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HPAI H5N1: A Global Pandemic Concern, with Implications for Pandemic Preperation and Public Health Policy

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HPAI H5N1: A GLOBAL PANDEMIC CONCERN, WITH
IMPLICATIONS FOR PANDEMIC PREPERATION AND PUBLIC
HEALTH POLICY

A thesis submitted in partial fulfillment of the

requirements for the degree of

Master of Science

By

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2013

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I hereby recommend that the thesis prepared under my supervision by Lauren Koontz entitled, HPAI H5N1: A Global Pandemic Concern, with Implications for Pandemic Preparation and Public Health Policy be accepted in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

Koontz, Lauren. M.S., Microbiology and Immunology, Wright State University, 2013. HPAI H5N1: A Global Pandemic Concern, with Implications for Pandemic Preparation and Public Health Policy

Research on highly pathogenic avian influenza A (HPAI) H5N1 has gained much attention in recent years due to its devastating impact within the bird population. While transmission to humans is rare, infections are fatal in more than half of the cases. One of the main concerns among the scientific community is the ability for this virus to mutate in a way much like seasonal influenza that would allow it to be transmitted efficiently among the human population. Two researchers were able to separately create mutant isolates of H5N1 influenza A virus that could be transmitted via the respiratory route between ferrets. The submission of these articles led to multiple public health and biosecurity/biosafety concerns. The purpose of this paper is to explore the current debate regarding restrictions on H5N1 research. H5N1 is a great risk to biosecurity/biosafety, and many believe it should be excluded from future research. However, with the viruses' high mutation rate and the possibility of genetic reassortment with other mammalian influenza viruses the risks to society are already high. Since the virus could become a pandemic within the human population, this paper will also provide an in depth exploration of current public health policy and pandemic preparedness operations underway.

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LIST OF ABBREVIATIONS

HPAI – Highly Pathogenic Avian Influenza

LPAI- Low Pathogenic Avian Influenza

HA – Hemagglutinin

NA- Neuraminidase

MS1- Matrix Protein 1

MS2- Matrix Protein 2

NP- Nucleocapsid Protein

PB1- Polymerase Basic Proteins 1

PB2- Polymerase Basic Proteins 2

PA- Polymerase Acidic Protein

NS1- Nonstructural Protein 1

NS2- Nonstructural Protein 2

SA- Sialic Acid

SP-D- Pulmonary Surfactant Protein

IL- Interleukin

TNF- α - Tumor Neurosis Factor alpha

IFN- γ –Interferon gamma

TRAIL- TNF-related apoptosis-inducing ligand

BSL- Biosafety Level

WHO- World Health Organization

HHS- U.S. Department of Health & Human Services

FDA- Food and Drug Administration

WT- Wildtype

URT- Upper Respiratory Tract

LRT- Lower Respiratory Tract

USDA- U.S. Department of Agriculture

DOI- U.S. Department of Interior

NSABB- U.S. National Science Advisory Board for Biosecurity

DURC- Dual Use Research of Concern Pathogens

APHIS- Animal Plant Inspection Service

UNICEF- United Nations Children’s Fund

NPI- Nonpharmaceutical Interventions

HI- Hemagglutination Inhibition

GSK-Glaxosmithklines H5N1 Vaccine

VRBPAC- Vaccines and Related Biological Products Advisory Committee

TAP- Transporter Associated With Antigen Processing Pathway

MHC- Major Histocompatibility Complex

CTL- Cytotoxic T Lymphocyte

APC- Antigen Presenting Cell

MDCK- Madin-Darby Canine Kidney Epithelia Cell

I. INTRODUCTION

Highly pathogenic avian influenza (HPAI) H5N1 is a bird-adapted strain that has reached enzootic status in many bird populations, especially in Southeast Asia where it is responsible for the death of millions of birds annually. HPAI H5N1 is considered an avian disease, although there is some evidence of transmission from infected birds to humans. The World Health Organization (WHO) data indicate that since 2003, HPAI H5N1 viruses have infected 603 people, with 356 of those infections resulting in mortality (1). Influenza A viruses circulating among poultry have the potential to recombine with human influenza A viruses and become more transmissible among humans. Therefore there is great concern that this virus may acquire the ability to be transmitted via respiratory droplets amongst humans, resulting in a future pandemic. Dr. David Nabarro, Chief Avian Flu Coordinator for the United Nations, and former Chief of Crisis Response for the World Health Organization, has estimated that the avian flu could kill up to 150 million people, comparing the potential impact of the virus to AIDS in Africa (2).

H5N1's high mortality rate and the possibility that it could mutate to become transmissible to humans make it a high priority research topic for public health preparedness and pandemic prevention efforts. Because HPAI H5N1 viruses are always changing, the CDC and other public health agencies look for genetic changes in HPAI H5N1 viruses that may impact how HPAI H5N1 viruses spread from person to person or their susceptibility to influenza antiviral drugs (1). Billions of U.S. dollars are spent to research H5N1 and prepare for a potential avian influenza pandemic. However, important scientific and public health questions regarding H5N1 remain unanswered, particularly the likelihood of such mutations arising and the mechanisms by which they may occur (1). In an attempt to explore this concern, two studies identified several mechanisms by which the virus might evolve to transmit efficiently in the ferret (1). Stemming from these transmission experiments came a need for policy to regulate biosecurity/biosafety hazards to prevent the accidental or potential release of this laboratory virus. Many argue that the risks of this research outweigh the benefits (18). However, with this virus already circulating in nature and constantly mutating, a credible threat already exists.

With the identification of genetic pathways by which H5N1 can better adapt to transmission among humans there is a great concern for public

health. With this sort of a crisis lurking around the corner there is urgency for policy analysis at both public and private levels. Exhaustive efforts were done to control the 2009 H1N1 pandemic, and with and a new pandemic potentially on the rise it will be important to implement both old and new public health policies such as prevention strategies, educational outreach, and vaccination (21) . Because HPAI H5N1 at the moment is not directly affecting the U.S., little is openly discussed as far as preparation for the future. This paper will discuss the HPAI H5N1 virus as a risk factor to human health, while also providing a detailed analysis of what is and can be done to mitigate the impact of a future pandemic within a public health perspective.

II. BACKGROUND

Highly Pathogenic Avian Influenza H5N1 (HPAI H5N1)

Influenza A viruses are enveloped RNA viruses with an eight-segmented, single-stranded, negative-sense genome belonging to the family Orthomyxoviridae. Aquatic birds are the natural hosts of type A influenza viruses (2). Occasionally, these viruses cross from aquatic wild birds to poultry or mammals, and new genotypes of the virus become established within the new host. Of the influenza viruses that cross to terrestrial poultry, one of the most important is the H5 subtype. Highly pathogenic Influenza A H5N1 viruses – also called “HPAI H5N1 virus”, is an influenza virus that occurs mainly within the bird population. The virus spreads rapidly through flocks of poultry, causing disease that affects multiple internal organs with a high fatality occurrence that can often reach 90 to 100% (2). In recent years H5N1 has reached epizootic and panzootic status, killing millions of birds (2). The HPAI H5N1 virus does not usually infect humans, but infections with the virus have been known to occur.

As seen in figure 1, eight gene segments of influenza A virus encode 10 proteins: hemagglutinin (HA), neuraminidase (NA), matrix proteins M2 and M1, nonstructural (NS) proteins NS1 and NS2, the nucleocapsid, and the three polymerases, the PB1 (polymerase basic 1), PB2 (polymerase basic 2), and PA (polymerase acidic) proteins. The segmented genome of influenza viruses allows for genetic reassortment to occur when two influenza viruses infect the same cell (3). This provides influenza viruses with the ability to generate genetic diversity to permit interspecies transmission and to evade host immune responses through a major antigenic change, as will be discussed later (3).

Viral Genes and Proteins

1. Hemagglutinin

Hemagglutinin (HA) is a glycosylated type I integral membrane protein that functions in both receptor binding and cell fusion events. The globular head portion mediates the attachment of the virus to host cells while the stem portion mediates the fusion of the viral membrane to the host cell membrane, allowing the viral genome to infect host cells. The HA protein binds to sialic acid -containing receptors expressed on the surface of host cells (4). After proteolytic activation of the precursor HA molecule into HA1 and HA2 the virus is able to fuse with the host cell, as can be seen in figure 2 (4). Once HA has bound to its receptor, the virus is able to enter the cell via receptor mediated endocytosis. With the virus now internalized, the low pH of the endosome allows a conformational change in the HA protein exposing the fusion peptide that functions to fuse the viral envelope and the cell membrane. After membrane fusion, the ribonucleoprotein particles vRNPs are released into the cytoplasm and traffic towards the nucleus for replication.

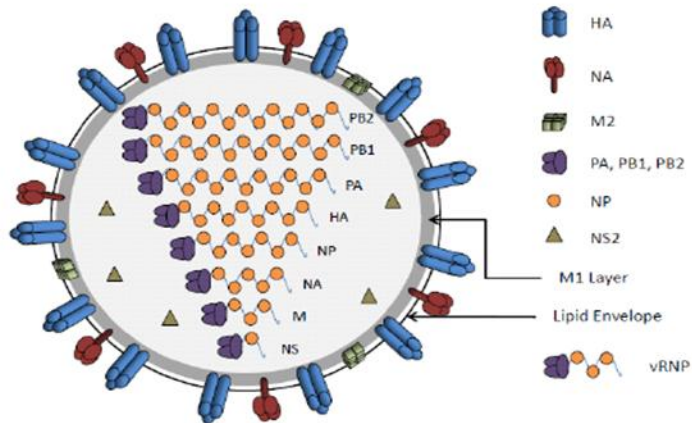


Figure 1. Representation of the Influenza A Viral Genome.

The enveloped RNA virus with its eight-segmented single stranded genome can be seen, along with the 10 viral protein products which are depicted as they are naturally associated within the virus.

Adapted from [5]



Figure 2. Representation of the HA Protein in its Monomeric Form.

