Identification and characterization of H2N3 avian influenza virus from backyard poultry and comparison to novel H2N3 swine influenza virus

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Identification and characterization of H2N3 avian influenza virus from backyard poultry and comparison to novel H2N3 swine influenza virus

by

Mary Lea Killian

A thesis submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Veterinary Microbiology

Program of Study Committee:
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ABSTRACT

There are currently 16 identified H subtypes of influenza A; all 16 are known to infect avian species. In 2006 a novel influenza subtype (H2N3) was identified in a swine herd in Missouri. The hemagglutinin (HA) and neuraminidase (NA) genes were found to be avian in origin, raising concern regarding the potential for inter-species transmission of avian viruses to swine and humans. In early 2007, H2N3 influenza virus was isolated from a duck and a chicken from two separate backyard poultry flocks in Ohio. The nearly coincidental isolation of the same subtype in unnatural hosts in the Midwest United States raised the question about the origin of the viruses and the potential for these viruses to adapt to a new host and easily spread to nearby flocks or herds. Therefore, the viruses were further characterized by DNA sequencing and in vivo chicken pathogenicity testing. The virus sequences were compared to those from the 2006 swine isolates. In addition, the avian viruses were tested for cross-reactivity by virus neutralization (VN) and hemagglutination-inhibition (HI) using a panel of H2 reference sera including serum from the Missouri swine virus. Serologic survey on swine herds and poultry flocks in Ohio for H2 virus infection was also conducted to assess virus spreading.

Sequence comparisons for the H and N genes demonstrated that the avian viruses were similar, but not identical, to the swine viruses. Accordingly, the avian and swine isolates were also antigenically related as determined by HI and VN assays, suggesting that both avian and swine viruses originated from the same group of H2N3 avian influenza viruses. Although serological surveys using the HI assay on poultry flocks and swine herds in Ohio did not reveal further spreading of H2 virus from the index flocks, continuous
surveillance will be necessary. Contemporary H2N3 avian influenza viruses appear to be
easily adaptable to poultry and swine, raising concern regarding the potential for inter-
species transmission of H2N3 avian virus to humans.
CHAPTER 1. INTRODUCTION

1.1 ORGANIZATION OF THESIS

This thesis consists of four chapters. Chapter 1 presents a general introduction and an overview of avian influenza virus (AIV). Chapter 2 (Identification and characterization of H2N3 avian influenza virus from backyard poultry and comparison to novel H2N3 swine influenza virus) contains the research paper detailing the project methods and results. The last chapter contains the general conclusions of the research studies and suggests possible areas of future research. Additional figures are presented in the Appendix following Chapter 3.

1.2 REVIEW OF THE LITERATURE: Overview of Avian Influenza Virus

1.2.1 Introduction

Influenza is the cause of seasonal respiratory illness in humans and animals each year. The illness is caused by a virus which at one time was thought to be species specific. In recent years, however, it has been discovered that the virus, at times, is able to rapidly mutate and cause infection in a variety of host species. Influenza viruses are classified into one of three types, Influenza A, B, or C (26, 72, 74). Influenza A is the most common, affecting the largest number of species. Influenza B and C are only infectious to humans (72). Avian species are only susceptible to influenza A viruses. It is accepted that avian species were the original, natural host for influenza viruses. Continued contact between avian influenza viruses and mammals has allowed certain subtypes of the virus to
establish itself in non-avian species (i.e. swine, horses and humans) (50, 72, 84).

Mammalian-adapted viruses have been maintained in their host species for centuries, creating new host lineages along species lines (84).

Avian influenza was first described in 1878 by Perroncito in Italy (75). At the time the disease was known as “fowl plague” and caused high rates of morbidity and mortality in chickens (5, 26, 75). The typical clinical symptoms associated with fowl plague include respiratory signs, excessive mucus production, sinusitis, cyanosis of the combs and wattles, edema of the head and face, diarrhea and central nervous disorders. The causative organism was identified as an ultra-filterable agent, or virus, in 1901. Despite prior circulation of influenza viruses in poultry, 1902 marked the first isolation of the agent from a chicken (26). Even though the human population had been plagued by “Spanish flu” throughout 1918 and 1919, human influenza virus was not isolated until the 1930s (5). In 1955, researchers identified the association between influenza viruses causing mild disease in birds and mammalian influenza A viruses (5).

Influenza outbreaks worldwide have devastating economic impacts on human and animal populations each year. Millions of people are infected with influenza, and approximately 250,000-500,000 people worldwide each year die from complications associated with the disease (89). Influenza also affects the food animal industry and racing industry significantly. Billions of dollars are spent each year vaccinating swine herds and horses for influenza, and the introduction of influenza into a poultry flock has been shown to cost the industry millions of dollars (75). Avian influenza is the primary focus of this review, as birds are the only species shown to be affected by all influenza A subtypes. The following
review addresses the etiology, diagnosis, transmission, and control of influenza in order to provide a more complete understanding of the impact associated with all types of influenza.

1.2.2 Avian influenza virus

Viral genome and proteins

Avian influenza virus belongs to the genus *Influenzavirus* A in the viral family *Orthomyxoviridae* (13, 14, 26, 42, 69, 74, 77, 84). The virion structure is pleomorphic early in infection, later developing into a mostly spherical shape which is approximately 80-120 nm in diameter (26, 84). The virion is enveloped in a host-derived lipid bilayer which displays two types of spikes which are approximately 16 nm in length (26, 74, 84). These spikes represent the hemagglutinin and neuraminidase proteins.

The virus contains eight segments of negative-sense (complementary to the mRNA), single-stranded RNA (13, 14, 17, 28, 42, 69, 73, 74, 77, 84). The eight segments code for eleven viral proteins. The gene products are: polymerase B2 (PB2), polymerase B1 (PB1 and PB1-F2), polymerase A (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix 1 (M1), matrix 2 (M2), non-structural 1 (NS1) and non-structural 2 proteins (NS2) (84).

PB1, PB2, and PA are polymerase proteins which combine together with the nucleoprotein and viral RNA’s to compose the ribonucleoprotein (RNP) complex (22, 24, 84). The RNP complex is exported from the cell nucleus to allow virion assembly and completion of the infectious cycle. The NP contains a C-terminal M1 binding domain on an N-terminal nuclear export signal to allow RNP export. The binding of PB1 to PB2 induces endonucleolytic cleavage of 5’-cap structures on cellular RNA (60). The PB2 also provides
RNA-dependent RNA polymerase activity. The PB1 is responsible for nucleotide addition to
the growing mRNA as well as the elongation protein for template RNA and viral RNA
synthesis. The PA is also part of the RNA-dependent RNA polymerase complex, but the role
in viral RNA synthesis is unclear. There is evidence that it may be involved as a protein
kinase or helix-unwinding protein (84). These are internal proteins and are subject to less
immune selective pressure than the surface proteins (i.e., HA and NA).

The HA protein is the major surface antigen and an integral membrane glycoprotein
(69, 84). It is responsible for attachment to the host cell receptor as well as fusion between
the virion envelope and host cell and plays a major role in determining virus pathogenicity.
The HA protein also influences host specificity by preferentially binding to one of two
different sialic acid receptors on the host cell surface (66). There are 16 known subtypes of
avian influenza based on the HA gene sequence (23). The HA gene is initially translated into
a single polypeptide precursor, HA0. Following translation of the HA0 it is processed into
two proteins, HA1 and HA2, by proteolytic cleavage (26, 36, 74, 84). The HA1 protein is
the amino-terminal fragment of the original HA0 protein (precursor form of HA) and is
responsible for virion attachment to the host cell receptor and is the major target of the host
immune response. The HA2, or the carboxy-terminal fragment of the HA0, then mediates
virus-cell fusion of the envelope and the cellular endosomal membrane. The HA and other
gene segments are prone to a high rate of mutation due to the nature of RNA viruses (42).
These mutations are introduced and become fixed into the viral population leading to
antigenic drift which accounts for much of the variation within and between subtypes. While
the amino acid sequence at the host cell receptor binding site among all H16 subtypes is
relatively conserved, the remainder of the HA gene is highly mutable. In addition to the high
rate of mutation, the HA is most susceptible to immune selective pressure (84), which also leads to antigenic drift.

The NA protein is the second major surface antigen (84). It is an integral membrane glycoprotein. There are currently 9 known NA subtypes (13, 14, 26, 69, 74, 77, 84). The function of the NA protein is to cleave the terminal sialic acid from glycoproteins or glycolipids which, in turn, free the progeny virus particles from the host cell. The NA also plays a role in viral pathogenicity (27). Virus particles with high NA activity are more efficiently released from infected cells releasing more progeny virions into the host. In addition, greater NA activity results in higher HA cleavage in multiple organs. The NA protein is subject to selective pressure from the host immune system and is prone to high levels of mutation, leading to antigenic diversity among the same subtype. Influenza treatment drugs target the NA activity to block infection (47). There is concern that selective pressure may drive mutations in the NA that would decrease the effectiveness of these drugs in treating influenza, and a spontaneous mutation in the NA has caused oseltamivir-resistant strains of influenza H1N1 to circulate throughout the world since 2004.

The matrix gene is highly conserved among avian influenza subtypes (28). The matrix gene codes for two proteins, the M1 and M2 proteins (84). M1 provides the shell surrounding the inside of the virion. There is no enzymatic activity associated with the M1 protein, but it plays an important role in progeny virus assembly (26, 28, 84). The M2 protein is spliced from the M1 and is the third integral membrane protein (in addition to the HA and NA). The M2 protein functions in signal transduction and as a proton pump causing decrease in pH in the viral endosome. When the pH of the endosome is decreased, M2 protein
tetramers on the surface of the virion are activated to acidify the inside of the virion and facilitate the uncoating of the virus and dissociation of the M1 from the RNP.

The NS gene encodes two overlapping proteins, NS1 and nuclear export protein (NEP) (32, 81, 84). The NEP was previously known as the NS2 protein. The NS1 contains two domains, one of which contains a nuclear localization signal to bind RNA and the second which interacts with host nuclear factors. The NS1 is abundant in the nucleus of the infected cell and is a multifunctional protein. Among the roles are the inhibition of nuclear export of mRNA and inhibiting type I interferon, both of which act to suppress innate immunity. NS1 is located primarily in the nucleus of the infected cell, but is not incorporated into the progeny virion. The NEP is abundant in the cytoplasm of the infected cell. Small amounts of NEP are packaged in the virion and appear to modulate the synthesis of the NS for the progeny virus.

**Biological properties**

Avian influenza viruses have been categorized into two groups based on their ability to cause disease in chickens. The virulent viruses are responsible for the fowl plague that was initially described, and are now termed “highly pathogenic avian influenza (HPAI)” virus. The less virulent viruses cause a milder, primarily respiratory disease termed “low pathogenic avian influenza (LPAI)” virus (5). Avian influenza virus infection begins by inhalation or ingestion of infectious virions (75). The trypsin-like enzymes in respiratory and intestinal epithelial cells permit cleavage of the hemagglutinin protein and initial replication cycles occur in the respiratory and/or intestinal tracts releasing more infectious virions. With LPAI, virus replication is limited to these sites. With HPAI, the virions invade the
submucosa, entering the bloodstream. The virus will replicate within endothelial cells and spread to other systems and infect a variety of cell types in visceral organs, brain, and skin. Macrophages may become infected with influenza virus resulting in suppression of phagocytosis and pulmonary microbicidal activity (72). The virus-infected macrophages show a decreased respiratory burst and chemotactic response making infected animals more susceptible to secondary infections.

