DISSERTATION

AVIAN INFLUENZA A VIRUS TRANSMISSION AND THE EMERGENCE OF DRUG RESISTANCE

Submitted by

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ABSTRACT

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As avian influenza A viruses (AIV) continue to circulate worldwide both naturally, within the reservoir host of wild waterfowl, and cross species barriers, eventually establishing itself in new host species, it is imperative to study the natural reservoir in respect to virus change and transmissibility. This dissertation will focus on the transmissibility of a mallard virus from mallards to other wild and domestic species as well as elucidate the possible outcomes of oseltamivir contamination in the environment and its effect on influenza A virus infected mallards.

Low pathogenicity (LP) AIVs of the H5N2 and H7N3 subtypes were utilized to evaluate the ability of transmission of a mallard derived virus to other species present in a co-habitation (barnyard) scenario. Other species in contact with the mallards were chickens, blackbirds, rats, and pigeons. Viral replication was assessed directly from ducks in the barnyard with assessment of the other animals in the barnyard through sero-conversion. Additional animals of each species were directly inoculated with these two viruses and assessed for viral replication. The H5N2 virus was transmitted to other ducks and chickens in the barnyard through either direct or environmental contamination, but not to rats or blackbirds. The H7N3 virus was transmitted to other ducks, chickens, pigeons, and rats.

Chickens and blackbirds directly inoculated with both virus strains shed significant amount of virus and seroconverted, but rats and pigeons (except for one pigeon) failed to shed virus but did develop antiviral antibodies. Knowing that both mallard viruses can directly transmit without adaptation, show the mallard to be a good model to further evaluate the outcome of oseltamivir contamination in the environment and its effect on AIV infected mallards.

The environment has been shown to be contaminated with significant amounts of oseltamivir carboxylate (OC) in an area of high drug prescription use. We analyzed the outcomes of AIV in infected mallards when they have access to OC in their drinking water. Two separate LPAIV H5N2 viruses were tested for their ability to mutate under drug pressure. One H5N2 virus did not demonstrate any altered sequence after 7-10 days of drug access and infection. The other H5N2 virus did show mutations in the neuraminidase gene that led to an increase in resistance to oseltamivir caused by a specific mutation at E119V. This resistant virus was further evaluated for its ability to transmit between infected and naïve mallards. While the resistant virus did transmit duck to duck, the mutation at position 119 was not detected after challenge or transmission showing instability of this mutation. This could either be a reversion to wild-type or possibly the low level presence of wild-type present in the resistant strain stock that outcompeted with the mutant strain to succeed in the host. This shows, that in these duck experiments, the E119V mutation is not stable in the absence of drug pressure and unlikely to succeed in the host.

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CHAPTER 1: REVIEW OF LITERATURE

Overview

Avian influenza has been known as a disease of birds since the late 1800's and the virus has continued evolving and adapting throughout the world to the present day. Avian influenza has not only affected the wild avian population and domestic avian species but has adapted to infect humans, pigs, horses, cats, dogs, sea mammals, and other land mammals, with certain strains establishing themselves in these new hosts. Since 1918, there have been five major influenza pandemics affecting humans, with the most recent in 2009 that left us with many more questions than answers in how to battle this disease. The recent outbreak of highly pathogenic H5N1 virus that was first seen in humans in 1997, with a resurgence in 2003, stimulated the scientific community to develop many vaccines to combat constantly evolving virus strains and to evaluate numerous antiviral medications. Creating a new vaccine at the start of a pandemic would take up to 6 months, thus management of the outbreak will require the use of antiviral drugs. Several anti-influenza antivirals have been evaluated for protection from highly pathogenic strains of avian influenza virus, but only a select few have been approved and stockpiled for use. In humans, the increase in drug resistant strains in response to the most commonly stockpiled drug is concerning, as is the predicted environmental buildup of these drugs if used worldwide in mass quantities. These concerns strongly argue for studying the potential outcomes of these problems before the next pandemic arrives.

Classification of Influenza Viruses

Influenza viruses are members of the *Orthomyxoviridae* family and classified into five genera: influenza A, influenza B, influenza C, Thogotovirus, and Isavirus (Kawaoka et al, 2005). All influenza viruses contain a segmented, linear, negative sense, single stranded RNA genome. The number of segments differs with influenza A and B viruses and Isavirus containing 8 segments (McGeoch et al, 1976, Palese & Schulman, 1976a, b, Palese et al, 1980, Mjaaland et al, 1997), influenza C virus having 7 segments (Palese et al, 1980), and Thogotovirus containing 6 segments (Clerx et al, 1983). This review will focus on influenza A viruses, and its 8 segment encoding properties are summarized in Table 1.1.

Table 1.1. Influenza A virus genome

Segment	Length (nt)	Encoded polypeptide	Protein name
1	**2341**	**PB2**	**Polymerase basic 2**
2	**2341**	**PB1**	**Polymerase basic 1**
		PB1-F2	
3	**2233**	**PA**	**Polymerase acidic**
4	**1778**	**HA**	**Hemagglutinin**
5	**1565**	**NP**	**Nucleoprotein**
6	**1413**	**NA**	**Neuraminidase**
7	**1027**	**M1**	**Matrix 1**
		M2	**Matrix 2**
8	**890**	**NS1**	**Nonstructural 1**
		NS2	**Nonstructural 2**

Structure of Influenza A Viruses

Influenza virus particles are considered pleomorphic and can appear spherical or filamentous in appearance (Bourmakina & Garcia-Sastre, 2003, Chu et al, 1949). All influenza viruses contain a protein and RNA core that is surrounded by a lipid layer, or

envelope. There are three viral integral membrane proteins, with two that extend externally from the lipid layer: the hemagglutinin (HA) and neuraminidase (NA). The HA protein resembles a spike shaped trimer extending outward from the lipid layer and is the most abundant viral surface protein (Compans et al, 1970). The HA protein has two major functions. First, it binds to sialic acid receptors on the host cell via the receptor binding site in a pocket located on each subunit, leading to the attachment of virus to the host cell. Second, the HA protein is required for fusion of the virus with the host cell membrane and penetration of the virus into the cell cytoplasm, which is triggered by low pH and conformational change of the HA protein leading to fusion of viral and endocytic membranes leading to the release of RNPs into the host cell. As described below, antigenic changes in the HA endow influenza viruses with a potent ability to evade host immunity.

The NA protein is a spike-shaped tetramer extending from the lipid layer, and is integral in both viral attachment and viral release from the host cell. The complete virion is released from the cell membrane by the enzymatic activity of NA which cleaves the α-ketosidic linkage between the terminal sialic acid and it's adjacent sugar residue to which the HA is bound (Gottschalk 1957). The cleavage of sialic acid leads NA to play a role in both viral attachment (Matrosovich et al, 2004) viral release, and viral spread by removing nearby sialic acid receptors from carbohydrates on the viral glycoprotein, thereby preventing aggregation of viral progeny (Palese et al, 1974, Palese and Compans 1976). This activity requires a delicate balance between HA and NA so that viral particles do not aggregate at the cell membrane and so released progeny can continue the cycle and infect other cells (Kaverin et al, 1998, Mitnaul et al, 2000, Wagner et al, 2002). NA,

like HA also plays a major role as an antigenic determinant that undergoes antigenic variation.

The third integral membrane protein is the matrix 2 (M2) protein that extends the entirety of the lipid layer and projects from the surface of the virion. The ectodomain of the M2 protein (M2e) that extends from the surface of the virion has become attractive as a vaccine prospect due to the high sequence conservation of this region across influenza A viruses in humans as well as sequence conservation between human and avian strains (Lamb et al, 1985). The M2 protein functions as a proton channel (Pinto et al, 1992) and is necessary for triggering viral uncoating. M2 has also been targeted by antivirals as a way to inhibit viral replication and is discussed in more detail below.

The matrix 1 (M1) protein lies beneath the lipid envelope in a layer extending the circumference of the virion and interacts with ribonucleoproteins (RNPs), forming a bridge between inner core components and membrane proteins, and allowing assembly of viral products and budding of the virion from the host cell (Gomez-Puertas et al, 2000, Latham & Galarza 2001). M1 not only promotes binding to RNA but it also acts as a nuclear localization signal (NLS) based on a specific signal sequence at amino acids 101-105 to promote transport from the cytoplasm to the nucleus (Elster et al, 1997, Ye et al, 1995).

The remaining proteins are all internal and involved with RNA replication and transcription. These are the nucleocapsid protein (NP) which coats the RNA, and the complex of three proteins which constitute the RNA dependent RNA polymerase. NP is a major structural protein that encapsidates viral RNA. NP protein is involved in RNA

synthesis and RNA nuclear export, and is required for the import of viral RNA (Cros et al, 2005, O'Neil et al, 1995).

The influenza virus RNA dependent RNA polymerase is a heterotrimer composed of three subunits designed polymerase basic 1 (PB1), polymerase basic 2 (PB2) and polymerase acidic (PA), so named based on their basic or acidic amino acid composition. PB1 displays catalytic activity, nucleotide polymerization, and chain elongation (Braam et al, 1983). PB1 possesses four conserved motifs that recognize it as a RNA-dependent RNA polymerase, and show the critical role of PB1 in RNA-transcription replication (Biswas & Nayak 1994). The PB1 gene also encodes a second protein PB1-F2 which regulates influenza A virus-mediated apoptosis by targeting the mitochondria, causing destabilization of the mitochondrial membrane with some H1N1 strains, but not in H5N1 strains possibly due to its cellular localization; it contributes to viral RNP activity and aids in viral RNA replication (Chen et al, 2001, Chen et al, 2010). PB2 binds to the 5' cap of host messenger RNA molecules, after which PB1 cleaves the cap for incorporation into viral RNAs (so called "cap snatching"). While most PB2 protein localizes in the nucleus, PB2 also localizes to the mitochondria and interacts with IPS-1, a mitochondrial antiviral signaling protein, but also shows differences in strain specificity like PB1, with seasonal strains targeting the mitochondria but nonmitochondrial targets in H5N1 viral strains (Graef et al, 2010). This strain-specific amino acid polymorphism in H5N1 strains leads to induction of higher levels of IFN-β, leading to attenuation in the animal model when there is overexpression of PB2, whereas the PB2 protein that targets mitochondria inhibits the production of INF-β (Graef et al, 2010). PA is the third component of RNA polymerase that interacts with PB1 and has protease activity (Sanz-

Ezquerro et al, 1995) with other functions still being investigated. Recent elucidation of the PA protein includes a more detailed understanding of its role, not only with protease degredation of both viral and host proteins but endonucleotic cleavage of capped RNA primers and transcript elongation (Fodor et al, 2002, Fodor et al, 2003). Like PB1-F2 and PB2, PA also has some involvement with mitochondrial proteins and regulation of apoptosis (Bradel-Tretheway et al, 2011).

The final set of proteins encoded by the genome of influenza A virus are the non-structural proteins. Originally they were given this designation because they had not been found to be present in virions but it has been discovered that NS2 has been isolated from purified virus (Richardson & Akkina, 1991). The nonstructural protein 1 (NS1) is considered multi-functional and works as a viral interferon antagonist by suppressing the host's immune response induced by the viral infection. Studies with NS1 mutants have determined that if NS-1 is not present, it increases the pathogenicity *in vivo* in animals lacking both STAT1 (Garcia-Sastre et al, 1998) or dsRNA-activated protein kinase, PKR (Bergmann et al, 2000, Kochs et al, 2007), both of which are antiviral mediators. NS1 can be divided in two parts, the RNA-binding domain (Chien et al, 2004, Hatada & Fukuda 1992, Qian et al, 1995) and the C-terminal effector domain, which mediates both the interactions with host cell proteins and functionally stabilizes the RNA-binding domain (Wang et al, 2002). Nonstructural protein 2 (NS2) which is also called the nuclear export protein is involved in nuclear export of viral RNPs (O'Neill et al, 1998). NS2 binds to M1 through ionic interactions in the C-terminal domain and is responsible for both the nuclear export of viral RNPs and for blocking re-entry of vRNPs into the nucleus by blocking the action of NLS of the M1 protein (Shimizu et al, 2011). NS2 also

has a nuclear export signal that interacts with the protein CRM1 which plays a role in trafficking.

Replication of influenza A viruses

Replication of influenza A virus is initiated through binding of the viral HA protein to the host cell via a specific sialic acid (SA) bound to a galactose. SA molecules are named based on both their chemical composition (N-acetylneuraminic acid or N-glycolneuraminic acid) and the sugar linkages to the α-2 carbon, and are designated as α2-3 or α2-6. There is diversity in the affinity of different HA proteins for the two SA structures. In general, avian influenza viruses prefer SAα2-3 linkages, with NeuSA-α-2,3-gal being present in duck intestinal cells (Ito et al, 1997, 2000), while human influenza viruses bind preferentially to SA with α2-6 linkages (Rogers & Paulson, 1983, Baum & Paulson, 1990, Conner et al, 1994). Cells of the upper respiratory tract of humans contain predominantly SA-α-2,6-gal (Baum & Paulson, 1990), but SA-α-2,3-gal is present on ciliated cells in the lower respiratory tract (Matrosovich et al, 1999, 2004, van Riel et al, 2006, Shinya et al, 2006). The differing affinities of HA molecules for different SA receptors is an important determinant of host range. For example, most mammals are relatively poor hosts for avian influenza viruses, as will be discussed in detail later.

Once the virus has bound, the virion is endocytosed into the host cell. During the endocytosis process, the M2 protein allows for the influx of protons leading to an acidic environment (Matlin et al, 1981). This low pH leads to a conformational change in the HA, which in turn triggers fusion of the viral and endocytic membranes and dissociation of M1 from the RNP complex (Matlin et al, 1981, Zhirnov et al, 1994). Once the two

vesicles are fused, the M1 protein promotes the expulsion of the RNP from the vesicle and its transport to the nucleus of the host cell (Martin et al, 1991). PB1 and PA have been shown to accumulate both in the cytoplasm and the nucleus, but do not accumulate in the nucleus without forming a dimer with each other before nuclear entry. PB2 enters the nucleus independently, then in that compartment forms a trimer with PA and PB1 (Fodor & Smith, 2004). The interaction with viral genomic RNA (vRNA) appears to occur with the PA/PB1 dimer before joining with PB2 (Deng et al, 2005). Transport into the nucleus is aided by karyopherin α and karyopherin β that bind to the complex of NP protein and vRNA. Karyopherin α recognizes a NLS-containing cargo protein on NP, which recruits karyopherin β which binds to the nucleus at the nuclear pore (Cros et al, 2003).