The cleavage site, known to be associated with HPAI virulence can be seen in green. HA1 and HA2 can be seen in blue and red. Post cleavage HA1 and HA2 will represent the dimeric form of the protein that will later allow the virus to fuse with its associated receptor. Within HA2, the transmembrane portion to be embedded within the viral envelope can be seen in orange.

Adapted from [5]

2. Neuraminidase

The neuraminidase (NA) protein is a type II integral membrane glycoprotein that functions as a sialidase. It functions in cleaving the HA of progeny virions from the sialic acid receptors, facilitating virus release from infected cells (4). The NA protein

additionally helps viral spread by also removing sialic acids from the cell surface (4). These cleavages prevent self-aggregation that would otherwise limit the infection to one round of replication. NA is inhibited by the antiviral neuraminidase inhibitors zanamivir and oseltamivir which interfere with the release of progeny virus from infected cells (4). Currently, the most prevalent strain of H5N1 has not adapted resistance and is still vulnerable to these drugs (4).

3. Polymerase Gene Complex and Nucleocapsid Protein

The polymerase complex is composed of three viral polymerase proteins: polymerase basic proteins 1 and 2 (PB1 and PB2) and polymerase acidic protein (PA) involved in viral RNA synthesis (4). PB1 functions as an RNA- dependent RNA polymerase, while PB2 is required for mRNA synthesis by binding and cleaving host 5' mRNA methyl caps (5). The PA protein has essential roles in the synthesis of both genomic and messenger RNA. The Nucleoprotein (NP) encloses all of the viral RNA gene segments and primarily serves as the structural protein of vRNPs. The NP is also involved in transcription by stabilizing template polymerase interactions and in intracellular trafficking of vRNPs (5).

4. M1 and M2 Proteins

The M gene encodes two proteins, matrix protein 1 (M1) and matrix protein 2 (M2), which are alternative splice products. M1 interacts with both surface glycoproteins and vRNPs and promotes assembly and budding (5). The M2 protein is a type III integral membrane protein that functions as a H⁺ ion channel, controlling the pH in the Golgi complex during HA synthesis and virion disassembly (4).

5. Nonstructural Proteins

The nonstructural proteins (NS1 and NS2) are also alternative splice products from the same gene segment, both having important roles in viral replication. NS1 has various functions within the viral replication cycle, including viral RNA replication, viral protein synthesis, and general host-cell physiology. It has also been known to inhibit host immune response, by decreasing production of interferon (IFN) and the antiviral effects of IFN-induced proteins, such as dsRNA-dependent protein kinase R (PKR) and 2'5'-oligoadenylate synthetase (OAS)/RNase L (5). The NS2 protein, also referred to as nuclear export protein (NEP), facilitates the nuclear export of vRNP complexes to the assembly site at the apical cell membrane (5).

Human Pathogenesis of HPAI H5N1

As can be seen in Figure 3, a combination of factors play a role in the pathogenesis of H5N1 (4). Similar to other human influenza infections H5N1 viral replication results in cell and organ damage via cytolytic and/or apoptotic mechanisms (4). Virus replication occurs mainly in the respiratory tract, allowing the virus to be isolated from throat and trachea aspirates, postmortem lung tissues, and viral RNA from nasal, nasopharyngeal, and tracheal specimens (4). Animal experiments have also shown human H5N1 isolates to be found in extra-pulmonary organs such as the intestines, brain and placenta.

The so called “cytokine storm” is thought to be one of the largest contributors to the pathogenesis of H5N1. H5N1 patients commonly show elevated serum levels of proinflammatory cytokines and chemokines (4). Immunohistochemistry analysis of the

lung from H5N1 autopsy cases has detected high expression levels of tumor necrosis factor- α (TNF- α), macrophage inflammatory protein -1 α , regulated on activation normal T cell expressed and secreted (RANTES), interferon- γ (IFN- γ), interferon- β (IFN- β), and interleukin-6 (IL-6)(4).

TNF-related apoptosis-inducing ligand (TRAIL) has been shown to be upregulated in macrophages infected with H5N1. This ligand binds to a death receptor present on a target cell, ultimately leading to cell death via apoptosis. Research comparing T lymphocytes apoptosis induction in the presence of macrophages infected with H5N1 or other influenza viruses showed that H5N1 infected macrophages can increase induction of apoptosis (4). These macrophages also displayed a delayed onset of apoptosis themselves, resulting in further T lymphocyte apoptosis and extended cytokine/chemokine synthesis (4). H5N1 induced apoptosis has also been seen in alveolar epithelial cells, leukocytes in the lungs, spleen, and intestinal tissues, therefore resulting in further injury of these organs, possibly through viral replication and/or up regulation of cytokines and chemokines. The HA of the H5N1 virus has also been shown in vitro to suppress the expression of cytotoxic perforin in CD8+ cytotoxic T lymphocytes (4). This impairment of cytotoxic T cells results in decreased lysis of infected target cells enabling the virus to survive intracellularly.

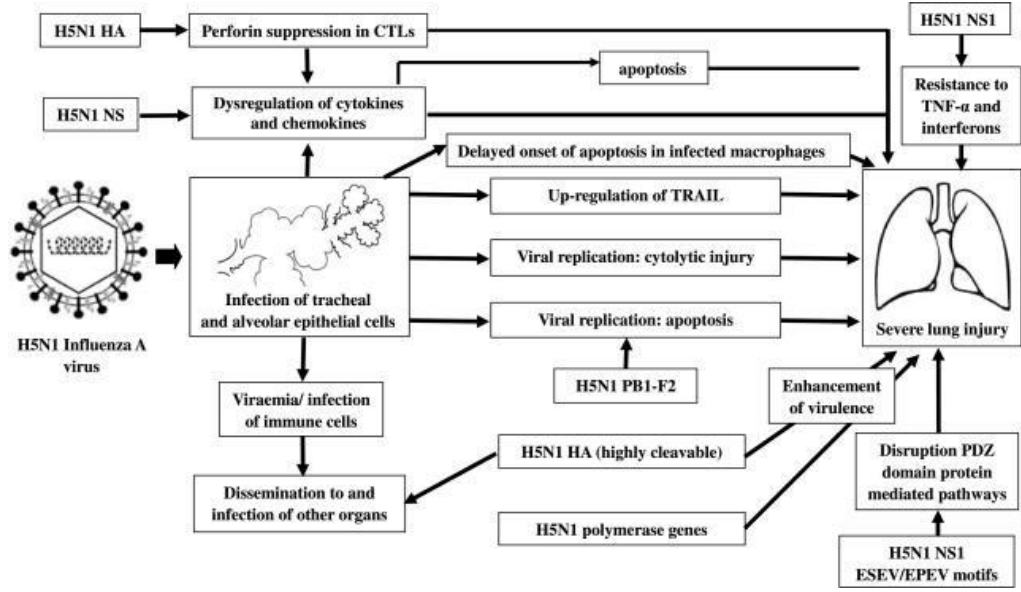


Figure 3. Human Pathogenesis of H5N1

Diagram depicting the key pathogenic mechanisms, viral genes, and gene products that may be involved in H5N1 influenza virus infection.

Adapted from [4]

Virulence of HPAI H5N1

Several viral genes and proteins contribute to the virulence of all influenza viruses. Differences in these proteins contribute to the high pathogenic state of H5N1. The cleavability of the HA protein and the distribution of HA-activating proteases in the host are the major virulence factors of H5N1. Low Pathogenic Avian Influenza (LPAI) viruses have a single arginine residue at the cleavage site that can only be cleaved by proteases in the upper respiratory tract and gastrointestinal tract leading to local

infections (4). HPAI has multiple basic residues at the cleavage site that can be activated by intracellular proteases such as Furin and PC6, and therefore can cause systemic infections (4).

The NS1 protein is also believed to be responsible for the enhanced virulence of the strain. A single amino acid substitution of serine for proline significantly increases the virulence of the virus strain (6). This substitution gives the virus an enhanced ability to antagonize the host IFN response while also preventing the dsRNA-mediated activation of the NF- κ B pathway and the IRF-3 pathway (6). This would allow the virus to block any cell mediated transcription in an effort to promote single stranded viral mediated transcription.

Using microarray analysis and quantitative RT-PCR, researchers were able to show a decreased expression of pulmonary surfactant protein (SP-D) in the lungs from two fatal cases of an H5N1 infection (7). SP-D's main function during an infection is to bind lectins on the surface of the pathogen and opsonize them for phagocytosis. However, research has shown that H5N1 is significantly less glycosylated than seasonal strains of influenza and therefore resistant to the effects of SP-D (7).

III. H5N1 A THREAT TO HUMANS

Risk of Human Transmissibility

In its present form the H5N1 virus can only be transmitted to humans through contact with secretions and excretions from an infected bird, such as saliva, nasal secretions and feces. The World Health Organization (WHO) data indicate that since

2003, HPAI H5N1 viruses have infected 603 people, with 356 of those infections resulting in mortality (1). These human infections cases may be a result of unusually high dose exposure to the virus, stemming from close contact with diseased poultry or rare individual susceptibilities. Alternatively, HPAI H5N1 viruses could be adapting to a human host.

There is great concern that this virus may acquire the ability to be transmitted via respiratory droplets among humans, therefore resulting in a future pandemic. One of the major host range determinants of any strain of influenza virus is the affinity of the viral HA protein for its host cell's sialic acid receptor. Avian influenza viruses bind to alpha-2, 3 sialic acid receptors, while human influenza viruses bind to the alpha-2, 6 isoform. Ferrets are used in influenza research because they are susceptible to infection with human and avian influenza viruses. Their receptor distribution resembles that of humans since they have alpha-2, 6 linked SA receptors present in the upper respiratory tract, with alpha-2, 3 linked SA receptors mainly present in the lower respiratory tract. While avian viruses replicate at temperatures around 41°C, for replication in a human host the virus must adapt to an internal temperature of 33°C (8). For H5N1 the amino acid substitution GLU627-LYS629 (E627K) in the polymerase complex protein (PB2) has been associated with increased viral replication in mammalian cells at lower temperatures (8). Another factor that the virus would need to overcome has to do with the NA protein that facilitates the release of virus particles from a previously infected cell. For H5N1 this process often results in the released viral particles forming aggregates with other viral progeny and therefore inhibiting their ability to infect other host cells (8).

