and Egypt. In an effort to bridge this gap, a duck enteritis virus vectored vaccine was developed to provide protection for both of these important diseases. The duck enteritis virus is a large double stranded DNA herpesvirus that causes an important disease in waterfowl. The attenuated duck enteritis virus was modified to express the A/duck/Anhui/1/06 (H5N1) hemagglutinin gene and the vaccine was shown to be protective in SPF ducks.[306] This vaccine has been licensed and used commercially in China. A variant of this vaccine using the hemagglutinin gene from A/duck/Guangdong/S1322/2010 (H5N1) was also shown to protect SPF chickens and was proposed as a useful vector in chickens because chickens are naïve to the virus and maternal or preexisting immunity to the vector will not interfere with the vaccine.[307] Other groups outside China have also developed duck enteritis vectored vaccines and also showed efficacy in experimental studies.[308] The limited availability of influenza HA gene inserts will likely limit the applicability of this vaccine.

Summary of what we know about commercially available AI vaccines

- Current vaccines will protect against morbidity and mortality, are estimated to reduce viral shedding 4-fold, and provide at least 20 weeks protection following a single vaccination for chickens. However, current vaccines do not completely eliminate virus replication in the respiratory and/or gastrointestinal tracts.
- Only two vaccine platforms are currently used for poultry: 1) oil-adjuvanted inactivated whole avian influenza virus vaccines and 2) recombinant viral vectored vaccine with an H5 AI gene insert. Both of these vaccine platforms have been shown to produce safe, pure, potent, and efficacious vaccines; however, both vaccine platforms require handling and injection of individual birds, with the exception of NDV vectored vaccines,.
- Currently, USDA has conditional licenses for inactivated AI vaccines for many of the 16-hemagglutinin subtypes. Full-licensure has been granted only to the recombinant viral vectors containing the H5 AI gene. Conditional licensure has been granted for both H5 and H7 vaccines; however, use of H5 and H7 AI vaccines is controlled by USDA and requires approval of the Deputy Administrator of Veterinary Services.
- The fowlpox virus vectored vaccine only works in chickens and can be administered to day-old chicks. Using this vaccine in older birds may not be recommended as prior exposure to fowlpox virus will induce immunity and inhibit replication of the vaccine virus and prevent development of effective immunity (see Appendix III).
- Experimentally, oil-adjuvanted inactivated vaccines have been shown to significantly reduce AI virus replication and shedding in domestic ducks and geese but the efficacy of vaccines in these bird species needs to be improved.
- Because of potential re-assortment of AI viral genes, live whole AI virus vaccines are not used.
- AI virus strains selected for manufacturing of inactivated vaccines have been based on LPAI viruses obtained from field outbreaks. HPAI strains are rarely used to manufacture inactivated

vaccines because such production requires specialized high biocontainment manufacturing facilities.

- LPAI strains protect against HPAI viruses of the same hemagglutinin subtype.
- Current vaccine strains have been shown to provide protection against diverse field viruses.
- The broad and long-term protection provided by poultry AI vaccines, as compared to human influenza vaccines that require frequent changes in the vaccine strains, is thought to be the result of: 1) the use proprietary oil-emulsion-adjuvant technology that elicits more intense and longer-lived immune response in poultry than alum-adjuvanted human influenza vaccines; 2) the AI virus immune response in poultry appears to be broader than in humans; 3) the immunity in the domestic poultry population is more consistent because of greater host genetic homogeneity than is present in the human population; and 4) vaccine use in poultry is targeted to a relatively young healthy population as compared to humans where vaccine is optimized for groups with the highest risk of severe illness and death.
- AI vaccines for poultry should still be evaluated every 2-3 years to ensure they are still protective against circulating virus strains. A recent study demonstrated the 1994 Mexican H5N2 vaccine strain is no longer protective against circulating H5N2 LPAI viruses in Central America.

Limitations of Current AI Vaccines

- Current vaccines do not cross-protect against the different hemagglutinin subtypes.
- Vaccines must be produced on demand and in large quantities with the appropriate hemagglutinin subtypes.
- Vaccination complicates trade in poultry and poultry products, with some countries refusing imports of such products from countries that vaccinate for AI.
- Differentiation of vaccinated from field exposed poultry is not currently possible using inactivated whole virus AI vaccines because vaccinated and field exposed birds will be positive by the type-specific agar gel precipitation (AGP) and the subtype-specific hemagglutinin inhibition (HI) tests.
- Adequate serological or virological surveillance must be done to determine if field virus is circulating in vaccinated flocks.
- Fowlpox-AI-H5 recombinant vaccine is ineffective in poultry previously exposed to fowlpox virus or vaccinated with fowl pox vaccines.
- All current AI vaccines require injection of individual birds, which is expensive and causes stress to the birds.
- Two or more injections are required to induce protective immunity in turkeys and older living birds such as egg-layers and breeder flocks.
- Inactivated whole AI vaccines require better potency standards to ensure a minimum immunizing dose in every batch of vaccine. This can be achieved by either establishing a minimum hemagglutinin protein content in the vaccine or by demonstration of a high level of protection as measured by ”in vivo” challenge studies or the presence of a minimal HI antibody titer in vaccinated birds (e.g. minimum of 1:32-1:40 HI test).

Assessment of Experimental AI Vaccines (see Appendix IV)

Several new vaccine platforms are being explored to rapidly develop emergency vaccines against new emergent strains of avian influenza viruses with epizootic and/or pandemic potential. These include a

wide range of vaccines have been made for both human and animal influenza viruses, many with successful results in challenge studies. As indicated in other sections of this report, protection against influenza viruses is primarily the result of neutralizing antibody produced to the hemagglutinin protein. [261] Although antibody to the neuraminidase and the M2 protein, if present at high levels can be protective, relatively speaking they play minor roles when antibody to the hemagglutinin protein is present. Cell mediated immunity through killer T cells to several internal proteins also can play an important role in protection, but in comparison to antigenically matched hemagglutinin antibody it plays a smaller role. Improving our understanding of what constitutes protective immunity, and how to apply this knowledge to design vaccines for the specific purpose control and eradication of a disease outbreak, has provided new opportunities for exploring novel vaccine technologies with proof of concept studies for protection against avian influenza viruses. In broad categories, these technologies include the use of viral-vectored vaccines, bacterial vectored vaccines, subunit vaccines, DNA vaccines, reverse genetics live attenuated influenza vaccines, and other new approaches such as synthetic vaccines. Many of these vaccine technologies cannot be practically implemented for production in animal medicine because of cost or application issues. It is also impractical to list every vaccine technology that has been described for avian influenza, but four technologies will be highlighted here that are more likely to be commercialized in the future.

Subunit vaccines

Subunit vaccines use various gene expression systems to produce hemagglutinin protein or occasionally other influenza virus proteins in cell culture, followed by protein purification steps, often formulated with an adjuvant and presented as a safe and purified "subunit" vaccine. The expression systems used have included bacteria, plant cells, insect cells, yeast cells, and mammalian cells as a source to produce the protein.[309-312] All these approaches have had some success for protection *in vivo*, but a major concern has been structural differences produced by the different cell culture systems. Likely the biggest difference is the protein glycosylation patterns used by the different systems. A major difference in bacterial and eukaryotic cell systems is that bacteria don't typically glycosylate proteins, particularly using the N-linked glycosylation system. This lack of glycosylation results in a greatly altered structure of the hemagglutinin protein that results in poor induction of neutralizing antibody.[309] Glycosylation of the hemagglutinin protein is important both for folding and for antigenicity,[313] and therefore bacterial systems are likely a poor choice as an expression system. Plant, yeast and insect cells have been proposed as systems that can produce large amounts of glycosylated hemagglutinin protein that can be used for vaccines purposes. The most mature of these expression systems is the baculovirus expression system using insect cells. Baculovirus is an insect virus that will infect and produce large amounts of viral protein in insect cells. As the system is widely available, considerable improvements in cell lines and expression vectors have been made. Although the glycosylation system is different than avian or mammalian cells, protein produced using this system is close enough to the native protein to be a good antigen for inducing a neutralizing antibody response in a number of hosts including chickens and humans.[314] In November of 2013, a commercial baculovirus expression system vaccine was licensed for seasonal influenza in the United States demonstrating that it is a viable technology.[315] In poultry, some modest progress has been made on the experimental use of this technology and veterinary vaccine companies have interest in the technology, but no commercial vaccines are on the near horizon.[316]

Viral vectored vaccines

The use of recombinant viruses to express hemagglutinin proteins for use as a live vaccine is an approach that has been shown to work experimentally for over 25 years.[289] Worldwide four different viral vectored systems have been developed for avian influenza virus with billions of doses of vaccines used in the field.

However, even with all this commercial activity, viral-vectored vaccines remain an active area of research. The reasons for further work are two-folds. One, the current vaccines although having value in the field are not greatly contributing to the eradication of the virus. Second, each of the viral vector vaccine technologies have unique attributes that could potentially be harnessed to develop improved next generation vaccines.

Some of the ongoing issues with current viral-vectored vaccines are briefly mentioned here to highlight some of the gaps and needs for specific improvements. For starters, the subtype and within subtype antigenic variation of avian influenza viruses potentially requires many different hemagglutinin genes to be expressed to adequately control the problem in the field. Often however, only a single hemagglutinin gene is commercially licensed for use, making availability of the proper vaccine a major issue. One impediment to making multiple vaccines is the cost to develop and then license the vaccines. Because the available viral vectored vaccines are live genetically modified organisms (GMO), regulatory authorities have required both increased scrutiny in licensure but each variant is treated as a new vaccine. Second, there are concerns a GMO vaccine virus could spread to non-target animal species and cause disease in other animals. An advantage of viruses like the fowlpox and Herpesvirus of turkeys (HVT) vaccines, previously discussed in this report, is that they have a naturally limited host range, which has aided there licensure in the United States. However, the Newcastle disease virus vectored vaccine has a wide host range in birds and is potentially zoonotic, which would need to be addressed if the vaccine was to be licensed and commercialized. It is unclear what regulatory requirements, if any, were needed to address safety for licensure in Mexico or China. A third major issue is the presence of either maternal antibody or an active immune response on suppressing replication of the viral vector, which reduces the immune response. It has previously been described that active immunity will suppress a fowlpox-vectored vaccine's immune response.[284] The same issue was observed for vectored vaccines with maternal antibody.[295] Since almost all commercial poultry are vaccinated with live Newcastle disease virus and for Marek's disease virus vaccines, this would greatly decrease the effectiveness of subsequent vaccination with viral vectored vaccines once the birds are placed on the farm.

Two fairly new viral vectored technologies have been selected here as examples because similar systems are licensed for other species. The first is the alphavirus vectored virus, which is already licensed and is used commercially with swine influenza in the United States.[317] The second is the human adenovirus technology has been conditionally licensed for foot and mouth disease virus in the United States but has not been used in the field.[318] Both technologies have similar attributes that overcome two of the issues mentioned previously. First, both technologies are replication-restricted virus that cannot complete the virus replication life cycle. This approach provides the advantages of a live vaccine that stimulates both humoral and cell mediated immunity, but it has a safety profile of an inactivated virus. This negates the host range issue and promises to make vaccine licensing easier because of the higher safety margin. Secondly, both viral vectored systems are not normally found in poultry, which means that maternal antibody or previous exposure eliciting active immunity to the vector will not be a problem with either system. Both viral vectors have been shown to provide solid protection for avian influenza in poultry.[108,319-321]

Reverse genetics (RG) platform

A new type of vaccine technology related to the traditional killed vaccines, which uses naturally occurring LPAI viruses for vaccine seeds, is to use modern biotechnology to produce a viral seed strain using reverse genetics (RG) technology. Reverse genetics uses cloned influenza gene segments in bacterial plasmids

that can be modified to produce engineered viruses with properties suitable for vaccine use. Most vaccine reverse genetics systems use the internal genes from a human vaccine strain and the hemagglutinin gene from the target virus to create a virus that can be finely targeted to the circulating outbreak strain and reliably grows to high titer in embryonating chicken eggs or cell culture, allowing for a cost effective emergency vaccine. The primary application for this technology for veterinary medicine has been to modify the hemagglutinin gene from a highly pathogenic avian influenza virus so as to attenuate the virus to a low pathogenic virus.[281,287] This change at the cleavage site does not affect the antigenic properties of the virus, and allows the virus to be handled and vaccine produced in vaccine production facilities that make traditional killed vaccines. This includes using an adjuvant to make them more cost effective. The use of RG technology has additional costs for production related to the patented technology needed to use the system. Currently in the U.S, only a single veterinary RG vaccine has been licensed for a H5N1 virus, but again, although licensed, it has not been used in the U.S. This vaccine has been used sparingly in other countries because of antigenic drift issues as well as higher cost issues. Up until just recently, any new reverse genetics vaccine would require a completely new application for licensure, which adds additional cost in the production of the vaccine. However, recently APHIS has opened the door for a simplified review process for previously approved RG vaccines that allows substitutions of the hemagglutinin gene when you continue to use the same vaccine backbone and production process (Veterinary Services Memorandum No. 800.213). This rule change potentially reduces one impediment for not updating vaccines because of antigenic drift issues.