Once inside the nucleus, the polymerase complex composed of PA, PB1, and PB2 initiates primary transcription of mRNAs from vRNA, beginning with the phenomenon of cap snatching. This involves the stealing of a 5′ capped primer from host pre-mRNA transcripts (Krug 1981). Transcription in then initiated when the 5′ end of vRNA binds to the PB1 subunit, which allows PB2 to recognize and bind to the pre-mRNA (Cianci et al, 1995, Fechter et al, 2005, Li et al, 1998). This change in polymerase leads to an increased affinity of PB1 for the 3′ end of the vRNA forming a duplex (Lee et al, 2003). The PB1 then exerts its endonuclease activity, cleaving pre-mRNAs initiating transcription and chain elongation. The synthesis of viral mRNA is completed with the polyadenylation on the 3′ terminus of the newly synthesized RNA. The vRNA segments also serve as templates for the production of cRNA but without the need for a capped primer; in this case, an exact copy of viral genomic RNA is produced. Once the positive

sense cRNA is produced, it serves as the template for the production of additional copies of negative sense vRNA. Once viral replication has occurred, the RNP complexes are transported out of the nucleus with the aid of M1, NEP/NS2 and an export receptor CRM1 (Neumann et al, 2000). CRM1 binds to the target protein that contains a leucine rich NES (Fornerod et al, 1997, Fukuda et al, 1991, Ossareh-Nazari et al, 1997) which M1 does not possess, but NS2 does, leading to a complex of NS2-M1-vRNP-CRM1 which exports the complex out of the nucleus into the cytoplasm. Once in the cytoplasm, the vRNP-M1-NS2 can be incorporated into the virion and assembly at the plasma membrane occurs with M1 playing a dual role in both trafficking and assembly. HA, NA and M2 proteins are processed in the endoplasmic reticulum into their appropriate configurations, then transported to the Golgi apparatus where cysteine residues of the HA and M2 proteins are palmitoylated (Steinhauer et al, 1991, Sugrue et al, 1990, Veit et al, 1991,1991,1993). These modified proteins are then transported to the plasma membrane to finish assembly of all eight viral segments into a complete viral particle before the budding process is initiated. The virion is released from the cell surface through the action of NA cleaving the appropriate sialic acid. Early studies were done with a transition state neuraminidase inhibitor 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA), which works to mimic the enzymatic substrate of NA and blocks viral release leading to inhibition of viral replication (Kilbourne et al, 1974, Palese et al, 1974, 1976). This valuable concept elucidating a major function of NA led to the later development of neuraminidase inhibitors in use today which utilize the same mechanism.

Evolution of influenza A viruses

Influenza viruses have the ability to infect and cause disease in a very broad range of avian and mammalian species due to the plasticity of their genome. Genomic diversity is acquired through two fundamental mechanisms: an intrinsically high rate of mutations and the ability of the virus to reassort gene segments. RNA viruses in general have high rates of mutation due to their lack of an exonuclease activity and inability to edit misincorporated nucleotides during RNA replication (Steinhauer et al, 1992). This high error rate leads to a quasispecies scenario, with many different genotypes generated that have the ability to succeed within the host depending on the level of fitness of the different viruses (Domingo et al, 1985). This is advantageous for the virus in allowing it to better evade the host's immune response and rapidly adapt to the new host (Manrubia et al, 2005). The low fidelity of RNA replication can also be a disadvantage in that many of the different genotypes produced will not be productive and will be eliminated from the host.

Changes in viral genomic sequence caused by many mutations over time, is referred to as antigenic drift. Protection is afforded in the host from antibodies generated by the immune response to viral infection, which in theory would provide protection against the HA of the same subtype but not between subtypes. Over time, it became clear that vaccines made for a specific subtype did not necessarily protect even against viruses of the same subtype due to alterations in the structure of antigenic sites. The changes in amino acid sequence over time are more likely in the HA and NA genes (Nobusawa et al, 1991). A consequence of antigenic drift of huge importance to public health is evident from the formulation of human seasonal influenza vaccines, which have

to be updated yearly due to best match the most common circulating strains of virus (Smith 2003). For the human influenza A H3 protein, there are five specific antibody-binding regions located on the globular head of the HA protein near the receptor binding site that are considered protective to the host (Webster & Laver, 1980, Wiley et al, 1981, Wilson et al, 1981). These regions accumulate amino acid changes that over time can alter a specific neutralizing epitope that prevents the antibody from binding (Webster & Laver, 1980). This allows the virus to escape the host immune response, thus perpetuating viral replication and transmission.

The ability of influenza virus gene segments to reassort is called antigenic shift. Antigenic shift is described as the introduction of an antigenically distinct virus within a population that is different from currently circulating strains, and to which the population has no immunity. This lack of immunity allows the virus to spread rapidly within a population, sometimes leading to a pandemic. Important examples of known human influenza pandemics due to antigenic shift are the H1N1 subtype in 1918, H2N2 in 1957, H3N2 in 1968, H1N1 reappearance in 1977 and most recently, the H1N1-swine origin virus from 2009. Pandemics are determined by the presence of three factors: 1) a novel virus must be present in the population, 2) the virus must have the ability to cause illness in the host, 3) there must be sustained human to human transmission. These events can occur by either the sudden introduction in the human population from an animal population, or a reassortment event between circulating strains of avian influenza and host-adapted human or swine influenza viruses (Wright et al, 2007, Dawood et al, 2009). Both the 1957 and 1968 strains are human and avian reassortants (Wright et al, 2007). The 1918 H1N1 virus was new to the human population, and introduced directly from an

avian source, either through adaptation of an avian H1N1 or a human-avian reassortment event (Reid & Taubenberger 2003, Reid et al, 2004). It has also been suggested that this virus was transmitted from humans to swine during the same time period when swine epizootics were occurring (Chun 1919, Koen 1919). Retrospective sequencing of the 1918 human H1N1 virus and swine viruses which were first isolated in the 1931 (Shope 1931) suggest that the H1N1 strain circulating in 1918 mixed between humans and swine and separated into two separate lineages; human-H1N1, which was isolated up until 1957 with a possible laboratory exposure causing the human H1N1 lineage to re-appear in 1977 (Nakajima et al, 1990, Taubenberger et al, 2007), or swine-H1N1 strains that are still circulating today (Kanegae et al, 1994). The most recent H1N1 pandemic resulted from generation of a reassortant virus with genes previously seen in a human-swine-avian triple reassortant present in North America with genes circulating in Eurasian swine (Dawood et al, 2009). H1N1 influenza virus first appeared in European swine in 1979 (Easterday & Van Reeth 1999) with H3N2 appearing in European swine in the mid 1980s and mid 1990s in the United States. Both H1N1 and H3N2 viruses still circulate worldwide in swine (Swayne 2008).

Influenza A virus has also established itself in horses; first with the H7N7 subtype, in the 1950's followed by a H3N8 subtype virus. It is this equine H3N8 influenza A virus that was transmitted to dogs and has since established itself widely in the canine population (Castleman et al, 2006, Crawford et al, 2005, Payungporn et al, 2008). More recently in South Korea, an avian influenza A H3N2 virus was detected in dogs (Song et al, 2008), suggesting direct interspecies transmission from birds to dogs.

The knowledge that waterfowl are the primary reservoir of influenza virus allows a complete study of evolution of the virus in this species. The conclusion that influenza is evolving at a much slower rate in wild avian species, and the consensus of conservation of sequence from many different avian sources, show adequate adaptation to the avian host and a continued perpetuation of the virus within wild avian species confirming their reservoir status (Suarez 2000). This is made possible in part due to the low pathogenic nature of influenza in wild ducks that allows for limited disease but high rates of shedding that lead to transmission within duck populations and ultimately to other susceptible hosts (Webster et al, 1978, Kida et al, 1980, Cooley et al, 1989).

Epidemiology of Avian Influenza A viruses

Avian influenza virus was first isolated from a common tern (*Sterna hirundo*) in South Africa in 1961, A/tern/South Africa/61 (H5N3), and was the first highly pathogenic avian influenza virus (HPAIV) isolate to be recovered from wild birds (Becker 1966). Viruses were subsequently isolated from wedge-tailed shearwaters (*Puffinus pacificus*) in Austrailia (Downie & Laver, 1973) and from wild ducks in California (Slemons et al, 1974). Since then, it has been determined that all known HA (H1-H16) and all known NA (N1-N9) subtypes are found in waterfowl (Hinshaw et al, 1980, Hinshaw et al, 1982, Suss et al 1994, Olsen et al, 2006). The waterfowl that harbor influenza A viruses can be divided into two separate orders, the Anseriformes, which include ducks, geese and swans, and the Charadriiformes, which include gulls, terns and shorebirds, both groups of which are distributed globally. While wild birds are generally accepted as the natural reservoir of all influenza viruses, wild ducks throughout North America and Northern Europe have a more limited range of carrying H1-H12, H14 and

N1-N9, while H13, H15 and H16 appear to be relegated primarily to shorebirds (Sharp et al, 1993, Hanson et al, 2000, Krauss et al, 2004, Olsen et al, 2006). While all subtypes appear in waterfowl, there appears to be a more limited subset of viruses for which wild ducks are the reservoir. Studies conducted in North America and Europe have shown that there are dominant subtypes in wild ducks that are isolated from both adults and juveniles and are of the HA subtypes H3, H4, and H6, along with NA subtypes N2, N6, and N8 (Sharp et al, 1993, Krauss et al, 2004, Munster et al, 2005, Hanson et al, 2003); H9 and H13 subtypes predominate in shorebirds (Kawaoka et al, 1988). Prevalence of AIV in ducks peaks during late summer and early fall and to higher numbers in juveniles due to increased numbers in pre-migration areas and possibly due to lack of immunity in juveniles (Hinshaw et al, 1985, Krauss et al, 2004, Wallensten et al, 2007). The rates of virus isolation decrease as birds migrate south (Stallknecht et al 1988, Krauss et al, 2004). Host age has also been shown to play a role in the quantity of virus shed from mallards, with birds 1 month of age shedding the most virus and on more collection time points than virus isolated from mallards tested at 2 weeks, 2 months, 3 months, and 4 months of age (Costa et al, 2010). Pathogenicity of AIV in mallards has also been reported to differ based on age (Pantin-Jackwood et al, 2007), suggesting that the age at which infection occurs could play an important role in the ultimate transmission of the virus. Long term studies in wild ducks have shown a clear periodicity in 2 year intervals for isolation rates with approximately 1 to 2 years between highs and lows (Hinshaw et al, 1985, Krauss et al, 2004).

Since AI viruses in wild ducks are primarily transmitted by the fecal/oral route, one must consider both the environment as well as shedding capacity of the duck. The

longer AIVs are shed from the host and persist in the environment, the higher the probability of transmission among ducks and other species. Webster and colleagues (1978) first demonstrated that experimentally infected ducks shed large quantities of virus, and others have shown that virus shedding can be detected from ducks for more than 28 days (Hinshaw et al, 1980). AIV has also been isolated from natural water sources, providing another source of infection (Hinshaw et al, 1980, Halvorson et al, 1983, Ito et al, 1995), and models have been constructed to better understand the potential role of contaminated water in yearly cycles infections (Roche et al, 2009). Several *in vitro* studies have also evaluated different strains of AIV to determine the environmental persistence of AIV in water and to evaluate the effects of temperature, pH and salinity on persistence (Webster et al, 1978, Brown et al, 2006, Stallknecht et al, 1990a and b, Negovetich & Webster, 2010, Achenbach & Bowen, 2011).

Mallards (*Anas platyrhynchos*) are the most commonly studied species of the Anseriformes and are infected with influenza virus more often than other birds (Olsen et al, 2006). Short term infection studies using mallards have shown some level of heterosubtypic and homosubtypic immunity does exist. This causes minimal illness on the mallards and allows their interactions with other birds and migration to be unhindered (Kida et al, 1980, Fereidouni et al, 2009, Jourdain et al, 2010). However, this transient immunity also decreases shedding, which may impact the ability to transmit (Latorre-Margalef et al, 2009). The concern remains that mallards continue to transmit AIV while undertaking long distance migration, leading to perpetuation and spread of the virus, particularly at stopover sites (Olsen et al, 2006, Wallensten et al, 2007).

Generally, it is wild birds that introduce LPAIVs to domesticated birds such as chickens, turkeys, quail and other game birds. These transmission events occur either through direct contact or contact with contaminated surfaces and water. Once the virus has established itself in domesticated birds through adaptation, the potential to spread to mammals is increased. This has led to the recent concerns with H5N1 HPAIV viruses.

An influenza virus considered to be a major concern to public health is highly pathogenic H5N1. This pathogen was first detected in 1997 in Hong Kong (Claas et al, 1998, Subbarao et al, 1998) and has continued to spread west through Asia, Europe, the Middle East, and Africa. The rapid evolution of H5N1 HPAIV, through both antigenic shift and antigenic drift, has generated 10 distinct clades (clades 0-9) (Donis et al, 2008). Initial fears of a pandemic centered on domestic poultry that had been in contact with humans, and there was little concern that this virus was spread from migratory species. Eventually, it was discovered that wild birds were also succumbing to infection from H5N1 HPAIV (Olsen et a, 2006, Liu et al, 2005, Ellis et al, 2004, Chen et al, 2005).

Early reports indicated that HP H5N1 viruses were non-pathogenic in mallards (Brown et al, 2006, Keawcharoen et al, 2008) or had the ability to become non-pathogenic through evolutionary adaptation in the duck host while remaining highly pathogenic to domestic poultry. Such a case would allow for the possibility that migrating ducks could transmit the HP virus to poultry without themselves suffering disease (Hulse-Post et al, 2005). A recent H5N2 HP virus was isolated in Nigeria from two healthy wild waterfowl (Gaidet et al, 2010), validating continued concern that wild birds with subclinical infection can be a significant source of virus to the poultry they contact. On the other hand, some recent H5N1 strains are clearly highly pathogenic to

wild ducks and other waterfowl, vividly demonstrating diversification of H5N1 strains and leading to concerns for the future evolution of these strains and their effects on humans. H5N1 AIV continues to be detected in wild birds and is endemic in domestic ducks and poultry in several regions of the world. This virus should continue to be viewed as a major threat to animal and human health.