Avian influenza has several mechanisms to overcome the H5N1 host transmission barrier. Due to the low fidelity RNA-dependent RNA polymerase, avian influenza viruses have a high mutation rate from 1×10^{-3} to 8×10^{-3} substitutions per site per year (5). This mechanism of mutation is referred to as antigenic drift (Figure 4 A). Mutations that alter amino acids found in antigenic regions of the HA and NA are likely to confer selective advantages via evasion of pre-existing immunity. Because the majority of the human population has never been exposed to H5N1 and therefore has not acquired prior immunity, this method largely results in seasonal epidemics and is not usually the cause of a pandemic virus emergence. The viruses that caused the major pandemics in the past emerged from reassortment of animal and human influenza viruses, such as the 2009 H1N1 virus. In this process known as antigenic shift an entire gene segment is exchanged (Figure 4 B). If a cell is infected with two different influenza viruses, it is possible that when the new virus particles are assembled at the plasma membrane, they could contain genomes from each parent strain, therefore resulting in a reassortment strain (5).

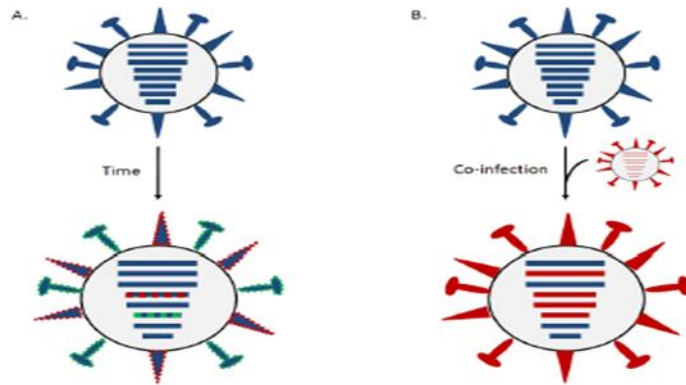


Figure 4. Antigenic Shift vs. Antigenic Drift

In figure A antigenic drift is represented as a function of time. Over time mutational changes occur that allow the virus certain selective advantages. Figure B represent s antigenic shift which occurs via co-infection with two different parent strains of influenza virus, resulting in a reassortment strain that is highly adaptable to new environments.

Adapted from [5]

This process of genetic reassortment is how one group of researchers was able to show respiratory transmission of the virus in ferrets. In order to study transmission ability the authors generated a virus containing the HA from an H5N1 virus and the seven remaining gene segments from a 2009 pandemic H1N1 virus (H5HA/pdm09)(9). Using receptor binding studies and animal experiments, they were able to identify a derivative of the reassortment virus. This virus (H5HA-mutant/pdm09) possessed four mutations in its HA protein (N158D, Q226L, N224K, and T138I) that enabled it to recognize sialic acid linked to galactose by alpha 2, 6-linkages (Figure 5) (9).

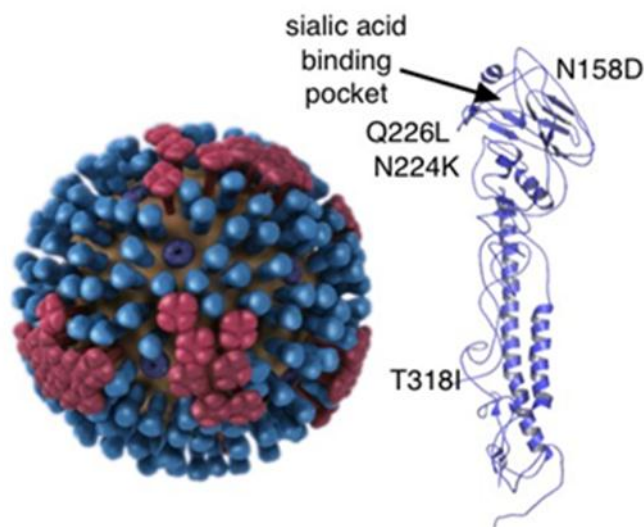


Figure 5. H5HA-mutant/pdm09 HA Mutations

A model of the H5N1 influenza virus and its HA protein possessing four mutations. These four single site insertion mutations (Q226L, N224K, N158D, and T318I), acquired by antigenic drifting, allowed the virus to change its receptor specificity from alpha 2,3-sialic acid receptors to the human alpha 2,6-isoform.

Adapted from [9]

Using an experimental setting that allowed the exchange of the virus between ferrets to occur only via respiratory droplets, the authors were able to show mutant virus (H5HA-mutant/pdm09) transmission. Naïve ferrets were placed in wireframe cages next to ferrets inoculated with 10^6 plaque forming unit of virus. In this manner all transmission stemming from direct or indirect contact was prevented. Similar to previous transmission experiments, a pdm09 virus was efficiently transmitted via respiratory droplets to all three contact ferrets, as evidenced by the detection of virus in nasal washes

in these animals (Figure 6)(9). As expected because of the lack of HA mutations, the H5HA/pdm09 virus was not transmitted (Figure 6). However, in the H5HA-mutant/pdm09-inoculated group, virus was recovered from 4 of the 6 contact ferrets between days 3 and 7 post-contact (Figure 6)(9). Moreover, seroconversion was detected in all six animals (9).

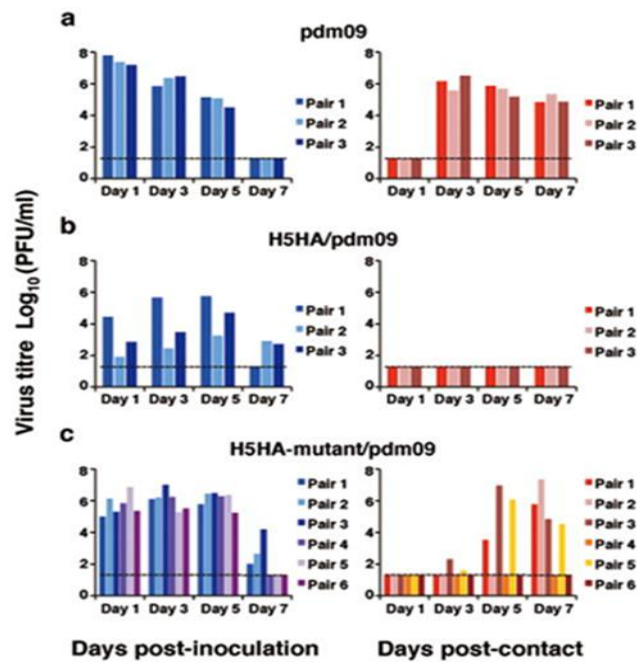


Figure 6. Respiratory Droplet Transmission of H5 Avian-Human Reassortment viruses in ferrets.

Groups of three or six ferrets were inoculated intranasally with 10^6 PFU of pdm09 positive control (a), H5HA/pdm09 negative control (b), or H5HA-mutant/pdm09 (c). After one day post infection, naïve ferrets were placed in

adjacent cages, with nasal washes being collected every other day from inoculated (left panel) and contact (right panel) ferrets for the purpose of virus titration.

Adapted from [9]

To ensure that the H5HA-mutant/pdm09 virus could bind specifically to a human type receptor, sections of human tracheal and lung tissues were exposed to viruses containing a human virus HA, a wildtype H5 HA which should bind exclusively to 2,3 siliac acid avian receptors, and the H5HA-mutant/pdm09 virus (9). As can be seen in figure 7, all viruses bound to the alveolar epithelial surface since this lung tissue contains both human and avian type receptors. However, within the tracheal epithelia where predominantly human type receptors are located, only the human virus HA and the mutant H5HA were bound (Figure 7)(9). This suggesting recognition of human type receptors by the virus plays an essential role in transmission.

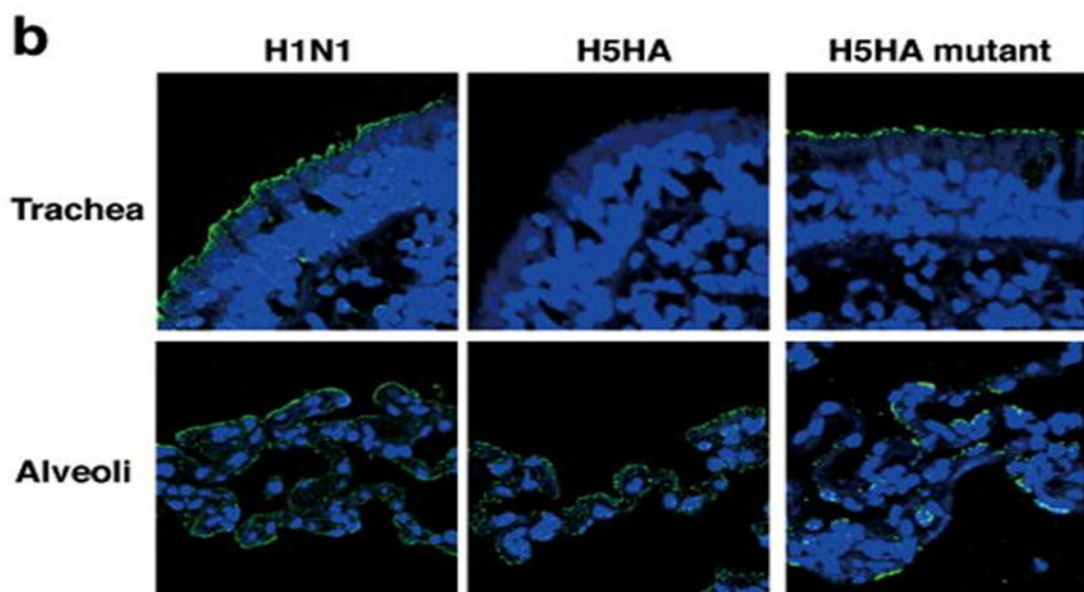


Figure 7. H5HA-mutant/pdm09 Virus Bound to Human Type Receptor

Human H1N1, wild-type H5, and mutant H5HA viruses were incubated with human trachea and alveoli tissue sections and then stained with the appropriate antibody to demonstrate receptor specificity. Nuclei are represented in blue (Hoechst dye) while virus binding can be seen in green (fluorescent-labeled secondary antibodies). Receptor binding can be seen with all viruses within the alveoli tissue which contains both alpha 2, 3 siliac and 2, 6 siliac acid receptors. The mutant H5HA virus was able to successfully bind to alpha 2, 6 siliac acid receptors within the human tracheal tissue, indicating receptor adaptation.