Live attenuated influenza vaccines

A classic approach to vaccination is to attenuate the target virus through chemical mutagenesis or passage in cell culture so that the virus becomes attenuated but can still elicit an immune response. This approach has been used with human influenza viruses with a few technologic enhancements. For example, using classical methods, the internal genes of a human influenza virus were cold adapted to grow only at lower temperatures. This temperature sensitive mutant therefore was restricted to viral replication to the upper air passages of humans, thereby delivering an attenuated phenotype. The specific mutations that account for the temperature sensitive mutants were identified and can serve as markers for the virus. Using this backbone virus in a reverse genetics system, new vaccine strains can be quickly made by inserting the hemagglutinin and neuraminidase gene from recently circulating strains to update the vaccine to ensure efficacy against relevant viral strains.[322] This system has resulted in licensed vaccine in the United States and many other countries to provide an alternative to the killed vaccine approach requiring injection by needle. Similar technology was used to produce a live attenuated vaccine for horses for equine influenza that has been licensed in the United States.[323] However, although the vaccine has been licensed, it has not been updated to include more recent field strains. This has been an issue with licensed vaccines for avian influenza, where companies have not been willing to update vaccines because of the high cost.

Live attenuated vaccines have been shown to be protective in poultry as well, and the same temperature sensitive mutants have been created using reverse genetics for avian influenza viruses.[324] An alternative approach has been to truncate the nonstructural protein 1, which suppresses the host immune response attenuates the virus.[325] Although both of these approaches work experimentally, the biggest concern about live attenuated avian influenza viruses is the potential of these viruses to mutate back to virulence and for the H5 and H7 subtypes to become highly pathogenic viruses. So although this appears to be a fruitful line of research, it is unlikely to be developed commercially because of the concern of back mutation and the dire implications on trade of poultry and poultry products this would have.

Conclusions

Several different vaccine technologies are poised to become commercialized because similar technology has already been licensed in the United States and proof of concept studies has already been published in the peer review literature for avian influenza viruses. Four promising technologies have been highlighted, each with their own advantages and disadvantages. While considerable time and effort continues to go into vaccine research, there always remains the issue of how to commercialize products without a ready market for the product. Although several next generation vaccines have been licensed in the United States, they have not been used here. The licensing process in the United States has aided licensure and use in other countries, but the issue remains of how to encourage innovation and commercialization of new vaccines in the United States for avian influenza when avian influenza vaccination is rarely performed.

Summary of experimental vaccines tested in the laboratory for potential use in the field

- ARS scientists at the SEPRL have tested experimental vaccines against the highly pathogenic H7N3 virus reported in Mexico in 2012. Two USDA-approved H7 isolates were used to develop conventional inactivated vaccines that showed 100 percent protection in vaccinated birds against a lethal challenge of the virus, showing that the vaccine derived from these isolates could protect U.S. poultry. SEPRL scientists also demonstrated that the Mexican 2006 low pathogenicity virus could be used as a vaccine. All birds vaccinated with the virus strain and challenged with the 2012 virus were protected.
- The recombinant fowlpox vaccine is a live, injectable vaccine for chickens and uses the same technology as the previously licensed recombinant-fowlpox-virus-AI-H5 vaccine, but includes inserted cDNA copies of AI hemagglutinin (H5) and neuraminidase (N1) genes (both from A/goose/Guangdong/3/96 [H5N1]).
- The other new vaccine is a traditional inactivated oil emulsion AI vaccine, but unlike current inactivated AI vaccines, the new vaccine virus is not an H5 LP or HPAI field virus. The vaccine virus was produced by reverse genetics using the 6 internal genes from a human influenza vaccine strain (PR8) and the hemagglutinin and neuraminidase genes from A/goose/Guangdong/3/96 (H5N1) AI virus. The use of PR8 internal genes imparts the characteristic of growth to high virus content in embryonating chicken eggs used in the manufacturing process and thus produces a high concentration of the protective hemagglutinin protein in the vaccine. Another change in the vaccine virus: the portion of the gene that codes the hemagglutinin proteolytic cleavage site has been changed from a sequence of an HP to an LPAI virus, thus, the vaccine virus is a LPAI virus and can be manufactured at a lower level of biosafety.
- Both vaccines require handling and injection of individual birds. Data published or presented at scientific meetings indicate that these new vaccines are as efficacious as the existing licensed vaccines, but no data have been presented to demonstrate they provide superior protection.
- Reverse genetics is a new research technology that is well suited to improving influenza vaccines. Intellectual property rights are one impediment to using reverse genetics because of the additional cost and it is currently unclear who owns the technology.
- Several recombinant vectored avian influenza vaccines are currently under development, including the human adenovirus-vectored platform. The licensing of recombinant vectored vaccines based on the HA gene currently requires separate licenses for each HA subtype inserted in the vector. The process could be improved with <u>one</u> license issued provided the insertion site remains the same. This would enable the rotation of the HA subtype on a licensed vector platform based on the

subtype associated with the outbreak. This is consistent with what is done with human vaccines where new strains can be added or deleted annually.

Assessment of Emergency Vaccine Strategies for Controlling New Variant AI Strains with Pandemic Potential

New and emerging zoonotic AI strains with pandemic potential such as the Chinese LPAI H7N9 require the rapid development and delivery of effective vaccines to stop the shed and spread in poultry populations at risk and, importantly, prevent further dissemination of the agent to people in contact with infected poultry.

The ideal vaccine for avian influenza would have a number of traits that current vaccines do not provide. The biggest challenge is the need for a mass administered vaccine that can be given to birds after they have been placed in the field. The current killed and viral vectored vaccines require individual administration of the vaccine, which makes vaccination for the birds in the field difficult and in most cases cost prohibitive except for high value birds such a breeders or genetic stock. Both the fowlpox and the HVT-vectored vaccines can be administered in the hatchery, which can be cost effective in the context of a planned vaccination program. However, it has been shown for the fowlpox vaccine that if the birds in the field are vaccinated or naturally exposed to fowlpox, they will not respond to the influenza part of the vaccination, which makes it even more unlikely that this type of vaccine could be used as part of an emergency vaccination response.[284] Currently there are no good options for a commercially available vaccine to be administered in the field as part of an emergency vaccination program.

A second feature of an ideal vaccination is the incorporation of a DIVA strategy. The DIVA principal, which stands for Differentiate Infected from Vaccinated Animals, for vaccination is not a new concept and has been used as part of eradication programs for Pseudorabies in swine, classical swine fever, and for avian influenza.[326,327] Interest in the use of a DIVA vaccination strategy as a possible tool for the control of avian influenza virus has increased as both LPAI and HPAI has become endemic in many countries. It is also a critical tool for recovery from a disease outbreak in AI-free countries, to assure trading partners that vaccinated animals have not been infected with the virus during production, which should facilitate poultry exports if the trading partner accepts the vaccination program. Several different DIVA strategies are possible and have recently been reviewed.[285] All of the vaccines that only express just the hemagglutinin gene should easily fit a DIVA strategy using existing avian influenza diagnostic tests, because influenza surveillance tests look for antibody to internal proteins like the nucleoprotein. Although the viral-vectored vaccines are prime candidates for a DIVA strategy, much works needs to be done to validate the system so that trading partners except the DIVA principals.

Additional ideal vaccine features for emergency response is the ability of a single vaccine to be used in multiple species is a practical field problem. Most of the viral vectored vaccines have some level of host adaptation that limits their use in other species. For example, the fowlpox recombinant vaccine works well in chickens, but it requires 10 times the dose for a comparable immune response in ducks.[263] Even the killed adjuvanted vaccines have large differences in response when administered to different species.[79] Other qualities such as a rapid immune response, ability for a single dose to provide long term protection, short withdrawal times required before processing, and vaccines that are stable for long term storage would all be favorable attributes. One ideal attribute is heterosubtypic immunity, where a single vaccine can provide protection for all influenza subtypes. This has been the stated goal for many vaccine studies,

but it appears increasingly unlikely that broad effective vaccine protection will ever be a practical goal for veterinary medicine.

Although there are worldwide a large number of vaccines available for avian influenza, on closer examination almost all of these vaccines are targeted to the H5 subtype. The H5 subtype, although an extremely important disease pathogen that is endemic (low pathogenic or highly pathogenic) on 3 continents, is not the only important influenza subtype in poultry.[260] The H7 subtype has caused many severe low pathogenic and highly pathogenic outbreaks in the last 50 years, although most of these outbreaks have been controlled. In addition several low pathogenic avian influenza viruses, including H9N2 and H6N1, are also endemic in many countries and cause serious disease losses.[328] For non-H5 influenza viruses, only the traditional killed adjuvanted vaccines are available to control these diseases with limited success. Commercial vaccine companies have been reluctant because of cost and profit issues to develop recombinant vaccines for these other influenza subtypes. In the U.S., routine vaccination of poultry for avian influenza is not allowed. Because of the lack of a guaranteed market for the use of vaccines, vaccine companies have no incentive to develop new cutting edge vaccines for a country that might use a vaccine only on an emergency basis. The currently licensed vectored vaccines have never been used in the U.S., although licensure in the U.S. has facilitated their use in other countries. Commercial vaccine companies have had little incentive to develop either new types of vaccines or to even adapt the current vaccines to include new HA subtypes. Currently in the U.S., if a vectored vaccine changes the hemagglutinin gene insert in a vector that is already licensed in another product, it is still considered a new vaccine and requires a separate license, which adds to the cost of the vaccine. This issue exists for other foreign animal diseases, including foot and mouth disease virus (FMDV). For FMDV the U.S. government has been the primary sponsor for research to produce a next generation vaccine leading to licensure of the vaccine.[329] Although this program of government support to get a license for a veterinary medicine product is unique, it does provide a blueprint of how to generate new vaccines that can be used for emergency use.

New vaccine technologies are available for the avian influenza field. Numerous vaccine approaches have been published that work in experimental studies, but few of these technologies are being further developed. One exception to this is the alphavirus vectored system that is licensed for swine influenza. This viral vector system has already been proven for avian influenza,[108,319] and the production capacity is available in the U.S., but the economics of licensing a new product with no defined market remains a major impediment. Identification of genetic and antigenic variants will be the basis for improved vaccines either as usage of the parent HPAI virus or use of reverse genetics (RG) to produce low pathogenicity avian influenza (LPAI) viruses as vaccine seed strains. The latter source is the best solution from a perspective of biosecurity in manufacturing, antigenic match and high production output.

Swine Influenza Vaccines

Influenza vaccines were introduced for use in swine in 1994 as inactivated, multivalent, whole virus preparations administered with oil in water adjuvant.[163] Since the introduction of the H3N2 triple reassortant virus, vaccines have been manufactured with an H3N2 antigen and a combination of H1 viruses that may or may not be currently circulating in swine. Over the past ten years, influenza vaccine usage in swine has increased, demonstrating the need to control this economically important swine pathogen.[163] The National Animal Health Monitoring System (NAHMS) reported 40% of large producers vaccinating breeding females in 2000 that increased to 70% in 2006.[105] Consistent with the

NAHMS report, a recent study evaluating management and production practices on 153 swine farms in Minnesota and Iowa between 2007 and 2009 reported vaccination usage in 71% of breeding females.[9] In contrast, vaccine use in growing pigs constituted less than 10% of the swine farms surveyed.[9]

Increasing genetic and antigenic diversity recognized among contemporary influenza viruses circulating in swine has created a challenge to produce efficacious vaccines. Inactivated vaccines effectively reduce clinical disease and lung lesions and provide partial protection when priming antigen and challenge viruses demonstrate similar HA proteins.[13,79,82,92,152,161,160,158] However, unlike natural infection, inactivated vaccines have demonstrated limited efficacy or inconsistent cross-protective immunity against heterologous homosubtypic or heterosubtypic viruses.[13,92,79,154,152,161,160,158] In one study, pigs that were naturally exposed to European H1N1 and H3N2 viruses demonstrated complete protection against a novel, heterologous H1N2 virus infection with an unrelated HA protein.[153] In contrast, inactivated vaccines containing H1N1 and H3N2 viruses did not confer protection against a heterologous H1N2 virus.[154] These data suggest live exposure, or vaccination that mimics natural infection, may provide optimal protection or reduction in clinical signs associated with IAV infection in swine. There are no commercially available modified live attenuated vaccines currently licensed for swine influenza. However, research using genetically altered, live attenuated influenza vaccines has demonstrated increased efficacy against heterologous infection. Modified live, attenuated vaccines have the advantage of enhancing cell-mediated immune responses typically directed against conserved internal proteins[182] and increasing heterologous cross-protection that may be lacking in inactivated vaccines.[180]

Due to the lack of cross-protection experienced through the use of commercial vaccines, many producers now rely on inactivated autogenous vaccines incorporating influenza viruses isolated from the farm of origin. In 2006, the NAHMS survey reported 20% of farms used autogenous vaccines in breeding females.[105] However, more recent data from a study of Minnesota and Iowa swine farms reported 72% of influenza vaccines used in breeding females on 153 swine farms surveyed were autogenous whereas only 24% were commercial vaccines.[9]

Assessment of Commercial SIV Vaccines (See Appendix V and VI)

Current SIV vaccines regulated by APHIS Center for Veterinary Biologics fall under 3 categories: 1) Commercial whole inactivated virus vaccines; 2) Non-living replication defective RNA virus vector; and 3) Autogenous inactivated vaccines.