Pathogenesis of avian influenza A virus infection

The ability of influenza A viruses to induce disease varies greatly among host species and even within the same subtype. For example, infection of humans with many H1N1 viruses induces a mild disease (seasonal influenza), whereas other H1N1 viruses have killed millions of people (e.g. the Spanish influenza epidemic of 1918). Analysis of the reconstructed 1918 influenza virus indicated that all original 8 gene segments together were required to recreate the virulence of the virus as a whole; replacing just one gene at a time greatly reduced virulence (Tumpey et al, 2005). Another excellent example of differing virulence among viruses of the same subtype is observed with avian influenza A viruses, which can be classified as HPAIV or LPAIV based on the responses of domestic chickens to infection. These differences in virulence appear to result from a complex interaction of several viral proteins acting in concert.

The HA gene and its encoded protein play an important role in virulence and pathogenicity. While the terms pathogenicity and virulence have been used interchangeably they are distinct from one another. Pathogenicity refers to the ability of the virus to infect and cause disease in a susceptible host and spread from host to host. Pathogenicity also involves the genetic component of the virus where the damage incurred on the host is due to host-virus interactions. Virulence refers to the degree of

damage caused by the virus which correlates with the ability of the virus to replicate in the host. Before the HA gene becomes fully functional it must be cleaved by a host protease that divides it into two separate subunits, HA-1 and HA-2 leaving the fusion peptide on the HA-2 region exposed. The cleavage and release of the fusion peptide is necessary for the initiation of a productive infection to occur (Klenk et al, 1975, Lazarowitz & Choppin 1975). To date, the only avian HA genes and proteins associated with a highly pathogenic phenotype are H5 and H7. The necessary molecular change from LP to HP generally occurs when the virus moves from its natural reservoir to poultry species (Webster, 1998). In multiple cases, a closely related progenitor LPAIV has been detected circulating immediately prior to an outbreak involving the corresponding HPAIV (Kawaoka et al, 1984, Horimoto et al, 1995, Garcia et al, 1996, Suarez et al, 2004, Bowes et al, 2004, Hirst et al, 2004).

The critical difference between LP and HP HA sequences is located at the proteolytic cleavage site (PCS) (Garten et al, 1981, Klenk 1980, Lazarowitz et al 1973). The consensus amino acid sequence of the PCS in LP viruses is PQRETR/GLFG for the H5 subtype and PEXPKXR/GLFG for H7 viruses (Perdue et al, 1997). In contrast, the PCS of HP viruses contains an increased number of basic amino acids (arginine and lysine); this change can occur through specific mutations or insertions of amino acids (Horimoto & Kawaoka 1994, Perdue et al, 1997, Senne et al, 1996, Wood et al, 1993). The additional basic amino acids at the PCS of HPAIV, allows cleavage to be completed with more widely found furin-like or subtilisin-like endoproteases (Garten et al, 1981, Horimoto & Kawaoka 1995, Rott et al, 1979). This increases the ability of the virus to be cleaved in more tissues, leading to widespread infection. In contrast, cleavage of the HA

molecule from LPAIV requires trypsin-like enzymes, limiting replication to the respiratory and intestinal tracts where enzymes are found.

LPAIV replicates in ducks predominantly in cells lining the gastrointestinal tract, leading to minimal or no clinical signs of infection, shedding of virus in high titers in the feces, and transmission via the fecal-oral route (Webster et al, 1978, Hinshaw et al, 1979). Initial observations of HPAIV infection in ducks revealed a lack of morbidity and mortality, but since 1999, both HP H7 and H5 viruses have been isolated that can induce systemic spread of the virus in wild ducks, leading to neurological disease and death within one week of infection (Capua & Mutinelli, 2001, Ellis et al, 2004, Sturm-Ramirez et al 2004, 2005, Tang et al, 2009). An additional feature of HPAIV infection in ducks is that higher titers of virus are shed from the respiratory tract than from the intestinal tract, indicating an evolution in tissue tropism (Sturm-Ramirez et al, 2004).

The NA gene has also been suggested to play a role in pathogenesis after increased pathogenicity was seen in chickens infected with different strains of H5N1 virus (Hulse et al, 2004). In chickens, typical waterfowl AIV has adapted to include both a deletion in the stalk region of the NA coupled with increased glycosylation in HA (Matrosovich et al, 1999). These nucleotide changes have been shown to precede increased pathogenicity in chickens (Perdue et al, 1995, Munier et al 2010, Giannecchini et al, 2010). These same changes, when tested in mice, show decreased virulence (Castrucci & Kawaoka 1993) showing variability in host range. Other changes in host directed glycosylation also lead to enhancement of host cell proteases that allow increased sialidase activity, leading to more efficient spread of virus through host tissues (Schulman & Palese, 1977).

The NS gene has also been implicated in virulence based on separate amino acid differences located in the NS2 protein, which show increased virulence in chicken embryos (Perdue 1992). Another amino acid change in the NS1 protein resulted in increased virulence in a H5N1 strain inoculated into chickens (Li et al, 2006).

The viral polymerase gene PB2 also plays an integral role in virulence and host range, particularly with regard to the specific amino acid found at position 627. It has been determined in mammalian-origin viruses that at position 627 the amino acid is lysine, where in avian-origin viruses that amino acid is glutamic acid (Subbarao et al, 1993). HP H5N1 and H7N7 viruses isolated from human patients have also shown a lysine at position 627, reflecting the adaptation from birds to humans (Puthavathana et al, 2005, Fouchier et al, 2004). This lysine contributes to the virulence of avian H5N1 viruses by increasing neurovirulence and systemic spread of the virus to non-respiratory organs in mice (Hatta et al, 2001, Shinya et al, 2004). Another amino acid change at position 701 of the PB2 protein of HP H5N1, proved to be non-lethal in ducks but was found to show increased replication and lethality in mice, confirming its ability to contribute to virulence and be a host range factor (Li et al, 2005). Overall, these observations show the many possibilities of separate influenza proteins having specific roles in increasing virulence and pathogenicity, and that those biologic properties are not the result of one gene acting alone, but a constellation of all the genes acting in concert.

Transmission of avian influenza A viruses

The ability of AIV to cross host species barriers depends on several factors. There needs to be direct interaction between 2 different species coupled with adequate exposure of virus either through direct or indirect contact. The virus needs to have the

ability to enter the new host cells and replicate, as well as adapt to the new host successfully in order to be shed and transmitted to others within the new host species. The closer the hosts are genetically related, the easier it seems to be for influenza virus to infect and persist. Intraspecies transmission is most common but interspecies transmission often occurs, and of course, then is the basis for the public health concern with HPAIV. Increasing interactions of wildlife and domestic animals on farms and in live bird and animal markets, as well as increased transport of commercial and exotic species within and between continents, has led to more interspecies transmission.

Some species are capable of being an intermediate host to transfer AIV from wild birds to other species. Chickens are a common intermediate for wild bird AIV strains with the most common subtypes found in chickens being H3, H5, H6, H7, and H9 (Liu et al, 2003). Chicken-origin viral sequences show distinct differences in their HA and NA genes that differ distinctly from wild waterfowl influenza A isolates, with increased glycosylation in the HA globular head region and NA protein, plus a deletion in the stalk region of the NA gene (Matrosovich et al, 1999, Hulse et al, 2004). These adaptations can make the virus isolate more pathogenic to chickens (Perdue et al, 1995, Hulse et al, 2004, Munier et al 2010, Giannecchini et al, 2010) and more transmissible to humans (Class et al 1998, Subbarao et al, 1998). Since 2002, a majority of the Z genotype H5N1 influenza viruses possess the same deletion in the NA stalk suggesting it has established itself in terrestrial poultry (Li et al, 2004).

Swine have also been considered a good intermediate due to the existence of both SA-α-2,3-gal and 2,6-gal receptors in respiratory epithelial cells that allow them to be infected with both avian and human influenza viruses. This also allows for the ability to

switch receptor specificity as the virus adapts in the swine host (Ito et al, 1998, Rogers & D'Souza 1989). These features have resulted in swine being labeled as the perfect 'mixing vessel' for both avian and human influenza strains that could combine and mutate to create a more infectious influenza virus to humans. Swine have also been responsible for transmitting swine H1 and H3 to turkeys housed on the same farm (Mohan et al, 1981, Suarez et al, 2003, Tang et al, 2005), providing yet another avenue for interspecies transmission.

Quail are also an effective intermediate for AIV. Characterization of sialic acid receptors present in quail have shown that they possess both SA-α-2,3 and α-2-6-gal (Matrosovich et al, 1999, Perez et al, 2003, Wan & Perez 2006, Kimble et al, 2010) allowing them to be infected with both avian and human viruses and adapt to switch receptors while replicating in the quail. Experimental and natural infections show that quail can be infected with and transmit multiple HA subtypes of AIV to terrestrial poultry (Guan et al 1999, Cameron et al, 2000, Marakova et al, 2003, Perez et al, 2003, Sorrell & Perez 2007, Hossain et al, 2008, Giannecchini et al, 2010, Lee et al, 2010). Quail can also support the replication of AIV subtypes H1-H14 as well as swine influenza viruses H1 and H3 (Marakova et al, 2003). Pheasants also carry both SA-α-2,3 andSA-α-2-6 receptors (Kimble et al, 2010) and are susceptible to infection with AIV HA subtypes H1-H15, and are capable of shedding virus for extended periods of time. This makes them a concern for transmission of AIV in live bird markets or on wild game bird farms (Humberd et al, 2006).

Equine influenza A viruses, like AIVs, prefer binding to SA-α2-3-gal (Rogers & Paulson 1983) and have directly transmitted equine influenza A H3N8 to dogs leading to

the establishment of this subtype in this species. Studies in dogs have detected the presence of SA-α-2,3-gal receptors on the surface of bronchial and bronchiolar epithelial cells, but a lack of SA-α-2.6 (Song et al, 2008). In this same study, avian-H3N2 virus was detected in dogs, showing their ability to be directly infected with both avian and equine influenza A (both SA-α-2,3-gal preference) viruses, but may limit the transmission from dogs to humans.

As outlined previously, humans have SA-α-2,3-gal on ciliated cells in the lower respiratory tract. This has allowed more recent avian H5N1 viruses, which still prefer SA-α-2,3-gal, to infect humans (Matrosovich et al, 1999, 2004, van Riel et al, 2006, Shinya et al, 2006), but not readily be transmitted human-to-human without the adaptation to SA-α-2,6-gal (Suzuki 2005). This is of considerable importance, in that avian H5N1 viruses have yet to become transmissible among humans. Adaptation of avian H5N1 virus to incorporate specificity for SA-α-2,6-gal may lead to the next human pandemic.

Research with reverse genetics, that allows for changing specific nucleotides in specific genes, has elucidated two specific amino acid changes in the HA gene that can convert the SA receptor specificity from SA-α-2,3gal to SA-α-2,6gal. These two specific amino acid changes are a serine to glycine at position 228, and a leucine to glutamic acid at position 226. Both of these changes appear essential to allow human influenza A viruses to successfully replicate in the intestine of ducks (Vines et al, 1998).

Another determinant of host range is the specific amino acid 627 of the PB2 protein. The change from a glutamic acid (avian) to a lysine (mammals) (Subbarao et al, 1993) enhances the transmission from birds to mammals. Once this change is established

in mammals it will present a threat for humans because it would enhance human to human transmission. Another aspect of position 627 in PB2 is its role in temperature sensitivity. When the PB2 627 is a lysine (human viruses), the viral polymerase complex is more likely to support replication from 33º C to 37º C, which is typical of human respiratory tract temperatures. If the PB2 627 amino acid is glutamic acid (avian viruses), viral replication is hindered at temperatures of 33º C since temperatures in the duck intestinal tract are higher at 41º C. This suggests the decreased ability of avian influenza viruses to efficiently replicate in humans, or human viruses in ducks, without adaptation (Massin et al, 2001).

The NA protein also plays a role in host range. The NA protein prefers to cleave the same sialosaccharides that the HA bound to initially, meaning NA prefers the same SA-α-2,3-gal for avian influenza viruses and SA-α-2,6-gal for human viruses (Baum and Paulson, 1991).

Resistance of Avian Influenza Viruses to Antiviral Drugs

While a multitude of vaccines have been made against different clades of H5N1 virus, there currently is not one individual vaccine that will universally protect against all H5N1 clades and subclades. Based on experience with the most recent pandemic of swine-origin H1N1 virus, we know there will be at least six months or more before a suitable vaccine can be created in the event of a pandemic. This leaves antiviral drugs at the forefront for treatment and control of a pandemic. Currently, there are only two groups of antiviral drugs that are approved for both treatment and prophylaxis of influenza virus infections: M2-ion channel inhibitors, the adamantanes, amantadine and

rimantadine, and the neuraminidase inhibitors, including oseltamivir, zanamivir and peramivir, the latter of which has been approved for use in certain medical situations.

The first antiviral studied for treatment of infection with influenza A, was amantadine hydrochloride, with its antiviral effects first published in 1964 (Davies et al, 1964) The adamantanes work to block the function of the influenza A virus M2 protein, which prevents viral uncoating in the infected cell (Wang et al, 1993). Adamantanes also cause an altered conformation of the hemagglutinin protein which prevents release of virus from the host cell (Grambas and Hay, 1992, Betakova et al, 2005). Amantadine hydrochloride continued to be the drug of choice for treating H3N2 infections and was licensed for this purpose in 1976. Another adamantane derivative, rimantadine was licensed in 1993 for use as an influenza antiviral. Both adamantine derivatives can be 80-90% effective and diminish symptoms by 1.5 days if taken within 48 hours after symptom onset (Oxford and Galbraith, 1984, Reuman et al, 1989, Younkin et al, 1983). Unfortunately, both amantadine and rimantadine are not effective against influenza B viruses and both have been shown to have significant side effects in humans during the course of treatment (Hayden et al, 1980, Dolin et al, 1982, Hayden et al, 1983). The widespread use of adamantanes led to the rapid development of resistant strains, either following drug treatment or naturally through evolution (Hayden and Hay 1992, Bright et al, 2005, Deyde *et al*, 2008). Since development of widespread resistance to adamantanes, neuraminidase inhibitors were evaluated to control influenza virus infections and currently provide the most effective antiviral treatment (Fiore et al, 2007).