Through 2007, there have been 25 outbreaks of HPAI worldwide; 16 caused by H7 influenza viruses, and nine caused by H5 viruses (3, 4, 5, 55, 56). In addition to the nine H5 outbreaks reported, an HPAI H5N1 outbreak was reported in 1996 in China (Asian H5N1), which spread to Hong Kong in 1997 (45). Multiple genotypes of the virus continued to circulate in live bird markets and farms throughout China and Hong Kong through 2003. In 2003 and 2004 the outbreak of Asian HPAI H5N1 became much more widespread and has been reported in poultry and wild birds in 61 countries between 2003 and 2008 (45, 90). To date, only subtypes H5 and H7 have been identified as both LPAI and HPAI. All other subtypes naturally occur as LPAI only. At this time, the reason that only H5 and H7 have occurred as HPAI is unknown. HPAI was one of the first diseases for which international control standards were developed and is reportable to the Office International des Épizooties (OIE), World Organization for Animal Health (49, 72, 88). Any HPAI is classified as “notifiable avian influenza” (NAI) to the OIE (92). Due to the risk of LPAI H5 and H7 viruses becoming virulent in poultry hosts, all H5 and H7 (LPAI or HPAI) are NAI viruses.
1.2.3 Disease and Pathogenesis

Clinical presentations

The clinical presentation of avian influenza is largely dependent on the pathotype of the virus (LPAI or HPAI), but is extremely variable depending on multiple factors including host species, strain subtype, age, sex, concurrent infections, acquired immunity, and environmental factors (75). Most infections of LPAI virus in wild birds are asymptomatic. Experimental infection of LPAI virus in poultry has shown decreased egg production and suppressed T cell function (75). LPAI viruses replicate primarily in the intestinal lining of wild ducks. This causes no apparent disease, but leads to high levels of virus excreted in the feces (84). Viability and severity of infection depends on strain, age of bird and concomitant infection with bacteria (26, 75). In contrast, LPAI in domestic poultry presents with respiratory signs and abnormalities in the digestive, urinary, and reproductive organs. Respiratory infection can be manifested by coughing, sneezing, rales, and excessive mucus production. Hens may exhibit increased broodiness and decreased egg production. Flocks may exhibit general clinical signs including depression, decreased food and water consumption, huddling, and ruffled feathers.

With the exception of Asian H5N1, HPAI viruses in wild birds replicate poorly and produce little or no clinical signs (35, 75). Clinical signs from experimentally infected ducks with different HPAI H5 viruses ranged from no clinical signs (A/tern/South Africa/61 H5N3) to blindness and continuous head-shaking (A/Duck/Yokohama/03 H5N1). In domestic poultry, HPAI viruses replicate systemically causing damage to multiple visceral organs as well as the cardiovascular and nervous systems (75). Virus replication occurs in multiple
cells types, e.g. neurons, cardiac myocytes, vascular endothelial cells, kidney tubules, skeletal myofibers, and adrenal or pancreatic acinar cells. Clinical presentation varies depending on the extent of visceral damage. HPAI viruses in poultry typically causes sudden death before clinical signs can be observed (75). HPAI produces systemic infection with central nervous system involvement, often leading to death within one week (13, 26, 84). If the disease is sustained for 3-7 days, individual birds may present with tremors, inability to stand, torticollis, and other unusual positioning of the head and appendages (75). Respiratory signs are less prominent in HPAI than LPAI but include sinusitis causing rales, sneezing, and coughing. Additional manifestations include diarrhea, depression, ruffled feathers, sinusitis, cyanosis of the comb and wattle, and edema of the head, neck, and face (13, 26, 75, 84). Breeders and layers exhibit an extreme decrease in egg production, typically leading to complete cessation of egg production (75). There is little evidence of vertical AIV transmission, however, virus has been recovered from eggshell surfaces and internal egg contents from hens infected with AIV.

**Pathogenesis**

Virulence determination of viral strains is polygenic, but the HA gene plays a major role. The amino acid sequence at the HA protein cleavage site determines whether the virus establishes local or systemic infection. The hemagglutinin requires post-translation cleavage from HA0 (precursor form) into HA1 and HA2 (5, 84). This enzymatic cleavage allows the virus-cell fusion mediated by the HA2 protein. It has been determined that the presence of multiple basic amino acids at the HA protein cleavage site is critical in the determination of the pathogenicity of the virus by affecting the specificity of the proteolytic enzymes capable of cleaving the protein into the HA1 and HA2 components (58). LPAI strains typically have
two basic amino acids, at positions -1 and -4 from the cleavage site for H5 viruses and at -1 and -3 for the H7 viruses (69). In low pathogenic strains, cleavage of the HA0 is limited to trypsin-like proteases (13). These are host enzymes which are limited to the linings of the gastrointestinal and respiratory tracts, causing a local, self-limiting infection (5, 13, 26, 69, 72, 79). In contrast, highly pathogenic strains have multiple basic amino acids at the cleavage site which allows cleavage by ubiquitous proteases that are proprotein-processing subtilisin-related endoproteases found in all cell types throughout the body. This leads to systemic infection and causes damage to vital organs. Postmortem examination of birds infected with HPAI virus shows hemorrhagic tracheitis, pneumonia, airsaccuulitis as well as duodenal and jejunal mucous enteritis due to such a systemic infection (46, 64). It has been determined that the minimal sequence at the HA cleavage site for a virus to be classified as highly pathogenic is B-X-B-R (B= basic arginine or lysine; X= any amino acid; R= arginine) (58). According to the OIE Manual of Standards, any virus is classified as highly pathogenic if it is lethal for six, seven or eight of eight 4 to 8 week old susceptible chickens within 10 days of inoculation via intravenous route (92). In addition, the amino acid sequence at the hemagglutinin protein cleavage site for an H5 or H7 virus that is low pathogenic in chickens must be determined. If the deduced amino acid sequence at the cleavage site is similar to that observed for isolates previously characterized as highly pathogenic, the virus being tested must be considered as highly pathogenic even if they do not cause mortality in chickens due to the risk of apparently low pathogenic viruses mutating into highly pathogenic forms.

The HA amino acid sequence is one major determinant of AIV virulence, but there are additional structural determinants as well. Sequence analyses of HPAI and non-HPAI isolates from the Pennsylvania H5N2 outbreak in 1984 are virtually indistinguishable (31,
Four of seven nucleotide substitutions resulted in amino acid changes, one of which resulted in the loss of a carbohydrate side chain affecting a glycosylation site near the peptide cleavage site. This mutation is felt to cause a structural change that allows ubiquitous proteases access to the cleavage site resulting in virus activation systemically as opposed to locally. However, the changes in glycosylation patterns are less reliable as markers of virulence than the multiple basic amino acid motifs (50).

Regardless of pathogenicity, the ability of a particular subtype of avian influenza virus to cause disease varies by host species (72). The same virus that is apparently low pathogenic in wild ducks may cause high morbidity or mortality in domestic chickens. Antibody production is also host species-specific. Chickens have a much higher antibody response than pheasants and turkeys, and the response is markedly decreased and sometimes absent in the wild duck population. The inability of ducks to produce hemagglutinating antibody is likely the result of the structure of the main type of avian serum antibody, IgY (7, 72). The IgY is a homolog to mammalian IgG primarily responsible for opsonization and neutralization. As opposed to IgG, chicken IgY only contains two constant region domains which eliminates effector functions associated with the Fc portion of the antibody, including hemagglutination.

**H5, H6, H7 and H9 Outbreaks**

As previously mentioned, H5 and H7 have been the only AIV subtypes identified as highly pathogenic. These strains are not commonly isolated from wild birds, but are the most important subtypes in chickens and turkeys (62, 70). The predominant subtypes isolated from ducks are H3, H4, and H6 while shorebirds primarily carry H4, H9, H11, and
H13 (72, 84). Common influenza infections in poultry include subtype H9 in Asia and subtypes H3 and H6 from turkeys and chickens, respectively, in the United States (41, 42, 51, 82, 87). Despite the circulation of these subtypes, H5 and H7 have been the ones that have been implicated in the largest outbreaks in galliformes (turkeys, grouse, chickens, quail, and pheasants) (62, 70). Because of their propensity to become highly pathogenic, it is important to conduct surveillance to identify H5 and H7 (63). These two subtypes are responsible for three outbreaks that have lasted for extended periods of time: H5N2 in Pennsylvania from 1983 to 1989, H5N2 in Mexico from 1993 to present, and H7N2 in the northeast United States live bird markets (LBM) from 1994 to 2006 (57, 68). The Pennsylvania outbreak began in 1983 as LPAI, and shifted to HPAI in October 1983. In all, 17 million birds were slaughtered and cost the United States more than $60 million (5). In 1986, a similar LPAI H5N2 virus was isolated in five northeastern US states, all of which traced back to live bird markets in New York City.

More recently, outbreaks in the United States belong to the LBM H7N2 lineage (68). This lineage has been shown to have a high rate of mutation, with the sequence at the HA cleavage site varying from 2 to 4 basic amino acids (70). This lineage has also been shown to be responsible for three outbreaks in commercial poultry, the largest being the H7N2 LPAI outbreak in Virginia and North Carolina in 2002 (19, 70). As with all large scale poultry outbreaks there were devastating economic consequences. More than 5 million birds were depopulated, with an associated cost of approximately $149 million (13). In past outbreaks, low pathogenic viruses, if allowed to circulate, have transformed into highly pathogenic strains (13). This emphasizes the need to survey for and control the LPAI forms before they have a chance to mutate into HPAI strains.
Besides subtypes H5 and H7, there have been 12 separate isolations of AIV subtype H6N2 from chickens associated with four different outbreaks in California between 2000 and 2002 (34, 82, 87). The isolates were all characterized as low pathogenic, causing mild disease symptoms and salpingitis (34). It is thought that the four separate introductions into commercial poultry reflect an increased adaptation of surface glycoproteins from natural reservoir viruses to replicate efficiently in chickens (82). Given time to continue to adapt to a new host (chickens), if this virus is not eradicated, it could increase in pathogenicity as well.

While subtype H9 has not been classified as highly pathogenic, it is associated with widespread, serious disease (5, 13). Since 1994, H9 infections have been common worldwide. In the United States in 1995-1996, 178 turkey farms in Minnesota were infected with H9N2 (5). This represents the worst economic loss to influenza in one year in Minnesota costing upwards of $6 million.