In the United States, there are currently 6 companies preparing SIV vaccines (see Appendix XIII): two companies prepare only commercial product; two companies prepare only autogenous products, and two companies prepare both. From these 6 companies there are a total of 30 commercially licensed products that include combination vaccines with other common swine pathogen antigens. There are also three autogenous product licenses. All of these vaccines are either whole inactivated influenza A viruses (WIV) or non-living replication defective RNA virus vector products. Inactivated vaccines can elicit strong homologous immunity and reduce both disease severity and virus shedding. However, in general, WIV afford little to no heterologous protection against drifted or reassortant virus strains. To address this concern, commercial companies utilize multiple (up to four) isolates representing different HA lineages.

Limitations of Current SIV Vaccines

- Whole virus inactivated vaccines provide little to no heterologous protection against drifted or reassortant virus strains
- Evidence of vaccine-associated enhanced respiratory disease (VAERD when pigs vaccinated with inactivated vaccines are exposed to heterologous SIV strains
- Poor efficacy of inactivated vaccines in young pigs due to maternal antibody interference
- Inactivated vaccines require single animal inoculations
- Multiple doses of vaccine required to achieve herd immunity
- Short duration of immunity

Assessment of Experimental SIV Vaccines (see Appendix VII)

Published research in swine evaluating live-attenuated IAV vaccines (LAIV) has repeatedly demonstrated their general safety and superior efficacy to inactivated vaccines.[164-166,330-337] DNA-based[338] and vectored vaccines have also demonstrated efficacy against IAV and have advantages over inactivated vaccines.[163,175,339,340] Current research is being conducted to investigate how efficacious these vaccines are in reducing shedding of circulating IAV when vaccinated pigs are challenged with homologous or heterologous IAV. Experimental data have demonstrated that attenuated vaccines are superior in their ability to protect against transmission of challenge viruses to naïve contact pigs. Further, these data have highlighted the importance of including contemporary strains in multivalent vaccine to increase antigenic coverage against the circulating influenza virus diversity. Research on next generation vaccines has focused on 3 key features: 1) providing the broadest possible heterologous immunity; 2) being efficacious in the face of maternal antibodies; and 3) providing a platform that new or emerging antigens can be rapidly incorporated (measured in a few weeks not months). The general categories of vaccines that possess the majority (if not all) of these qualities include: 1) live attenuated influenza vaccines (LAIV); 2) Replication-defective vector influenza vaccines; and 3) DNA vaccines.

Live attenuated influenza vaccines (LAIV)

Three distinct LAIV platforms have been evaluated and described in the peer-reviewed literature. The first platform described mutations in the NS1 protein of swine influenza virus that impaired the anti-interferon activity of NS1 and therefore conferred attenuation in pigs. This vaccine has been shown to be efficacious when administered intranasally. It primes T cells and confers cross-protection against a heterosubtypic challenge in pigs; and it provides superior protection from heterologous infection in pigs with maternal antibodies without inducing vaccine-associated enhanced respiratory disease (VAERD).[162]

The second LAIV platform to be described involves modification of the cleavage site of the hemagglutinin which confers attenuation in pigs.[337] As with the NS1 truncated mutants, HA cleavage site mutants provided pigs with complete protection from homologous H1N1 infection and partial protection from heterologous subtypic H3N2 SIV infection.[166,335,336]

The third LAIV platform described in the literature is a temperature-sensitive mutant virus based on selected mutations in the viral polymerase gene segments.[165,334] This vaccine was also shown to be efficacious when administered intranasally, primed T cells and conferred cross-protection against a heterosubtypic challenge in pigs. It provided superior protection from heterologous infection without inducing vaccine-associated enhanced respiratory disease (VAERD).

Replication-defective vector influenza vaccines

A second category of next generation swine influenza vaccines that has been evaluated in pigs involves vectored antigens. The first swine influenza vaccine vectored platform described in the literature was the replication defective human adenovirus 5 vector.[175,339,340] Although various constructs of this particular vector have been described in the literature, the basic aspect of the replication defective nature are deletions in the E1 and E3 regions of the adenovirus genome. Reverse engineering is used to insert the desired hemagglutinin genes of IAV into the genome of the vector. A cell-line that provides the missing products from the deleted genes is used to grow the virus that once purified is replication defective. Among the experimental vaccines, some have distinct advantages over others from a reverse engineering, manufacturing and potential mass administration point of view. The ease and shorter time required for reverse engineering of the alphavirus vector is an apparent advantage over the Ad5 vector.[341] In the published research, alphavirus derived replicon particle (RP) vaccine expressing an IAV HA gene induced protective immunity against homologous influenza virus challenge.[317,342,343] Vaccinated animals showed significantly elevated specific antibody response, reduced lung lesions and viral shedding. While the alphavirus vector has efficacy against homologous viruses and a distinct advantage in time to market,[344] there remain research gaps around its ability to induce mucosal immunity, to work in the face of maternal antibody and to provide heterologous protection.

SIV DNA vaccines

The third category of experimental vaccines tested in swine is the DNA vaccine approach.[338] In published reports, a DNA plasmid vaccine expressing a HA elicited robust serum antibody and cellular responses after three immunizations and conferred significant protection against influenza virus challenge. However, the 3 doses needed to achieve a significant HI titer were a drawback.

The key to success for any of these next generation vaccines will be if the regulatory process changes to allow contemporary HA's and NA's to be substituted into previously approved next generation vaccine platforms. For example, if a vaccine platform is shown to be efficacious, it would be a tremendous improvement over the currently approved vaccines because of the ability to rapidly reverse engineer the platform with the appropriate HAs and scale it up for use in swine production systems.

Assessment of Emergency Vaccine Strategies for Controlling New Variant SIV Strains with Pandemic Potential

New and emerging zoonotic SIV strains with pandemic potential such as vH3N2 require the rapid development and delivery of effective vaccines to stop the shed and spread in swine populations at risk and, importantly, prevent further dissemination of the agent to people in contact with infected pigs.

There were at least 49 distinct human to swine transmission events with the H1N1pdm09 virus globally (there were probably far more that were undocumented).[345] This resulted in a situation where a human pandemic virus became an endemic swine virus around the world in a matter of weeks. Eradication of this kind of a pandemic virus is impossible because it is maintained in two equally susceptible host species in frequent contact with one another.

All surveillance and clinical evidence, as well as experimental studies published to date, indicate that the HPAI viruses don't replicate and transmit efficiently in pigs or humans. Moreover, research attempting

to identify mutations enabling a more transmissible HPAI virus have usually demonstrated a loss of virulence as it gained transmissibility.

The key for success in the future event of a pandemic virus is to dramatically revise the current regulatory process to mimic the WHO model for human influenza vaccine strain selection. The new regulations should allow for the science-based usage of the next generation of vaccines that that can be rapidly engineered to immunize against contemporary strains found in the population of pigs being vaccinated.

The emergence of a virus that has never been shown to exist - i.e., a highly pathogenic and highly transmissible virus impacting humans and pigs is always possible. It is more likely that we will again see recycling of previous subtypes between human and swine and perhaps experience a pandemic virus akin to the 2009 H1N1 pandemic virus - i.e., a virus that becomes endemic in both species virtually overnight. However, the emergence of a novel pandemic strain or a new endemic strain in swine are both best managed by a regulatory environment that approves a vaccine platform that can then be reverse engineered at the speed of molecular virology. A third challenge for the swine industry is managing our current endemic SIV situation to prevent/reduce zoonotic events. Each of these scenarios can be managed far better if a new WHO-style model for rapidly updating HA's & NA's on a previously approved backbone is in place. Under the current regulatory environment, commercial vaccine manufacturers struggle to keep their vaccines current with contemporary antigens.

DIAGNOSTICS

The AICWG determined that the effectiveness of available diagnostics is high but several obstacles need to be addressed to ensure diagnostics are available, strategically deployed, and used effectively. Table 1 summarizes the most relevant diagnostic tests that are available now or under development. The U.S diagnostic system for detecting animal influenza viruses consists of strategic links between the National Animal Health Laboratory Network (NAHLN) and the National Veterinary Services Laboratories (NVSL). The mission of the NAHLN includes surveillance, response (surge), and recovery from high consequence agricultural diseases. High-throughput semi-automated robotic systems are beginning to be deployed and utilized in the NAHLN laboratories. These systems are compatible with sample processing and rapid nucleic acid detection technologies. The following section provides current obstacles for detecting animal influenza viruses, specific information on diagnostic strategies (surveillance, response, and recovery), and a detailed analysis of available commercial diagnostic and laboratory tests as well as assays under development (see Appendices VIII-XI).

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Diagnostics for Avian Influenza

AI surveillance in the U.S. is accomplished through a combination of passive and active surveillance programs. Passive surveillance relies on the testing of clinical cases where AI is suspected, e.g., in cases of respiratory disease and/or reproductive failure. The USDA also supports an aggressive active surveillance program through the National Poultry Improvement Plan (NPIP) and the Live Bird Marketing System (LBMS).

Assessment of Commercial and Laboratory Avian Influenza Diagnostic Tests (Appendix VIII)

Virus isolation

Performed in 9-11 day embryonating hens eggs and is considered the gold standard for detection of all subtypes of AI virus.[20,346,347] Isolates causing hemagglutination of erythrocytes are confirmed as type A influenza virus by AGID, antigen capture tests, or RT-PCR,[348-350] and subtyped by HI and NI tests.[20,351,352] Pathogenicity testing of isolates is done by inoculation of eight 4-week-old susceptible chickens by the intravenous route. Isolates killing 75% (six) of the eight chickens are classified as HPAI (OIE, 2008).[20] Additionally, the pathogenicity potential of H5 and H7 subtype viruses can be determined by sequencing the cleavage site of the hemagglutinin protein. A virulence marker, associated with the presence of multiple dibasic amino acids at the cleavage site of the H protein, has been associated with high pathogenicity.[48,353,354]

Real time RT-PCR (rRT-PCR) assay

rRT-PCR has been used for AI virus detection since the early 2000's for routine surveillance, during outbreaks, and for research. Some of the advantages of rRT-PCR are: high sensitivity, high specificity, rapid time-to-result, scalability, cost, and its inherently quantitative nature. Furthermore, rRT-PCR can be used with numerous sample types, is less expensive than virus isolation in chicken embryos, and since infectious virus is inactivated early during processing, biosafety and bio-security are also easier to maintain. However the high genetic variability of AI virus may decrease sensitivity and increases the chances of a false negative result.

The rRT-PCR assay has three components: a matrix protein (M) assay,[349] an H5 assay and an H7 assay.[348,349] The M assay is designed to detect most North American type A influenza viruses whereas the H5 and H7 assays are designed to identify most North American H5 and H7 strains; the subtypes that are associated with HPAI. These tests have been field validated. The M assay, when evaluated on a flock basis (all specimens from a single submission) has a diagnostic sensitivity of 95.1% and diagnostic specificity of 99.5% when compared to virus isolation. Sensitivity of the H5 and H7 assays are slightly lower.

The Agar Gel Immunodiffusion (AGID) test

AGID is the most widely used test in surveillance for antibodies to avian influenza in serum.[355] It can also be used to identify viral nucleoprotein or matrix proteins of all type A influenza viruses. This is a generic test with reagents provided by USDA to State diagnostic laboratories. This test is considered to be the gold standard for detection of type-specific antibodies in serum.

The Hemagglutination Inhibition (HI) test

HI is used to determine subtype specificity of antibodies or hemagglutinin proteins (H1-16 subtypes).[351] This test is usually performed by a reference laboratory such as the National Veterinary Services Laboratories (NVSL), Ames, Iowa. The HI is considered to be the gold standard test for H subtype determination.

The Neuraminidase Inhibition (NI) test

NI is used to determine subtype specificity of antibodies or neuraminidase proteins (N1-9).[352] The NI test is usually performed by a reference laboratory such as the NVSL. The NI is considered to be the gold

standard test for N subtype determination. A new more reliable and user-friendly lectin-based NI assay is being developed for swine influenza and will be adapted for avian influenza (Spackman unpublished data).[356,357]

Antigen capture tests

Several commercial solid phase (flow through or lateral flow) enzyme-linked immunosorbent assay (ELISA) tests have been developed to detect influenza A virus in laboratory samples and in clinical specimens. Originally these products were developed to diagnose influenza A infections in humans, and were adapted to veterinary use. Two are currently licensed for veterinary use, Flu Detect (Zoetis) and VetScan (Abaxis), see Appendix XIII). Studies have shown that the Directigen test (Becton-Dickinson) will detect AI virus in clinical samples. The Directigen test is not licensed for veterinary use, but was used extensively during an outbreak of LPAI H7N2 in Virginia in 2002[358] and was shown to have a diagnostic sensitivity of approximately 70 % and diagnostic specificity of 95% compared to virus isolation. These studies were conducted prior to the veterinary licensing of the Flu Detect and VetScan kits, both of which have been in wide use for the past few years.