Neuraminidase inhibitors were designed to be a valuable drug target because NA is directly involved in the propagation of influenza virus. Also, the specific amino acids

in the NA active site that interact with the substrate or that are in the general area of the active site are strictly conserved among influenza A and B strains (Burmeister et al, 1993, Varghese et al, 1998). The important amino acids at the active site can be divided into two groups: the catalytic residues (R-118, D-151, R-152, R-224, E-276, R-292, R-371, and Y-406) that have direct contact with the substrate, and the framework residues (E-119, R-156, W-178, S-179, D-198, I-222, E-227, E-277, N-294, and E-425) that play a role in the stabilization of the active site structure (Coleman et al, 1983). The idea that using an inhibitor that so closely resembled the natural substrate, was expected to avoid the selection of drug-resistant mutants (Varghese et al, 1998). Those drugs currently licensed for treatment are inhaled zanamivir (2,4-dideoxy-2,3-didehydro-4-guanidino-sialic acid) (Relenza^{TM}) and oral oseltamivir phosphate (OP) (Tamiflu®). OP is the prodrug which is converted in the liver of humans to the active metabolite oseltamivir carboxylate (OC) (ethyl-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate). Alterations in design of the two drugs allow them to interact with different amino acid residues in the NA active site. OP has an affinity for a positively charged and hydrogen binding environment created by the catalytic amino acid residues R118, R292, and R371. Zanamivir interacts with a negatively charged region formed by the framework residues E227 and E119 (Ferraris & Lina 2008). Both oseltamivir and zanamivir interact with the catalytic residue R152 but zanamivir is more likely to interact with R224 and E276 based on a specific glycerol side chain (Yen et al, 2006, McKimm-Breschkin 2000, Smith et al, 2002, Stoll et al, 2003). Neuraminidase inhibitors (NAIs) work to interfere with the normal function of influenza virus neuraminidase and to limit viral infection by blocking the enzyme active site leading to inhibition of its sailidase

activity. NAIs also work by preventing the release of virus from the infected host cell (von Itzstein et al, 1993, Hsieh *et al*, 2007, Gubareva et al, 2000) NAIs can also cause aggregation of the virus if released from the host cell preventing the virus from penetrating into mucous secretions and spreading to nearby cells (Roberts and Govorkova 2009).

Problems with Antiviral Drug Resistance

In vitro studies have revealed that continued passage of virus in the presence of NAIs lead to the emergence of resistant strains of virus (McKimm-Breschkin 2000, Bantia et al, 1998, Molla et al, 2002, Hurt et al, 2009). Cell culture testing of NAIs have shown variable propensity to evolve resistance among different viruses (Woods et al, 1993) which makes comparison testing between laboratories difficult, and animal models valuable tools.

Development of resistance *in vivo* was detected in a ferret model following H3N2 challenge and treatment with amantadine but not zanamivir (Herlocher et al, 2003) *In vivo* resistance was also detected following oseltamivir treatment and H5N1 infection in the ferret model (Govorkova et al, 2007). This suggests the potential of developing resistance but not the probability of occurrence. Finally, studies from human clinical trials, treatment during natural infection, natural infection without treatment, and surveillance in avian strains have shown an increase in oseltamivir resistance from both LPAIV as well as HPAIV such as H5N1 (Gubareva et al, 2001, Kiso et al, 2004, Le et al, 2005, deJong et al, 2005, McKimm-Breschkin et al, 2007, Sheu et al, 2008, Hauge et al, 2009, Boltz et al, 2010). The increased detection of seasonal human influenza virus strains showing resistance to oseltamivir is of great concern. The predominant mutation

of H274Y in N1 subtypes, detected from America to Europe to Asia, is puzzling in that regional drug use is not suspected to be the problem (Sheu et al, 2008, Dharan et al, 2009, Meijer et al, 2009, Hurt et al, 2009, Hauge et al, 2009).

Current usage statistics do not show clear connections between oseltamivir usage and the increase in oseltamivir resistant strains. For example, Japan, which had the highest reported prescription rates (70.9 for every 1,000 people in 2005, Yasui et al, 2007), reported that only 3% of tested H1N1 strains were oseltamivir resistant (WHO, 13 June 2008). The next highest prescription usage in 2005 was in Germany (~5.5 prescriptions per 1,000 people, but dropped to usage of <2 prescriptions/1,000 people by 2007) where it was reported that 13.1% of tested strains were oseltamivir-resistant (Kramarz et al, 2009). Norway was one of two countries with the highest rates of oseltamivir resistance, reporting resistance to oseltamivir during the 2007-8 season at 67.4% of tested strains, despite the fact that less than 1 prescription/1000 people was reported in 2006 and 2007. The second highest increase in resistant strains was reported in Belgium, where a rate of 53.1% resistance was reported, but with less than 1/1,000 prescriptions in 2006 and less than 2/1,000 in 2007 (Kramarz et al, 2009). Since oseltamivir resistance is showing a natural evolution of resistance rather than resistance primarily driven by drug usage, it is important to understand what molecular changes are taking place within the NA gene that are associated with this functional change.

It is important to both evaluate the affinity of NA for its substrate and inhibitors, as well as its relation to the receptor binding affinity to HA. Once there is a better understanding of the kinetics, the role of genetic changes can be better elucidated. Recent analysis of H1N1 virus strains with the H274Y mutation from the 2007-08 flu

season report an increase in affinity of N1 NA for its substrate and inhibitor and moderate genetic changes that may play a role in this change. The changes of importance are that, except for one resistant virus, all sensitive and resistant strains were in the same clade, thus sharing their evolutionary background. Other mutations new to the 2007-08 season were H45N, K78E, E214G, R222Q, G249K, T287I, K329E, and D344N (Rameix-Welti et al, 2008). Amino acids at position 45 and 78 are located in the NA stalk region and are not suspected to play a significant role in resistance. In contrast, those at positions 222, 249, and 344 are located near the catalytic site (H5N1 structure, Russell et al, 2006) and may alter NA substrate affinity (Rameix-Welti et al, 2008). This shows that there are increasing evolutionary changes taking place within the NA that, in conjunction with HA and the other six genes, are contributing to an increase in oseltamivir resistance.

This documented increase in resistance has lead researchers to evaluate other options for development of new antiviral compounds to combat influenza virus infections, as well as re-evaluate the current drugs that are stockpiled for a potential pandemic.

Investigational compounds for Influenza

Peramivir, a newer NAI, was designed utilizing a novel approach to create a more orally bioavailable drug than oseltamivir. Based on protein crystallography, peramivir was created as cyclopentane derivative with additions including a negatively charged carboxylate group, a positively charged guanidino group and lipophilic side chains (Babu et al, 2000). Peramivir was shown to be more beneficial than oseltamivir in animal studies when given orally and exhibited more potent viral inhibition by decreasing death rates and lowering viral lung titers (Babu et al, 2000, Bantia et al 2001, Drusano et al,

2001, Smee et al, 2001, Sidwell et al, 2001, Govorkova et al, 2001, Sweet et al, 2002) Initial clinical trials in humans discovered low bioavailability when administered orally, (Barroso et al, 2005) opening the prospect of parenteral administration of the drug. Parenteral administration of the drug would also be beneficial in reducing the dosing levels when compared to traditional oral oseltamivir dosing. Intramuscular treatment of mice with Peramivir demonstrated reduced weight loss and mortality after infection with H1N1 and H3N2 subtypes (Bantia et al, 2006), and was effective against H5N1 in both mice and ferrets (Boltz et al, 2008, Yun et al, 2008). Phase II and phase III trials have been carried out with intravenous administration of peramivir in humans, and this treatment was shown to reduce clinical symptoms and show a significant reduction in viral titers (Kohno et al, 2009, Ison et al, 2009). Peramivir is not recommended for treatment when oseltamivir resistance is known or suspected as it follows the same resistance patterns (Baz et al, 2007). Intravenous zanamivir is currently in phase II clinical trials and can currently be used in emergency situations with critically ill patients. Zanamivir has also been shown to effectively treat oseltamivir resistant viruses (Roberts and Govorkova, 2009) as a second line of defense. Laninamivir, another NAI was designed to be a high-potency drug to minimize the high number of doses needed with traditional treatment with oseltamivir or zanamivir, and works as a long lasting neuraminidase inhibitor that can be administered once weekly. Laninamivir has been shown to be more effective than zanamivir in a mouse model and was found to be longer acting, with higher retention rates, in tissues following inhalation (Honda et al, 2009, Koyama et al, 2009).

Ribavirin (Virazole), a nucleoside analogue, which has limited use in certain countries, is a polymerase inhibitor that inhibits RNA synthesis against both influenza A and B viruses (Witkowski 1972, Sidwell et al, 1972, Oxford 1975, Scholtissek 1976, Eriksson et al, 1977). One limitation of ribavirin is that at high doses or chronic use, hemolytic anemia has been induced (Canonico et al, 1984, Page and Connor, 1990). Viramidine, a prodrug of ribavirin is being investigated further and was found to have lower toxicity, better targeting of the liver for conversion, and also limits the amount of drug localizing in red blood cells (Lin *et al*, 2003, Sidwell et al, 2005). Favipiravir (T-705), a pyrazine derivative, is a novel polymerase inhibitor that is orally active, and has shown anti-influenza activity both *in vitro* and *in vivo* (Furuta et al, 2002, Takahashi et al, 2003, Furata et al, 2005, Sidwell et al, 2007, Furuta et al, 2009, Smee et al, 2009, Kiso et al, 2010, Sleeman et al, 2010) against influenza A, B, and C viruses. Favipiravir differs from ribavirin in that it does not interfere with host DNA or RNA synthesis, rather inhibits the viral RNA polymerase during early to middle stages of infection, and it is less cytotoxic (Furata et al, 2002, Furuta et al, 2005). Fivipiravir was found to be more therapeutic in the mouse model against influenza A than oseltamivir, with increased survival and decreased viral lung titers (Furuta et al, 2002, Takahashi et al, 2003). Mice treated with high doses of fivipiravir were also protected against HP H5N1 viruses including oseltamivir-resistant HP H5N1 viruses, even when drug treatment was delayed up to 72-96 hours post infection (Sidwell et al, 2007, Kiso et al, 2010).

Cyanovirin-N (CV-N) is a virucidal protein that comes from the cyanobacterium *Nostoc ellipsosporum.* CV-N targets the high mannose oligosaccharides on influenza glycoprotein HA1 directly, via protein-carbohydrate interactions that inactivate the viral

particle thus preventing it from entering the host cell (O'Keefe et al, 2003). CV-N is known to have potent inhibitory activity against many influenza A and B strains but may be limited in use with influenza strains that acquire certain mutations in the hemagglutinin gene that lack glycosylation patterns leading to reduced CV-N binding (O'Keefe et al, 2003, Smee et al, 2007). CV-N studies in mice show reduced mortality and pneumonitis and in ferrets, reduced viral titers in nasal washes collected following intranasal delivery of drug (Smee et al, 2008). DAS181 is a recombinant fusion protein containing a saliadase derived from *Actinomyces viscosus* and a respiratory epithelium-anchoring domain (Malakhov et al, 2006). The saliadase works to remove sialic acids in respiratory epithelium cells, which normally serve to bind the virus, thus inhibiting or causing a reduction in infection. Mice studies have shown protection from death using intranasal treatment of DAS-181 (Triana-Baltzer et al, 2009).

Thiazolides are a class of drugs initially discovered to treat parasitic infections. Nitazoxanide, is a thiazolide liscensed to treat entiritis of *Cyrptosporidium parvum* and *Giardia lamblia*, but was recently tested against several influenza A strains *in vitro*. Nitazoxanide works by a novel mechanism that appears to interfere with the maturation process and blocks intracellular transport of the viral hemagglutinin, keeping the virus from being transported to the cell surface (Rossignol et al, 2009).

Small interfering RNAs (siRNA) have also been evaluated as a treatment to control influenza virus infection. RNA interference (RNAi) occurs when double stranded RNA (dsRNA) is injected into a subject and specifically silences sequence-specific regions of a gene (Fire et al, 1998) or causes sequence specific degredation of homologous mRNA (Hannon 2002). siRNA treatment has proven beneficial in influenza

infection both *in vitro* by showing potent inhibition of influenza virus production with influenza A H1N1 suptypes (Ge et al, 2003), and *in vivo* with increased survival and inhibition of viral replication with treatment prior to lethal challenge from H1N1, H5N1 and H7N7 (Tompkins et al, 2004). Sequence homology between the gene target and the siRNAs was crucial for protection and may not be valuable with certain influenza viruses that would contain mismatches from the siRNAs (Tompkins et al, 2004). While these aforementioned drugs are still in developmental and clinical trial phases, it is necessary to focus on better understanding the abilities and outcomes of the usage of the most commonly stockpiled drug, oseltamivir.

Efficacy of Oseltamivir in Human and Animal Models

Oseltamivir was initially designed following development of zanamivir in order to create a more orally bioavailable drug for treatment of influenza virus infections. Initial studies were carried out with both oseltamivir carboxylate and its ethyl ester prodrug oseltamivir phosphate, with the latter being much more orally bioavailable in humans (Li et al, 1998, Mendel et al, 1998). Initial treatment studies in mice led to a significant reduction in viral titers in the lung and enhanced survival when infected with influenza A and B viruses. Studies using ferrets also demonstrated reduced viral titers in nasal washes and elimination of typical signs of distress seen during influenza virus infection (Mendel et al, 1998). OP was also well tolerated in treated animals and showed no signs of toxicity. Along with efficacy, oseltamivir was also tested in mice to evaluate the immune effects associated with this specific neuraminidase inhibitor. Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells were evaluated for their presence both in influenza infected mice and uninfected mice during treatment with oseltamivir. Results confirmed

that oseltamivir treatment had no adverse effects on the primary cellular immune response to influenza infection (Burger et al, 2000). Mice that survived were also re-challenged with influenza virus and were able to survive due to adequate neutralizing antibodies, showing that oseltamivir did not interfere with the ability of the mice to develop an ample humoral response to the virus (Burger et al, 2000).