Co-infection of AIV H9N2 and *Escherichia coli* has been reported to increase the pathogenicity to a more moderate level (6). The *E. coli* may provide the necessary enzymes to cleave the hemagglutinin enabling widespread replication and mimicking a highly pathogenic virus. Analysis of the H9N2 viruses circulating in Asia provide evidence that viruses established in chicken and quail populations have been transmitted back to domestic ducks (40). This two-way transmission between aquatic birds and terrestrial birds results in the generation of multiple genotypes and the potential for interspecies transmission of the reassortant. The species barrier has suddenly become permeable and the theory of evolutionary stasis can be viewed as unsound.
Influenza in other species

Influenza viruses have also caused outbreaks in animal populations. Swine influenza was first identified in 1918 (1). The virus was serologically identified as H1N1. The symptoms exhibited by the pigs were very similar in nature to those in the human population, and the genetic sequence of the HA gene provides strong evidence to support the virus was transmitted from humans to swine (8). In addition to this genetic information, the disease was observed in humans prior to being described in pigs. This H1N1 virus, which is commonly referred to as “classical H1N1 SIV”, remained stable in the swine population for approximately 70 years until the late 1990’s when a new subtype (H3N2) was introduced (80). Subsequent reassortment between the H3N2 virus and the classical H1N1 virus has resulted in the generation of novel subtypes (e.g., H1N2, H3N1), as well as antigenically different strains bearing the same H and N type (e.g., variant or reassortant H1N1 or H1N2).

In 2006, yet another subtype was identified from two swine farms in Missouri (43). The virus was identified as H2N3 (novel H and N subtypes). It appears this virus was introduced via reassortment with the contemporary swine viruses and one or more avian viruses (H2N? and H?N3). The presence of swine-origin internal genes suggests this virus may have the potential to establish itself in the swine population. This occurrence is of particular concern because the researchers discovered receptor binding site changes associated with increased affinity for NeuA(α2,6Gal), which is found in mammalian hosts. The H2 subtype has not circulated in the humans since 1968, so the current human population has little to no immunity to this virus strain. The H2 subtype is sporadically detected in poultry in the live bird market system in the Northeast United States (56).
In 1956, subtype H7N7 influenza virus was isolated from a horse in Prague (former Czechoslovakia) (1). The HA antigen of this equine influenza was closely related to the fowl plague virus H7N7. A second subtype (H3N8) emerged in the United States in 1963. This H3N8 virus spread throughout the US and into Canada, causing severe disease in naïve populations. Since 1963, the virus has undergone major antigenic variations, and during an outbreak in 1978-79, animals that were vaccinated with the prototype strain (A/equine/Miami/1/63) were not protected against the active strain (A/equine/Kentucky/1/1978). In the US, the H3N8 virus is the only subtype continuing to circulate in equine species.

In 2004, subtype H3N8 influenza was isolated during an outbreak of hemorrhagic respiratory disease in racing greyhounds in Florida (20, 48). The virus was subsequently associated with clinical illness in dogs from racing parks in 9 different states suggesting sustained dog-to-dog transmission. Sequence analysis of the eight gene segments showed significant similarity to contemporary H3N8 equine influenza viruses. Since the initial isolation of this virus, the virus has been isolated and continues to circulate among all breeds of dogs worldwide (15). Serologic surveillance conducted on sick animals since 2005 has identified H3N8 antibody in canine populations in 25 states and Washington D.C. (48).

In 2008 a novel subtype of influenza, H3N2, was identified in canine populations in South Korea. These viruses appear to be genetically distinct from the equine and canine H3N8 viruses circulating in the U.S. and are more closely related to Asian lineage avian influenza viruses (61). In this instance it appears the entire avian influenza virus genome infected the affected dogs. It is hypothesized that the virus was transferred by feeding minced poultry meat to dogs for fattening in kennels. However, it is also possible that there
was interspecies transmission by aerosol or by close contact between the avian and canine species. The avian-like H3N2 also appears to be spread dog-to-dog but surveillance indicates a low seroprevalence in both farmed and pet dogs in Korea (38).

### 1.2.4 Epidemiology

**Pandemics and Epidemics**

Pandemic influenza can occur when a novel HA is introduced to a population that lacks immunity to that subtype. While pandemics of influenza probably have been occurring for centuries, three large pandemics have been documented to date (1). The largest pandemic (“Spanish flu”) occurred in 1918-1919 and caused more than 21 million fatalities. This outbreak was later found to be caused by the H1N1. Full genome sequencing and phylogenetic analysis has shown that the viruses are the most avian-like among all of the mammalian sequences, indicating the virus was entirely avian in origin (78). In particular, the HA and NA genes are more similar to the avian viruses at 37 of 41 and 21 of 22 amino acid positions that are known to be subject to immune pressure, respectively, indicating the viruses had not been circulating in mammalian hosts for an extended period of time prior to the 1918 pandemic. The 1918 influenza virus is now known to be the common ancestor of human and swine H1N1 lineages. Approximately 38 years later, in 1957, a second pandemic (“Asian flu”) occurred when a novel influenza virus, subtype H2N2, was introduced to the human population (1). In 1957-58 there were approximately 62,000 deaths in the US caused by the new strain of flu in addition to the mortalities caused by the circulating seasonal influenza. A third influenza pandemic (“Hong Kong flu”) occurred in 1968 caused by the introduction of subtype H3N2 which caused approximately 34,000 deaths in the US. In each
pandemic, the previous circulating subtype was replaced by a new subtype in the population. In 1977, another H1N1 outbreak occurred. At this time, the H3N2 subtype that was introduced in 1968 was still circulating in the human population, and these two subtypes continue to co-circulate with influenza B causing yearly epidemics of influenza in the human population.

**Interspecies transmission**

Influenza viruses are typically host-specific (9, 54, 83, 93). In the United States, birds, swine, horses, dogs and humans are the only species in which there is an established influenza A lineage (54). While many other species have been shown to be affected, especially by avian influenza (cats, mink, seal, and whale), these viruses tend to be pathogenic to the new host, and perhaps for this reason have not been able to establish a stable lineage. Equine influenza A viruses appear to have been derived from an avian influenza antecedent (as it is believed that all influenza viruses are). The virus established itself in the equine population and has remained relatively stable, as it is the most distantly related to all other mammalian influenza A viruses.

Multiple influenza viruses have also recently been identified in coastal and aquatic mammals (seals and whales) (84). An H7N7 virus identified in harbor seals in 1980 was genetically similar to avian viruses, however, it appeared to act more like a mammalian virus *in vivo*. Experimentally this virus replicated to high titers in mammals and very poorly in avian species. This characteristic likely indicates a very rapid adaptation of the virus to its mammalian host with the resulting virus unable to re-infect an avian host. Subsequently, multiple other subtypes have been isolated: H4N5, H4N6, and H3N3 (10). Like the H7N7, all eight segments of the H4N5 virus were genetically related to avian influenza, however,
the H3 viruses showed close relationship to both avian and human isolates. Multiple subtypes of avian influenza (H13N2, H13N9, and H1N3) have also been isolated from tissues of whales. The influenza epidemics that have occurred in these species have been self-limiting, and the viruses have not been maintained in these populations or species.

Contradictory to the equine influenza A viruses, swine, human, and avian lineages appear to be growing closer and closer together. Influenza A has typically been thought to be species specific, however, recent events show that interspecies transmission is becoming more and more common. As previously stated, avian species are known to carry combinations of all 16 HA types and all 9 NA types. Whole viruses as well as viral gene segments may occasionally be transferred to a different host species where there is selective pressure to establish a new, stable lineage (75).

Host specificity of influenza A viruses has been determined to be polygenic with a particular emphasis on the HA gene product as a receptor binding protein (29). Typically, avian origin viruses do not replicate well in human populations and vice versa. A large part of this specificity is due to the preferential binding of the virus to sialyloligosaccharides with specific linkages of N-acetyleneuraminic acid (NeuAc) to galactose (Gal) (9, 29, 93). Avian influenza viruses bind to NeuAcα2,3Gal receptors, where human viruses prefer NeuAcα2,6Gal receptors. With this receptor specificity, avian viruses cannot effectively bind and replicate in humans and vice versa. It has been postulated that swine may be considered a “mixing vessel” for new lineages of influenza viruses. Swine appear to be genetically susceptible to avian and human influenza virus infection due to the presence of both NeuAca2,3Gal and NeuAca2,6Gal viral receptors on swine tracheal cells. While there appears to have been infrequent transmission of swine influenza virus to human populations
and vice versa, the maintenance of viruses in the swine population as well as the frequent introduction of novel virus subtypes (avian origin) into swine suggest that swine populations may be increasingly viewed as a potential source for a new pandemic strain of influenza (84). Outside of this theory, there are historic instances of outbreaks in humans where there is no swine intermediate, such as the 1957 and 1968 influenza pandemics that were the result of a reassortment event between avian and human influenza viruses, and the more recent chicken to human transmission of the highly pathogenic H5N1 avian influenza virus (9, 83). There appears to be no consistent rule for introduction and transmission of novel influenza viruses between species, especially swine, avian, and human.

Swine influenza viruses in the United States were historically stable until the late 1990’s (18, 80, 83, 93). There was a single circulating subtype, referred to as classical H1N1 (cH1N1). In 1998 a new subtype of influenza virus was identified in swine populations across the US (H3N2) that contained swine (internal genes) and human (HA and NA), or swine (NP, M, NS), human (HA, NA, PB1) and avian genes (PA, PB2). With the outbreak of this new subtype, there were also concurrent recombination events occurring between this new virus and the classical virus. This resulted in the appearance of two additional viruses: recombinant H1N1 (rH1N1) where the HA and NA proteins were derived from cH1N1 and the internal genes from the emergent H3N2 virus; and a new subtype, H1N2 where the HA and NA were derived from cH1N1 and H3N2, respectively, and the internal genes from the H3N2 virus. From this time on, the mixture of infections in swine herds has caused a dramatic increase in the rate of genetic variation among the H1 and H3 swine influenza viruses. Likewise, in Europe in the late 1970s/early 1980’s an avian influenza H1N1 was
transmitted to swine and established itself as a stable swine lineage that continues to circulate (11).

Following the outbreak of subtypes H3N2 and H1N2 in swine, these subtypes (H3N2 and H1N2) were eventually identified in turkey flocks across the United States (18, 71). Sequencing analysis performed on the swine and turkey H3N2 isolates showed greater than 97% similarity among the viruses, indicating that there was interspecies transmission as opposed to a separate recombination or evolution event occurring in the turkey flocks. The transmission of H3N2 from swine to turkeys is reportedly the first documented transmission from mammals to birds, as opposed to the typical flow of infection from birds to mammals. Similar transmission was documented in 1999 with the spread of H1N2 from swine to turkeys. These two new reassortant viruses have continued to circulate in the swine and turkey populations, and vaccination against these subtypes is commonly practiced.

In addition to the rH1N1 and H1N2 viruses, a novel subtype, H3N1, was isolated from a swine herd in Minnesota in 2004 (44). This virus has been characterized as a second-generation reassortant taking the HA gene from the H3N2 triple-reassortant viruses and the NA gene from the contemporary H1N1 viruses. The same subtype was also isolated in Taiwan from a reassortment between classical H1N1 and H3N2 SIV. To date, no isolations of this virus subtype have been isolated from poultry although it continues to be identified in swine from time to time.