AI Antibody Test Kits

Two commercial ELISA test kits are currently licensed for antibody detection for avian influenza in the U.S.: IDEXX, Westbrook ME and Synbiotics, San Diego, CA. These test kits can only be used to test serum from chickens or turkeys. AGID is needed to confirm positive ELISA test results. These tests have been field validated but the specificity and sensitivity have not been disclosed by the firms. A blocking ELISA, which is not species specific has been recently licensed by IDEXX and has been used for poultry and non-poultry species.

Assessment of Experimental Avian Influenza Diagnostic Tests

Little information is available and companies that were contacted prior to the meeting were unwilling to share information regarding products in the pipeline or their diagnostic discovery research programs. Because of the importance of influenza A, the virus is typically a target for novel diagnostic technologies and/or is used as a proof of concept agent. Therefore, the KT decision model was not used as insufficient information was available for a valid analysis. One must also take into account that many new diagnostic technologies are never implemented, because they are impractical, don't out-perform validated tests, or most often are just never adequately validated (an expensive and time consuming process). Finally, innovations in influenza diagnostics are typically meant for human influenza, and therefore are not always appropriate for animal use. Fit for purpose is critical to fulfill for the implementation of any new diagnostic test.

Enzyme linked lectin assay

Current NA inhibition tests are difficult to run, expensive and generate hazardous waste. A more reliable lectin based NI assay is being developed for swine influenza (Spackman unpublished data),[356,357] which is more environmentally friendly, faster and easier to use. A full validation for SIV not been published, but the method appears promising and the optimization for application to AIV has very recently been initiated.

Recommended tests for Avian Influenza Surveillance

Clinical diagnosis

The first line of defense against an outbreak relies on reporting of suspicious cases by personnel handling or observing susceptible animals such as farmers, technicians, farm hands, and veterinarians. Education of farm personnel is a critical element of early detection. However, tests are necessary to confirm influenza infection, since the clinical signs are non-specific.

Assays for detection of influenza virus-infected animals

Current tests have high sensitivity and specificity. However molecular tests need to be continually monitored in reference to new virus isolates to assure that they will detect all influenza viruses since rapid genomic changes can decrease the effectiveness of molecular tests. Tests for poultry are virus isolation and real-time RT-PCR. Pen-side antigen detection lateral flow devices are available, but have lower sensitivity than other tests.[327,358-360] Therefore, antigen detection kits are recommended to be used only with sick animals.

Improvements in sample processing are where the most change is needed. Cheaper and faster RNA extraction, optimized sample collection methods, a way to stabilize the virus in samples without refrigeration and an internal positive control in the real-time RT-PCR test would be beneficial. No novel technologies that substantially improve current tests are clearly in development; cost per assay and utility for a veterinary diagnostic laboratory are common barriers to adoption.

Identification of subtypes is relatively poor with most rapid tests for avian influenza virus because of the high variability (Spackman, unpublished data). Sequencing is the only 100% accurate method, but can be slow and is more technically demanding than HI or real-time RT-PCR. Development of a rapid sequencing method is critical. Subtype identification for swine, canine and equine influenza is more reliable with RT-PCR because of the limited subtypes associated with these species. However, there is always the potential for variants to emerge, particularly in swine.

Differentiation of Newcastle disease virus detection from avian influenza

Newcastle disease (ND) is a highly contagious and often fatal disease that affects over 250 bird species worldwide, and is caused by infection with virulent strains of avian paramyxovirus-1 (APMV-1) of the family *Paramyxoviridae,* genus *Avulavirus*.[361,362] Newcastle disease is widespread in many poultry producing areas of the world. Infections of poultry with virulent strains of APMV-1 (Newcastle disease virus) are reportable to the World Organisation for Animal Health (OIE). Vaccination of poultry species with live non-virulent strains is a key measure in the control of ND. Other APMV-1 viruses of low virulence, which are not used as vaccines, are also often isolated from wild bird species.[362]

Because the clinical presentation of ND cannot be distinguished from AI without confirmatory tests, techniques for differential diagnosis are essential to correctly identify which virus is present, as well as to detect concomitant infection with both viruses. Further, the high incidence of both virulent and vaccine strains of APMV-1 in the field complicates laboratory detection of AI virus because the APMV-1 virus, like AI virus, is a hemagglutinating virus (HA) and is able to agglutinate chicken red blood cells (RBC). Therefore, when an HA positive virus is detected by virus isolation, additional assays must be performed to determine which virus is present.[347] However, when specimens may contain isolates from wild birds, both classical and molecular methods may be necessary as some virulent ND viruses from cormorants in the United States after 2002 have lost their ability to hemagglutinate chicken RBCs and molecular methods are needed for identification.[362,363] In general the available starting material from the diagnostic specimen (e.g., live virus from allantoic fluid, cell culture medium, or extracted RNA), reagents, and

equipment will determine the method used to identify or exclude the presence of APMV-1. Hemagglutinating fluid from cell culture supernatants or egg fluids may be further identified by the hemagglutination inhibition (HI) assay or by RT-PCR.[347] Currently available serologic assays (HI, ELISA) are not able to distinguish antibodies induced by vaccination from those due to infection with virulent virus.

Recommended tests for Response to Avian Influenza Outbreak

Tests in the early stages of an avian influenza outbreak and sustained response

In the early stages of an outbreak surveillance is substantially increased, which requires a scalable rapid test; real-time RT-PCR and antigen detection kits. The matrix rRT-PCR is used as a highly sensitive screening test, and then positive samples are further characterized for subtype by rRT-PCR and are confirmed by virus isolation. Antigen detection kits have much lower sensitivity, but may be used in the field and are very rapid.[327,358-360] A positive is reasonably assured to be a true positive although it will be confirmed by other methods. A negative antigen detection test is not definitive and should be confirmed by a more sensitive method.

Virus isolation is necessary to obtain an isolate for characterization, full genome sequencing for epidemiological investigation and to develop a targeted rRT-PCR test if needed (e.g., if the current tests will not detect the new lineage causing the outbreak). Virus isolates also provide a way to select vaccines if needed, by conducting challenge studies. If necessary the virus itself may be used as a vaccine seed strain. *In vivo* pathogenicity testing is conducted with an early H5 or H7 isolate to determine the pathotype of the virus, although eradication ("stamping out") will likely be used for control regardless of the pathotype.

Full genome sequencing is used to confirm the isolate subtype and to gain data for molecular epidemiology to trace the original of an isolate. Molecular modeling can be conducted post- outbreak to evaluate spread patterns and genetic changes can be assessed. This provides valuable information on the epidemiology and evolution of the virus.

Tests to monitor flock immunity

Immunity of poultry is monitored through antibody levels. Numerous commercial ELISA kits are available for antibody and AGID (current reference standard test).[355] HI assay may be used to identify subtype specificity, but only reference laboratories have the skill and reagents to conduct this test.[351] Commercial ELISA assays have good sensitivity and specificity, are easy to use and fast, but are relatively expensive per animal. Most reliable tests are type based (i.e., antibody is detected regardless of HA and NA subtype), it is not clear that subtype based tests would offer an advantage since most are not broadly reactive and are less sensitive. No commercial subtype based tests for antibody are available in the U.S. at this time.

Recommended tests for Recovery from an Avian Influenza Outbreak

Tests to demonstrate absence of infection

These are essentially the same tests that are used to detect infected birds with the addition of antibody based assays. Numerous commercial ELISA kits are available for antibody and AGID[355] and HI[351] are

commonly used as in-house tests. Commercial ELISA assays have good sensitivity and specificity, are easy to use and fast, but are relatively expensive per animal (see flock immunity section above).

Tests for vaccine matching (see Appendix XI)

Assays that measure the immune response of an exposed animal and, depending upon policy, the ability to discriminate infected from vaccinated animals, may be important. Currently, HI assay is used as the standard for comparing antigenic relationships among AIV isolates.[119,351,364-366] The amino acid sequence of antigenically important region is also used for comparisons. The most accurate method to confirm a vaccine match is by conducting in vivo challenge studies since there is no accurate way to evaluate the full protection of an isolate *in vitro* (e.g., immunogenicity varies among isolates and there as aspects of vaccinal immunity that are not adequately understood to develop in vitro tests).

Diagnostic Test Needs for Avian Influenza

- There is a need for developing internal controls to validate the performance of diagnostic test procedures across laboratories.
- More sensitive pen-side influenza A tests or commercial test kits that can be used in the field are needed. Even with simultaneous identification of the most important HA and NA subtypes.
- If vaccines are to be used in an eradication campaign, companion diagnostic tests will be needed to implement a DIVA (differentiating infected from vaccinated animals) strategy. Surveillance will need to be conducted on vaccinated flocks to determine whether the field virus is circulating and the control strategy is working. This should be done by both serological and virological surveillance of vaccinated and non-vaccinated flocks. The use of sentinel birds in vaccinated repopulated flocks should be considered as appropriate.
- Improve identification of hemagglutinin and neuraminidase subtype.
- Develop a reliable test for the HA and NA subtype specificity of AIV antibody in avian serum.
- Develop a rapid full genome sequencing method (e.g. with next generation methods).
- Develop a viral transport media that can stabilize live influenza virus without refrigeration or maintenance of the cold chain.
- Continue to monitor current molecular diagnostic tests for sensitivity and specificity with novel influenza isolates.
- Continue to monitor and evaluate genome sequence from novel influenza viruses for diagnostic needs.
- A rapid molecular test that is not easily affected by genetic mutations would be helpful.
- Develop *in vitro* tests to evaluate vaccine efficacy and potency with variant isolates.

Diagnostics for Swine Influenza

A national surveillance system was established in the U.S in 2009 by the USDA in response to the 2009 H1N1 pandemic, the growing diversity of swine viruses, and increasing number of detections of zoonotic events in humans.[8] The number of isolates with sequence data from this surveillance stream has grown significantly, building the foundation for systematic sequence analyses to pair with antigenic assessment. Phylogenetic analysis of contemporary H3 suggested increasing evolution since the emergence and subsequent reassortment with the H1N1pdm09.[8]

Assessment of Commercial and Laboratory Swine Influenza Diagnostic Tests (Appendix VIII)

Virus isolation

Typically performed with cells culture or less frequently in 9-11 day embryonating hens eggs and is considered the gold standard for detection of all subtypes of SIV virus.[367] Virus isolation is critical to obtain isolates for in depth characterization.

Real time RT-PCR (rRT-PCR) assay

Commercial and in-house tests are used. The primer target is for type A influenza (i.e., the M gene). Additional tests for the H1 and H3 subtypes may also be used. A commercial H1 and H3 subtype rRT-PCR test is being licensed in the U.S. by Life Technologies.

The Hemagglutination Inhibition (HI) test

HI is used to determine subtype specificity of antibodies to the HA protein (e.g., H1 or H3 specific).[351] This can be used to evaluate prior exposure or response to vaccination. Hemagglutination inhibition assay is also the current standard for characterizing antigenic differences among SIV isolates.

The Neuraminidase Inhibition (NI) test

NI is used to determine subtype specificity of antibodies or neuraminidase proteins (N1-9).[352] The NI test is usually performed by a reference laboratory such as the NVSL. The NI is considered to be the gold standard test for N subtype determination. A new more reliable and user friendly lectin based NI assay is being developed for swine influenza (Spackman unpublished data).[356,357]

Antigen capture tests

A commercial solid phase (flow through or lateral flow) enzyme-linked immunosorbent assay (ELISA) tests have been developed to detect influenza A virus. Originally these products were developed to diagnose influenza A infections in humans and were later validated for animal influenza viruses. For SIV the Flu Detect (Zoetis) kit is currently licensed for veterinary use with SIV in the US.

SIV Antibody Test Kits

Commercial ELISAs are available in the US for SIV H1 and H3 antibody detection; however, according to the manufacturer there can be cross-reaction, therefore results are not sufficiently accurate.

Diagnostics for Emerging Variant Swine Influenza Viruses

Current tests have high sensitivity and specificity. Both a commercial and USDA rRT-PCR test are in wide use for SIV detection. Both currently perform well, however molecular tests need to be continually monitored in reference to virus isolate to assure that they will detect all influenza viruses since rapid genomic changes can decrease the effectiveness of molecular changes. Tests for swine are virus isolation and real-time RT-PCR (see above). Pen-side antigen detection lateral flow devices are available, but have lower sensitivity than other tests.

Subtype identification for swine influenza is more reliable with RT-PCR than for avian influenza because of the limited subtypes associated with pigs. However, there is always the potential for variants to emerge, particularly in swine. Currently a commercial N1 and N3 test are available (Life Technologies) and has

been in use in some swine diagnostic labs experimentally, although the product is still undergoing licensing with the USDA Center for Veterinary Biologics (CVB). Other tests are available although HI and NI are still in use.

Although tests are available it is critical to maintain a program to monitor and characterize SIV isolates (in the United States and abroad). This is critical to identify genomic and biological changes that could affect the ability to detect SIV and identify SIV infected animals.