Following the outbreak of HP H5N1 viruses, oseltamivir was re-evaluated to confirm its efficacy for treatment against these HP strains. In animal models, varying levels of protection have been shown through the use of oseltamivir to protect mice and ferrets from death after challenge with HP H5N1 viruses (Govorkova et al, 2001, 2007, 2009, Yen et al, 2005, Ilyushina et al, 2008). Some concerns that arose during these studies with oseltamivir focused on the need for appropriate timing of drug delivery and a dose dependency required for different HP H5N1 strains (Ilyushina et al, 2008). These studies show that the longer the treatment (8 days versus 5 days), and the higher the dose (10mg/kg/day vs. 1 or 0.1mg/kg/day), the better the survival rate and more significant the decrease in viral replication in the host. Similar studies to confirm the optimal dosage of oseltamivir necessary to protect against lethal infection and to minimize the severity of disease were also determined for HP H5N1 in the ferret model (Govorkova et al, 2007). Ferrets in this study received either a prophylactic dose or delayed treatment of oseltamivir; it was confirmed that the dosage of oseltamivir needed to protect the ferrets from lethal challenge was higher than the current recommendations for humans, depending on strain of H5N1 used for challenge. This is of some concern since delayed treatment is commonly reported for humans and, depending on the strain of virus, frequently has led to decreased viral loads but not full protection from death. Further

evaluation of differing treatments in ferrets led to the observation that oseltamivir resistance can arise following drug treatment. Evaluation of three different samples (lung, brain, and nasal wash; 10 individual clones from each) from ferrets revealed 1 of 10 clones in the lung of animals treated with 10mg/kg/day, and 1 of 10 clones in the brain of those treated with 25mg/kg/day, to have acquired the H274Y mutation which is known to confer resistance to oseltamivir. The authors of this study concluded that direct sequencing and plaque reduction assays were not adequate by themselves to detect the mutations, but that analysis of individual plaques was adequate to determine that mutations occur (Govorkova et al, 2007). This is only a small representation of the possible clones recovered and needs to be further addressed.

Resistance to oseltamivir has been observed in humans infected with HP H5N1 following treatment with oseltamivir (de Jong et al, 2005, Le et al, 2005. Le et al, 2008). Some concerns with these observations are both the timing of drug treatment and the timing of collection of samples. First, one patient was not given oseltamivir until at least 48 hours after infection. The other cases had treatment started well after the optimal time, which would be within 48 hours of onset of symptoms. Unfortunately, an initial specimen was not collected before treatment from the one patient with optimal drug dosage, so confirmation of whether she had the resistance mutation of H274Y before treatment or whether it evolved during treatment could not be confirmed. Another patient that did show the resistance mutation after treatment but not before showed a possible small subset of the wild-type sequence along with the mutant sequence, suggesting that the mutant sequence may not be fit enough to continue adequate replication and lead to effective transmission, which is of concern if it is to become a pandemic. In a recent

reverse genetics study, the replication efficiency and pathogenicity of HP H5N1 strains in mice documented an alteration to include the H274Y and N294S mutations; the mutated virus retained its lethality to mice and retained replication efficiency *in vitro* (Yen et al, 2007). Another NA resistant mutation, E119V, has been shown to retain replicative ability and transmissibility as efficiently as wild type virus (Herlocher et al, 2004, Yen et al, 2005), whereas another mutation, R292K, appears to be compromised both *in vitro* and *in vivo* and had limited ability to transmit in the ferret contact model (Herlocher et al, 2002, Yen et al, 2005). Seasonal H1N1 viruses with the H274Y mutation have shown restriction in replicative ability and transmission in ferrets (Ives et al, 2002), but the virus has the ability to be transmitted if infectious doses are increased (Herlocher et al, 2004).

Viral fitness has been analyzed utilizing a competitive-mixtures model in ferrets (Hurt et al, 2010). The premise was to evaluate the outcome of infecting ferrets with both wild-type and oseltamivir resistant virus of the same subtype at differing percentages to determine if one virus or the other will win out while in competition within the host. Results show that the R292K mutated virus was outgrown by the R292 wild-type virus and was not transmissible which is consistent with previous studies with this mutation. The H274Y mutated virus was only marginally outgrown by the H274 wild-type virus, but was equally transmissible among ferrets which has been variable in previous studies. Recent evidence has also shown that those strains resistant to oseltamivir can be efficiently transmitted by direct contact through guinea pigs. (Bouvier et al, 2008) Current research indicates that mice, when infected with swine H1N1 followed by treatment with OP 1 hour post infection, had reduced viral titers in the lung at days 3 and

6 post-infection (Itoh et al, 2009). Analyzing the virus after treatment was not addressed in this experiment, nor was the effect of delayed treatment post-infection.

Chickens have also been evaluated for protection from HP H5N2 virus following treatment with oseltamivir and zanamivir (Meijer et al, 2004). Oseltamivir was able to reduce infection and transmission levels but did not completely prevent it while zanamivir proved to have no effect on infection or survival (Meijer et al, 2004). One interesting aspect of the study showed that 3 contact chickens in the oseltamivir group become positive after cessation of treatment, suggesting length of treatment may be important and play a role in transmission.

All this is of some concern as the stockpiling of the most orally bioavailable drug oseltamivir is at a peak. Since human patients are now more aware of antivirals coupled with recent concerns regarding the most recent pandemic of swine-H1N1, we can expect that this will ultimately lead to a higher use of the drug. It is imperative that we better understand the possible outcomes of resistance from antiviral treatment including the possibility of oseltamivir contamination in the environment from excessive use.

Oseltamivir in the Environment

Recent interest has been shown in the inability of OC to be removed or degraded by natural environmental conditions or sewage treatment plants (Singer et al, 2007, Bartels and von Tumpling jr, 2008, Fick et al, 2007, Straub 2009, Hutchinson et al, 2009, Ghosh et al, 2010a) suggesting a potential for environmental build-up. The prodrug Tamiflu is given orally as OP. OP breaks down in the liver of humans to the active metabolite OC. OC is not broken down any further and 80% is renally excreted as OC (Ward et al, 2005). While OC has been shown to be poorly orally bioavailable, it is not

known to what extent the interaction in the gastrointestinal tract of infected animals may have on the selective pressure of the virus.

The hypothesis of this outcome is that OC will enter the regional aquatic environment, such as lakes, streams and rivers, and will be found at high levels in areas of high human treatment use. Proposed suggestions show that OC can remain in the environment for up to 18 days and remain at levels of 0.08-0.23 μg L-1.(Singer et al, 2007, Bartels and von Tumpling jr 2008) A suggestion of the highest predicted environmental concentration (PEC) was also proposed for Japan, a country which currently uses a large amount of oseltamivir, to be 0.028 μg L-1.(Bartels and von Tumpling jr 2008) These are proposed values only, as different issues such as water size, prescribed amounts of oseltamivir, and the actual spread of a pandemic would all play a role in actual numbers and outcomes.

More recently, actual OC levels were determined in areas of Japan that show high levels of prescribed Tamiflu®. Values of OC measured before the 2007-2008 influenza season were undetectable in the Yodo river system, but levels of 2-58 ng/L were measured during the influenza season (Söderström et al, 2009). The highest levels detected in Japan during the 2008-2009 influenza season were 293.3 ng/L of OC at its peak from sewage treatment plant discharge, with river water levels of OC ranging from 6.6-190.2 ng/L (Ghosh et al, 2010b). These proposed and actual outcomes show the need for research into what extent the overuse of oseltamivir and build-up of OC or OP in the environment contributes to the emergence of resistant virus strains, and the impact that these resistant strains will have on avian and human populations.

Relevance

We know that influenza viruses are evolving in many species alarmingly fast. While great strides have been made in increasing surveillance in both humans and avian species, there are still a vast number of unknown factors present. Since wild birds are the only known reservoir to harbor all subtypes of influenza A viruses, it is valuable to study and evaluate issues that directly concern wild birds, including the viruses they carry and the impact that environmental contamination with antiviral drugs has on the species as a whole. Understanding these factors will help predict the eventual impact on humans in terms of virus transmission. Influenza A viruses resistant to oseltamivir have been detected at an increasing rate in humans, forcing us to re-evaluate the potential dangers associated with stockpiling only this drug, as well as possible outcomes of mass human use in the event of a pandemic. The knowledge that oseltamivir has already been detected in the environment formulated the basis for the proposal to study what may happen to viruses in our avian reservoirs when they ingest varying quantities of oseltamivir from the environment. We sought to determine whether emergence of more resistant viruses in wild birds might eventually pose a threat to humans.

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CHAPTER 2: TRANSMISSION OF AVIAN INFLUENZA A VIRUSES AMONG SPECIES IN AN ARTIFICIAL BARNYARD

Summary

Waterfowl and shorebirds harbor and shed all hemagglutinin and neuraminidase subtypes of influenza A viruses and interact in nature with a broad range of other avian and mammalian species to which they might transmit such viruses. Estimating the efficiency and importance of such cross-species transmission using epidemiological approaches is difficult. We therefore addressed this question by studying transmission of low pathogenic H5 and H7 viruses from infected ducks to other common animals in a quasi-natural laboratory environment designed to mimic a common barnyard. Mallards (*Anas platyrhynchos*) recently infected with H5N2 or H7N3 viruses were introduced into a room housing other mallards, in addition to chickens, blackbirds, rats and pigeons. Transmission was assessed by monitoring virus shedding (ducks) or seroconversion (other species) over the following 4 weeks. Additional animals of each species were directly inoculated with virus to characterize the effect of a known exposure. In both barnyard experiments, virus accumulated to high titers in the shared water pool. The H5N2 virus was transmitted from infected ducks to other ducks and chickens in the room either directly or through environmental contamination, but not to rats or blackbirds. Ducks infected with the H7N3 virus transmitted directly or indirectly to all other species present. Chickens and blackbirds directly inoculated with these viruses shed significant amounts of virus and seroconverted; rats and pigeons developed

antiviral antibodies, but, except for one pigeon, failed to shed virus at detectable levels.

Introduction

Gaining a more detailed understanding of the transmission potential of different avian influenza viruses among co-habitating species will enhance our ability to develop accurate models for disease spread, develop control strategies and, in some cases, assess risk of transmission to humans. Influenza A viruses are a common concern among many animal species including, birds, horses, pigs, sea mammals and humans, as the effects of infection can range from asymptomatic to severe respiratory distress leading to death. While avian influenza viruses (AIVs) are maintained in wild water birds, they occasionally spread to other animals and humans and can lead to public health concerns, as currently is the case for highly pathogenic H5N1 viruses. All 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes of influenza A virus are found in wild waterfowl and shorebirds (Webster et al, 1992, Rohm et al, 1996, Fouchier et al, 2005), but a much more restricted subset of these viruses is found in other birds and mammals.

Most strains of AIV are designated low pathogenic (LP) and cause minimal illness in chickens as well as in wild waterfowl and shorebirds, but infection results in high levels of virus shedding, efficient spread among susceptible hosts, and perpetuation of the agent. Other AIV strains are classified as highly pathogenic (HP) and are restricted to members of the H5 and H7 subtype. HPAIV classification comes from the ability to cause severe morbidity and mortality in domestic fowl, and more recently has caused mortality in wild waterfowl, mammals, and humans (Chen et al, 2004, Sturm-Ramirez et al, 2004). In several outbreaks of HPAIV, circulation of a H5 or H7 LPAIV was detected shortly before the HPAIV outbreak of the same subtype, and was

determined to have evolved from the LPAIV strain either through a recombination event (Suarez et al, 2004, Hirst et al, 2004) or a gradual increase in virulence over time through the insertion or substitution of basic amino acids at the HA cleavage site (Kawaoka et al, 1984, Horimoto et al, 1995).

Among water birds, mallards are of great interest due to their widespread distribution, reservoir for subtypes H1-H12, and ability to shed large amounts of virus with minimal pathology and disease (Munster et al, 2007, Keawchareon et al, 2008). Mallards can also travel large distances and have been implicated as carriers of AIVs from one region to another (Nagy et al, 2009, Brochet et al, 2010). While it has been shown that shedding time decreases during a season of sampling, likely due to transient immunity (Latorre-Margalef et al, 2008), the concern remains that while infection and shedding continue to occur, the ability of LPAIV to mutate or evolve into HPAIV remains. Furthermore, some HP H5N1 viruses are non-pathogenic in mallards (Keawcharoen et al, 2008) or can become non-pathogenic through evolutionary adaptation in the duck host, while remaining highly pathogenic to other domestic poultry. This would allow for the possibility of ducks transmitting the virus to other poultry without themselves suffering from disease (Hulse-Post et al, 2005).

The relative roles of direct contact versus environmental contamination in the transmission of AIVs remains poorly understood; both mechanisms likely occur based on experimental and field studies (Markwell & Shortridge 1982, Sivanadan et al, 1991, Laudert et al, 1993, Forrest et al, 2010). Understanding routes of transmission is important to modeling spread of virus (Breban et al, 2009, Rohani et al, 2009, Roche et al, 2009). AIVs have been shown to persist in water sources (Stallknecht et al, 1990,

Brown et al, 2007) and may provide a source of contamination to other species sharing the same source. It has also been shown that over a 4 year period in Hong Kong, virus was isolated throughout the year from domestic ducks (Shortridge 1982).