Besides reassortment between H1N1 and H3N2 SIVs, there have been reassortment events between SIV and human or avian influenza viruses. Between 2003 and 2005, researchers in Canada reported the isolation of three novel H1 viruses from swine herds in Ontario (30). One virus, subtype H1N2, was wholly human-origin. Subsequent H1N2
isolates also contain an HA with similar phylogeny to the human virus and the remaining genes are mixed human and swine lineage. During the same time period, a novel H1N1 was also isolated in Ontario. The PB1 gene in this isolate appears to be human-origin, with the remaining 7 genes showing similarity to classical H1N1 swine viruses. In 2006, a novel subtype (H2N3) of influenza virus was isolated from two separate swine herds in Missouri (43). The hemagglutinin, neuraminidase, and polymerase A genes were identified as avian-origin, and the remaining five genes were homologous to the triple reassortant internal genes found in currently circulating swine influenza viruses. Serologic surveillance of swine herds in Missouri indicate that the H2N3 virus continues to circulate but has not spread outside of the affected premises.

**Reservoirs**

Wild waterfowl and shorebirds have been shown to be the natural reservoir for avian influenza with the highest number of different viruses being isolated from Order Anseriformes compared to the other Orders of birds (5, 21, 29, 41, 42, 53, 59, 68, 70, 72, 73, 85). Wild birds carry many subtypes of influenza with infection varying from asymptomatic to mild respiratory or gastrointestinal disease. This stability is frequently described as evolutionary stasis (41, 42, 50, 59, 73, 84, 85). Once the virus is introduced to a new species, however, it rapidly evolves and may cause a wide range of infections in the new host (42, 50, 85). Symptoms can range from mild upper respiratory infection to a rapidly fatal disease. Avian influenza can be spread between species in a variety of ways. Primarily, infected ducks can shed virus for up to 14 days post infection which is excreted in high concentrations in the feces. Contaminated water supplies can subsequently infect other wild or domestic birds as well as non-avian species (26, 42, 59).
Another reservoir for avian influenza viruses in the U.S. and other countries (especially Asia) are live bird markets (LBMs). There are approximately 120 markets in the northeastern United States with roughly 80 markets in and around New York City (70, 75). These markets pose a high risk for the introduction and perpetuation of avian influenza virus by mixing many different types of birds (chicken, pigeon, quail, pheasant, duck, guinea fowl, chukar partridge, etc.) in very high density and very close proximity (52, 70). This combination of birds creates a new source of infection for domestic poultry. The first isolation of avian influenza virus in the US LBMs was made in 1986 and was characterized as low pathogenic H5N2 (70, 75). This virus was subsequently linked to outbreaks in five New England states (5). Since 1994, avian influenza virus subtype H7N2 has been the dominant virus circulating in LBMs (62, 70, 75). This lineage has been linked to three outbreaks in commercial poultry since then, two in Pennsylvania (1997-1998 and 2001-2002) and one in Virginia (2002). Up to 60% of the markets during this period could be found to be positive for H7N2 at any one time. Also, as many as nine other subtypes have been isolated in the LBMs since 1994. As of 2006, the H7 subtype avian influenza viruses have been eradicated from the LBMs in the Northeastern U.S. (57).

Serologic surveillance and experimental infections of mink, raccoons, mice, and other rodents indicate that these species can be infected with influenza viruses (25). Raccoons experimentally infected with avian and human influenza A viruses have been shown to develop infection, transmit the virus to non-infected animals, and seroconvert. The increased comingling of these rodents may serve to transmit influenza viruses to poultry and livestock either by direct transmission or by acting as fomites.
1.2.5 Laboratory Diagnostics

Virus Isolation

Suitable samples for the identification and isolation of avian influenza viruses include swabs collected from the tracheal or oropharyngeal and/or cloacal area as well as tissue samples from the respiratory tract (trachea, lung) or digestive tract (33, 76). Tracheal or oropharyngeal swabs are the preferred sample for virus isolation from poultry since influenza viruses preferentially replicate in the respiratory tract of gallinaceous species. Likewise, cloacal swabs are the preferred sample for isolation from wild birds since virus replication occurs primarily in the gastrointestinal tract in these species. In the case of HPAI, virus may be isolated from both collection sites as well as any organ system due to the systemic nature of virus replication. Nine to eleven day old embryonating chicken eggs are inoculated with the above mentioned suspensions via the allantoic sac (86). Eggs are incubated at 37 °C for 3-7 days. Some viruses are more lethal for the chicken embryo, and for some viruses embryonic death may or may not be observed during the incubation period. Embryonic death within 24 hours is usually considered to be non-specific, however, highly pathogenic viruses may kill embryos within 24 hours. Typically, increased egg passages will increase the potential for embryonic death.

Amnionic-allantoic fluid (AAF) from the embryos (both dead and alive) is collected and tested for hemagglutinating activity. The HA protein on the surface of influenza will bind to receptors on erythrocytes from a variety of species (76). Other hemagglutinating agents include Newcastle disease virus (NDV), avian paramyxovirus types 2-9, adenovirus 127 (egg-drop syndrome), and hemagglutinating bacteria (e.g. Neisseria gonorrhoeae) (37, 76). Once these agents are excluded as being responsible for the hemagglutination, the virus
may be characterized by HA and NA subtyping (76). The hemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) tests are performed using monospecific reference sera produced against each of the 16 known HA types and the 9 known NA types. There are also a variety of molecular subtyping tools (i.e. sequencing and real-time reverse transcriptase polymerase chain reaction). Virus isolation (VI) is considered the standard method to which all other methods are compared, however, it is a labor intensive procedure and can take up to 2 weeks for completion of the test (14, 19, 63, 65). The period of time field diagnosticians and poultry producers might spend waiting for virus isolation and characterization raises issues related to the welfare of the diseased animals (14). Similarly, the extended period of time places limitations on the ability to move birds between commercial premises in surveillance situations. Despite these limitations, VI cannot be abandoned as a standard procedure, as it is the only procedure which yields a viable virus. Live virus is required for the in vivo pathogenicity testing outlined by the OIE and is the only method by which enough viral copies are generated for sequencing or antigen production (76). All other diagnostic methods are merely designed to detect the presence of viral antigen, antibody, or nucleic acid.

**Antigen-capture enzyme immunoassay (AC-EIA)**

An AC-EIA (e.g. Directigen™, FluDetect®, BinaxNOW®) takes significantly less time than virus isolation, 15 minutes compared to 7 days (21). The assay is analytically 100% specific (no cross-reaction to infectious bronchitis, Newcastle disease, infectious bursal disease, or infectious laryngotracheitis), however, the sensitivity is only 79% compared to virus isolation (21). The rapid antigen detection test was originally designed for
use with human specimens, and has since been adapted for use with veterinary specimens. The kits detect nucleoprotein antigen present in the sample (tracheal swab for Directigen; tracheal/oropharyngeal or cloacal swab for FluDetect and BinaxNOW). Due to the limited sensitivity of the assay, the test should only be performed on specimens collected from clinically ill animals, and should be used to determine the infection status of a flock rather than an individual bird (76). The tests are simple and can be used at pen-side in order to get a rapid diagnosis. Another advantage of the commercially available AC-EIA kits is the use of an internal control which indicates the sample was suitable for testing and the assay was performed correctly.

**Antibody detection**

There are four major tests available to detect antibody: agar gel immunodiffusion (AGID), enzyme-linked immunosorbent assay (ELISA), HI and NI (76). The serum-virus neutralization (SVN) test also detects antibody, however, the HI provides essentially the same information and is much less labor-intensive. For this reason, HI is usually performed on avian specimens rather than SVN. AGID is a highly specific test that will detect all subtypes of influenza A. The test utilizes the antigenically conserved matrix protein, and is relatively easy to perform. A positive precipitation line will form between test antigen and positive serum with 18-24 hours at room temperature. There are also commercially available indirect ELISA kits that detect influenza A RNP and M antibodies. Positives obtained by either the AGID or ELISA methods should also be confirmed by HI and NI subtyping.
An important emerging diagnostic tool in antibody detection is the differentiation of infected from vaccinated animals (DIVA). This differentiation is important for economic trade reasons because historically, domestic and international trade has been restricted based on antibody detection in flocks. However, if antibody to killed vaccine can be distinguished from antibody induced by a natural infection, trade may not be affected. There are multiple ways to accomplish this differentiation (67). One method is the use of subunit vaccines target to the HA gene that allows serologic surveillance to internal proteins such as NP or M. A second method is to vaccinate with a homologous hemagglutinin subtype to the circulating field strain paired with a heterologous neuraminidase subtype (67). With this method, serologic surveillance can be performed for the homologous neuraminidase subtype as evidence of natural infection. Thirdly, killed vaccines are produced with whole influenza virions and a differential antibody response can be measured against the NS1 protein, no or small amounts of antibody being produced with a vaccine, and much larger amounts produced by a natural infection (67). Each of these surveillance strategies provides advantages and disadvantages; the overall advantage is the ability to assure the safety of poultry and poultry products for international trade. The disadvantages of the assay range from poor test sensitivity due to low seroconversion rates to lack of high throughput capabilities for surveillance testing.

**Nucleic acid detection methods.**

There are several methods to detect viral nucleic acids. Standard reverse-transcription polymerase chain reaction (RT-PCR) coupled with gel electrophoresis has been developed to amplify the matrix gene (63, 65). Following amplification, the cDNA is
visualized using agar gel electrophoresis. In addition to the matrix gene this method has been used to detect the HA for all 16 subtypes of avian influenza. RT-PCR can be applied directly to viral RNA extracted from clinical specimens, but is probably better utilized as a supplement to VI. Subtype-specific primers to H5 and H7 HA genes allow determination of the amino acid sequence at the HA cleavage site for characterization of pathogenicity.

Another nucleic acid detection technique, nucleic acid sequence-based amplification (NASBA) is a subtype-specific assay targeted to either H5 or H7 (19, 77). It has been shown to be 10-100 times more sensitive than the AC-EIA procedure previously described. The test is designed to be performed in a high throughput situation. It can be completed in four hours in the presence of DNA contaminants as well as heparin, EDTA, citrate, hemoglobin, albumin, and lipids. It is a continuous amplification step at a constant temperature in a single mixture. This allows for rapid detection of potentially highly pathogenic strains (H5, H7) on a large-scale surveillance basis. As with all other rapid detection methods, findings should be confirmed and further characterized by virus isolation. The microarray and Luminex® technologies for detecting nucleic acid were designed to test multiple targets (up to 100 analytes in a single well) which would allow the user to test for all 16 H types and all 9 N types, including variants of each gene, with a single test. At the current time, these technologies have not been developed and optimized for avian influenza diagnostic use.