Adapted from [9]

Since the mutant virus contains seven segments from a human H1N1 virus there are several other factors that may have contributed to the mutant viruses' successful transmission. As previously mentioned, one example of this includes amino acids in the PB2 polymerase protein that allow for lower temperature replication. This specific sequence of amino acids allows for efficient replication in mammalian, but not avian cells (9). Most avian influenza virus PB2 proteins lack these amino acids and therefore lack the ability to replicate within mammalian cells. However, the change, of a glutamic acid to lysine mutation at position 627 has been found in H5N1 viruses circulating in the Middle East (10). Another study was able to show that a human virus NA gene was necessary to allow transmissibility to a mutant H5 avian-human reassortmant virus (11). In this study, four amino acid substitutions (S227N, D187G, E190G, and Q196R) were found within the H5N1 virus head domain (11). The mutant virus proved to be transmissible among ferrets through direct contact, however it was not susceptible to

respiratory transmission. Using a reassortant virus with the mutant HA, a human N2 and internal genes from an H5N1 the virus was transmitted via respiratory droplets (11). Therefore the mutant H5 HA recognizing human alpha 2, 6 receptors may not be the sole contributor to mammalian transmissibility of the virus.

The three influenza pandemics in the past 80 years have all evolved from reassortments of preexisting human/mammalian adapted viruses with genes of an avian influenza virus. No purely avian influenza virus has acquired the ability to infect humans through adaptation or mutation, causing a pandemic outbreak (1). However, avian influenza viruses have been able to adapt to other mammals so we cannot rule out their ability to adapt to a human host. It has been proposed that the 1918 H1N1 virus was not a reassortant virus, but an entirely avian-like virus that adapted to humans (35). Ten amino acid changes in the polymerase proteins differentiate the 1918 and subsequent human influenza viruses from avian virus sequences (35). Since we cannot exclude the possibility that a future pandemic could be triggered by a wholly avian virus without the requirement of reassortment, researchers investigated whether HPAI H5N1 could change its transmissibility characteristic without genetic reassortment. In order to do this a combination of targeted mutagenesis followed by serial virus passage in ferrets was done. The virus strain chosen was a complete A/Indonesia/5/2005 virus isolated from a human case of HPAI A/H5N1 infection. The authors had previously located several amino acid substitutions (N128K, Q22L/G224S, or N128K/Q22L/G224S) in the receptor binding site of the HA surface glycoprotein of A/Indonesia/2005 (8). These mutations efficiently changed the virus binding preference from the avian-2, 3-link SA receptors to the human

alpha-2,6-linked SA receptors, providing them with a mutant virus to be used in future transmission based studies.

The authors used multisite-directed mutagenesis to introduce these specific amino acid substitutions into a wildtype A/Indonesia/5/2005 virus, giving rise to the mutant A/H5N1 HA Q222L, G224S strain (8). This strain was proven to yield high virus titers in the ferret upper respiratory track (URT), and was further used in other experiments related to transmissibility. The amino acid substitution E627K in PB2 is an important host range determinant of all influenza viruses. The authors introduced this amino acid substitution into the mutant strain, giving rise to A/H5N1 Q222L, G224S PB2 E627K. When these mutations collectively did not allow transmission when tested amongst ferrets using the cage method previously described, the authors then forced the virus to mutate on it's on through serial passages that would allow the virus to adapt to replication within the mammalian respiratory tract (8). As the passage number increased up to 10 serial passages the authors saw a continuous increase in virus titers in the nasal turbinates of ferrets inoculated with the mutant virus. As can be seen in Figure 8A these titers ranged from 1×10^4 TCID₅₀/gram tissue at the start of the experiment to 3.2×10^5 to 1×10^6 TCID₅₀/gram tissue in the final passages (8). Figure 8 B shows that ferrets inoculated with the mutant virus also showed an increase of viral titers within nose swabs, peaking at 1×10^5 TCID₅₀ (8). Collectively, data indicated that the mutant virus was adapting in order to successfully replicate in the ferret URT after repeated passage.

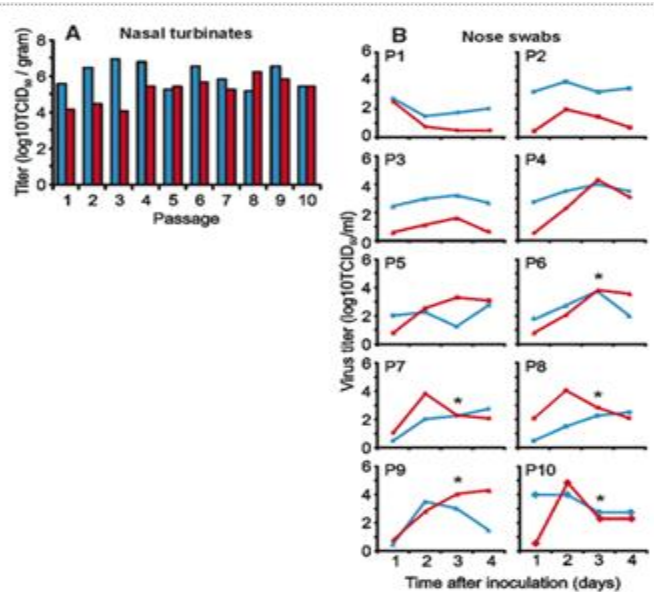


Figure 8. Ferret Nasal Turbinates and Nose Results during Serial Dilution

(A) Virus titers in the nasal turbinates collected at day 4 from ferrets inoculated with A/H5N1 wildtype (blue) and A/H5N1 mutant (red) throughout 10 serial passages. (B) Virus titers in nose swabs collected daily until day 4 from ferrets inoculated with A/H5N1 wildtype (blue) and A/H5N1 mutant (red) throughout 10 serial passages. As can be seen in both images mutant virus titers increased significantly during serial passages, indicating that a pattern of adaptation was being developed.

Adapted from [8]

To test airborne transmission nasal wash samples collected 3 days post infection (DPI) from wild type and mutant infected ferrets at passage 10 were used to inoculate six naïve ferrets intranasally (8). The next day a naïve recipient ferret was placed in a cage adjacent to an inoculated donor ferret through only which air flow was capable, as to

prevent any direct or indirect contact. Even though mutations had acquired within the A/H5N1 wildtype strain, airborne transmission of this virus did not occur (Figure 9A and B)(8). However, virus was detected in recipient ferrets paired with those inoculated with passage 10 mutant viruses, as can be seen in Figure 9 (8). Three out of four naïve recipient ferrets became infected as can be seen in figure 9 C and D with the presence of replicating virus in collected nasal and throat swabs (8). Transmission was further verified when virus obtained from a throat swab from recipient ferret 2 was used to again successfully infect two additional ferrets. This study was able to show that as few as five amino acid substitutions (four in the HA and one in PB2) may be sufficient to confer airborne transmission of HPAI H5N1 virus between mammals.

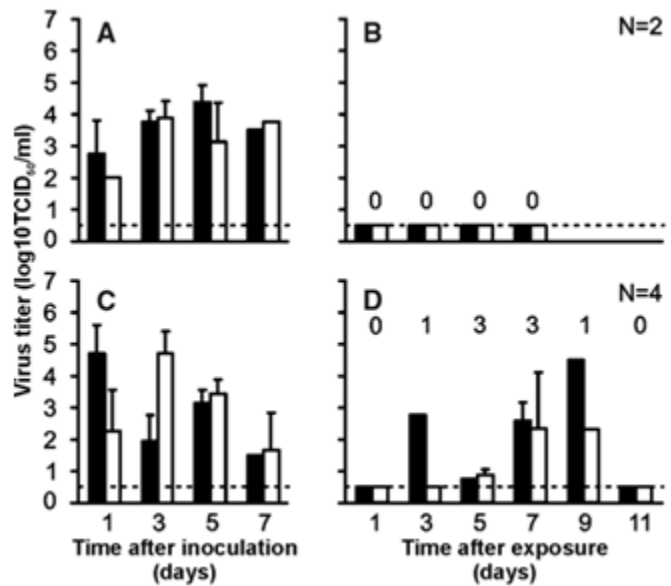


Figure 9. Wildtype and mutant H5N1 Virus Airborne Ferret Transmission Results

Transmission experiments for A/H5N1 wildtype (A) and (B) and A/H5N1 mutant (C) and (D) after 10 serial passages. Groups of ferrets were inoculated intranasally with nasal wash samples collected from P10 virus of both wildtype and mutant types and housed in transmission cages (A and C). A naïve recipient ferret was added to a cage adjacent to the transmission cage and transmission rates were measured via virus titer (B and D). Virus titers in throat (black bars) and nose swabs (white bars)

Adapted from [8]

Current Containment Level/Biosafety Considerations

While research on H5N1 viral transmission and vaccine development is critical, it is important to conduct such research using appropriate biocontainment and biosafety conditions. Currently, studies with mammalian transmissible H5N1 are being done at BSL3 or higher (12). However because of the 60% fatality ratio there is a push for virus to be moved into BSL4 facilities (2). According to Laboratory Biosafety Manual (BMBL) four questions must be considered when choosing a containment level. First, does the pathogen cause disease in healthy humans, animals, and plants (13)? Second, if so, how severe is the disease (13)? Third, how transmissible is the pathogen, and what is its primary route of transmission (13)? Fourth, are preventative vaccines and therapeutic antivirals available, and if so, how widely (13)? The main point of concern here is, does

making the H5N1 virus transmissible among mammals change its biosafety profile. To explore the issue one must begin with answering the questions listed above. H5N1 clearly causes disease in otherwise healthy humans and animals. The disease itself is severe, with a case fatality rate of approximately 60% (1). As far as transmission, H5N1 is spread through a respiratory route, and is therefore categorized by the BMBL as a serious pathogen (13). While H5N1 is not currently transmissible among mammals, we must take the results of the previously discussed ferret studies in consideration, as the virus consistently evolves. Finally, the issue of treatment and prevention is to be considered. To date, a vaccine against H5N1 is not publicly available, ruling out the possibility of immunizing laboratory workers and the general public. The virus has been shown to be sensitive to the antivirals zanamivir and oseltamivir (14). However, if there were an H5N1 pandemic, drug resistance would undoubtedly evolve. Collectively these considerations lead to the push for BSL4 containment of the virus.