Assessment of Experimental Swine Influenza Diagnostic Tests

There is limited information available on SIV tests in development. Because of the structure of the SIV testing and limited advantages for the swine industry the market is relatively limited. Experimental tests are often developed in-house by diagnostic laboratories and information is only widely available upon publication. Some limited information is available on a few subtypes identification tests. A new format of neuraminidase inhibition assay based on lectin binding is under development. This assay is easier to use than the old NI assay and does not generate hazardous chemical waste. A commercial rRT-PCR N1 and N3 identification test (Life technologies) is in the process of being licensed in the US. This assay has been used successfully by several diagnostic labs during development and validation.

Recommended tests for Swine Influenza Surveillance

Because of the high sensitivity and specificity as well as practicality rRT-PCR is used frequently as well as virus isolation. These are reliable and accurate tests. Virus isolation provides an isolate that can be characterized in depth by sequencing and pathogenesis studies. The USDA SIV Surveillance System was initiated in 2009 and over 2000 SIV isolates have entered into the system with their associated sequences. A consistent and continued assessment of viruses from the surveillance system is required for identifying those that require further experimental analysis.

Recommended tests for Response and Recovery to Swine Influenza

Same as the tests used for surveillance. SIV is not a reportable disease, which is reflected in the diagnostic paradigm. Virus isolation and rRT-PCR are used.

Diagnostic Test Needs for Swine Influenza

- Develop a better test for the H and N subtype specificity of SIV antibody in serum.
- Develop better methods to evaluate antigenic relationships among SIV isolates.
- Develop a viral transport media that can stabilize live influenza virus without refrigeration or maintenance of the cold chain.
- Continue to monitor current molecular diagnostic tests for sensitivity and specificity with novel influenza isolates.
- Continue to evaluate genome sequence from novel influenza viruses for diagnostic needs.

RECOMMENDATIONS

COUNTERMEASURES PRIORITIES

Based on the results of the assessment conducted by the AICWG, the following needs were identified:

Vaccines

- There is a need to develop and license live-vectored vaccines to include the H7 hemagglutinin subtype and antigenic important variants for H5 subtype avian influenza
- To develop next generation modified live vectored-influenza virus vaccines that can be cost effectively used on poultry after they have been placed into the field
- To explore the use of reverse genetics technology to develop a range of vaccine seed strains with hemagglutinin genes matching likely H5 and H7 threats from around the world with an egg adapted high growth reassortant.
- To explore the use reverse genetics technology to develop a live-attenuated IAV vaccine for use against new variant SIV strains with pandemic potential.

Diagnostics

- There is a need to improve rapid identification molecular diagnostics of hemagglutinin and neuraminidase subtypes.
- There is a need for developing internal controls to validate the performance of diagnostic test procedures across laboratories.
- More sensitive pen-side tests or commercial test kits that can be used in the field are needed. Even with simultaneous identification of the most important HA and NA subtypes.
- If vaccines are to be used in an eradication campaign, validated companion diagnostic tests will be needed to implement a DIVA (differentiating infected from vaccinated animals) strategy.
- There is a need to develop a reliable test for the HA and NA subtype specificity of avian influenza virus antibody in avian serum
- There is a need to develop a reliable test for the HA and NA subtype specificity of swine influenza virus antibody in swine serum
- There is a need to develop a rapid and cost effective full genome sequencing method (e.g. with next generation methods).
- There is a need to develop a viral transport media that can stabilize live influenza virus without refrigeration or maintenance of the cold chain.
- Continue to monitor current molecular diagnostic tests for sensitivity and specificity with novel influenza isolates.
- Continue to evaluate genome sequence from novel influenza viruses for diagnostic needs.
- A rapid and robust molecular test that is not easily affected by genetic mutations.
- Most state and regional diagnostic laboratories all already participating in the NAHLN for animal influenza, this program should be maintained to assure that all laboratories meet the minimum diagnostic requirements, pass proficiency testing and are notified of test updates.

Drugs

- The use of antiviral drugs to control animal influenza viruses in swine and poultry are not recommended.

Disinfectants

- Commercially available disinfectants are effective but there is a need to develop methods to collect and dispose of disinfectants after use to minimize negative environmental impact
- There is a need to develop new generation disinfectants that degrade rapidly in the environment to innocuous materials.

PPE

- There is a need to improve respirators for working under field conditions in hot contaminated environments.
- There is a need to ensure availability of FDA-approved drugs and vaccines to protect workers.

RESEARCH RECOMMENDATIONS

Research priorities were identified that were determined to be critical to address the gaps in our scientific knowledge and importantly, advance the development of countermeasures to effectively control and mitigate an animal influenza virus outbreak with epizootic and/or pandemic potential. Overall, research studies applied to 1) the understanding of viral evolution in animal populations, 2) viral pathogenesis, 3) understanding transmission and epidemiology, and 4) development of improved countermeasures such as vaccines and diagnostics are likely to yield significant improvements in our ability to control influenza virus outbreaks in animals, domestically and internationally.

Virology

There are important gaps in our understanding of influenza virology and studies addressing these gaps are especially critical to support prediction and forecasting initiatives, as well as the development of therapeutic and vaccine interventions. Research priorities include studies to:

1. Identify the molecular determinants of host specificity
2. Identify the molecular determinants of tissue tropism
3. Identify molecular determinants of virulence in target animal species

Pathology

Although the role of the multi-basic cleavage site in poultry is well understood, the role of other determinants contributing to virulence is less clear. For instance, in poultry it is not clear why HPAI are limited to H5 and H7 subtypes. The basis for the lack of virulence of H5N1 in pigs is unclear, as is its virulence in some species of aquatic birds. Research priorities include studies to:

1. Identify mechanisms of innate immune evasion across target animal species.
2. Identify virus and host determinants of host range restriction to identify mechanisms by which viruses adapt to new host species, e.g., from birds to mammals, and vice versa.

3. Investigate whether increased virulence of influenza viruses in susceptible hosts is due to enhanced viral replication or systemic spread, or to altered innate responses.
4. Explore possible influence of virus on host gene expression by novel mechanisms.
5. Investigate the influence of co-infecting immunosuppressive viruses and other respiratory viruses on influenza transmission and pathogenesis.

Immunology

Although the role of antibody induced protection from disease is fairly well understood, the role of other contributing factors to immunological protection is less clear. It is not clear why some animals are resistant to influenza viruses and others are highly susceptible. The likely genetic basis for this resistance must be identified and applied where possible. Research priorities include studies to:

1. Understand the innate immune response to influenza virus infection in different animal species.
2. Determine the source and contribution of unregulated cytokine expression following HPAI virus infection.
3. Determine antibody response profiles against influenza B-cell epitopes to identify those involved with virus neutralization.
4. Determine the role and importance of T-cell epitopes to protection following infection, and how cell-mediated immunity contributes to immunity in wild birds versus poultry.
5. Determine the immunological response to recombinant live vaccines and define the humoral and cellular protection associated with cross protection.
6. Determine the contribution of host immunogenetics on innate protection.
7. Identify conserved B- and T-cell epitopes within and between virus subtype to target in new vaccine platforms or improvement of existing vaccines
8. Identify antibody epitopes important for antigenic drift in the swine host and development of models to predict based on HA sequence evolution.
9. Characterize the humoral and cellular immune response to wild-type infection, and compare it to attenuated and inactivated vaccines to identify correlates of protection.
10. Identify unique B- or T-cell epitopes that are non-overlapping between subtypes to support development of virus and subtype specific diagnosis of previous infection using blood or mucosal specimens.

Epidemiology

Gaps exist in our understanding of virus evolution and population dynamics. There continues to be insufficient surveillance of equine, swine and avian populations for influenza viruses worldwide to have a clear picture of what is circulating in the field. There is a need for systematic analysis of field viruses, particularly those that display variation in their genetic and phenotypic traits, and to investigate pathogenesis, transmission within and between animal species, and mathematical modeling and molecular epidemiology. Research priorities include:

Pathogenesis

1. Research programs that maintain the characterization of new animal influenza virus isolates *in vitro* (e.g., genetically) and *in vivo* (e.g., pathogenesis in important species).
2. Identify the function and consequence of mutations that emerge during replication of influenza viruses in different avian species.

3. Evaluate the genetic information available for AI reservoirs and poultry and generate data on the genomics characteristics that confer resistance to avian influenza viruses in some species.
4. Determine if some village chicken breeds contain genes that confer AI resistance.
5. Explore the impact of variable host susceptibility on avian influenza virus persistence in different ecosystems (i.e., domestic ducks in South East Asia).
6. Provide the poultry community with improved predictive tools for how viruses are likely to increase in virulence over time
7. Determine the drivers of virulence associated with antigenic variants.
8. Determine the role of mutations at receptor binding sites on replication and pathogenesis, especially which mutations are important in changing host specificity.
9. Determine host-virus factors that influence infection outcome in different poultry hosts.
10. Determine viral and host factors that contribute to the successful transmission of swine influenza virus to other species.
11. Identify markers of swine influenza viruses with human pandemic potential.
12. Characterize swine influenza virus isolates from humans, birds or other species, and identify potential factors involved in interspecies transmission of swine influenza viruses.

Transmission

1. Develop improved transmission models that are more applicable to the complexities of virus in the field.
2. Develop standarized procedures and tools for conducting transmission studies in experimental settings (interhosts, intrahosts, fomites, and aereosol).
3. Develop appropriate systems for measuring shedding of model viruses in target species. Develop a better understanding of ecological factors and risk pathways for interaction between wild birds and poultry.
4. Determine risk of transmission of virus in markets or in flocks of vaccinated poultry.
5. Evaluate population dynamics of swine influenza viruses to determine virus persistence at the farm level and the major routes of virus spread between farms.
6. Determine virus survival characteristics, persistence, and underlying factors in a range of settings relevant to animal production.
7. Establish the mode of transmission and mechanisms of persistence of LPAI compared with HPAI, identifying virus-host factors that influence virus transmissibility.
8. Develop appropriate systems for detecting and measuring survival of model viruses in the environment.
9. Identify risk factors in poultry production that favor transmission and spread of avian influenza to poultry.
10. Identify virus-host correlates of virus transmissibility both within and between host species.

Mathematical modeling and molecular epidemiology

1. Use modern evolutionary methods to evaluate the effect of distinct hosts on virus evolution and selective pressure.
2. Link phylogeny with socio-economic information, poultry trade data to better understand spread and movement of viruses within and across countries and continents.
3. Develop standards for data collection and placement on GIS maps/GPS databases for locations where isolates from wild birds and poultry are collected to enhance traditional and molecular epidemiologic investigations.

4. Develop computer simulation modeling methods to study multi-strain interaction (e.g., H9N2 and H5N1) and possible outcomes of multiple strain circulation.
5. Apply next generation sequencing tools to better elucidate the intra-host and outbreak genetic diversity and how this can affect evolutionary and intra and interspecies transmission dynamics.
6. Develop means to detect and characterize emergent influenza viruses and use these data to generate models that predict future outbreaks.
9. Identify epidemiological and ecological factors affecting disease outbreaks

Vaccines

There is an ongoing need for research studies to support vaccine development. These should explore approaches to improved and sustainable vaccination. Conserved, protective epitopes need to be identified to support the development of vectored vaccine approaches. Candidate live attenuated influenza viruses should be developed and their safety and genetic stability as vaccines evaluated. Characterization of antigenic structure and evolution is still rudimentary in livestock and poultry (although antigenic cartography is being used experimentally in the analysis of swine isolates to assess matching with vaccines). In poultry, there is a need for antigenic analysis of H5 and H7 viruses to ensure good (neutralizing) matching with potential vaccines. In swine, there is a need for evaluating the risk associated with long-term drift away from human H3N2 strains that could provide a potential source for emergence of new variant viruses for humans. The possibility of developing an approach to vaccine selection that is coordinated with selection of human vaccines to minimize divergence of swine and human vaccines should be considered.

Vaccine research priorities include:

1. Evaluation of novel technologies that reduce the time required to produce a vaccine.
2. Development of novel vaccine technologies to produce a broader or universal clinical protection.
3. Development of vaccine platforms that can be used in multiple species.
4. Improvement in the regulatory process for vaccine selection and production.

Diagnostics

Influenza is a highly variable virus, which complicates diagnostic tests. Tests for type A influenza viruses are generally reliable, but serological test require improvement. The key animal influenza diagnostic gap is in subtype identification and in the identification of the subtype specificity of sera. Better serologic tests are needed, both to determine what subtypes has an animal been infected with and to characterize the antigenic differences among animal influenza isolates. Characterization of the antibody response and antigenic differences among animal influenza isolates are critical for updating vaccines and evaluating vaccinal protection. Hemagglutination inhibition (HI) assay is the current standard test for identifying subtype specificity but it is a cumbersome test that lacks precision.

Other areas where improvement would be beneficial are: 1) a rapid molecular test that is not easily affected by genetic mutations; 2) a transport media that stabilizes the virus without refrigeration; and 3) at the very least, efforts to characterize new isolates should be continued to assure that current tests will have optimal sensitivity and specificity.

Diagnostic research priorities include:

1. Better tests to identify the subtype specificity of antibody in animal sera (e.g., to identify what HA and NA subtypes an animal has been exposed to.