Common concerns with transmission of AIV from waterfowl to other species arise when one observes interactions of multiple species, both domestic and wild, present within a single small farm virtually anywhere in the world. Transmission to species such as rodents would not likely result in disease spread, due to lack of detectable shedding, but could be exploited to monitor disease incursion via serosurveillance. Rats and mice are found in abundance on small farms and are of concern due to their ability to move freely from outside into enclosures, and their propensity to eat and drink from common containers of poultry feed. Although rats are not considered reservoir hosts for influenza viruses, both laboratory and cotton rats have been shown to replicate unadapted avian and human influenza A viruses (Ottolini et al, 2005, Eicherberger 2007, Shortridge et al, 1998). Similarly, pigeons are not generally considered an important host for transmission of influenza viruses, but are ubiquitous on small farms and undoubtedly exposed to these viruses on a routine basis. Their susceptibility to experimental infection with both LPAIV or HPAIV has been variable (Fang et al, 2006, Werner et al, 2007, Jia et al, 2008, Brown et al, 2009).

It is clear that wild and domestic ducks harbor and shed influenza A viruses and recurrently interact in nature with a broad range of other avian and mammalian species to which they might transmit such viruses. Estimating the efficiency and importance of such multispecies transmission using epidemiological approaches is difficult. We therefore addressed this question by studying transmission of LP H5 and H7 viruses

from infected ducks to other common animals in a quasi-natural laboratory environment designed to mimic a common barnyard.

Materials and Methods

Ethics Statement

All experiments were approved by the Institutional Animal Care and Use Committee of Colorado State University, Fort Collins, Colorado, USA, under approval number 09-168A.

Animals

Mallard ducks and chickens were purchased from local producers at 2-4 months and 3-4 weeks of age respectively. Red-winged blackbirds and pigeons were captured locally. Sprague Dawley rats, 6-8 weeks of age were obtained from Charles River Laboratories. All animals tested negative for group-specific antibodies to influenza A virus by ELISA and strain specific antibodies (H5 and H7; <10) by hemagglutination inhibition (HAI) assay prior to infection.

Viruses, Virus Assays and Serologic Assays

The viruses used in this study were A/Mallard/MN/346250/00 (H5N2) and A/Ruddy turnstone/ReedsBeachNJ/00 (H7N3). Both viruses were propagated to passage three in 10 day old specific pathogen free embryonated chicken eggs (Sunrise Farms, NY). Allantoic fluid was harvested 48 hours after inoculation, aliquoted, and stored at -80 C until use. Both viruses were titrated by plaque assay using MDCK cells. (ATCC, Manassas, VA)

Subtype-specific antibodies were assayed by hemagglutination-inhibition assay (Kendal et al, 1982), using sera treated with receptor destroying enzyme (Denka Seiken, Tokyo, Japan) as previously described (Tyrrell & Horsfall, 1952). Serial 2-fold dilutions of sera in PBS were prepared in 96-well V-bottom plates and mixed with 0.5% chicken red blood cells; titers of 10 or greater were considered positive. Group specific antibodies were detected using a commercial ELISA test (Flu DETECT® BE, Synbiotics Corporation, Kansas City, MO) based on detection of antibodies to a recombinant AIV nucleoprotein antigen.

Barnyard Transmission Experiment

Two independent experiments were conducted using different influenza viruses. In the first experiment, the barnyard contained 8 ducks, 8 chickens, 8 rats and 10 blackbirds. The second experiment consisted of 8 ducks, 8 chickens, 6 pigeons, 5 blackbirds and 7 rats. In both experiments, animals were allowed to freely range inside a room within an ABSL3 facility. The room had dimensions of 12 (width) x 18 (length) x 12 (height) feet and basic illumination was provided through a skylight in the roof. The barnyard rooms contained a plastic children's swimming pool (4 feet diameter, cut to 6 inches height) and 2 large bowls that contained commercial duck and chicken layer feed (Figure 2.1A). Three smaller bowls filled with songbird mixed grains were suspended approximately 6 feet off the floor from pipes that ran longitudinally across the room; these pipes also served as perches for the blackbirds and pigeons. Straw was spread across the floor sparsely and a sawhorse was present to provide additional perching opportunities for blackbirds and pigeons. Two or three cardboard boxes were

provided as nest boxes for the rats. The pool was filled daily (but not emptied within the first week of the trial) with 5 gallons of water that had been sitting at room temperature and aerated with an aquarium pump for 24 hours to dechlorinate and thus prevent inactivation of any influenza virus (Rice et al, 2007). This was also done to better mimic the natural state of water such as lakes and streams and farm water, which are typically not chlorinated. In the first experiment, night vision (infrared) cameras were installed in the room to allow monitoring of behaviors in the dark (Figure 2.1B).

Figure 2.1. Barnyard room layout observed during the day (A) and at night (B).

On day 0 of each experiment, four ducks were removed from the barnyard room, placed in a separate room, and inoculated orally, intranasally and ocularly with 10^6 PFU/0.5 ml of the respective virus. After 4 hours, the 4 inoculated ducks were returned to the barnyard where all animals were free to move about the room and interact. All 8 ducks (4 inoculated and 4 non-inoculated) were sampled on days 0-7 by collecting oral and cloacal swabs into 2 ml BA-1 medium (MEM, 1% bovine serum albumin, 350 mg/L sodium bicarbonate, 50ml/L 1M Tris, pH7.6, 5mg/L phenol red) supplemented with

antibiotics (gentamicin 50 ug/ml, polymyxin B 100 U/ml, nyastatin 50 U/ml, penicillin, 100 U/ml and streptomycin 50 ug/ml), refrigerated and tested within 2 days for virus titer by plaque assay on MDCK cells; titers are reported as PFU/ml. The only barnyard animals to have daily swab samples collected for virus titration were ducks as we wished to minimize the stress on remaining barnyard species and use seroconversion as the determination of virus infection. All animals in the barnyard room were bled on days 0, 14, 21 and 28, and those sera were tested for seroconversion using HAI and ELISA.

Two to 3 water samples were collected daily before the addition of new water. Samples were collected by skimming the top of the pool with a tube, by running the tube along the bottom of the pool to collect sediment, and, collecting splashed water from the floor near the pool if present. To assist in interpreting pool water virus titers, we conducted an *in vitro* experiment in which pool water from a room containing non-infected ducks was spiked with the H5 or H7 viruses, maintained at room temperature and tested by plaque assay at intervals up to 42 days.

Direct Inoculation of Control Animals

For both experiments, groups of each of the animals in the barnyard except ducks were housed in cages in a separate room and directly inoculated with virus to determine the effects of known exposure. Chickens, pigeons, blackbirds and rats were inoculated intranasally with 10^6 PFU in 0.1 ml. Once daily on days 0-7, oropharyngeal and cloacal swabs were collected from the birds, and oral swabs from rats; these samples were processed as described above for duck samples. Sera were collected on

days 0, 14, 21, and 28, and tested for anti-influenza antibodies by ELISA and for challenge virus-specific antibodies by HAI

In vitro detection of persistence of virus

Water was collected from a pool that had non-infected ducks swimming in it for 24 hours prior to water collection. This was done to mimic the natural state of the water from the barnyard study where water would also contain feces and food particles. Pool water was then placed in a 50 ml conical tube and spiked with either $1x10^6$ PFU/ml of H5N2 or H7N3 virus and placed at room temperature. A tube of the same water not spiked with virus served as the negative control and was collected and tested for virus. Samples were collected once daily on days 0 through 7 then weekly for 6 weeks. One ml aliquots were collected at each time point and stored at -80 until all samples were collected. Samples were then tested for virus titer utilizing the plaque assay.

Results

Clinical signs of disease were not observed in any of the birds or rats in the barnyard environments nor among those caged and directly inoculated with either virus. Animals in the barnyard were observed several times daily. The ducks and chickens tended to cluster and move about in their own groups. Blackbirds and pigeons spent much of their time perched above the floor, but were frequently observed walking on the floor or perched on the side of the pool. All of the birds and rats were observed drinking from the pool and eating out of common feed bowls on the floor. The rats were almost never seen out of their houses during daylight, but were confirmed by video to be

exceptionally active in running around the room and through the pool of water during the dark (Figure 2.1B).

Infection and Transmission: H5N2 virus

Virus was shed by all 4 inoculated ducks and transmitted to all 4 contact ducks either through direct contact or environmental contamination of the floor and shared pool (Table 2.1). As would be expected with LPAIV in ducks, virus was shed to higher titers by the cloacal versus oral routes. Contact ducks did not begin shedding detectable virus until at least 1 day after inoculated ducks began shedding. Detectable shedding of virus from ducks ended on day 5 post inoculation. H5N2 virus was first detectable in sampled water on day 2 post inoculation and continued until day 7 which was the last day samples were collected before the pool was emptied completely and refilled (Figure 2.2). Titers of virus were comparable in all three samples on each day except the floor sample from day four was 100-fold greater than either the sediment or surface pool water sample, likely due to a concentration of feces in that area on that day. *In vitro* testing of H5N2 virus stability in pool water demonstrated a steady decline in virus titer with viable virus detected out to day 35 (Figure 2.3).

Table 2.1. Virus shedding from inoculated and contact ducks.

Virus	Exposure	Sample*	Duck	Virus titer in swab sample (log_{10} PFU/ml)						
				1§	2	3	4	5	6	7
H5N2	Inoculated	CLO	2	<1.0	<1.0	<1.0	1.0	2.0	<1.0	<1.0
			3	<1.0	4.7	2.8	1.5	<1.0	<1.0	<1.0
			4	<1.0	3.0	3.6	3.1	2.9	<1.0	<1.0
			6	<1.0	5.9	3.8	3.0	2.0	<1.0	<1.0
		OP	2	<1.0	1.3	<1.0	<1.0	<1.0	<1.0	<1.0
			3	2.3	1.3	<1.0	1.0	<1.0	<1.0	<1.0
			4	<1.0	<1.0	<1.0	2.0	<1.0	<1.0	<1.0
			6	<1.0	1.0	<1.0	1.0	<1.0	<1.0	<1.0
	Contact	CLO	1	<1.0	<1.0	3.1	4.6	3.1	<1.0	<1.0
			5	<1.0	<1.0	4.6	3.3	2.7	<1.0	<1.0
			7	<1.0	<1.0	4.1	3.9	3.3	<1.0	<1.0
			8	<1.0	<1.0	3.0	4.3	2.9	<1.0	<1.0
		OP	1	<1.0	<1.0	<1.0	1.0	<1.0	<1.0	<1.0
			5	<1.0	<1.0	1.9	1.5	1.5	<1.0	<1.0
			7	<1.0	1.5	1.8	2.3	<1.0	<1.0	<1.0
			8	<1.0	<1.0	1.5	<1.0	1.8	<1.0	<1.0
H7N3	Inoculated	CLO	1	<1.0	4.4	3.7	2.5	<1.0	<1.0	<1.0
			2	3.3	3.5	3.0	2.9	1.0	1.6	<1.0
			3	2.3	3.5	3.0	2.5	1.3	<1.0	<1.0
			7	<1.0	3.5	2.9	3.3	2.3	<1.0	<1.0
		OP	1	1.3	3.0	1.5	2.4	1.7	2.3	<1.0
			2	2.3	2.5	2.5	2.9	1.3	1.0	<1.0
			3	1.0	<1.0	1.0	3.3	1.0	<1.0	<1.0
			7	2.7	2.3	2.6	2.9	1.7	<1.0	<1.0
	Contact	CLO	4	<1.0	2.6	3.6	3.3	<1.0	<1.0	<1.0
			5	<1.0	3.3	3.0	3.6	2.0	<1.0	1.0
			6	<1.0	4.6	3.7	2.0	3.7	1.0	<1.0
			8	<1.0	6.3	6.5	3.5	3.7	1.0	<1.0
		OP	4	<1.0	2.5	1.8	1.6	2.5	<1.0	<1.0
			5	<1.0	1.0	2.0	1.6	1.5	<1.0	<1.0
			6	<1.0	2.3	2.0	<1.0	1.3	<1.0	<1.0
			8	<1.0	2.0	2.3	2.3	2.9	<1.0	<1.0

***** Samples are cloacal (CLO) or Oropharyngeal (OP); § Numbers represent days post challenge

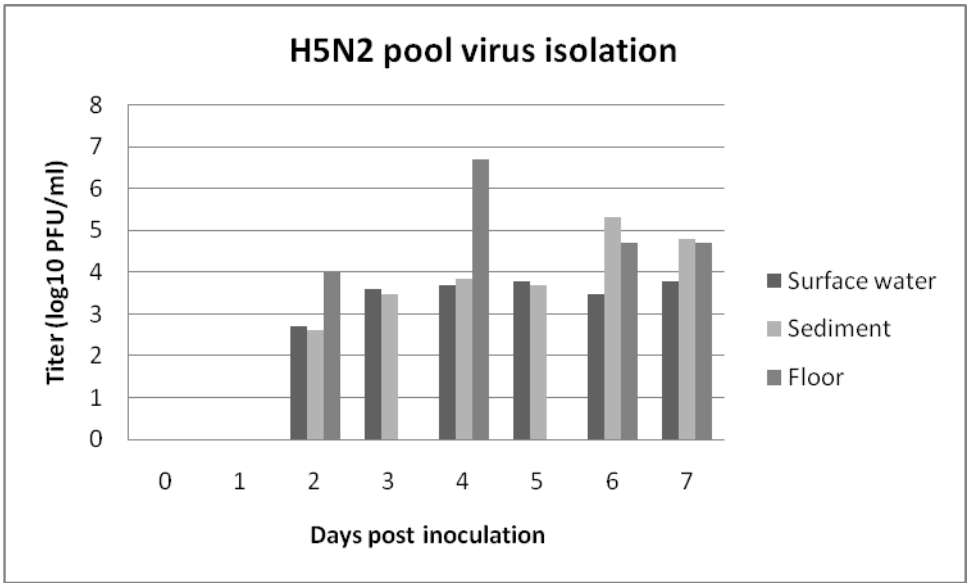

Figure 2.2. Accumulation of H5N2 viruses in barnyard pool water. Water samples skimmed from the surface of the pool, off the bottom (sediment-rich) or splashed onto the floor were assayed for infectious virus by plaque assay on MDCK cells.