While maintaining the virus at BSL4 conditions might seem to decrease biosafety and biosecurity concerns, ultimately it is doing just the opposite. Since BSL4 laboratories are not only very sparse in number but also engaged in research with other dangerous pathogens (12). To minimize the possible risk of accidental virus release into the environment, research should be conducted in a facility equipped with interlocked rooms with negative pressure and high efficiency particulate air (HEPA) filtered air circulation and using appropriate decontamination and sterilization practices for any material exiting the facility (14). Since human infection with influenza occurs via the respiratory route, infection of laboratory workers can be prevented by the use of powered air-purifying respirators (14). Flu scientist Adolfo Garcia-Sastre of the Mount Sinai School

of Medicine argues that BSL3 facilities offer the necessary security measures, such as interlocked rooms with negative pressure, air circulation systems, and adequate decontamination and sterilization practices (15). If H5N1 research would become restricted to BSL4 containment it would restrict research of viral transmission and vaccine development to few facilities while significantly decreasing the future knowledge of this pathogen.

IV. Public Policy: A Public Health Response

Dual use Research Policy

In 2011 the U.S. National Science Advisory Board for Biosecurity (NSABB) came to the conclusion that the benefits of H5N1 research did not outweigh the risks (17). Their concerns were stemmed from the two studies previously mentioned that were able to successfully create mutant strains of the H5N1 virus that could be transmitted via the respiratory route between ferrets. The authors were asked to withhold several key methodologies used in their research (16). In a debate the World Health Organization (WHO) stated that open publication of the studies would only serve to benefit public health. However, the NSABB brought up two significant issues that sealed the decision not to fully publish the articles. The first issue was based on the high human case-fatality rate and concerns of a future pandemic, while the second had to do with the ability of our current influenza vaccines and their ability to react against the mutated virus (17). Nevertheless, in April of 2012 due to a suggestion from the U.S. Department of Health & Human Services (HHS), the NSABB recommended to permit full publication of the articles (17).

As a result of this debate the U.S. government instituted a policy to oversee research concerns that pose both beneficial and harmful concerns to society. The H5N1 virus, along with many other viruses make up a category of dual-use research of concern pathogens (DURC), which this policy regulates. The new policy requires all research agencies to review both proposed projects and those already funded. If a review identifies DURC potential, the funding agency, the institution, and the lead scientist are instructed to develop a "risk mitigation plan" (18). This policy will include efforts to modify how the research is conducted, move it to a more secure laboratory, and communicate it to the public and other scientists responsibly. For viruses such as H5N1 that raise a large risk to the human population, agencies will determine whether to release the content of their publications or withhold information to reduce public health and biosecurity risks (18).

An Industrial Policy Perspective

The U.S. Department of the Interior and U.S. Department of Agriculture have adopted strict policies for monitoring bird health within the United States. The Interagency Strategic Plan for monitoring of wild birds has implemented the majority of their efforts within the state of Alaska. Within this plan local biologists sample live birds, hunter -taken birds, sentinel flocks, and the environment used by these targeted populations for HPAI H5N1(19). Alaska serves as a crossroad for migratory birds that annually return from winter migration in Asia, and come in contact with other North American migratory birds that return to Alaska in the spring from wintering areas in the southern United States and Central America (19.). Migratory birds are also being tested by state and local agencies so that early detection of H5N1 may be forwarded to the agriculture, public health and wildlife communities. The USDA and DOI plan to test

75,000 to 100,000 live and dead birds annually (19). Of these samples approximately 11,000 will be initially screened by USGS at its National Wildlife Health Center in Madison, WI, with the remaining samples being tested at labs certified by USDA in the National Animal Health Laboratory Network (19). If any of these samples test positive for H5N1, they will automatically be sent to the USDA National Veterinary Services Laboratory in Ames, Iowa, for further diagnostic testing (19). U.S. domestic and wild bird populations also undergo enhanced monitoring regimes. The USDA monitors live bird markets, commercial flocks, backyard flock and migratory bird population (19).

The USDA also maintains strict trade restrictions on the importation of poultry and poultry products from countries such as Europe, Asia and Africa where H5N1 has been detected in commercial poultry (19). The USDA's Animal Plant Health Inspection Service (APHIS) and Interior's Fish and Wildlife Service also work with the U.S. Department of Homeland Security's U.S. Customs and Border Protection at major U.S. air and seaports to inspect and regulate the importation of live poultry, commercial birds, pet birds and hatching eggs (19). In order for live birds to be imported into the United States post examination, they are required to be quarantined for thirty days at a USDA quarantine facility and tested for H5N1, before entering the country (19).

Reaching an optimal level of disease control will require a combination of private and public activities. Poultry producers play a key role in implementing control measures to reduce the probability of HPAI infection. These individuals may unknowingly lack information on HPAI and appropriate disease prevention and control strategies. HPAI education is one way of improving awareness of the disease; clinical signs, infection prevention, and the importance of outbreak containment are all essential components of

this education (20). Another factor involved in controlling the spread of disease is surveillance of poultry operations to monitor birds for disease and immediately report any suspected cases of H5N1. So far the DOI and USDA have tested more than 13,000 birds (19). Of these tested, 113 have tested positive for some form of avian influenza. However, all were negative for the highly pathogenic H5N1 virus (19). Surveillance has been made efficient through regular of tests such as HA/HI, FAT ELISA, RT-PCR, real time RT-PCR, in-situ hybridization, and gene sequencing methods, although direct virus isolation from fecal samples are still widely used (19). Because poultry contamination results in great economic loss to farmers, it is important to have proper incentives available for reporting suspected cases of disease to public health agencies. This could include discounted vaccinations and/or reward money for communicating with public health agencies regarding the condition of poultry. Proper incentives are also necessary for encouraging proper disposal of infected carcasses.

H5N1 disease control is also being done at regional and national levels. Along with a proper investigation to identify the source of disease, strategies to track the movement of poultry and poultry products are also important in identifying the origin in the event of an outbreak. Strict biosecurity practices that prevent exposure to animals or other items potentially contaminated with H5N1 are vital for preventing and controlling the spread of this virus. To help backyard and smaller poultry producers, the USDA developed the Biosecurity for the Birds Program (21). This program provides important information about reducing the chances of birds becoming infected with H5N1. Key considerations for greater biosecurity are preventing exposure of poultry to wild birds,

casually introducing new poultry into existing flocks and preventing exposure to infectious agents transported by people and/or equipment (20).

Vaccination of poultry is a highly effective way to reduce the probability of infection. In the event of an H5N1 outbreak in the United States, USDA maintains a bank of 40 million bird vaccines. If an outbreak were occur, the vaccines would protect healthy birds outside the targeted quarantine areas, preventing spread of the virus. Fortunately the vaccine has been shown to reduce avian influenza viral transmission. According to the FAO Avian Influenza Disease Emergency Situation Update, H5N1 pathogenicity is gradually rising in endemic areas; however, the disease is being contained in farmed birds through vaccination efforts (2). However poultry vaccination does have several limitations. Along with implications for cost, there are concerns that vaccination may suppress the symptoms of the virus in vaccinated birds that become infected. This would allow the virus to continue spreading into an endemic, which could increase the probability of a naturally occurring mutation enabling human transmissibility. Therefore clinical and laboratory surveillance are necessary.

The Public Health Response to an Emerging Pandemic

Due to the high lethality and virulence of HPAI H5N1, its endemic presence, its increasingly large host reservoir, and its ongoing mutational adaptations, the H5N1 virus has become the world's largest pandemic threat. Planning for the control of H5N1 in poultry and minimizing the potential of a pandemic influenza in humans is essential. The Global Influenza Program within the World Health Organization (WHO) plays a central role in monitoring global public health, via ongoing influenza monitoring, virus

sampling, and regular flu shots (21). The global health response to H5N1 would ideally consist of two systems, a technological system including drugs and vaccines and health systems which are responsible for delivery and infrastructure (21). Within these two broad systems three public health responses are consistently seen in response to a pandemic threat such as H5N1. First is the technological response, which focuses on drug and vaccines and their delivery. The WHO has stock piled donated antiviral drugs such as oseltamivir for rapid containment purposes, while the U.S. has about two million treatment courses of anti-virals in federal and state stockpiles. Vaccination policy is an especially controversial topic. While the regular seasonal influenza vaccine is highly recommended by the WHO, not everyone chooses to receive the vaccine. Approximately 16 manufacturers globally are currently in the advanced stages of producing H5N1 vaccines through both egg and cell based manufacturing techniques (21). The WHO has committed to stockpiling 50 million doses for the event of a global pandemic. However with the influenza viruses being variable and prone to antigenic shift and drift it is extremely difficult to elicit an efficient strategic vaccination response. It is believed by many that the only feasible vaccine solution would involve a global commitment to an infrastructure for universal provision of the influenza vaccine (21). Unfortunately, aside from the fact that this strategy would require an immense amount of public money, the likelihood that seven billion vaccines could be produced, stored and distributed is very low (21).