2. Develop rapid pen-side virus detection tests that are highly sensitive and that are economically feasible for animal production systems.
3. Develop a transport media that stabilizes the virus and maintains virus viability in the absence of the cold chain.

Biotherapeutics

Continued research on viral/host protein interactions leading to potential anti-viral drugs will continue to be critical for public health, especially if new pandemic strains emerge that are also resistant to available anti-viral drugs. In addition, anti-virals could play a role in controlling disease outbreaks in animal agriculture in particular situations, e.g., outbreak control (under "vaccination with the intent to slaughter" disease eradication plans) or protecting important genetic stocks, where residues, resistance and cost would not be an issue. Biotherapeutic approaches with cytokines or therapeutic antibodies need to be assessed in pigs in the context of IAV. Particularly for how effective they might be at reducing viral shedding for purposes of stamping out the spread of an emerging pandemic virus of heightened virulence.

Depopulation, Disposal, and Decontamination

There is a need for continuing the development and validation of methods to ensure they are humane, effective, and result in stopping the spread of high-consequence agents:

- Validate the effectiveness of common disposal processes and identify potential exposure pathways so risks can be mitigated
- Develop new technologies and perform feasibility studies to determine their effectiveness under field conditions
- Validate efficacy of generic disinfectants against high-consequence pathogens, optimize cleaning technologies for cost-effectiveness, and evaluate environmental risk of disinfectant use

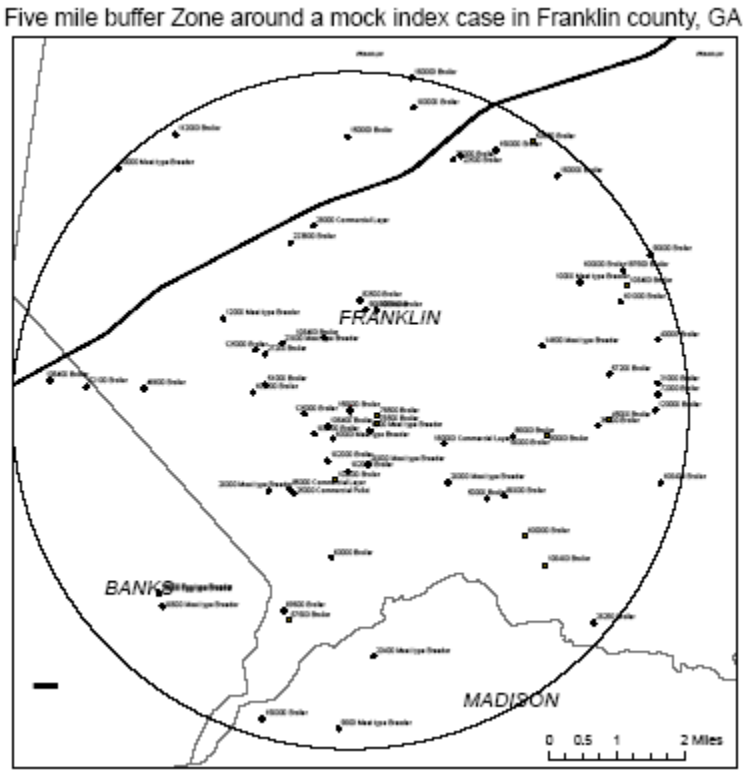
CONCLUSION

The citizens of the United States and the world are vulnerable to a disease outbreak caused by animal influenza virus with epizootic and/or pandemic potential. This disease outbreak would not only produce significant economic loss in the animal agricultural sectors but also affect the health and well being of millions of people around the world. The magnitude of such an outbreak is dependent on many variables but there is no question that a "One Health" response plan is needed that integrates effective forecasting, surveillance, countermeasures, and strategies for the rapid control and elimination of novel influenza viruses in people and animals, especially livestock and poultry.

In the United States, the major factors that drive animal influenza viruses preparedness and response plans are the AIV-free status in poultry and the endemic SIV status in swine. Modern production practices limit the transmission of AIV, which complements current AIV eradication policy in the poultry industry. In contrast, most swine herds are SIV positive and novel influenza viruses can be transmitted from man to swine making SIV eradication untenable. Consequently, there is a need for vaccines to control SIV on a daily basis, and for a vaccine stockpile that can be rapidly deployed in case of a widespread AIV outbreak. Current vaccine technology needs to keep pace with the rapidly changing animal influenza viruses, demonstrating the need for improved vaccine platforms and revision of regulations on manufacturing AIV and SIV vaccines. In addition, there is a critical need for maintaining basic research programs to investigate how influenza viruses are evolving and adapting to new animal species, including humans. Importantly, the stream of scientific information generated from these basic research programs are needed to predict and forecast disease outbreaks and inform the development of robust surveillance and countermeasures development programs.

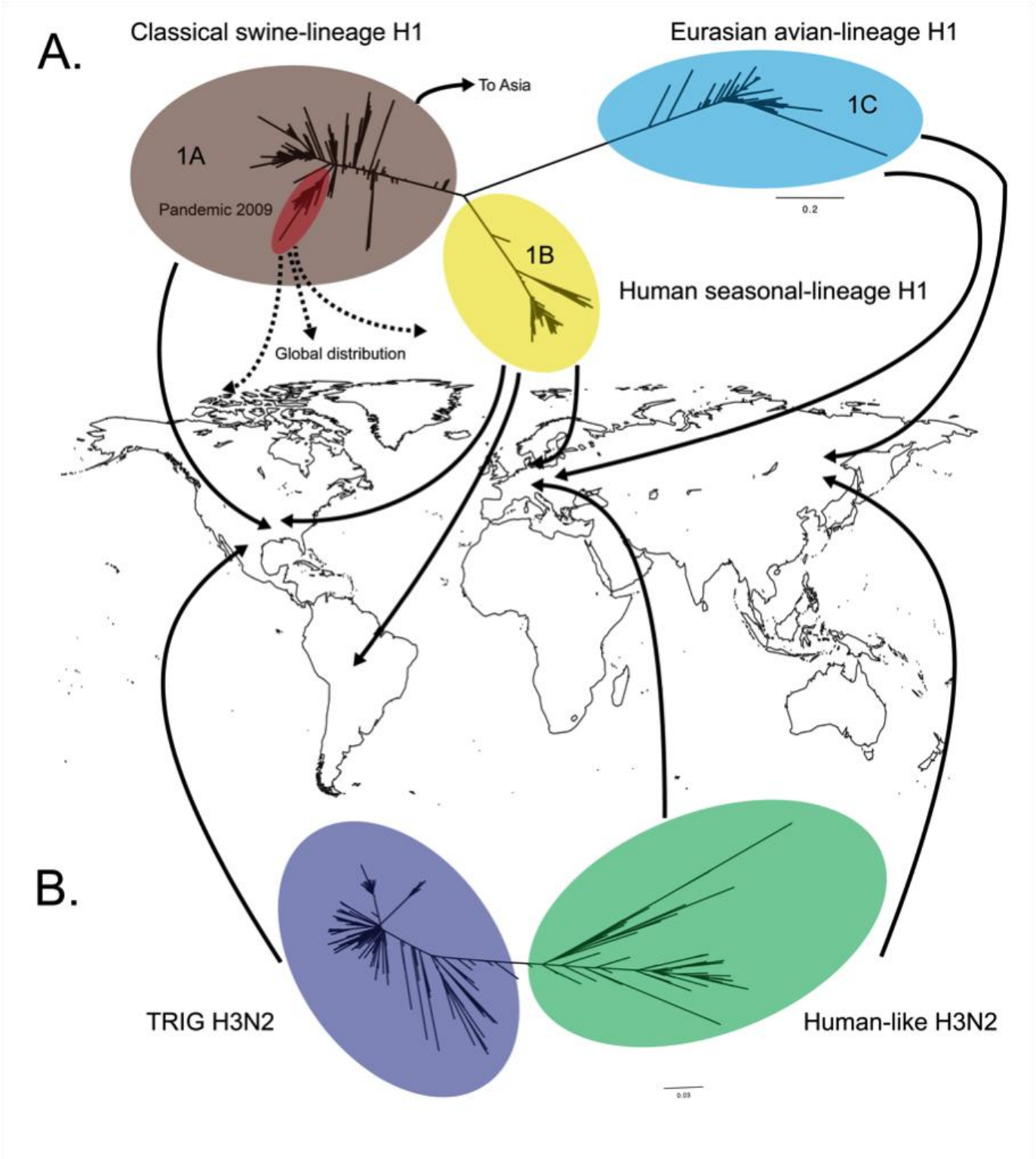
FIGURES

Figure 1: Poultry Farms in within a Five Mile Radius



Source: Wayne Farms LLC

Figure 2: Global swine influenza lineage situation



TABLE

Table 1: Animal influenza virus diagnostic tests available now or under development

Test	What does it detect?	Development status	Capability	Utilization
Agar Gel Immuno Diffusion (AGID)	Antibodies against Type A influenza	In service	Positive v. negative	Type-Specific Flock screening
ELISA	Antibodies against AIV	In service	Positive v. negative	Type Specific Flock screening
Hemagglutination Inhibition (HI)	Antibodies against AIV	In service	HA Subtype specific	HA subtype determination
Neuraminidase Inhibition (NI)	Antibodies against AIV	In service	NA subtype specific	NA subtype determination
Antigen Capture	Influenza Type A antigen	In service	Positive v. negative	Rapid diagnostics Pen side test
Molecular (RRT-PCR)	Virus RNA.	In service	Pan AIV, and subtype specific	Rapid testing and subtype determination
Virus isolation	Infectious virus	In service	All replicating AIV	Gold standard and confirmatory test
Nucleotide sequencing	Virus RNA	In service	Requires viral RNA	Phylogenetic analysis of virus

APPENDIX I: AGENDA - ANIMAL INFLUENZA VIRUSES GAP ANALYSIS AND COUNTERMEASURES ASSESSMENT WORKSHOP

Agenda

March 25-27, 2013

Introduction

An international group of experts on animal influenza viruses (AIWG) will meet at the University of Georgia, Riverbend South Laboratory, on March 25-27, 2013, to conduct a gap analysis and assess countermeasures to effectively control and mitigate the impact of an outbreak of an animal influenza virus with zoonotic and/or pandemic potential. The AIWG will prepare a report that will 1) define the threat, 2) provide a gap analysis of our knowledge of animal influenza viruses, 3) identify priority research needs, 4) and provide an in-depth analysis of available countermeasures to contain and mitigate the threat.

Workshop Objectives

1. Gap analysis
2. Countermeasures assessment
3. Research priorities
4. Workshop Report

Organizing Committee

Cyril G. Gay, DVM, Ph.D (Chair)
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March 25, 2013
Knowledge and Gaps

09:00 – 09:15

Welcome

Carl Bergmann, Ph.D
Assistant Vice President for Research, Associate Director CCRC, Executive Director AHRC
University of Georgia, Athens, Georgia

09:15 – 09:30

Workshop goals, objectives, methods, and outcomes

Cyril G. Gay, DVM, Ph.D
National Program Leader, Animal Production and Protection, USDA-ARS

09:30 – 11:30

Introduction: The threat of animal influenza viruses to animal agriculture and public health

09:30 – 10:00

Poultry Production

Bruce Stewart-Brown, DVM, dipl ACPV
Vice President of Food Safety and Quality, Perdue Farms

10:00 – 10:30

Swine Production

Terry Coffey, Ph.D
Chief Science & Technology Officer, Murphy-Brown LLC

11:00 – 11:30

Public Health

Ruben Donis, Ph.D
Centers for Disease Control and Prevention (CDC)

11:30 – 14:30

Introduction: Obstacles to preventing and controlling animal influenza viruses

11:30 - 12:00

Avian Influenza

David E. Swayne, DVM, Ph.D
Laboratory Director
Southeast Poultry Research Laboratory (SEPRL)
USDA -Agricultural Research Service, Athens, Georgia

13:00 - 13:30

Swine Influenza

Kelly Lager, DVM, Ph.D
Veterinary Medical Officer
Virus and Prion Research Unit
National Animal Diseases Center (NADC)
USDA-Agricultural Research Service, Ames, Iowa

13:30 - 14:00

Equine Influenza

Thomas M. Chambers, Ph.D
Associate Professor and Head, OIE Reference Laboratory for equine influenza
University of Kentucky, Lexington, Kentucky

14:00 – 17:30

Session 1: Epidemiology - Avian, swine, and wildlife

14:00 - 15:00

Avian Influenza

Mary Pantin-Jackwood DVM, Ph.D
Veterinary Medical Officer
Exotic and Emerging Avian Viral Diseases Research Unit, SEPRL

Group Discussion: Leader Isabella Monne, Ph.D, Istituto Zooprofilattico Sperimentale delle Venezie, Italy

- Gaps and research needs

15:30 – 16:30

Swine Influenza

Amy Vincent, DVM, Ph.D
Veterinary Medical Officer
Virus and Prion Research Unit, NADC

Group Discussion: Leaders Jennifer Koeman, DVM, National Pork Board, and **Harry Snelson**, **DVM**, American Association of Swine Veterinarians