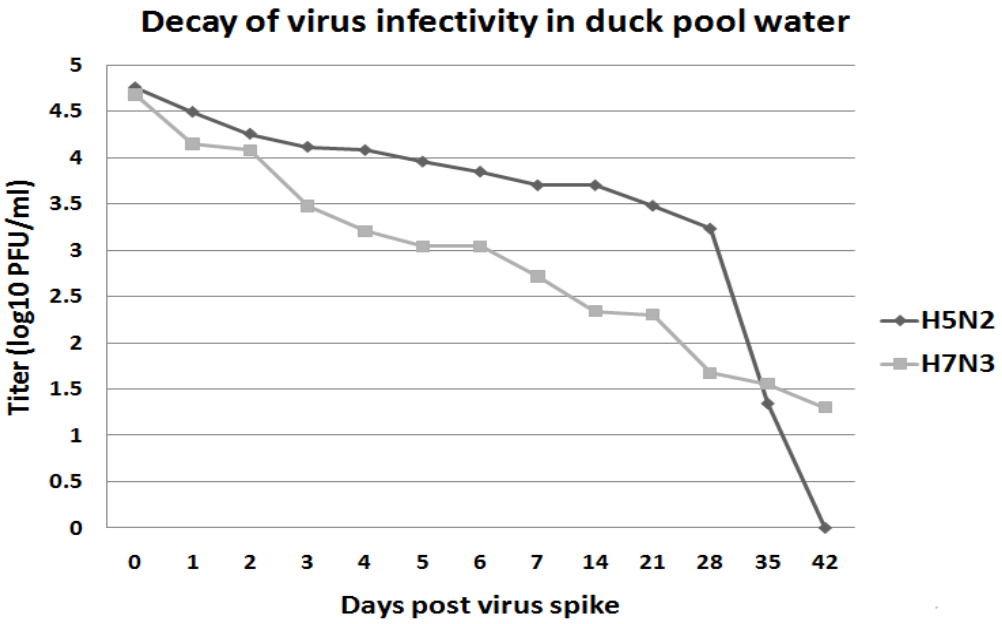

Figure 2.3. Survival of H5N2 and H7N3 viruses added to duck pool water and maintained at ambient temperature. Water from a pool used by non-infected ducks was spiked with virus, sampled over time and assayed by plaque assay on MDCK cells.

In the experiment in which control animals were directly inoculated, 83% of chickens and 100% of blackbirds shed detectable virus orally on days 1 through 5 and 1 through 6 respectively (Table 2.2). Small amounts of virus were detected sporadically on cloacal swabs from 1 chicken on day 4. Virus was not recovered from rats or pigeons that were directly inoculated with virus.

Table 2.2. Virus isolation from oropharygeal swabs taken from directly inoculated control animals.

Virus	Species	Number Shed	Days shed	Peak day of shedding	Peak virus titer (log10 PFU/ml)
H5N2	Chicken	5/6	1-5	3	2.6
	Blackbird	6/6	1-6	2	3.6
	Pigeon	0/6	NA	NA	NA
	Rat	0/6	NA	NA	NA
H7N3	Chicken	5/6	1-7	2	2.3
	Blackbird	4/4	1-7	4	4.0
	Pigeon	1/6	1-3	1	3.5
	Rat	0/6	NA	NA	NA

In order to determine infection rates of all animals exposed in the barnyard or those directly inoculated, sera were collected on days 0, 14, 21 and 28 post-inoculation or exposure.

The contact chickens in the barnyard had seroconversion rates of 63% by HAI and 100% by ELISA (Table 2.3). None of the barnyard contact rats and blackbirds seroconverted by either HAI or ELISA. For the directly inoculated control animals, there was 100% seroconversion in the chickens and rats by both HAI and ELISA but only 50% (1/2) in the blackbirds by HAI (Table 2.3). The ELISA failed to detect

antibody in any blackbird, regardless of HAI titer or virus isolation. One caveat to the experiment relevant to the blackbirds is that repeated daily handling to obtain the cloacal and oral swabs proved too stressful, and 3 of the 6 blackbirds died due to non-influenza complications before serum collection was initiated on day 7.

Table 2.3. Seroconversion following virus exposure in directly-inoculated (caged) and contact (barnyard) animals.

Species and exposure	H5N2 virus		H7N3 virus	
	HAI*	ELISA	HAI	ELISA
Duck, Inoculated	3/4 (75%)§	4/4 (100%)	1/4 (25%)	4/4 (100%)
Duck, Contact	3/4 (75%)	4/4 (100%)	2/4 (50%)	4/4 (100%)
Chicken, Inoculated	6/6 (100%)	6/6 (100%)	4/6 (67%)	6/6 (100%)
Chicken, Contact	5/8 (63%)	8/8 (100%)	8/8 (100%)	8/8 (100%)
Blackbird, Inoculated	1/2 (50%)*	0/2 (0%)*	1/1 (100%)*	0/1 (0%)*
Blackbird, Contact	0/10 (0%)	0/10 (0%)	4/5 (80%)	0/5 (0%)
Pigeon, Inoculated	0/6 (0%)	5/6 (83%)	0/6 (0%)	2/6 (33%)
Pigeon, Contact	ND	ND	0/6 (0%)	5/6 (83%)
Rat, Inoculated	6/6 (100%)	6/6 (100%)	4/5 (80%)	5/5 (100%)
Rat, Contact	0/8 (0%)	0/8 (0%)	0/7 (0%)	6/7 (86%)

*HAI titer $\geq$ 10 were considered positive.
§ Number of birds positive/total (% positive) at any one timepoint from days 14, 21, or 28

Infection and Transmission: H7N3 Virus

Virus was shed by all 4 inoculated ducks and transmitted to all 4 contact ducks (Table 2.1). Virus was shed longer, and to slightly higher titers than with the H5N2 virus isolate, which was only detectable to day 5. Virus titers from oral samples were also higher than oral viral titers seen in the H5N2 experiment. As seen with the H5N2 experiment, contact ducks did not shed detectable virus until at least 1 day after inoculated ducks began shedding, as would be expected in cases of transmission (Table 2.1). Virus shedding was detected in at least 1 duck on all days 1 through 7. For the

H7N3 experiment the number of days of water sample collection was extended from 7 days to 9 before cleaning out the pool. H7N3 virus was detected in samples from day 1 through day 9, with floor samples showing the highest levels of virus at all time points. (Figure 2.4) H7N3 virus was also tested *in vitro* for stability, and behaved similarly to the H5N2 virus with viable virus detected out to day 42 (Figure 2.3).

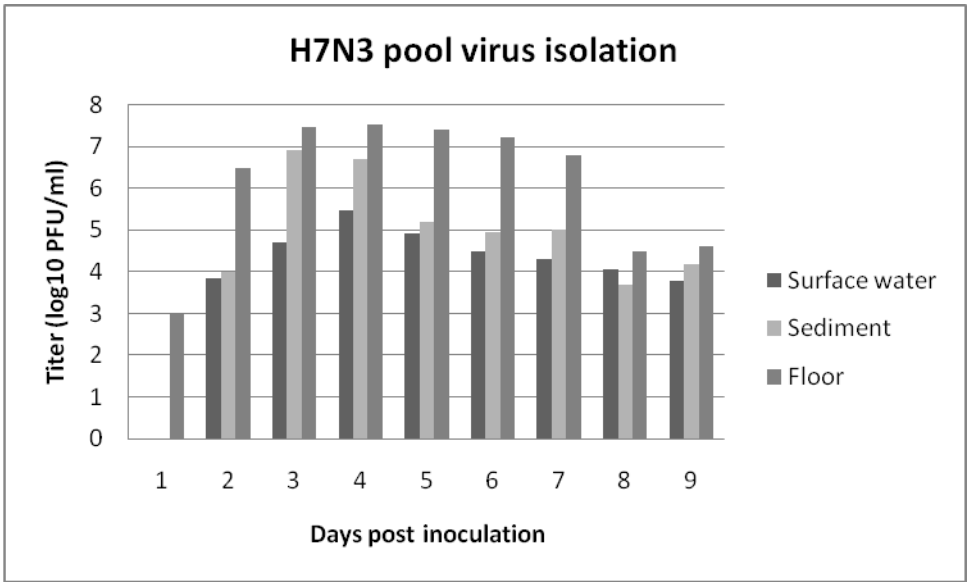

Figure 2.4. Presence of H7N3 viruses in barnyard pool water. Water samples skimmed from the surface of the pool, off the bottom (sediment-rich) or splashed onto the floor were assayed for infectious virus by plaque assay on MDCK cells.

Virus isolation was also performed on all control animals directly inoculated with H7N3 virus, including blackbirds, pigeons, chickens and rats. Virus was isolated from oropharygeal swabs at various time points from 83% of chickens, 16% of pigeons, and 100% of blackbirds (Table 2.2), but was not isolated from any of the oral swabs collected from rats.

Transmission rates in the barnyard were assessed serologically on days 0, 14, 21 and 28 post inoculation. Only 25% of virus-inoculated ducks seroconverted based on

HAI results but 100% were positive by ELISA, correlating with the fact that 100% shed virus to varying degrees in the experiment (Table 2.3). Of the 4 contact ducks, 50% seroconverted by HAI and 100% by ELISA. Also in the barnyard, contact chickens, rats, blackbirds, and pigeons seroconverted at 100%, 0%, 80%, and 0% respectively as detected by HAI, and 100%, 86%, 0%, and 83% by ELISA (Table 2.3).

For the directly inoculated chickens, rats, blackbirds, and pigeons we observed seroconversion rates of 67%, 80%, 100%, and 0% by HAI respectively, and 100%, 100%, 0%, and 33% by ELISA respectively (Table 2.3). As with the H5N2, the experimentally infected blackbirds did not all survive to day 7 for serum collection, so the percentage represents only one of the original four blackbirds that survived to the end of day 21 (Table 2.3).

Discussion

In both barnyard experiments, introduction of recently-infected mallards was followed rapidly by infection and shedding of virus by contact ducks, and the presence of substantial quantities of virus in water from the shared pool. Based on detection of seroconversion, ducks infected with either virus efficiently transmitted the virus to other species either through direct contact, which would be most likely with the contact ducks, or through contamination of the environment such as the pool and floor water where high virus titers were recovered in titers high enough to infect the other species. The H7N3 virus was transmitted to a large fraction of other animals in the room, including blackbirds, pigeons and rats, but transmission of the H5N2 virus to blackbirds and rats was not detected. This apparent difference in cross-species transmission may reflect, in part, differences in transmissibility between the two viruses, but it is more

likely that transmission of the H7 virus was enhanced due to its higher magnitude and duration of shedding, and higher levels of accumulation in the shared water source compared to that of the H5 virus. As anticipated, neither of the two viruses induced noticeable signs of disease in any of the exposed animals, including those directly inoculated with virus.

The high titers of virus that accumulated in water of the shared barnyard pools undoubtedly were in excess of what might typically be expected in natural situations involving wild mallards, but may not be altogether unrealistic for low pathogenic AIVs in small bodies of water. Moreover, it seems likely that such titers may occur in ponds associated with high density domestic duck production facilities, although studies attempting to measure virus titers in such situations are lacking with ducks. The presence of environmental AIV in water habitats of turkeys supports the need for increased environmental sampling along with avian surveillance studies (Sivanandan et al, 1991). It is evident, however, from this and other experimental studies, that efficient transmission via contaminated water can occur among ducks and between ducks and other birds [Forrest et al, 2010, VanDalen et al, 2010).

Rats were included in the barnyard transmission experiments because they or other rodents are inevitably present in variable numbers on small farms, are in direct contact with ducks and poultry and are able to move freely among enclosures. Live markets in Asia, where H5N1 influenza is prevalent, are an additional setting where large numbers of rats live in close contact with ducks and chickens (Shortridge et al, 1998). The role of rodents in facilitating spread of AIVs is essentially unknown. Rats housed in the H7N3 contaminated barnyard room seroconverted to that virus, as did

caged rats inoculated with both H5N2 and H7N3 viruses. Neither virus was shed from infected rats as measured in our study, supporting the idea that they do not play a significant role in transmission to other species.

Sero-surveys in natural settings (Arenas et al, 1990, DeMarco et al, 2003, Niqueux et al, 2010) as well as in experimental studies (Fereidouni et al, 2010) have provided valuable insights into the infection rates of mallards and other wild birds. In the current study, seroconversion was used to evaluate virus transmission among the barnyard animals and to assess infection in the animals directly inoculated with virus. Further, it would have been extremely stressful to capture the blackbirds and pigeons daily to obtain samples for virus isolation. Results obtained from the nucleoprotein blocking ELISA more accurately reflected the results of virus isolation and known virus exposure than did the HAI test, and allowed detection of virus transmission to rats and pigeons in the H7 barnyard trial. An interesting exception to this finding was that the ELISA consistently failed to detect antibodies to either influenza virus in blackbird sera despite positive findings by HAI testing. Results reported here suggest that multiple serologic tests are necessary to accurately conduct serosurveillance for influenza viruses when diverse species are involved. Additional research to identify factors responsible for these serologic discrepancies would clearly be beneficial to surveillance efforts and allow an enhanced understanding of the ecology and evolution of avian influenza viruses worldwide.

Both LPAIVs tested efficiently spread from ducks to chickens within the shared environment, and a majority of chickens directly inoculated with these viruses shed them at reasonable levels from the intestinal tract; we did not test whether chicken-to-

chicken transmission occurred. Transmission of LPAIV from ducks to chickens, if accompanied by mutations in the hemagglutinin gene encoding the HA-1:HA-2 cleavage site, could lead to generation of a HPAIV and a subsequent outbreak in poultry. It is not known whether either of these viruses replicating in chickens, blackbirds, or pigeons might evolve and adapt to those hosts, allowing the new host to better transmit the virus, or whether the virus would encounter a dead end in the new host.

Small farms, live and wet markets and many poultry shows provide abundant opportunities for interactions among free ranging and domestic species which may result in transmission and perpetuation of AIVs, particularly when ducks are involved. The studies reported here indicate that introduction of ducks infected with LPAIV into a room designed to mimic a typical barnyard resulted in efficient dissemination of virus to a number of other species, including other birds and rodents. This model system should be extended to investigate more refined questions, such as transmission from passerines to ducks or chickens, multiround transmission and transmission involving additional viruses, including H5N1 AIV.