Fluorogenic RT-PCR, or real-time RT-PCR (rRT-PCR) is similar to conventional RT-PCR; however, there is no need for post-PCR processing because amplification is visualized as a measure of probe degradation (63). Several types of probes are available for use in the fluorogenic RT-PCR assays, the most common of which is the TaqMan or hydrolysis probe (76). The elimination of the post-PCR step dramatically decreases the
chances for cross-contamination between samples. Compared to virus isolation, there is also less handling of potentially infectious material required. rRT-PCR is very rapid, taking 3-4 hours to obtain results from a single assay. When reagent and labor costs are analyzed, it is less expensive on a cost-per-sample basis than virus isolation.

The matrix gene primer and probe set was designed to detect all subtypes of influenza A and targets a conserved region of the M gene. The matrix PCR assay is able to detect $10^{-1}$ EID$_{50}$ of virus (63). This is more sensitive than virus isolation at times. Even though one-tenth or less of the sample material is used than for virus isolation, the procedure is optimized to detect fewer virions than what is required to cause hemagglutination.

Virus isolation is only able to detect live virus, where rRT-PCR detects viral RNA, viable or not (63). This can be beneficial or detrimental. It will allow the detection of influenza strains that may not be adapted to grow in embryonating chicken eggs, but it will also detect viral RNA from specimens where the virus is no longer actively replicating. The presence of PCR inhibitory substances, inefficient RNA extraction, or RNA degradation will potentially yield false negatives. The potential for false positives is minimized by the use of a sequence-specific probe in the reaction mix, however, primer and/or probe incompatibility with the target virus will also result in a false negative.

1.2.6 Prevention and Control

**Economics**

The economic impact of avian influenza varies in significance based on the species infected, the strain of virus, and the number of infected animals or farms (75). The
greatest economic losses have typically occurred during commercial poultry outbreaks on farms with intensive production units or in large live bird market (LBM) systems. Direct losses include depopulation and disposal costs, mortality losses, quarantine and surveillance costs, and indemnities paid for elimination of market birds from infected premises. Historically, low pathogenic AI outbreaks that do not involve subtype H5 or H7 viruses incur lower economic losses because infected flocks have been depopulated through a controlled marketing program and lower mortality rates. It is current practice in the United States to depopulate poultry flocks that are infected with subtype H5 or H7 viruses in order to reduce the risk of virus mutation into a highly pathogenic form. Therefore, these subtypes are associated with higher economic costs.

For example, the 2002 LPAI H7N2 outbreak in Virginia cost approximately $130 million USD (2). Approximately $14 million was spent on a task force response by the US government, and the Commonwealth of Virginia contributed $1 million. The United States Department of Agriculture (USDA) also paid approximately $67 million in indemnities. The indemnity value reimbursed growers 100% of their cost for flocks, followed by the poultry producers, with the total indemnity paid equal to 50% of the losses for the industry.

**Vaccination**

Influenza vaccines are commonly used in the human population (16). These vaccines are composed of combined human influenza strains: H3N2, H1N1, and influenza B virus. In contrast, influenza vaccines are much less commonly used in poultry. The usual method used for control is the culling of infected poultry (41). Vaccination has not been used in the past because field viruses may still be able to replicate undetected. Additionally,
antibody detected as a result of outbreak and post-outbreak surveillance activities will not indicate whether the antibody is a result of vaccination or natural infection. Until recently, there has not been a method available to differentiate between natural and vaccine infection (12). Naïve and vaccinated birds have been shown to be susceptible, in one specific study, to H7N3 virus at the same dose; however, the vaccinated birds show decreased viral shedding and a shorter duration of infection. Additionally, in this particular study the challenged birds did not present any clinical signs of infection while the unvaccinated controls showed depression associated with mild diarrhea and respiratory signs. Consequently, vaccination may reduce the viral load in the surrounding environment and may well decrease the potential of a low pathogenic virus to mutate into a highly pathogenic form due to the decreased virus replication and length of infection period. Recently, oil-emulsion vaccines have been created with the same H type and a different N type (i.e. H7N1 vaccine). This allows differentiation between natural infection and vaccine by doing an assay specific for anti-N antibodies. In this situation, the N type of the virus that is currently circulating must be different than the N type used in the vaccine. If they are not different, the DIVA technique cannot be used for differentiation.

Killed vaccines are produced in egg culture, and the birds develop antibodies to both internal and external antigenic determinants (72, 75). Antibody to these proteins can still be detected by AGID and ELISA, preventing efficient differentiation between naturally infected birds and vaccinated birds. The inability to differentiate is one of the primary reasons whole killed vaccines are rarely used in an outbreak situation. Another disadvantage to vaccination is the need to vaccinate against many different subtypes. In 1987, Minnesota turkey flocks were infected with at least six different HA types. Current vaccines are only specific to one
subtype, and efforts are being put forward to develop a universal vaccine to protect against all HA subtypes.

Live attenuated vaccines have a distinct advantage over killed vaccines by triggering mucosal immunity and a cell-mediated immune response (81). This gives the vaccine more potential for cross protection and longer-lasting immunity. In addition, the viruses can be administered by aerosol or in drinking water, making them a much cheaper alternative to killed vaccine that must be administered by injection. If a portion of the NS1 gene is deleted, the action of the NS1 is blocked and a virus can be effectively attenuated. Studies with this vaccine show that the virus cannot be efficiently transmitted from animal to animal and, because the truncated protein is not packaged in progeny virions, the antigenicity of the virus is not changed. These modified viruses stimulate a strong dendritic cell response and are potent immunogens. In the occurrence of a natural infection, the vaccine strain can be differentiated from a field strain by analysis of the NS1 protein.

**Antiviral chemotherapy**

Anti-influenza drugs approved for use in humans include the M2 blockers amantadine and rimantadine and the neuraminidase inhibitors oseltamivir and zanamivir (91). Amantadine blocks the migration of hydrogen ions into the endosomal interior of the virus via the M2 ion channel. Amantadine prevents the acidification of the endosome and therefore the uncoating of the viral particle. The neuraminidase inhibitor drugs oseltamivir and zanamivir work by preventing the viral hemagglutinin from binding to the host cell
receptor containing N-acetylneuraminic acid and inhibiting the release of the progeny virus from the infected cell.

The approved antiviral medications for influenza are currently only approved for human use. There is currently no practical chemotherapy for the large-scale treatment of poultry or other livestock (75). There is evidence indicating the extensive use of amantadine on poultry farms in Asia may have contributed to the increase in amantadine-resistant influenza (39). Supportive care and antibiotic use may be used to reduce the amount of disease seen in concurrent infections with bacteria and influenza.

**Control of Avian Influenza**

Avian influenza virus within a flock is spread from bird-to-bird via aerosol inhalation and ingestion (75, 91). Contaminated poultry feces is likely responsible for transmission between flocks. The spread of influenza virus from an infected commercial flock can be controlled by implementing appropriate biosecurity including movement control, the use of artificial and natural boundaries, and husbandry practices. Equipment that comes in direct contact with birds or their manure should not be moved from farm to farm without disinfection.

One industry program that has been implemented in the United States includes education, preventing exposure, monitoring, reporting, and a “responsible response” (75). After the detection of disease, the affected producer is responsible for promptly reporting the outbreak in order to prevent unnecessary spread to nearby producers. In order to eradicate the virus using the above mentioned plan, producers voluntarily quarantine the flock to prevent transmission. The flocks may be marketed in a well-timed manner after
seroconversion when the virus is not associated with a high risk of contamination. After the last flock on a farm becomes infected the farm should impose a four week delay before repopulating the farm. New flocks should be managed separately to prevent infection of the newly added birds.

OIE defines international standards designed to control the spread of avian influenza (49). List A diseases are transmissible and carry the potential to cause widespread disease, affecting international trade. HPAI is a list A disease. Countries are required to make information regarding HPAI outbreaks available to OIE, which is responsible for making other countries aware of the HPAI infection status. In addition, OIE has required the notification of any isolation of subtype H5 or H7 from domestic poultry, regardless of pathogenicity, due to the risk of mutation into highly pathogenic forms of the disease. This provides a high level of protection to importing nations, upholding obligations and ethics in international trade.

1.2.7 References


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1.3 STATEMENT OF PROBLEMS AND OBJECTIVES

The increased availability of molecular tools for the characterization of pathogens has provided scientists and researchers the means to investigate and identify biological occurrences that were previously thought to happen infrequently or not at all. Increased in-depth study of influenza viruses has shown that inter-species transmission of viruses occurs more frequently than believed, even though the establishment of the virus in a new host as a stable lineage is infrequent. The outbreak of Asian H5N1 highly pathogenic avian influenza has increased the awareness of the threat of the pandemic potential of animal pathogens. As of 2008, these viruses have not been able to establish themselves in a human host long enough to efficiently transmit from human to human. However, given enough time, it is a possibility that the virus may adapt to the human host and cause a pandemic.

In 2006, an influenza virus novel to swine populations (H2N3) was identified in two independent swine herds in Missouri. Molecular characterization of these isolates revealed that the hemagglutinin and neuraminidase surface proteins were avian origin while the internal proteins were swine origin, demonstrating reassortment between avian influenza virus (AIV) and swine influenza virus (SIV). As swine are felt by many to be a “mixing vessel” for the transmission of influenza viruses between birds and humans, this isolation raises the question for potential transmission to humans. The H2 influenza subtype has not been isolated from humans since the late 1960s, leaving a naïve human population susceptible to the introduction of this virus.

At approximately the same time (early 2007), the same influenza subtype (H2N3) was isolated from two independent backyard poultry flocks in Ohio. This isolation
immediately raised questions: a) whether both swine and avian isolates have originated from similar or the same viruses and b) how widely the virus is spread. Therefore, molecular and antigenic characterization and comparison of the H2N3 AIV and the H2N3 SIV was necessary to determine their relatedness.
CHAPTER 2: Identification and characterization of H2N3 avian influenza virus from backyard poultry and comparison to novel H2N3 swine influenza virus

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2.1 Abstract

In early 2007, H2N3 influenza virus was isolated from a duck and a chicken in two separate backyard poultry flocks in Ohio. Since the same subtype influenza virus with hemagglutinin (HA) and neuraminidase (NA) genes of avian lineage was newly identified in a swine herd in Missouri in 2006, the objective of the study was to characterize genetic, antigenic and biological properties of the avian isolates and compared to the swine isolates. Avian isolates were low pathogenic by in vivo chicken pathogenicity testing. Sequencing and phylogenetic analyses revealed that all genes of the avian isolates were comprised of avian lineages whereas the swine isolates contain contemporary swine internal gene segments, demonstrating that the avian H2N3 viruses were not derived from the swine virus. Sequence comparisons for the HA and NA genes demonstrated that the avian viruses were
similar, but not identical, to the swine viruses. Accordingly, the avian and swine isolates were also antigenically related as determined by hemagglutination-inhibition (HI) and virus neutralization (VN) assays, suggesting that both avian and swine viruses originated from the same group of H2N3 avian influenza viruses. Although serological surveys using the HI assay on poultry flocks and swine herds in Ohio did not reveal further spreading of H2 virus from the index flocks, continuous surveillance will be necessary to ensure that transmission has not occurred. Contemporary H2N3 avian influenza viruses appear to be easily adaptable to poultry and swine, raising concern regarding the potential for inter-species transmission of avian viruses to humans.