- Gaps and research needs

16:30 – 17:30

Influenza Viruses in Wildlife

Hiroshi Kida, DVM, Ph.D
Head, Research Center for Zoonosis Control
Head, OIE Reference Laboratory for Avian Influenza
Head, WHO Collaborating Centre for Zoonoses Control
Hokkaido University, Sapporo, Hokkaido Japan

Group Discussion: Leader David Stallknecht, Ph.D, College of Veterinary Medicine**,** University of Georgia

- Gaps and research needs

March 26, 2013
Knowledge and Gaps

09:00 – 10:30

Session 2: Virology – Determinants of virulence, host range, and host-pathogen interactions

09:00 – 09:45

Determinants of virulence and host range

Richard Webby, Ph.D
St. Jude Children's Research Hospital

Group Discussion: Leader Ruben O. Donis, Ph.D, Center for Disease Control and Prevention, U.S

- Gaps and research needs

09:45 – 10:30

Host-pathogen interactions

Daniel Perez, Ph.D
University of Maryland

Group Discussion: Leader Mike Skinner, Ph.D, Imperial College London, London, UK

- Gaps and research needs

11:00 – 15:00

Session 3: Diagnostics - Surveillance, response, and recovery

11:00 – 11:45

Avian Influenza Surveillance

Mia Kim
USDA-APHIS, National Veterinary Services Laboratory (NVSL)

Group Discussion: Leader Sharon M Brookes, Ph.D, Virology Department, Animal Health and Veterinary Laboratories Agency (AHVLA), Surrey, UK

- Gaps and research needs

11:45 – 12:30

Swine Influenza Surveillance

John Korslund, DVM
USDA- APHIS, National Center for Animal Health Programs (NCAHP), Swine Health

Group Discussion: Leader Amy Vincent, DVM, Ph.D, National Animal Disease Center, Ames, IA

- Gaps and research needs

13:30 – 14:30

Response and recovery

John Pasick, DVM, Ph.D
National Centre for Foreign Animal Disease, Canadian Science Centre for Human & Animal Health, Canadian Food Inspection Agency, Winnipeg, Manitoba, Canada

Group Discussion: Leader Christian Grund, Ph.D, Friedrich-Loeffler-Institut, Insel Riems, Germany

- Gaps and research needs

15:00 - 17:00

Session 4: Vaccines - Ideal vaccine profile, vaccinology, what's in the pipeline

15:00 - 16:00

Swine Influenza

Marcus Kehrli, DVM, Ph.D
Research leader
Virus and Prion Research Unit, NADC

Group Discussion: Leader Wenju Ma, Ph.D, Kansas State University, Manhattan, Kansas

- Gaps and research needs

16:00 - 17:00

Avian Influenza

David Suarez, DVM, Ph.D
Research Leader
Exotic and Emerging Avian Viral Diseases Research Unit, SEPRL

Group Discussion: Leader Erica Spackman, Ph.D, Exotic and Emerging Avian Viral Diseases Research Unit, SEPRL

- Gaps and research needs

March 27, 2013
Gap Analysis and Countermeasures Assessment

09:00 – 15:00 (Working lunch)

Breakout Sessions – Epidemiology, Virology, Diagnostics, and Vaccines

Instructions: Provided by **Dr. Cyril G. Gay**

Each breakout group will review available scientific information and countermeasures, identify gaps, assess strength and weaknesses, and identify research priorities to address gaps and weaknesses. For diagnostics and vaccines, the breakout groups will use a decision model to assess potential countermeasures to stockpile. These countermeasures must significantly improve our ability to control, and when feasible, eradicate animal influenza viruses from poultry and pork production. When finished with their objectives, the epidemiology group will join the diagnostic group to contribute to the assessment of diagnostics for surveillance and epidemiological investigations. When finished with their objectives, the virology group will join the vaccine group to determine priority research areas that will contribute to vaccine discovery research.

Breakout Group 1: Epidemiology

Session leaders: Dr. Mary Pantin-Jackwood and Dr. Carol J. Cardona

- Transmission
- Susceptible hosts
- Swine versus avian production
- Wildlife-domestic animal interphase
- Research gaps and priorities assessment

Breakout Group 2: Virology

- **Session leaders:** Dr. Mike Skinner and Dr. Ruben Donis
- Functional genomics
- Determinants of virulence and host range
- Host-pathogen interactions
- Research gaps and priorities assessment

Breakout Group 3: Diagnostics

- **Session leaders:** Dr. Mia Kim and Dr. John Pasick
- Review criteria for selecting diagnostics
- Review available and new diagnostics for surveillance, response, and recovery
- Decision model analysis of available diagnostics
- Decision model analysis of experimental diagnostics
- Rank diagnostic tests
- Research gaps and priorities assessment

Breakout group 4: Vaccines

Session leaders: Dr. Mark Tompkins, Dr. David Suarez, and Dr. Marcus Kehrli

- Review criteria for selecting vaccines
- Review list of available vaccines
- Review most promising technologies in the pipeline
- Decision model analysis of commercial and experimental vaccines
- Rank vaccines
- Research gaps and priorities assessment

15:00 - 16:00

Reports from Section Leaders

- **Epidemiology**
- **Virology**
- **Diagnostics**
- **Vaccines**

16:00 - 16:30

Wrap up, conclusion and next steps

Cyril G. Gay, DVM, Ph.D

National Program Leader, Animal Production and Protection, USDA-ARS

APPENDIX II: ANIMAL INFLUENZA VIRUSES COUNTERMEASURES WORKING GROUP INSTRUCTIONS

Decision Model

We will use a decision model to assess potential countermeasures to stockpile. These countermeasures must significantly improve our ability to control and eradicate an outbreak of an animal influenza virus with zoonotic and/or pandemic potential in the United States. The decision model is a simple tool that will allow us to focus on critical criteria for the National Veterinary Stockpile, and rank the available interventions relative to each other. The decision model is available as a Microsoft Excel spread sheet has been prepared to quantitatively assess the rankings we assign to a set of selected criteria that will lead to the selection of the highest cumulative option. We can use as many criteria as we want but the objective is to get down to the ones that will make or break success. The criteria for each intervention will be selected by the Animal Influenza Viruses Countermeasures Focus Group March 25-27, 2013, but a preliminary set has been identified to expedite the process. You are encouraged to review the criteria prior to coming to the meeting and be prepared to modify the criteria as needed with the focus group on March 25, 2013. The following provides an example of criteria and assumptions for assessing vaccines.

Criteria

If a vaccine is going to be used as a emergency outbreak control tool in swine or poultry, then we need to know: 1) is it efficacious (does it effectively eliminate virus amplification or just reduce amplification by a known log scale); 2) does it work rapidly with one dose (probably do not have time for a second dose); 3) whether it is available today from the perspective of having a reliable & rapid manufacturing process (need to know it can be up & running rapidly and will yield a predictable amount of vaccine; 4) can we get the product to the outbreak site rapidly & safely; 5) once at the site, can we get it into the target population rapidly; 6) type of administration-mass or injected, people and equipment to do the job become important); and 7) are diagnostics available to monitor success and or DIVA compliant. While cost is important, the cost of the vaccine in an outbreak may be small in comparison to the other costs. In addition, how fast the product can be made is important because that will have a big impact on how big a stockpile will be needed. Accordingly, you will see from the Excel sheets that have been prepared for vaccines that the following critical criteria and assignment of weights for each criterion are proposed.

Weight	Critical Criteria
10	Efficacy
2	Safety
8	Available Today
10	Speed of Scale up
2	Storage
6	Distribution
8	Mass Administration
4	All Ruminants
6	DIVA Compatible
8	Dx Available
4	Cost to Implement

APPENDIX III: COMMERCIAL VACCINES FOR AVIAN INFLUENZA

Rank each Intervention (2,4,6,8, or 10) as to its importance to making a decision, only one "10" rankings allowed

Weight	Critical Criteria	H5 Oil (U.S)	H7 Oil (U.S)	Trovac H5) (Merial)	H5H7 Oil (Italian)	H5 Oil (Mexico)
10	Efficacy	6	6	4	6	6
6	Safety	6	6	10	6	6
8	One dose	6	4	6	6	6
8	Speed of Scaleup	6	6	10	6	6
2	Storage	6	6	4	6	6
2	Distribution/Supply	6	6	6	4	8
10	Mass Administration	2	2	4	2	2
6	In ovo	0	0	2	0	0
8	DIVA Compatible	4	4	8	4	4
2	Withdrawl	2	2	6	2	0
4	Cost to Implement	4	4	6	4	4

Rank each Criteria 2,4,6,8 or10 on each criterion -- no more than two "10" rankings allowed

Critical Criteria	H5 Oil (U.S)	H7 Oil (U.S)	Trovac H5) (Merial)	H5H7 Oil (Italian)	H5 Oil (Mexico)
Efficacy	60	60	40	60	60
Safety	36	36	60	36	36
One dose	48	32	48	48	48
Speed of Scaleup	48	48	80	48	48
Storage	12	12	8	12	12
Distribution/Supply	12	12	12	8	16
Mass Administration	20	20	40	20	20
In ovo	0	0	12	0	0
DIVA Compatible	32	32	64	32	32
Withdrawl	4	4	12	4	0
Cost to Implement	16	16	24	16	16
Value	288	272	400	284	288

Major Assumptions:

Vaccine Profile

1. Highly efficacious: prevent transmission; efficacy in all bird species; cross protection across subtypes; one dose; quick onset of immunity; one year duration of immunity
2. Safe to workers and birds
3. DIVA compatible
4. Manufacturing method yields high number of doses
5. Mass vaccination compatible to eliminate individual bird inoculation
6. Rapid speed of production and scale-up
7. Reasonable cost
8. Short withdrawal period for food consumption

Vaccine Administration

1. Commercial poultry firms can logistically vaccinate 1 million birds in 20 days.
2. Federal and state vaccination crews can vaccinate 1 million birds in 4 weeks.
3. If mass vaccine available (water/spray delivered) the entire target population could be vaccinated.

APPENDIX IV: EXPERIMENTAL VACCINES FOR AVIAN INFLUENZA

Rank each Intervention (2,4,6,8, or 10) as to its importance to making a decision, only one "10" rankings allowed

Weight	Critical Criteria	Reverse genetics Killed Fort Dodge	Reverse genetics MLV (Medimmune/ARS)	NDV-vectored (ARS)	Adeno-vectored (Vaxin)	Alphavirus-vectored (Alphavax)	Plasmid DNA (Vical Merial)	Baculovirus-exp. Protein Science/Intervet	Plant-exp. (Dow Agro.)
10	Efficacy	8	10	6	8	8	6	6	6
6	Safety	6	6	6	8	8	8	6	6
8	Time to availability	8	2	4	6	4	2	2	8
8	One dose	6	6	4	6	6	2	6	6
8	Speed of Scaleup	6	8	8	8	6	4	6	6
2	Storage	6	4	4	4	4	6	6	6
2	Distribution	6	4	4	4	4	6	6	6
10	Mass Administration	2	8	8	4	4	2	2	4
6	In ovo	0	6	6	6	6	2	0	0
8	DIVA Compatible	6	6	8	8	8	8	8	8
2	Withdrawl	2	6	6	6	6	6	6	6
4	Cost to Implement	2	6	6	4	4	2	4	6

Rank each Criteria 2,4,6,8 or10 on each criterion -- no more than two "10" rankings allowed

Critical Criteria	etics Killed	cs MLV (Me	vectored (	-vectored	us-vectored (A	DNA (Vica	xp. Protein Sc	xp. (Dow
Efficacy	80	100	60	80	80	60	60	60
Safety	36	36	36	48	48	48	36	36
Time to availability	64	16	32	48	32	16	16	64
One dose	48	48	32	48	48	16	48	48
Speed of Scaleup	48	64	64	64	48	32	48	48
Storage	12	8	8	8	8	12	12	12
Distribution	12	8	8	8	8	12	12	12
Mass Administration	20	80	80	40	40	20	20	40
In ovo	0	36	36	36	36	12	0	0
DIVA Compatible	48	48	64	64	64	64	64	64
Withdrawl	4	12	12	12	12	12	12	12
Cost to Implement	8	24	24	16	16	8	16	24
Value	380	480	456	472	440	312	344	420

Major Assumptions:

Vaccine Profile

1. Highly efficacious: prevent transmission; efficacy in all bird species; cross protection across subtypes; one dose; quick onset of immunity; one year duration of immunity
2. Safe to workers and birds
3. DIVA compatible
4. Manufacturing method yields high number of doses
5. Mass vaccination compatible to eliminate individual bird inoculation
6. Rapid speed of production and scale-up
7. Reasonable cost
8. Short withdrawal period for food consumption

Vaccine Administration

1. Commercial poultry firms can logistically vaccinate 1 million birds in 20 days.
2. Federal and state vaccination crews can vaccinate 1 million birds in 4 weeks.
3. If mass vaccine available (water/spray delivered) the entire target population could be vaccinated.