Clearly at the present time a solely technological response is not enough. Therefore the most efficacious response may be through an educational approach to change behavior. In the past the United Nations Children's Fund (UNICEF) has been

responsible for a variety of communication messages for public education all over the world. Their strategy has been largely focused towards preventing animal to human transmission. The program voiced standard health issues such as washing hands, cooking meat specifically poultry properly, and safe handling of chickens in both work and domestic environments (21). Currently UNICEF is involved in pandemic mitigation, which involves proper routines for health etiquettes, quarantine and social distancing (21). Two studies, independently done in 2007 showed just how effective rapid social containment can be. The first study found that in the 1918 pandemic social containment measures cut weakly death rates by up to fifty percent (21). In this study, nonpharmaceutical interventions (NPIs; i.e., voluntary quarantine, school closure, reduced public activities) were proven to decrease virus transmission (30). Seventeen U.S. cities, in which multiple interventions were implemented at an early phase of the epidemic (6 weeks), showed 50% lower death rates than those cities who engaged in limited social containment (30). The second used mathematical models to reproduce the pattern of the 1918 pandemic and found that areas that had relaxed their containment restrictions after the peak of the pandemic often saw a second re-emergence of infection (21). During this pandemic the U.S. engaged in various NPIs, while cities in Europe either did not or ended efforts early (31). In the 23 cities, within the U.S. and Europe for which data was obtained on timing of interventions, a correlation was found between excess mortality and early removal of NPI strategies (31). Therefore the timing of public health interventions has great influence on the pattern of the 1918 pandemic.

It is essential that these types of behaviors are implemented. In reality a pandemic will most likely hit months before a pharmaceutical response can be initiated

and these behaviors will have to serve as the front line in pandemic defense. The US Centers for Disease Control and Prevention, together with the Departments of Commerce, Transport and Health and Human Services published Interim Pre-Pandemic Planning Guidance: Community Strategy for Pandemic Influenza Mitigation in the United States-Early, Targeted, Layered, use of Non-Pharmaceutical Interventions (21). This document involves plans for closing schools, cancelling public gatherings, organizing work leave and various telecommunication working strategies.

The third public health response is based on the systems within which the measures are to be delivered. It is thought by the medical professionals within the WHO that a good health system should be structured, ordered, well resourced, state funded, and run by doctors (21). However, in the current health setting where hybrid public-private-traditional systems exist in highly unregulated and poorly resourced setting, and where services can be purchased in pharmacies and on the internet, the likelihood of this sort of health system evolving is unlikely (21). In order for a system like this to be effective, information, prediction, and early warning methods are essential. The WHO has generated a series of guidelines to be followed in the event that a human H5N1 infection should occur here in the United States. These guidelines consist of confirming the diagnosis of recent infection with influenza A (H5N1), reducing morbidity and mortality through isolation and treatment, reducing further spread through identification of potential human, animal, and/or environmental sources of exposure, determining key epidemiological, clinical, and virological characteristics for cases, and lastly exchange of information among clinicians, investigators of public and animal health, and government officials (21).

V. Pandemic Preparations

Challenges for H5N1 Vaccine Development

Influenza vaccines are possibly the most effective way of mitigating the impact of an influenza pandemic by potentially reducing the risk of infection by avian viruses in humans during the current interpandemic period. Unfortunately, the H5N1 virus presents several challenges that must be overcome in order to make an efficient vaccine. Due to the high virulence of H5N1 strains, the vaccine manufacturing process must take place under higher biosafety conditions than those used during seasonal influenza vaccine development. Currently licensed influenza vaccines are derived by growing viral stocks in embryonated chicken eggs. However, a new avian influenza pandemic will result in a lack of embryonated eggs due to virulence in domesticated poultry. Cell culture based systems could be used to overcome this challenge if a pandemic did arise. Vero cells have been widely used for human vaccine production over the past 30 years and are the only cell line fully accepted by regulatory authorities for production of whole virus vaccine (10). However, are Vero cells susceptible to an H5N1 viral infection? Fluorescence-activated cell sorting analysis has shown that even though Vero cells possess predominately alpha 2, 3 sialic acid receptors, they are fully susceptible to infection with influenza A viruses, indicating that linkages other than alpha 2, 6 and 2, 3 might be involved in the attachment of influenza virus to host cells (34). The madin-darby canine kidney cells (MDCK) have been previously shown to sustain viral growth due to their abundance of alpha 2, 6 sialic acid receptors, and therefore served positive control for this study (34). All influenza A virus specific proteins were synthesized in the same proportions in Vero cells as in MDCK cells (34). Electron microscopic and

immunofluorescence studies showed that infected Vero cells undergo the same morphological changes as other polarized epithelia cells (34). As for the issue of biosafety, cell culture vaccine development can be done at BSL-3 standards.

Much is still unknown about antigenic sites in H5N1 surface proteins and immune correlates of protection are inconclusive (5). Typically hemagglutination inhibition (HI) assays are used to determine influenza immunity post vaccination. In this assay, serum antibodies to the influenza virus will interfere with the virus's ability to attach to red blood cells, therefore inhibiting HA, as seen through inhibition of erythrocyte agglutination (5). Agglutinating chicken or turkey erythrocytes are commonly used; however, because H5N1 viruses have different receptor specificity than the seasonal influenza virus, these cells are not useful to the H5N1 HI assay (5). Horse erythrocytes are agglutinated by H5N1 viruses and are therefore a useful substitute (5). The neutralization assay is another method used for evaluating serum antibody protection. In this assay, serum antibody is evaluated for its ability to inhibit the virus from infecting tissue culture cells (5). Both of these assays require live virus and since, H5N1 viruses pose a strong biosafety risk, high containment facilities must be used. To counteract the need for high containment facilities while performing these assays, noninfectious virus particles expressing H5N1 surface molecules and reporter gene are currently being developed (5).

In addition to challenges with vaccine development, H5N1 antigenic diversity represents further challenges. H5N1 viruses are divided into ten clades based on phylogenic relationships within the HA genes (5). Human infections have resulted from clades 0, 1, 2, and 7, with clade 2 causing more infections than any other clades (5).

Antigenic heterogeneity exists between clades and has even begun to show within clades; clade 2 contains subclade and sub-subclade divisions (5). Although the sequence identity between clades is high, there is little cross reactivity in receptor blocking antibodies across the various clades and subclades (5). A recent study was able to show that twenty residues critical for antigenicity resulted from antigenic profiling of the H5N1 virus. Identity patterns across these twenty amino acids were approximately 30%, indicating high levels of clade diversity (5). Since it is impossible to predict exactly which clade could cause a pandemic, it is important to develop a vaccine that will elicit immunity to all or multiple clades.

The HA of H5N1 is poorly immunogenic and requires higher doses of antigen or the use of adjuvants to elicit an effective antibody response (5). In Fact, a vaccine most recently approved by The Food and Drug Administration's Vaccines and Related Biological Products Advisory Committee (VRBPAC) is backed up by the use of an adjuvant as will be discussed in the following section.

Current Stockpile Vaccines

On April 17, 2007, FDA licensed the first vaccine in the United States for the prevention of H5N1 influenza. This vaccine manufactured by Sanofi Pasteur Inc is derived from the A/Vietnam/1203/2004 influenza virus. As an inactivated monovalent influenza virus vaccine, it is for use in people 18 through 64 years of age who are at increased risk of exposure to the H5N1 influenza virus subtype contained in the vaccine. One multi-center, randomized, double-blinded, placebo-controlled, dose-ranging study in healthy adults, 18 to 64 years of age, investigated the safety and immunogenicity of the

vaccine (22). A total of 103 healthy adults received a 90 microgram dose of the vaccine by injection, followed by another 90 microgram dose, 28 days later (22). The 90 microgram two-dose regimen produced levels of antibodies expected to reduce the risk of getting H5N1 influenza in 45% of those who received it (22). This was concluded based on a 4 fold increase of serum antibodies in both HAI and neutralization tests (22).

In an aim to strengthen the U.S. stockpile for the threat of an H5N1 pandemic, the Food and Drug Administration's (FDA's) Vaccines and Related Biological Products Advisory Committee (VRBPAC) voted that the immunogenicity and safety data on Glaxosmithklines's (GSK's) H5N1 vaccine were sufficient to support its licensure for use in adults (23). In order to generate a vaccine with antigen sparing potential, the squalene adjuvant AS03 was used. The vaccine contains 3.75 micrograms (mcg) of antigen, as compared to 90 mcg of antigen that are seen in the previous H5N1 vaccine (24). The fact that this vaccine was approved even with its need to be coupled with an adjuvant is monumental. No seasonal flu vaccines used within the United States contain adjuvants, nor does the existing stockpiled H5N1 vaccine. In fact, ASO3 used in European GSK vaccines during the 2009 H1N1 pandemic was found to be linked with an increased risk of narcolepsy in children of Sweden, Finland, and Ireland (23). Research regarding the safety of adjuvants in vaccines remains inconclusive. However, it seems as though the risks are statistically minimal. A study done in Quebec city during the H1N1 pandemic showed that vaccination with an inactivated monovalent AS03 adjuvanted vaccine was associated with two cases of Guillain- Barre syndrome for every million doses administered (32). During the 6-month study period, there were 83 confirmed cases of Guillain-Barre syndrome (32). Of those, 25 were related to the receipt of the influenza

vaccine 8 weeks prior to onset. After adjusting for age and sex, the risk of developing the disease post vaccination was 1.80% (32).

Two key trials demonstrated the effectiveness of the GSK vaccine. The first trial compared use of the GSK vaccine with or without adjuvant amongst 680 adult volunteers (24). The 3.75mcg dose of antigen was sufficient to generate an adequate immune response when two doses were used (24). The second trial involving 4,561 adults compared GSK with a saline placebo vaccine. In order for the vaccine to be considered successful it had to generate an antibody titer of 1:40 (24). Results of the study were a success, with 70% of younger adults (18-50) and 60% of older adults (51-65) meeting the required FDA standard (24). The study also showed high seroconversion rates of 90.8% and 74.0% after two doses in both groups of adults. The GSK vaccine is more immunogenic than the H5N1 vaccine already in the nations stockpile, whose seroconversion rate is 45% compared to the 90% seen for the GSK vaccine.