2.2 Introduction

There are currently 16 identified hemagglutinin (HA) subtypes of influenza A viruses. All 16 subtypes have been detected in wild birds. These avian influenza viruses (AIVs) cause little to no disease in their natural hosts, with virus replication primarily occurring in the gastrointestinal tract. Poultry and swine are unnatural hosts for AIVs that are found in wild birds, and infection with wild bird AIV in these species typically cause mild to severe respiratory disease.

Major HA subtypes circulating in human and swine populations have been restricted to H1 and H3 for approximately 40 years; H2 has been absent in the human population since 1968 and in the US poultry population since 2005. In 2006, however, a novel influenza subtype (H2N3) was identified in a swine herd in Missouri. The HA and neuraminidase (NA) genes of the virus were found to be avian in origin, raising concern regarding the potential for inter-species transmission of avian viruses to swine and humans. Shortly after
these isolations, the same subtype was identified in a duck and a chicken from two separate backyard poultry flocks in Ohio in early 2007. The nearly coincidental isolation of the same subtype in unnatural hosts in the Midwest raised questions about the origin of the viruses and the potential for these viruses to adapt to a new host and spread to nearby flocks or herds. An outbreak of a novel subtype could have a severe impact on the poultry and swine industries across the United States. The following study was conducted to characterize biological, genetic and antigenic properties of the avian H2N3 isolate in comparison to the swine H2N3 isolates. In addition, a serological survey was conducted to assess spreading of the virus.

2.3 Materials and Methods

Viruses. Avian influenza (AI) viruses [A/duck/OH/492493/07 (H2N3) and A/chicken/OH/494832/07 (H2N3)], tracheal and cloacal swabs from backyard poultry flocks, were routine surveillance samples collected from live bird markets in Ohio. Swab samples were screened for type A influenza viruses using a real-time RT-PCR test targeting the influenza matrix gene (10) at The Ohio Department of Agriculture ADDL and PCR-positive samples were submitted to NVSL and processed for virus isolation in embryonating chicken eggs (ECE) per standard procedures (13). Swine influenza viruses [A/Swine/Missouri/4296424/2006 (H2N3) and A/Swine/Missouri/2124514/2006 (H2N3)] (5) were kindly provided by Dr. Marie Gramer at the University of Minnesota.

Animal Studies and Antiserum Production. All animal studies were conducted in BSL-3 Agriculture laboratory space as required for work with AI virus. All animal care was provided as directed by the Institutional Animal Care and Use Committee of the National
Veterinary Services Laboratories. In all studies the birds were housed with *ad libitum* access to feed and water.

*In vivo* pathogenicity studies on the avian H2N3 isolates were performed in accordance with the World Animal Health Organization (OIE) procedures (15). In brief, ten 6-week old specific-pathogen-free chickens were inoculated by the intravenous route with 0.1 ml of amniotic-allantoic fluid (HA titer $2^5$, diluted 1:10 in sterile 0.1 M phosphate-buffered saline (PBS), pH 7.2). The animals were held and observed daily for sickness or death for 10 days post inoculation (PI) to determine the intravenous pathogenicity index (IVPI). At each observation each animal was scored 0 if normal, 1 if sick, 2 if severely sick, and 3 if dead. Birds were determined to be sick if one of the following signs were observed, and severely sick if two or more of the signs were observed: respiratory distress, sneezing, coughing, diarrhea, cyanosis of exposed skin or wattles, edema of the face or head, and nervous signs. Birds found to be too sick to eat or drink were euthanized humanely and scored dead at the next observation. The IVPI is calculated as the mean score per bird per observation over the 10-day period. Viruses with an IVPI greater than 1.2 are considered to be highly pathogenic.

Four 7-week old chickens were inoculated with 4 ml of undiluted amniotic-allantoic fluid [A/chicken/OH/494832/07 (10$^{6.9}$ EID$_{50}$/ml) or A/Swine/Missouri/4296424/2006 (10$^{7.1}$ EID$_{50}$/ml)] by the intravenous route for antiserum production. Serum was collected from each animal after 14 days PI. The presence of antibody was confirmed by the agar gel immunodiffusion (AGID) test and ELISA (AI MultiS-Screen Ab ELISA, IDEXX, Westbrook, ME).
Subtyping. The viruses were tested for hemagglutinating activity. Hemagglutination-inhibition (HI) and neuraminidase inhibition (NI) assays (7, 8) were performed with a panel of standard antigens and sera for H1-15 and N1-9 produced at the National Veterinary Services Laboratories (NVSL). The viruses were diluted to a standard concentration of 4 hemagglutinating units (HAU) for the HI test (25 µl/well). Serum was diluted two-fold and incubated with antigen at ambient temperature for 30 minutes. Rooster red blood cells (0.5%) were added and incubated for 15-20 minutes at ambient temperature before reading.

The NI procedure was performed as previously described (14). In brief, viruses were diluted and mixed with subtype-specific standardized neuraminidase antiserum. Following incubation, fetuin from fetal calf serum was added to each sample and incubated for 3 hours. Periodate reagent, arsenite reagent and thiobarbituric acid were added to induce a chromatic reaction if neuraminidase activity was not inhibited by subtype-specific neuraminidase antibodies.

Cross-Reactivity Studies. Antigenic relatedness among the H2N3 isolates was assessed by HI and virus neutralization assays. Standard HI assay was performed using a panel of standard chicken sera (H1-H15), chicken serum produced against avian H2N3 (A/chicken/OH/494832/07) and chicken serum produced against swine H2N3 (A/Swine/Missouri/4296424/2006). Each virus was diluted to 4 HAU in PBS and tested. HI tests were each conducted in duplicate with chicken and turkey erythrocytes and repeated once.
Virus neutralization (VN) tests were performed using MDCK cells as previously described with minor modifications (4). The viruses were diluted to a target concentration of 100-300 TCID$_{50}$/ml for the VN test. Chicken sera generated against H2N3 avian and swine isolates were employed in the test. The sera were diluted 1:10 in infection media (1X minimum essential media with 1:1000 TPCK trypsin) and then by serial 2-fold dilution method. Each diluted serum (50 µl) was mixed with an equal volume of each virus preparation. After incubation at 37°C for 60 min, each virus-serum mixture (100 µl) was inoculated onto MDCK monolayer. Cells were then incubated at 37°C with 5% CO$_2$ supply. Viral infection of the MDCK cell monolayer by swine influenza virus (SIV) or avian influenza virus (AIV) was detected by observation of cytopathic effect and confirmed by indirect immunocytochemistry using a mouse monoclonal antibody that detects type A influenza virus nucleoproteins (ATCC). A rabbit anti-mouse IgG labeled with horseradish peroxidase (HRP) was used as a secondary antibody (SouthernBiotech). Staining was carried out using 3-amino-9-ethylcarbazole (AEC) substrate (Sigma). Back titrations were performed to confirm a working virus concentration of 100-300 TCID$_{50}$/0.1 ml. The VN titer of each serum against each virus was determined as reciprocal of the highest dilution in which no staining (i.e., no replication) was observed. VN tests were each conducted in duplicate and repeated once.

**RT-PCR and Sequencing.** All eight gene segments from the virus isolates were sequenced after no more than 3 passages in ECE. RNA was extracted with the MagMAX™ AI/ND Viral RNA Isolation Kit (Ambion/Applied Biosystems, Austin, TX) in accordance with manufacturer instructions. Individual influenza genes were amplified by RT-PCR as
previously described (11). The amplicons were purified directly with the QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA) or from agarose gels with the QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, CA). The purified PCR products were submitted to and sequenced at the DNA Facility of the Iowa State University Office of Biotechnology.

Sequencing primers are listed in Table 1.

Table 1. Primers used for amplification and direct sequencing.

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<th>Gene</th>
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</tr>
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</tr>
<tr>
<td></td>
<td>H-T7†</td>
<td>TAATACGACTCATAAGTAGAAACAAGGGTG</td>
</tr>
<tr>
<td>H2</td>
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<tr>
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<td>H2-528</td>
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<td>H2</td>
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<tr>
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<td>H2-1120</td>
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<td>M+5†</td>
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<td></td>
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<td>M+605</td>
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<td></td>
<td>PA+1224†</td>
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Table 1. (continued)
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<td>PB1-1128</td>
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<td>GCAAGCTAGTCCGAATCAA</td>
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<td>PB1-1840</td>
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<td></td>
<td>PB2+1630</td>
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†Indicates primer used for RT-PCR amplification of RNA
**Phylogenetic and Sequence Analysis.** Sequences were aligned with the Clustal W method (Lasergene 6.0, DNASTar, Madison, WI). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (www.megasoftware.net) using the Neighbor-Joining tree building method, with 1000 bootstrap replicates. For the HA gene, separate phylogenetic groups were defined by less than 96.0% identity among the genes. Analysis with all genes was done with genes from viruses which were phylogenetically representative of other lineages and included genes from viruses from all possible sources; wild birds, poultry, mammals and from all geographical regions including North America, Europe and Asia (2).

**Surveillance.** A total of 1272 serum samples from swine herds randomly located throughout Ohio were collected between May 2008 and September 2008 and were tested at the NVSL for H2 antibody by the standard influenza HI assay (7) against A/swine/Missouri/4296424/2007. A serum control was also performed on each specimen. Equal parts of serum and PBS (25 µl) were mixed and 50 µl of 0.5% RBC were added. Samples were assessed for hemagglutination. Specimens that demonstrated hemagglutination (HA) on the serum control were subsequently treated with receptor-destroying enzyme from Vibrio cholerae (Lonza, Walkersville, MD), heat inactivated at 56°C for 30 min, and tested again for HA. Specimens that continued to demonstrate hemagglutination were identified as no test and disregarded in the data analysis.

Statewide backyard and pre-slaughter poultry surveillance was conducted at the Ohio Department of Agriculture Animal Disease Diagnostic Laboratory (ADDL) in 2007 and 2008. All specimens (n=81,706) were initially tested by the AGID assay. AGID-positive
serum samples (n = 865) were submitted to the USDA National Veterinary Services Laboratories (NVSL) and tested by HI and NI assays to determine the subtype of the virus.

2.4 Results

Virus Characterization. The viruses isolated were subtyped as H2N3. The viruses isolated were determined to be low pathogenic avian influenza (IVPI = 0). No clinical signs were observed by the animal care staff and all birds were scored as normal during the 10 day observation period. All 8 genes of the avian H2N3 viruses were sequenced and analyzed for similarity. The identities of the viral genes between the two avian H2N3 viruses ranged from 99.6% to 100% (Table 2).