APPENDIX V: COMMERCIAL INFLUENZA VIRUS VACCINES FOR ADULT PIGS

Rank each Intervention (2,4,6,8, or 10) as to its importance to making a decision, only one "10" rankings allowed

Weight	Critical Criteria	Current inactivated vaccines	Autogenous
10	Efficacy	8	6
8	Transmission	7	7
8	Speed of Scaleup	4	6
10	Safety	6	6
6	Number of doses & cost of goods	6	6
8	MDA	6	6
6	Cost to Implement/regulatory issues	5	7
2	Combo Vaccine compatible	10	8
2	DIVA	0	0
2	Withdrawal	8	8

Rank each Criteria 2,4,6,8 or10 on each criterion -- no more than two "10" rankings allowed

Critical Criteria	Current inactivated vaccines	Autogenous
Efficacy	80	60
Transmission	56	56
Speed of Scaleup	32	48
Safety	60	60
Number of doses & cost of goods	36	36
MDA	48	48
Cost to Implement/regulatory issues	30	42
Combo Vaccine compatible	20	16
DIVA	0	0
Withdrawal	16	16
Value	378	382

APPENDIX VI: COMMERCIAL INFLUENZA VIRUS VACCINES FOR YOUNG PIGS

Weight	Critical Criteria	Current inactivated vaccines	Alphavirus vector	Autogenous
10	Efficacy	6	6	4
8	Transmission	4	4	4
8	Speed of Scaleup	4	8	6
10	Safety	8	9	8
6	Number of doses & cost of goods	6	4	6
8	MDA override	0	0	0
6	Cost to Implement/regulatory issues	5	9	7
2	Combo Vaccine compatible	10	0	8
2	DIVA	0	8	0
2	Withdrawal	8	8	8

Rank each Criteria 2,4,6,8 or10 on each criterion -- no more than two "10" rankings allowed

Critical Criteria	Current inactivated vaccines	Alphavirus vector	Autogenous
Efficacy	60	60	40
Transmission	32	32	32
Speed of Scaleup	32	64	48
Safety	80	90	80
Number of doses & cost of goods	36	24	36
MDA override	0	0	0
Cost to Implement/regulatory issues	30	54	42
Combo Vaccine compatible	20	0	16
DIVA	0	16	0
Withdrawal	16	16	16
Value	306	356	310

APPENDIX VII: EXPERIMENTAL INFLUENZA VIRUS VACCINES FOR YOUNG PIGS

Rank each Intervention (2,4,6,8, or 10) as to its importance to making a decision, only one "10" rankings allowed

Weight	Critical Criteria	LAIV-NS1	LAIV-ts	LAIV-HA cleavage	Ad5 Vector	Alphavirus Vector	DNA
10	Efficacy	8	8	8	7	7	4
8	Transmission	8	8	8	7	7	4
8	Speed of Scaleup	6	6	4	4	8	8
10	Safety	5	5	5	7	9	9
6	Number of doses & cost of goods	3	3	3	2	4	1
8	MDA override	8	6	6	4	0	0
6	Cost to Implement/regulatory issues	3	3	3	5	9	5
2	Combo Vaccine compatible	0	0	0	0	0	0
2	DIVA	8	2	2	8	8	8
2	Withdrawal	8	8	8	8	8	8

Rank each Criteria 2,4,6,8 or10 on each criterion -- no more than two "10" rankings allowed

Critical Criteria	LAIV-NS1	LAIV-ts	LAIV-HA cleavage	Ad5 Vector	Alphavirus Vector	DNA
Efficacy	80	80	80	70	70	40
Transmission	64	64	64	56	56	32
Speed of Scaleup	48	48	32	32	64	64
Safety	50	50	50	70	90	90
Number of doses & cost of goods	18	18	18	12	24	6
MDA override	64	48	48	32	0	0
Cost to Implement/regulatory issues	18	18	18	30	54	30
Combo Vaccine compatible	0	0	0	0	0	0
DIVA	16	4	4	16	16	16
Withdrawal	16	16	16	16	16	16
Value	374	346	330	334	390	294

APPENDIX VIII: DIAGNOSTICS FOR ANIMAL INFLUENZA VIRUS DETECTION

Weight	Critical Criteria	Virus isolation (ECE)	Virus isolation (cell culture)	Seq as dxg.	Type A rRT-PCR	H5/H7 rRT-PCR	SIV subtype PCR	lateral flow antigen immunoassays (Type A only)	Ag ELISA (not licensed in US)
10	Sensitivity	**8**	**8**	**6**	**8**	**6**	**6**	**2**	**6**
8	Specificity	**4**	**4**	**10**	**10**	**6**	**10**	**8**	**6**
10	Validation for purpose	**10**	**10**	**8**	**8**	**10**	**8**	**6**	**4**
8	Throughput	**4**	**4**	**4**	**8**	**8**	**8**	**4**	**8**
8	Deployable to NAHLN	**6**	**6**	**4**	**8**	**8**	**8**	**6**	**2**
8	Rapid Result	**4**	**4**	**4**	**8**	**8**	**8**	**10**	**6**
4	Viral characterisation	**6**	**6**	**8**	**6**	**8**	**6**	**4**	**4**
6	Easy to perform	**4**	**4**	**4**	**6**	**6**	**8**	**8**	**6**
2	Cost to Implement	**4**	**6**	**2**	**6**	**6**	**6**	**8**	**6**

Rank each Criteria 2,4,6,8 or10 on each criterion -- no more than two "10" rankings allowed

Critical Criteria	Virus isolation (ECE)	Virus isolation (cell culture)	Seq as dxg.	Type A rRT-PCR	H5/H7 rRT-PCR	SIV subtype PCR	lateral flow antigen immunoassays (Type A only)	Ag ELISA (not licensed in US)
Sensitivity	80	80	60	80	60	60	20	60
Specificity	32	32	80	80	48	80	64	48
Validation for purpose	100	100	80	80	100	80	60	40
Throughput	32	32	32	64	64	64	32	64
Deployable to NAHLN	48	48	32	64	64	64	48	16
Rapid Result	32	32	32	64	64	64	80	48
Viral characterisation	24	24	32	24	32	24	16	16
Easy to perform	24	24	24	36	36	48	48	36
Cost to Implement	8	12	4	12	12	12	16	12
Value	**380**	**384**	**376**	**504**	**480**	**496**	**384**	**340**

commercially available

in use in reference laboratories

APPENDIX IX: DIAGNOSTICS FOR FREEDOM OF H5-H7 AVIAN INFLUENZA VIRUS INFECTION WITH VACCINATION

Weight	Critical Criteria	Virus isolation	Type A rRT-PCR	H5/H7 rRT-PCR	lateral flow antigen immunoassays (Type A only)	Ag ELISA (not licensed in US)
10	Validation to purpose	**6**	**8**	**2**	**0**	**0**
10	Specificity	**6**	**8**	**4**	**8**	**6**
8	Sensitivity	**6**	**8**	**4**	**0**	**0**
8	Throughput	**4**	**10**	**10**	**6**	**8**
6	Pan-species use	**8**	**8**	**8**	**8**	**8**
6	Deployable	**6**	**8**	**8**	**8**	**2**
4	Cost to Implement	**4**	**6**	**6**	**6**	**6**

Rank each Criteria 2,4,6,8 or10 on each criterion -- no more than two "10" rankings allowed

Avian specific DIVA

Critical Criteria	Virus isolation	Type A rRT-PCR	H5/H7 rRT-PCR	lateral flow antigen immunoassays (Type A only)	Ag ELISA (not licensed in US)
Validation to purpose	60	80	20	0	0
Specificity	60	80	40	80	60
Sensitivity	48	64	32	0	0
Throughput	32	80	80	48	64
Pan-species use	48	48	48	48	48
Deployable	36	48	48	48	12
Cost to Implement	16	24	24	24	24
0	0	0	0	0	0
0	0	0	0	0	0
Value	**300**	**424**	**292**	**248**	**208**

commercially available (could be stockpiled)

in use in reference laboratories

APPENDIX X: DIAGNOSTICS FOR FREEDOM OF INFLUENZA VIRUS INFECTION WITHOUT VACCINATION

Weight	Critical Criteria	Ab cELISA (type A)	lateral flow antigen immunoassays	Ag ELISA (not licensed in US)	AGID chicken/ turkey	VI	HI (SIV export)	Type A rRT-PCR
10	Validation to purpose	**8**	**2**	**6**	**8**	**8**	**6**	**8**
10	Specificity	**8**	**8**	**6**	**8**	**4**	**8**	**10**
8	Sensitivity	**8**	**2**	**6**	**6**	**8**	**2**	**8**
8	Throughput	**8**	**6**	**8**	**4**	**4**	**4**	**8**
6	Pan-species use	**6**	**2**	**8**	**4**	**8**	**2**	**8**
6	Deployable	**8**	**6**	**2**	**6**	**4**	**6**	**8**
4	Cost to Implement	**8**	**8**	**6**	**10**	**4**	**4**	**8**
Rank each Criteria 2,4,6,8 or10 on each criterion -- no more than two "10" rankings allowed								

Critical Criteria	Ab cELISA (type A)	lateral flow antigen immunoassays	Ag ELISA (not licensed in US)	AGID chicken/ turkey			Type A rRT-PCR
Validation to purpose	80	20	60	80			80
Specificity	80	80	60	80			100
Sensitivity	64	16	48	48			64
Throughput	64	48	64	32			64
Pan-species use	36	12	48	24			48
Deployable	48	36	12	36			48
Cost to Implement	32	32	24	40			32
0	0	0	0	0			0
0	0	0	0	0			0
Value	**404**	**244**	**316**	**340**			**436**

commercially available (could be stock piled)

in use in reference laboratories

APPENDIX XI: TESTS FOR INFLUENZA VIRUS VACCINE MATCHING

Weight	Critical Criteria	Sequencing	HI assay	VN	*in vivo* testing
10	Validation for purpose	**4**	**4**	**4**	**6**
10	correlation to cross-protection	**4**	**4**	**4**	**6**
8	Repeatability	**10**	**2**	**2**	**4**
6	ability to detect multiple strains				
6	Pan-species use				
2	Cost to Implement	**6**	**4**	**2**	**2**

Critical Criteria	Sequencing	HI assay	VN	in vivo testing
Validation for purpose	40	40	40	60
correlation to cross-protection	40	40	40	60
Repeatability	80	16	16	32
ability to detect multiple strains	0	0	0	0
Pan-species use	0	0	0	0
Cost to Implement	12	8	4	4
Value	**172**	**104**	**100**	**156**

in use in reference laboratories

APPENDIX XII: INFLUENZA VACCINE STOCKPILES

WHO finalized in 2012 its *Guidance on Development and Implementation of a National Deployment and Vaccination Plan for Pandemic Influenza Vaccines* - Guidance available at: http://www.who.int/influenza_vaccines_plan/resources/deployment/en/. It is designed for public and private sector officials at all levels with responsibility for planning and managing deployment and vaccination operations.

Human H5N1 Vaccine Stockpile

An international stockpile of 150 million doses of H5N1 vaccine was established in 2008 through pledges from two manufacturers. In response to the 2009 H1N1 pandemic, these two manufacturers agreed to convert their H5N1 pledges into H1N1 pandemic vaccine. They also increased their pledges to a total of 160 million doses. Of the 78 million doses of pandemic H1N1 vaccine that WHO deployed to low- and middle-income countries, 40 million doses came from the H5N1 vaccine stockpile. This reduced the remaining number of pledged pandemic vaccine doses to 120 million. Manufacturers subsequently agreed to reconvert the 120 million doses of pledged pandemic vaccine back to H5N1 vaccine, or other pandemic influenza vaccine, if needed.

The Strategic Advisory Group of Experts (SAGE) Working Group on Influenza Vaccines and Immunizations was tasked in February 2011 to reconsider options for the nature, deployment and storage of the 120 million doses of pledged pandemic H5N1 vaccine. The Working Group proposed three options and considered that a virtual stockpile with a small physical stockpile of filled doses of H5N1 vaccine for outbreak control may provide flexibility, minimum costs and simplify the logistics of storage. Discussions are ongoing to explore other options and technical aspects of implementing the various options for the final recommendations to SAGE.

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GRAND ISLAND, NY 14072

MERK INC
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MILLSBORO, DE 19966-0318

LOHMANN ANIMAL HEALTH INTERNATIONAL
2285 EAST LANDIS AVENUE
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MEDIMMUNE, VACCINES
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MERIAL, INC
SUBSIDIARIES: MERIAL SELECT, INC.
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ZOETIS, INC.
100 CAMPUS DRIVE
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APPENDIX XIV: PRODUCT INFORMATION

AIV Antibody Test Kits:

http://www.idexx.com/view/xhtml/en_us/livestock-poultry/poultry/avian-influenza.jsf

http://www.synbiotics.com/Products/ProductionAnimals/Avian/AvianInfluenzaDiagnosticKits/AvianInfluenzaVirusPlus.html

SIV Antibody Test Kits:

http://www.idexx.com/view/xhtml/en_us/livestock-poultry/swine/swine-influenza-virus.jsf

AIV Antigen Detection Kits:

http://www.abaxis.com/veterinary/products/avian-influenza-rapid-test.html

http://www.synbiotics.com/Products/ProductionAnimals/Avian/AvianInfluenzaDiagnosticKits/FluDETECT-AI-TypeAAntigenTestKit.html

SIV real-time RT-PCR kits

http://www.lifetechnologies.com/order/catalog/product/4415200?ICID=search-product

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