Options to Improve Pandemic Preparation: What Went Wrong During the 2009 H1N1 Pandemic?

While the 2009 H1N1 pandemic showed that pandemic vaccines could be successfully developed and deployed, vaccines became available in significant quantities only after the peak of the infection, and only in high income countries. Figure 10 shows the extended delay from the onset of the first human case in March of 2009 and the distribution of mass vaccine months later (25).

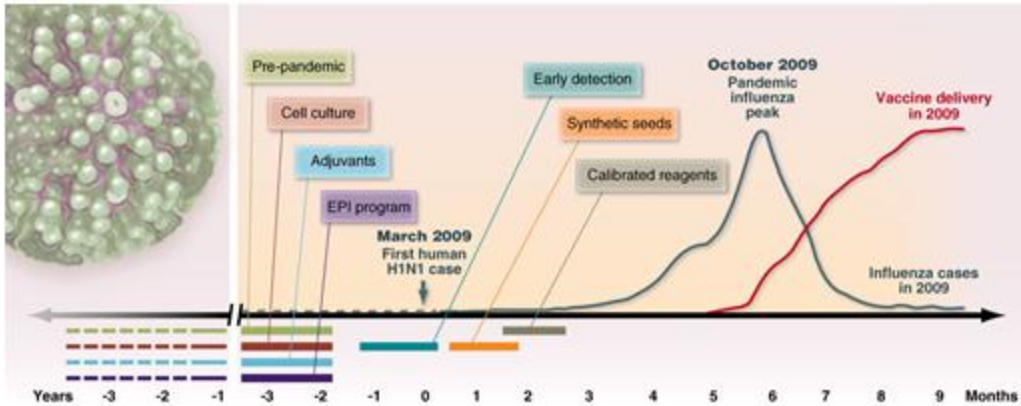


Figure 10. H1N1 Response Timeline

Summary and timeline of the activities during the 2009 H1N1 influenza pandemic from day 0 when the first case of H1N1 was reported through 8 and 9 months later when large quantities of the pandemic vaccine became available.

Adapted from [29]

With H5N1 potentially resulting in a pandemic there are several factors that can be improved upon in order to lessen the negative impact. The first option, prepandemic vaccination would undoubtedly improve vaccine efficiency over developing, manufacturing, and distributing a vaccine as the pandemic rapidly spreads. While it is highly unlikely that the vaccine would represent a perfect match there is hope that the vaccine would elicit a cross reactive immune response. Policy makers and public health officials hope that it would offer at least some protection to people who are essential to maintaining security, health care providers, those who provide essential products and services, infants, young children and pregnant women (25). During the 2009 H1N1 pandemic, most individuals' immune systems had been previously exposed via infection and/or vaccination with distantly related H1N1 antigens and did not develop severe

infections (25). Unfortunately, the mass- majority of the human population has never been exposed to an H5 HA, and therefore are more susceptible to the severe disease caused by H5N1. Given that licensed H5N1 vaccines are available in the nation's stockpile, we have the option to vaccinate individuals at greater risk to infection and also to expand vaccinations to populations around the globe. It has been suggested that this theory backed by investment and public policy could result in a global population being vaccinated with a pre-pandemic vaccine within 3 to 5 years, which could dramatically reduce the risk of an H5N1 pandemic (25).

The second option is generation of a higher quantity of vaccines. There are approximately 7 billion people in the world, and the H5N1 vaccine currently requires 2 doses, meaning that 14 billion vaccines would have to be made available in order for global vaccination to occur. This would undoubtedly be a daunting task, but, as mentioned before, possible. While not every individual is willing or able to receive vaccines, the majority of the population could become vaccinated in order to enable herd immunity. Since H5N1 could mutate to become highly transmissible amongst a population with little preexisting immunity, a large number of people could become infected. Modeling has indicated that the proportion of the population that must be immunized in order to generate herd immunity is roughly 80% (25). Cell culture vaccine production allows for faster production of vaccines and is ideal for avian outbreaks where egg production is vulnerable. The use of an adjuvant would also reduce the amount of antigen needed and increase the production of bulk vaccine.

The third option to improve pandemic preparation, deals with acceleration of vaccine manufacturing during the actual pandemic. Global estimates suggest that, given

current manufacturing capacity, only 500 million doses of vaccine would be available 12 months after a pandemic outbreak. In 2009 it took three months for the manufacturing of an H1N1 vaccine to begin (25). Vaccine manufactures rely on the WHO to identify and distribute live reference viruses to create vaccines, which adds weeks to production time. Recent studies have shown that this three month time lapse could be reduced to a few weeks by early detection and the use of synthetic seed viruses for vaccine production (25). This field of study is referred to as synthetic genomics. In this approach genomes are designed using the computer and constructed in the laboratory using chemical techniques (25). When the genome of a potential influenza vaccine seed virus is synthesized and placed into a cell, the essential starting material for an influenza vaccine can be produced (25). The ability to synthesize vaccine seed viruses would allow them to begin developing pandemic vaccines without waiting for a vaccine virus to arrive from the World Health Association Laboratory (25).

Long Term Pandemic Preparation

As for long term pandemic preparation, universal vaccine development is the Holy Grail approach. By targeting part of the virus that rarely mutates, such as the stem region of the HA protein or the NP protein, researchers believe that they can develop a vaccine that will provide immunity to various strains of influenza viruses. In contrast to the HA head, which is highly mutated, mutations in the HA stem are not as frequent due to the essential functions that occur within this region. After the virus is endocytosed, the low pH in the endosome induces a complex conformational change in the HA stem (26). This conformational change exposes a peptide that mediates fusion of the viral and endosomal membranes, enabling the viral genome to gain access to the cytoplasm (26).

Therefore, antibodies targeted against the HA stem can block this conformational change and membrane fusion, enabling the virus access to the cells replication machinery. A vaccine like this would allow people to be vaccinated as children and then simply receive boosters. Research led by National Institute of Allergy and Infectious Diseases (NAID) scientist Gary J. Nabel used a two-step immunization approach to elicit infection fighting antibodies capable of attacking a diverse array of influenza strains. This two-step immunization approach is capable of inducing not only humoral immunity, but also cell mediated immunity. The subject is first primed with a DNA vaccine which elicits a cell mediated immune response, and then boosted with an inactivated subunit vaccine which not only boosts the cell mediated memory response, but also promotes an active humoral response. Since the DNA vaccine often encodes a conserved influenza protein, a broad cell mediated response is initiated towards a variety of virus strains. The boost with an inactivated subunit vaccine creates a specific immune response that will provide immunity towards the particular strain of virus used in the vaccine. This is very different from current flu vaccines which do not generate such broadly neutralizing antibodies, requiring seasonal influenza vaccines to be reformulated annually to match the existing virus strains.

DNA vaccines seem to be the key to future vaccine development. While under control by a specific promoter a plasmid vector expressing a viral protein, in this case HA, is injected into the host (33). After uptake of the plasmid, the protein is produced endogenously and enters the transporter associated with antigen processing (TAP) pathway (33). Through this pathway the proteins enter the lumen of the endoplasmic reticulum (ER) via the TAP membrane associated transporters (33). In the ER, peptides

bind to MHC class I molecules and then are shuttled to the cell surface (33). The MHC class I subclass is recognized by CD8⁺ cytotoxic T cells (CTL) and stimulates cell mediated immunity (33). DNA vaccines also work in the same way as traditional vaccines with respect to humoral immunity. In this case the foreign protein is presented by the MHC class II pathway via antigen presenting cells (APCs) which elicit CD4⁺ helper T cell responses. CD4⁺ T cells are able to recognize the peptides formed from exogenous proteins that were endocytosed/phagocytosed by APC, then degraded into peptide fragments and presented as MCH class II molecules (33). B cells can then be stimulated by CD4⁺ T cells, resulting in antibody production (33).

In a set of experiments done in 2010, Dr. Nabel and his team primed monkeys, mice, and ferrets immune systems with a vaccine made from DNA encoding the influenza virus HA surface protein (27). In the second “boosting” step the animals received a booster dose of the 2006-2007 seasonal influenza vaccine or a vaccine made from a weakened adenovirus containing the HA influenza protein (27). The monkeys were only boosted with the seasonal influenza vaccine. Though the DNA in the priming vaccine was derived from a 1999 circulating influenza strain, all animals made antibodies capable of neutralizing virus strains from several other years (27). Mice and ferrets produced antibodies not only against strains previous to 1999, including a strain that emerged in 1934, but also against strains that emerged in 2006 and 2007. (27). More interestingly, even though the prime-boost vaccines were both made from H1 subtypes of the influenza A virus, the antibodies they generated neutralized other influenza subtypes, including H5N1 (27). This observation indicated that a prime-boost vaccine strategy could potentially confer immunity to many or all subtypes of influenza A viruses. In an

attempt to see if the prime-boost vaccine could protect from potentially lethal amounts of virus the researchers infected mice with high levels of a 1934 flu virus. They saw that 80% of mice that had received the prime-boost vaccine survived, while mice receiving DNA only, seasonal flu vaccine or a mock prime-boost vaccine all died (27). The same results were also seen in ferrets, which are more comparable to the expected results for humans, due to their sialic acid receptor localization.