Table 2. Sequence homologies of each gene segment between 2 avian H2N3 viruses (A/duck/OH/492493/07 and A/chicken/OH/494832/07) from backyard flocks in Ohio

<table>
<thead>
<tr>
<th>Gene Segment</th>
<th>Percent Identity, nt</th>
<th>Percent Identity, aa</th>
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</thead>
<tbody>
<tr>
<td>Hemagglutinin</td>
<td>99.8</td>
<td>99.6</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>99.9</td>
<td>99.8</td>
</tr>
<tr>
<td>Non-Structural</td>
<td>99.9</td>
<td>99.5</td>
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<tr>
<td>Matrix</td>
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<td>100</td>
</tr>
<tr>
<td>Nucleoprotein</td>
<td>99.9</td>
<td>99.8</td>
</tr>
<tr>
<td>Polymerase A</td>
<td>99.9</td>
<td>100</td>
</tr>
<tr>
<td>Polymerase B1</td>
<td>100</td>
<td>99.9</td>
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<tr>
<td>Polymerase B2</td>
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</tr>
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</table>
A BLAST search revealed that HA genes from both H2N3 viruses most closely matched other North American wild bird H2 viruses (Table 3). The NA genes of both viruses were similar to that of a Northern Shoveler isolate from California in 2008. The internal genes were highly similar to recent wild bird isolates from Ohio, Maryland, and California.

Phylogenetic analyses of all 8 gene segments indicated the avian H2N3 viruses belong to the North American avian lineage which is distinct from the Eurasian lineage of H2 viruses and the human lineage of H2 viruses isolated during and after the 1957 outbreak (1, 2) (Figures 1-2). Dendograms for the remaining 6 gene segments are located in the Appendix.

In comparison to the recent H2N3 viruses isolated from swine (5), the identity of the HA gene ranged from 95.9% to 96.4% between the avian and swine viruses and the identity of the NA gene ranged from 97.9% to 98.1% (Table 4). The internal genes (M, PB1, PB2, PA, NP, and NS) of the swine H2N3 isolates were consistent with contemporary triple reassortant swine influenza viruses while all 8 gene segments of the avian H2N3 viruses were consistent with avian lineages.
Table 3. Influenza A viruses with highest nucleotide sequence identity to recent H2N3 AIV isolated in backyard poultry in OH (A/duck/OH/492483/07/H2N3 and A/chicken/OH/494832/07/H2N3) as determined by BLAST search in the GenBank® and the Influenza Virus Resource Database®.

<table>
<thead>
<tr>
<th>Gene</th>
<th>% Identity</th>
<th>Virus Designation</th>
<th>Subtype</th>
<th>% Identity</th>
<th>Virus Designation</th>
<th>Subtype</th>
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</thead>
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<tr>
<td>PB1</td>
<td>99</td>
<td>A/mallard/Maryland/897/2004</td>
<td>H5N2</td>
<td>99</td>
<td>A/mallard/Maryland/897/2004</td>
<td>H5N2</td>
</tr>
<tr>
<td>PB2</td>
<td>98</td>
<td>A/environment/Maryland/251/2006</td>
<td>H7N3</td>
<td>98</td>
<td>A/environment/Maryland/251/2006</td>
<td>H7N3</td>
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</table>

Table 4. Sequence comparison of avian and swine H2N3 hemagglutinin and neuraminidase genes.

<table>
<thead>
<tr>
<th>Gene Segment</th>
<th>Virus 1</th>
<th>Virus 2</th>
<th>% Identity, nt</th>
<th>% Identity, aa</th>
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<tbody>
<tr>
<td>Hemagglutinin</td>
<td>A/duck/OH/492493/07</td>
<td>A/Sw/MO/2124514/2006</td>
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<td>97.9</td>
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<td>A/chicken/OH/494832/07</td>
<td>A/Sw/MO/4296424/2006</td>
<td>95.9</td>
<td>97.7</td>
</tr>
<tr>
<td>Neuraminidase</td>
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<td>98.1</td>
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<td>97.9</td>
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<td>98.2</td>
<td>98.5</td>
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<td></td>
<td>A/chicken/OH/494832/07</td>
<td>A/Sw/MO/4296424/2006</td>
<td>98.0</td>
<td>98.3</td>
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</table>

Further sequence comparisons of HA genes between avian and swine H2N3 isolates demonstrated that the amino acid sequence of the HA gene differed at codon 226, a key position in determining receptor binding specificity between mammals and birds (5). Both of the swine H2N3 viruses contained a leucine (L) at position 226 whereas the avian H2N3 viruses contained glutamine (Q). Aside from residue 226, there were no other differences between the avian and swine isolates at positions thought to be responsible for HA receptor binding. Amino acid (AA) positions 344 and 466 have been shown to affect host specificity by affecting the pH at which sialidase is active (12). There were no differences between the swine and avian viruses in these residues or other conserved residues in the active site of NA (AA positions 119, 292, and 294). Tables 5 and 6 show the amino acid differences in the HA and NA genes, respectively.
Figure 1. Phylogenetic analysis with hemagglutinin gene
Figure 2. Phylogenetic analysis with matrix gene

A chicken OH 494832 07 H2N3
A duck OH 492493 07 H2N3
A chicken PA 298101-4 2004 H2N2
A northern shovel California HKWF1...
A pintail Alberta 293 1977 H2N9
A mallard duck Alberta 323 1998 H2N1
A mallard ALB 202 1996 H2N5
A mallard duck ALB 894 1984 H2N5
A green-winged teal Ohio 175 1986 H2N1
A mallard Ohio 30 1986 H2N1
A mallard Alberta 79 2003 H2N3
A mallard duck ALB 376 1985 H2N3
A mallard ALB 205 1998 H2N3
A mallard ALB 226 1998 H2N3
A semi-palmated sandpiper Brazil 43 1...
A mallard duck ALB 353 1988 H2N3
A laughing gull NJ 75 1985 H2N9
A blue-winged teal ALB 16 1997 H2N9
A mallard ALB 201 1996 H2N3
A chicken New York 13828-3 1995 H2N2
A guinea fowl New York 20221-11 1995 ...
A mallard Alberta 77 1977 H2N3
A Duck Nanchang 20486 2000 H2N9
A duck Nanchang 4 184 2000 H2N9
A duck Hong Kong 319 1978 H2N2
A duck Germany 1215 1973 H2N3
A mallard Postdam 178-4 83 H2N2
A duck Hong Kong 278 1978 H2N9
A gull MD 19 1977 H2N9
A herring gull Delaware 471 1986 H2N7
A laughing gull NJ 798 1986 H2N7
A ruddy tumstone Delaware 34 1993 H2N1
A sanderling NJ 766 1986 H2N7
A herring gull DE 692 1988 H2N8
A herring gull DE 698 1988 H2N1
A herring gull DE 703 1988 H2N8
A herring gull Delaware 670 1988 H2N9
A turkey Ohio 313053 04 H2N2
A swine North Carolina 2003 H3N2
A swine MI PU243 04 H3N1
A swine IN PU542 04 H3N1
A swine Mississippi 2124514 2006 H2N3
A swine Missouri 4296424 2006 H2N3
A swine Tennessee 109 1977 H1N1
A swine Wisconsin 1 1961 H1N1
A Swine Colorado 1 77 H3N2
A Albany 1 1968 H2N2
A Berkley 1 68 H2N2
A Berlin 3 1964 H2N2
A North Carolina 1 1968 H2N2
A Moscow 1019 1965 H2N2
A Albany 26 1957 H2N2
A Ann Arbor 23 1957 H2N2
Table 5. Amino acid (AA) changes in the hemagglutinin gene among the avian and swine H2N3 viruses

<table>
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<tr>
<th>AA position</th>
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<th>Av/494832&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Sw/2124514&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Sw/4296424&lt;sup&gt;d&lt;/sup&gt;</th>
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<td>46/36</td>
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<td>172/162</td>
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<td>P</td>
<td>P</td>
<td>S</td>
</tr>
<tr>
<td>188/178</td>
<td>I</td>
<td>I</td>
<td>V</td>
<td>V</td>
</tr>
<tr>
<td>192/182</td>
<td>V</td>
<td>V</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>236/226</td>
<td>Q</td>
<td>Q</td>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td>284/274</td>
<td>T</td>
<td>T</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>291/281</td>
<td>R</td>
<td>R</td>
<td>K</td>
<td>K</td>
</tr>
<tr>
<td>326/316</td>
<td>V</td>
<td>V</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>331/321</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>V</td>
</tr>
<tr>
<td>399/389</td>
<td>I</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
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<td>429/419</td>
<td>L</td>
<td>L</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>456/446</td>
<td>R</td>
<td>R</td>
<td>K</td>
<td>K</td>
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<td>500/490</td>
<td>P</td>
<td>P</td>
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<td>P</td>
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<tr>
<td>520/510</td>
<td>D</td>
<td>D</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

<sup>a</sup>A/duck/OH/492493/2007 (H2N3)
<sup>b</sup>A/chicken/OH/494832/2007 (H2N3)
<sup>c</sup>A/swine/Missouri/2124514/2006 (H2N3)
<sup>d</sup>A/swine/Missouri/4296424/2006 (H2N3)
Table 6. Amino acid (AA) changes in the neuraminidase gene among the avian and swine H2N3 viruses

<table>
<thead>
<tr>
<th>AA position</th>
<th>Av/492493</th>
<th>Av/494832</th>
<th>Sw/2124514</th>
<th>Sw/4296424</th>
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</thead>
<tbody>
<tr>
<td>30</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>I</td>
</tr>
<tr>
<td>47</td>
<td>H</td>
<td>H</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>49</td>
<td>T</td>
<td>T</td>
<td>I</td>
<td>T</td>
</tr>
<tr>
<td>52</td>
<td>I</td>
<td>T</td>
<td>T</td>
<td>T</td>
</tr>
<tr>
<td>78</td>
<td>T</td>
<td>T</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>81</td>
<td>A</td>
<td>A</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>135</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>T</td>
</tr>
<tr>
<td>253</td>
<td>H</td>
<td>H</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>262</td>
<td>R</td>
<td>R</td>
<td>K</td>
<td>K</td>
</tr>
<tr>
<td>357</td>
<td>N</td>
<td>N</td>
<td>D</td>
<td>D</td>
</tr>
</tbody>
</table>

**Assessment of antigenic relatedness.** Cross-reactivity studies were conducted with the avian and swine viruses and serum to assess antigenic relatedness. The virus inhibition or neutralization titers were highest with the homologous virus/serum combinations (Table 7 and 8). There was significant difference (p<0.05) between HI titers using heterologous virus and serum (avian virus with swine serum or swine virus with avian serum) when chicken RBC were used in the test. There was less difference between the titers when turkey RBC were used. HI titers were significantly higher (p<0.05) when turkey RBC were used compared to the HI titer with chicken RBC except for the swine viruses tested against the avian serum. The VN titers were significantly different from the HI titers (p<0.05) using either chicken RBC or turkey RBC, with the exception of the avian virus/serum homologous
combination. Assay sensitivity decreased according to the following order: HI turkey RBC > VN > HI chicken RBC.