The nucleoprotein (NP) of the Influenza A virus is another highly conserved protein capable of inducing cross-protective immunity against different influenza A viruses. For this reason it is a feasible option for universal vaccine production. The amino acid sequence similarity of NP is above 90% within influenza A viruses (28) The NP is a conserved internal protein and a good target for eliciting cell mediated immunity. Immunologically, NP is the major antigen recognized by cytotoxic T cells (CTL) during a viral infection (28). At least 14 human NP peptides have been identified as epitopes of CTLs (29). Epitopes in random coil regions are susceptible to mutations and may be associated with escape from CTL mediated immunity. Regions that are structurally and/or functionally important are useful to vaccine development due to their limited mutations. Two epitopes NP 265-274 and NP 174-184 within the RNA binding groove have been shown to generate a robust CD4+ T cell response (29). NP-specific CTLs can promote lysis of infected cells by recognizing the NP peptide-MHC complex presented by the virus-infected cells. Therefore they are a valuable contributor to the clearance of virus from an infected host and an essential component to universal vaccine development. In a recent study, the NP protein was used as a target antigen and a DNA prime-intranasal protein boost strategy was used to immunize mice (28). The conserved NP protein

genome was used to construct the DNA vaccine which was injected for the purpose of inducing cell mediated immunity. Twenty eight day later a rNP vaccine was intranasally administered to promote a natural humoral immune response. They were able to show that the NP DNA prime-intranasal protein boost was able to induce systemic and local mucosal immune responses, which could effectively provide a cross-protection against homologous and heterosubtypic influenza virus, due to the conserved NP DNA priming vaccine and the strain specific rNP boost vaccine.

This prime-boost immunization strategy effectively enhanced the CD8+ T cellular immune response induced by DNA preimmunization. Three weeks after the last immunization, splenocytes were isolated from mice and stimulated with MHC-I epitope peptide from NP in vitro, with the amount of cells secreting IFN- γ measured post stimulation via ELISpot. (28). As shown in Figure 11, significant amounts of IFN- γ secreting CD8+ T cells were induced by NP DNA vaccination alone, either once or twice (28). However, single i.n. administration of rNP alone (Group P1) induced fewer IFN- γ secreting CD8+ T cells. An intranasal boost with rNP after once or twice DNA vaccinations could effectively increase the number of IFN- γ secreting CD8+ T cells (28).

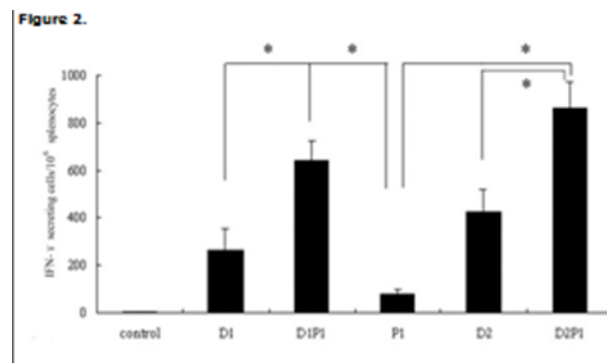


Figure 11. IFN- γ Secreting Splenic CD8+ T cells in Vaccinated Mice

Three weeks post immunization the number of IFN- γ secreting CD $^{+}$ T cells in the spleen from different groups of mice was evaluated by ELISpot. D1 and D2 represent NP DNA vaccination alone, either once or twice. P1 represents a single i.n. administration of rNP alone. D1P1 and D2P1 represent an intranasal boost with rNP after once or twice DNA vaccinations.

Adapted from [28]

To evaluate the efficacy of the NP DNA prime-intranasal protein boost strategy against a lethal dose challenge of heterologous virus, 57 mice were randomized into 3 groups with 19 mice in each group. Two groups received one or two doses of NP DNA vaccine followed by an intranasal boost with rNP at an interval of 2 weeks and the rest were unimmunized for control. All mice were i.n. challenged at week 3 after the last immunization with $5 \times LD_{50}$ of A/Chicken/JiangSu/07/2002 (H9N2). As can be seen in figure 11 A-B, mice receiving the vaccine displayed significantly less weight loss and an increased survival rate over those mice who received a control (28).

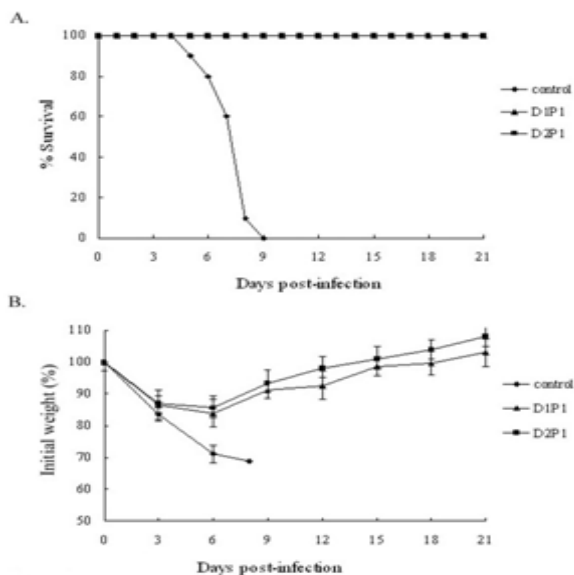


Figure 12. NP-DNA Vaccine Protection Against Heterologous Virus

Three weeks post vaccination mice were challenged with a lethal dose (5xLD50) of influenza A/Chicken/JiangSu/07/2002(H9N2). Survival rate (A) and weight loss (B) were then monitored for 21 days.

Adapted from [28]

While all of these vaccines need additional development before commercialization is possible, it is exciting to think that one vaccine could protect us from such a wide variety of influenza strains. It has been estimated that by 2015 the universal vaccine approach might be in full affect and diminishing the rate of 250,000 to 500,000 lives taken annually by this disease.

VI. Conclusion

Collectively, HPAI H5N1 is cause for great concern. With its ability to mutate rapidly and spread through various species, the amount of damage that this virus could inflict is of disastrous capacity. However the risks that this virus possesses can be minimized through combined efforts pertaining to public policy and pandemic preparedness. While research with this virus is a risk, the knowledge obtained from doing such research is essential in preparing for a pandemic outbreak. In order to lessen the possible impact of an H5N1 influenza pandemic, we need more research and further development of improved vaccines and antivirals with cross reactivity against multiple strains. With proper BSL3 biosafety restrictions and surveillance provided through the Dual Use Research Policy, H5N1 research can be done in a safe manner that can benefit society.

As far as public health policy, these efforts must be done simultaneously with those governing poultry policy at both public and private sectors. It is imperative that viral infection be contained and regulated amongst livestock poultry. By not doing so we are simply inviting the virus into a human host and giving it permission to adapt to its surroundings. This is why policies regarding poultry quarantine, euthanize procedures, private farm industries and wild bird migration are so important. By limiting the infection to poultry we are buying ourselves valuable time to research and publicly prepare for a future pandemic. Obviously it is not certain that policy regulations will prevent a pandemic attack, and with an inadequate H1N1 response in the past, we must be prepared for the worst. Pandemic preparedness would require the United States to work with other nations to detect human cases of H5N1 and contain any outbreaks. Supporting the manufacturing and testing of influenza vaccines, including finding more reliable and quicker ways to make large quantities of vaccines, while developing a national stockpile of antiviral drugs to help treat and control the spread of disease if an outbreak should occur is essential. Also important is working with federal agencies to prepare and to encourage communities, businesses, and organizations to plan for pandemic influenza. Education and H5N1 awareness are essential to this last point. It is not intended to use fear to gain an individual's understanding of the virus; however people need to know that it is a cause for concern. By simply informing the public of the basics of the virus, such as mode of transmission, symptoms, and the importance of seeking medical attention, public awareness would be achieved. It is also important to educate the population on what procedures should be carried out in the instance that a pandemic should arise. This should consist of avoiding public places through acquiring

an abundant food and water storage, also having nonprescription drugs and other first aid supplies on hand, educating ones children on proper illness etiquette such as washing your hands, covering your mouth, and being involved in a community emergency plan.

Future research should focus on improved vaccines that can confer broad protection against multiple viral strains and various influenza subtypes. While extensive research has been done with the HA protein, it seems only a few studies have looked at the NP as a potential vaccine candidate. It would be beneficial to further investigate the NP for its use in DNA vaccines combined with HA protein. DNA vaccines are highly advantageous over traditional live attenuated or killed vaccines. Because proteins do not have to be altered to inhibit pathogenesis, the vaccine is able to induce the expression of antigens that resemble the original viral epitopes, making the vaccine more efficient. As far as vaccine manufacturing, rapid and large scale production can be done at a much lower cost than traditional vaccination, which makes them a necessity under pandemic conditions. This could be done through the development of a DNA vaccine that encodes both the surface HA protein and the internal NP protein from a highly pathogenic H5N1 strain. It can be assumed that a vaccine of this sort would support both humoral and cellular immune responses. This approach has been tested using HA and NA proteins. However, since both of these proteins are highly prone to mutations, little cross subtype protection was elicited. A combination of surface and internal proteins should be effective against the circulating strain and other antigenic variants. The surface HA protein expressed from the vaccine would induce a specific H5N1 antibody response, while the internal conserved NP protein should not only induce a homologous and

heterosubtypic antibody response but also aid in viral clearance through a cell mediated immune response.

The pandemic potential of H5N1 calls for the productions of large quantities of vaccine in a short amount of time. While all vaccines mentioned in this paper do provide an adequate immune response, there are limitations such as dose size, dose regimes, and production efficiency. An interesting approach would be to develop a vaccine that could be administered in a single dose. This would cut vaccine production time in half, which is essential during a pandemic. Also patient compliance is more likely to improve, as some patients most likely will not receive dose two of the two dose regime. An inactivated whole –virus vaccine and adjuvant would be ideal. With whole virus vaccines being more immunogenic, using one instead of two injections will shorten the time to develop immunity by 4 weeks. As far as dosage, an 8 microgram injection of antigen should be sufficient, as that was the total combined dosage used with adjuvant in the GSK vaccine (24). An HI assay using horse erythrocytes could be used as positive indication of antibodies towards various strains of H5N1 (5). A second neutralization assay using human respiratory tissues could also be used to determine antibody neutralization (8).

Individually, a virus like this could be devastating, however in a collective effort between research agencies, government policies and the public itself, we can defeat the pandemic threat this virus brings. The key to success in an intrapandemic period is research, pandemic preparation and education of the public.

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