Table 7. Cross reactivity of avian and swine H2N3 viruses as determined by hemagglutination-inhibition (HI) assay using antisera produced in chickens against A/chicken/OH/494832/2007/H2N3 (A), A/swine/Missouri/24296424/2006/H2N3 (S), A/swine/IA/31/H1N1 (R1), or A/pintail/Alberta/293/77/H2N9 (R2). The HI assay was done using both chicken (top panel) and turkey (bottom panel) erythrocytes. Each serum was run in duplicate and the test was repeated once.

<table>
<thead>
<tr>
<th>SERUM</th>
<th>Avian 1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Avian 2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Swine 1&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Swine 2&lt;sup&gt;d&lt;/sup&gt;</th>
<th>REF 1&lt;sup&gt;e&lt;/sup&gt;</th>
<th>REF 2&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5.50±0.29</td>
<td>5.50±0.29</td>
<td>6.50±0.29</td>
<td>6.00±0.00</td>
<td>0.00±0.00</td>
<td>4.00±0.00</td>
</tr>
<tr>
<td>S</td>
<td>4.50±0.29</td>
<td>4.50±0.29</td>
<td>6.50±0.29</td>
<td>5.50±0.29</td>
<td>0.00±0.00</td>
<td>3.00±0.00</td>
</tr>
<tr>
<td>R1</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>7.50±0.29</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>R2</td>
<td>5.50±0.29</td>
<td>5.25±0.25</td>
<td>7.00±0.00</td>
<td>5.75±0.48</td>
<td>0.00±0.00</td>
<td>6.50±0.29</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>SERUM</th>
<th>Avian 1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Avian 2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Swine 1&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Swine 2&lt;sup&gt;d&lt;/sup&gt;</th>
<th>REF 1&lt;sup&gt;e&lt;/sup&gt;</th>
<th>REF 2&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>7.00±0.00</td>
<td>7.00±0.00</td>
<td>7.75±0.25</td>
<td>8.00±0.00</td>
<td>0.00±0.00</td>
<td>5.00±0.00</td>
</tr>
<tr>
<td>S</td>
<td>6.00±0.00</td>
<td>6.00±0.00</td>
<td>8.00±0.00</td>
<td>8.00±0.00</td>
<td>0.00±0.00</td>
<td>3.50±0.29</td>
</tr>
<tr>
<td>R1</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>8.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>R2</td>
<td>6.00±0.00</td>
<td>5.50±0.29</td>
<td>8.00±0.00</td>
<td>5.00±0.00</td>
<td>0.00±0.00</td>
<td>7.50±0.29</td>
</tr>
</tbody>
</table>

<sup>a</sup>A/duck/OH/492493/2007 (H2N3)
<sup>b</sup>A/chicken/OH/494832/2007 (H2N3)
<sup>c</sup>A/swine/Missouri/2124514/2006 (H2N3)
<sup>d</sup>A/swine/Missouri/4296424/2006 (H2N3)
<sup>e</sup>A/swine/IA/31 (H1N1)
<sup>f</sup>A/pintail/Alberta/77 (H2N9)
Table 8. Cross reactivity of avian and swine H2N3 viruses as determined by serum-virus neutralization (SVN) assay using antisera produced in chickens against A/chicken/OH/494832/2007/H2N3 (A) or A/swine/Missouri/4296424/2006/H2N3 (S). Each serum was run in duplicate and the test was repeated once.

<table>
<thead>
<tr>
<th>SERUM</th>
<th>SVN titer (mean±SEM in log$_2$) against the following virus:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avian $^a$</td>
</tr>
<tr>
<td>A</td>
<td>7.32±0.41</td>
</tr>
<tr>
<td>S</td>
<td>6.82±0.29</td>
</tr>
</tbody>
</table>

$^a$A/duck/OH/492493/2007 (H2N3)
$^b$A/swine/Missouri/4296424/2006 (H2N3)

**Surveillance.** Out of 1272 serum samples collected from swine, there were five samples that demonstrated hemagglutination on serum control so they were eliminated from the data analysis. None of the specimens (n=1267) were positive for H2N3, indicating there has not been any infection of this subtype in swine herds across Ohio.

The Ohio Department of Agriculture ADDL tested 36,604 poultry specimens (chicken and turkey) in 2007 and 45,102 poultry specimens in 2008 by the AGID test. All AGID positive specimens (n=865) were submitted to the NVSL for confirmation and subtyping. None of the specimens were positive for H2N3, indicating that H2N3 AIV is not currently circulating among backyard or commercial poultry in Ohio.

#### 2.5 Discussion

Molecular and phylogenetic analyses of the recent influenza viruses isolated from poultry revealed that the H2N3 viruses were similar to North American avian lineage H2 viruses, demonstrating no introduction of a Eurasian strain. All eight gene segments from these viruses were avian lineage, clearly demonstrating that the virus was derived from a
wild bird H2N3. Each gene segment of the avian isolates was also compared molecularly and phylogenetically to the corresponding segment of the recent H2N3 swine isolates. The internal genes (M, PA, PB1, PB2, NP and NS) of the swine H2N3 isolates were consistent with contemporary triple reassortant swine influenza viruses (i.e., swine-human-avian) while HA and NA genes were of avian lineage in origin, demonstrating that the SIV is a reassortant between AIV and SIV. When the HA and NA genes of the swine and avian viruses were compared, there was a high degree of homology (approximately 96% and 98% for HA and NA genes, respectively), however, they were not identical. Both infected premises in Missouri were using surface water collected in ponds as a water source for cleaning barns and watering animals (5). These ponds may have been used by migrating waterfowl infected with H2N3 AIV, consequently contaminating the water and introducing the novel HA and NA to the animals creating the potential for reassortment with influenza viruses already present in the herds. The observed divergence of HA and NA nucleotide sequence between the swine and avian H2N3 viruses suggests that the HA and NA genes of the H2N3 SIV were derived from a common predecessor of the current H2N3 AIV or was circulating undetected in swine or other susceptible species for some time after reassortment.

Antigenically, the avian and swine isolates showed strong cross-reactivity on both the HI test and the VN test. All viruses were able to be subtyped using standard avian origin antiserum in the HI test. The overall antibody titers were higher when tested by the VN assay than HI assay. The molecular analysis of the avian viruses indicates a wild bird lineage origin. It has been reported that the HI assay using chicken erythrocytes has a decreased sensitivity for antibodies to Asian H5N1 influenza from wild birds (3). The wild bird H2N3 viruses may also have a decreased ability to bind chicken erythrocytes causing a lower HI
titer, and likewise the swine viruses may have a decreased affinity for chicken erythrocytes. It appears the decreased affinity can be overcome by using turkey erythrocytes in the assay. The HI titers are more similar to the VN titers when turkey erythrocytes were used. It is possible that the antibody to antigen ratio required for hemagglutination-inhibition and virus neutralization may be different for the two assays. The assays both target the hemagglutinin surface protein, however, multiple epitopes are present and it is likely that the results of each assay react with different epitopes or regions of the protein causing slight variations in titer. In addition, the virus in each assay is diluted to a different standardized concentration which may affect the endpoint of detection. Further studies remain to be carried out to determine if the antibodies are cross-protective in vivo.

The nearly coincidental isolation of a novel influenza subtype in swine and an influenza subtype that has sporadically been isolated from poultry suggests that there has been a shift in the H2N3 wild bird viruses that are currently circulating in the United States. There may be an increased prevalence of wild birds affected with H2N3 AIV causing increased exposure to livestock and poultry reared outdoors. Information published by Panigrahy, et.al. and Slemons et.al suggest that while H2 influenza viruses are found in wild birds, the prevalence of this subtype in the past has been much less than other common subtypes such as H3, H4, and H6 (6, 9). Virus isolation from ongoing North American wild bird surveillance remains to be completed in order to determine the current prevalence of H2N3 viruses in wild birds. There may also have been an antigenic shift in the virus which has increased the susceptibility of unnatural hosts to the virus or increased the virus’ ability to adapt to such a host. Although additional studies should be carried out to determine the susceptibility and transmissibility of these viruses within and among species, a few
observations suggest that the H2N3 SIV or AIV would not be an immediate threat to poultry and swine, respectively, at this moment. Despite the evidence of limited transmission to poultry and swine, animals raised outdoors for commercial or hobby purposes are at an increased risk of acquiring AIV due to increased exposure to wild birds infected with AIV. First, the amino acid sequence of the HA gene differed between the avian and swine viruses at codon 226, a key position in determining receptor binding specificity between mammals and birds. While both of the swine H2N3 viruses contain a leucine (L) at position 226, the avian H2N3 viruses contain glutamine (Q) as consistent with typical avian influenza viruses. The Q→L substitution has been shown to accompany virus adaptation from avian to human hosts (5). Second, serological survey of the swine population spanning May 2008 through September 2008 did not indicate that there has been any avian H2 infection in Ohio swine herds. There were two specimens that reacted with the swine H2N3 virus, however, these results are considered equivocal considering the number of specimens tested. Nonetheless, continuous monitoring for interspecies jump or the emergence of a new subtype should be in place since: a) a single amino acid change may alter the host specificity; b) most contemporary swine influenza viruses contain polymerase genes of avian lineage or human lineage; and c) spatial proximity between swine herds and poultry flocks continue to decrease due to growth of both industries.

2.6 References


CHAPTER 3. GENERAL DISCUSSION AND CONCLUSIONS

A novel subtype of influenza A virus, H2N3 was isolated from swine herds in Missouri in 2006. Shortly afterwards, the same subtype was isolated from two backyard poultry flocks in Ohio. Molecular and antigenic analysis was conducted to assess the similarity of the swine and avian viruses. The molecular analysis indicates that the hemagglutinin (HA) and neuraminidase (NA) genes are highly similar with amino acid differences in at least one position that is critical to HA binding in avian vs. mammalian hosts. The HA and NA genes from both species were found to be derived from a common predecessor avian influenza virus of wild bird origin. This suggests that this particular virus may be easily transmitted to aberrant host species. Wild birds shed the virus in feces contaminating environments that may be shared with livestock if the animals are reared outdoors or if ponds are used as a watering source. As evidenced by the infection of the swine herds, reassortment events are likely to occur if the herd is also infected with a naturally occurring influenza virus (i.e. H1N1 or H3N2).

Infections of two unnatural host species with a wild bird origin avian influenza virus reinforces the need for good biosecurity practices by commercial swine producers in order to prevent introduction of new viruses into herds. In addition, swine influenza surveillance should include tests that will detect subtype H2 influenza in order to detect continued circulation of the virus. The National Poultry Improvement Plan has already incorporated a surveillance system that will detect these novel subtypes in poultry. In addition to the work that is presented in this paper, future studies should be conducted to determine the transmissibility of these viruses within and among species as well as the pathogenicity of each virus to the other host species (i.e., swine virus to poultry and avian virus to swine).
Figure 3. Phylogenetic analysis with non-structural gene.
Figure 4. Phylogenetic analysis with neuraminidase gene
Figure 5. Phylogenetic analysis with polymerase A gene
Figure 6. Phylogenetic analysis with polymerase B1 gene
Figure 7. Phylogenetic analysis with PB2
Figure 8. Phylogenetic analysis with nucleoprotein gene.
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