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CHAPTER 3: THE EFFECT OF OSELTAMIVIR CARBOXYLATE CONSUMPTION ON THE EMERGENCE OF DRUG RESISTANT AVIAN INFLUENZA VIRUS IN DUCKS

Summary

Oseltamivir carboxylate (OC) has been detected in environmental waters at varying levels during typical flu seasons in humans, reflecting high level usage of this drug. This prompted us to evaluate the effect of consumption of low doses of OC on development of oseltamivir-resistant mutants in mallard ducks (*Anas platyrhynchos*) infected with two different low pathogenic H5N2 influenza viruses. We found that oseltamivir resistance did emerge repeatedly in OC-exposed mallards infected with 1 of the 2 strains of influenza virus, but not in ducks that were not exposed to the drug. The E119V mutation that arose, conferring resistance to oseltamivir, was found to be stable *in vitro* and had similar replication kinetics when compared to wild-type, but was unstable *in vivo* in the absence of OC. Only the wild-type parent strain 182742WT was transmitted to naïve ducks. The ducks infected with 182742-MUT-E119V showed a 1 day delay in shedding with only the wild-type virus being transmitted. Sequencing of isolates recovered from both inoculated and contact naïve ducks detected only the wild-type strain present in all ducks, indicating the instability of this E119V mutation in mallards. These results show the differing capabilities of the E119V mutation to replicate and transmit as efficiently as wild-type between mammals, humans, and water fowl.

Introduction

Oseltamivir is one of the most widely prescribed antiviral drugs in the world and is used primarily for treatment of human influenza virus infections. This antiviral is administered orally as oseltamivir phosphate (OP), which is readily absorbed and has high oral bioavailability. In humans, OP is metabolized in the liver to the active metabolite oseltamivir carboxylate (OC), which is not metabolized further and is excreted primarily in urine (He et al, 1999), leading to large quantities of the drug entering wastewater when drug usage in the population is high. Importantly, OC is poorly removed or degraded by natural environmental conditions or sewage treatment (Singer et al, 2007, Bartels and von Tumpling jr, 2008, Fick et al, 2007, Straub 2009, Hutchinson et al, 2009), resulting in significant retention in waterways. These same waterways often support large numbers of waterfowl, the primary reservoir of avian influenza A viruses (AIV), and waterfowl in several parts of the world are likely exposed over lengthy periods to at least low doses of OC in their drinking water. In contrast to mammals, AIVs replicate predominantly in the intestinal tract of ducks and other waterfowl, and there is reason for concern that the presence of OC in the intestinal lumen may promote selection of AIVs that are resistant to oseltamivir.

In order to obtain a better understanding of the risk for emergence of resistance to oseltamivir due to environmental contamination with the drug, we infected mallard ducks with two different low pathogenic avian influenza (LPAI) H5N2 strains of mallard virus, while giving them access to drinking water containing low doses of OC. We used a concentration of OC that approximated concentrations that have been found naturally in

water. Furthermore, if resistance was detected, we hypothesized that the oseltamivir resistant virus would be as transmissible as the wild type progenitor.

Materials and Methods

Ethics Statement

All experiments were approved by the Institutional Animal Care and Use Committee of Colorado State University, Fort Collins, Colorado, USA, under approval number 09-168A.

Animals

Mallard ducks were purchased as hatchlings from either Stromberg's Chicks and Gamebirds Unlimited (Pine River, MN) or Murray McMurray Hatchery (Webster City, IA). Ducklings were housed in an ABSL3 facility until four to six weeks of age at the start of each experiment. All ducks tested negative by ELISA for group specific antibodies to an influenza type A virus antigen (recombinant nucleoprotein), (Flu DETECT BE, Synbiotics Corporation, Kansas City, MO) and strain specific antibodies to H5N2 by hemagglutination inhibition (HAI) assay prior to infection.

Viruses and Chemicals

The viruses used in this study were A/mallard/Minnesota/346250/00 (H5N2) and A/mallard/Minnesota/182742/98 (H5N2). Both viruses were propagated by passage in 10 day old specific pathogen free embryonated chicken eggs (Sunrise Farms, NY). Eggs were incubated at 37º C and allantoic fluid was harvested 48-72 hours after inoculation,

aliquoted, and stored at -80º C until use. Both viruses were titrated by plaque assay on MDCK cells and titers expressed as plaque-forming units (PFU)/ml.

Viruses utilized in the neuraminidase inhibition assay (NAI) were obtained from the Centers for Disease Control, Atlanta, GA, grown once in MDCK cells, and used as the sensitive and resistant controls. The virus strains used included A/Texas/36/1991 (H1N1) and its H274Y resistant strain, and A/Wuhan/395/95-like (H3N2) and it's E119V resistant strain.

Viruses used in the transmission study included the previously mentioned stock of A/mallard/Minnesota/182742/98 (H5N2) and its resistant counterpart 182742-MUT-E119V that was recovered from a duck cloacal swab as a picked plaque, grown up in eggs, and passaged one additional time in eggs to obtain a stock virus. All viruses were sequenced prior to infection of ducks to verify the presence or absence of the E119V mutation.

Oseltamivir phosphate (Ro-64-0796) and oseltamivir carboxylate (Ro-64-0802) were provided by F. Hoffman-La Roche (Roche Diagnostics GmbH, Mannheim, Germany) and were utilized in both the NAI assays and mallard exposure studies.

Plaque Assay

The plaque assay was performed as previously described (Achenbach and Bowen, 2010). Plaques were either visualized with an overlay containing neutral red or with crystal violet staining. Briefly, for crystal violet staining, 48-72 hours after cells were inoculated, the primary overlay was removed, and cells were fixed with 70% ethanol for

30 minutes, followed with staining with 1% crystal violet for 15 minutes. Plates were rinsed with tap water and allowed to air dry before counting plaques.

Replication Kinetics

Single-step and multistep growth curves were determined for both the 182742WT and 182742-MUT-E119V viruses in MDCK cells. Cells were grown in 6-well tissue culture plates until ~90-95% confluency. Based on cell count, the cells were infected at a multiplicity of infection (MOI) of 2 PFU/cell for the single-step growth curve and an MOI of 0.01 PFU/cell for the multistep growth curve. Following one hour incubation at 37° C and 5% CO2, cells were rinsed two times with PBS and overlayed with DMEM supplemented with antibiotics, 0.5% bovine serum albumin (BSA) and 1µg/ml of TPCK trypsin. Supernatants were collected at 2, 4, 6, 8, and 10 hours post-infection for the single-step growth curve and at 12, 24, 36, 48, 60, and 72 hours post-infection for the multistep curve, both in duplicate, and stored at -80° C until titrated by plaque assay.

Detection of Oseltamivir-Resistant Viruses from Ducks

The inhibition of viral neuraminidase (NA) by oseltamivir was analyzed using the NA-*Star*® influenza neuraminidase inhibitor resistance detection kit (NAI, Applied-Biosystems, Foster City, CA) according to the manufacturer's protocol. Initially, duck cloacal swabs were diluted in 10-fold dilutions for the plaque assay, and 50 individual plaques were picked for each duck from virus shed at the latest possible time point. Virus from each plaque was inoculated into eggs to obtain stock viruses. The egg grown samples were tested in the NAI assay. Virus samples were diluted at least 5-fold to

obtain a signal to noise ratio of 10:1 to 60:1. Luminescence was measured using the Victor™ X5 Multilabel Plate Reader (Perkin-Elmer, Waltham, Massachusetts) equipped with automatic injectors for the addition of NA-*Star* Accelerator. IC_{50}s were determined using the JASPR v1.1 (beta) software for curve fitting and analysis, provided by the Virus Surveillance and Diagnosis Branch at the Centers for Disease Control, Atlanta, GA. Statistical analysis of IC_{50}s from picked plaques followed the outline described by Sheu et al (2008), which required an $IC_{50} \pm 3SD$ as a cutoff for outliers of resistance to minimize the number of samples tested by sequencing. This proved to be acceptable when compared to final sequencing results which confirmed resistance.

RT-PCR and Sequencing of the Neuraminidase Gene

RNA was extracted from either allantoic fluid or directly from duck cloacal swabs using TRI Reagent® (Molecular Research Center, Inc, Cincinnati, OH) according to manufacturer's directions. Both amplification of PCR product and sequencing of the NA N2 gene utilized primers based on Chander et al (2010) and named NA-2.1, NA-2.3, NA-2.5, NA-2.6 (forward and reverse). We also designed one additional primer 1413R (5´-ATA GGC ATG AAG TTG ATA TTC GC-3´) to amplify the entire NA gene from the 3′ end. RT-PCR was performed using a one step RT-PCR kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Amplification involved an initial RT step of 50° C for 30 minutes, followed by heating at 95° C for 15 minutes, 3-step cycling of 35-40 cycles with denaturation at 95° C for 1 min, followed with annealing at 53° C for 1 minute, and extension at 72° C for 1 min, with one final extension of 72° C for 10 minutes. For sequencing, PCR amplicons were electrophoresed in a 1-2% agarose gel

and DNA was extracted using the QIAquick® gel extraction kit (Qiagen, Valencia, CA) according to manufacturer's directions. PCR fragments were sent to the Proteomics and Metabolomics Facility at Colorado State University, Fort Collins, CO. Sequencing was done in both directions utilizing the same primers described above. Sequences were aligned and analyzed using the MEGA 5 software (Tamura et al, 2011).

Site specific RT-PCR to Differentiate Wild-type from Mutant Viruses

RNA was extracted as previously described and was subjected to RT-PCR utilizing a specific set of primers designed to detect the specific point mutation of nucleotide 356 A to T which changes the amino acid glutamic acid to valine (E119V) (Table 3.1). The same forward primer was used in both RT-PCR assays with two different reverse primers to differentiate between the wild-type and mutant viruses. The mutant reverse primer had the specific point mutation on the 3′ end as well as an additional point mutation 3 base pairs from the terminus on the 3′ end. This additional mismatch allowed for a destabilization of the primer to minimize anomalous products from forming (Newton et al, 1989, Latorra et al, 2003, Renaud et al, 2010). The RT-PCR cycling parameters are the same as above with the annealing temperature raised to 61º C to differentiate between wild-type and mutant using the wild-type primers. The annealing temperature of 53º C was used with mutant primers.

Table 3.1. Primer sequences for site-specific RT-PCR

	Forward primer	Reverse Primer
182742WT_E119	5′-GCAGATTGCCATCCTAGC -3′	5′-GGTGTCACATGACACATAAGGTT-3′
182742MUT_E119V	5′-GCAGATTGCCATCCTAGC -3′	5′-GGTGTCACATGACACATAAG**a**T**A**-**3′**

- Nucleotide in red is the specific point mutation change, and the nucleotide in red lowercase is the intentional mismatch

Pharmacokinetics of Oseltamivir in Mallards

Six juvenile mallard ducks were gavaged with 5mg of oseltamivir phosphate (Tamiflu, Ro 64-0796) dissolved in 2.5ml sterile water. Approximately 1 ml of blood was collected in lithium-heparin tubes from each duck at pre-dose, 0.25, 0.5, 1, 2, 3, 4, 6, 12, 24, and 48 hours post-dose. Blood samples were centrifuged and plasma was collected and stored at -80º C until assayed by PRA International (Assen, the Netherlands). Samples were extracted using a C18 solid phase extraction method and analysis was performed utilizing LC-MS/MS tandem mass spectrometry. Internal standards included deuterated oseltamivir and oseltamivir carboxylate (Ro 64-0802).

Pharmacokinetic Statistics

The plasma concentrations of OP and OC were supplied by Dr. Nicolas Abello of PRA International to Roche Pharmaceuticals (Nutley, NJ), who performed all of the pharmakokinetic stastical analyses and provided us with the final data. The final data was kindly summarized by Clinical Pharmacologist, Dr. James R. Smith, Roche. Noncompartmental pharmacokinetic parameters were calculated using WinNonlin Enterprise, version 5.2.1 (Pharsight Corporation, St. Louis, MO). The values of the maximum plasma concentration (C_{max}) and time to C_{max} (T_{max}) for each duck were the observed values. The area under the plasma concentration time curve was determined using the linear trapezoidal method for the time periods of 0 to 12 h post-dose (AUC_{0-12h}) and from 0 to the last measurable plasma concentration (AUC_{0-t}). The area under the curve from the last measurable concentration (C_t) to infinity was calculated as C_t/λ_z, where λ_z is the elimination rate constant, which was calculated using at least 3 time points

of the log linear portion of the elimination curve. AUC from 0 to infinity ($AUC_{0-\infty}$) was calculated as $AUC_{0-t} + C_t/\lambda_z$. The elimination half-life was calculated as ln 2/ λ_z. Oral clearance (CL/F) was determined as the dose divided by $AUC_{0-\infty}$ and the oral volume of distribution (Vz/F) was calculated as dose/($AUC_{0-\infty}$ x λ_z). CL/F and Vz/F were divided by the weights of ducks to facilitate comparison to human data.

Oseltamivir Carboxylate Treatment of Mallards and AIV Infection

These studies were designed to simulate exposure of ducks to low levels of OC in drinking water prior to infection with AIV. Four separate groups of mallard ducks aged 4-6 weeks were divided into separate rooms as outlined in Table 3.2, based on the concentration of drug provided in water and were allowed to free range within a 12 foot x 18 foot sized room. Groups of mallards given OC in their drinking water were started on drug 24-48 hours before challenge while control ducks were supplied water with no OC. The concentration of OC in water ranged between 10 and 10,000 ng/L.

Table 3.2. OC in the mallard model experimental groups

Group	1	2	3	4
Virus inoculum*	346250	182742	182742	346250
10,000ng/L OC	2 ducks	2 ducks		
1,000ng/L OC	2 ducks	2 ducks		
100ng/L OC	2 ducks	2 ducks	4 ducks	5 ducks
10ng/L OC	2 ducks			
No OC	2 ducks	2 ducks	4 ducks	5 ducks

* A/Mal/MN/346250/00 (346250), A/Mal/MN/182742/98 (182742)

All water dishes were re-filled daily at the time of cloacal swab collection. All ducks were inoculated on day 0 with 10^6 PFU of either H5N2 virus orally and intranasally in the first two groups and through their choanal cleft in groups 3 and 4. The change in inoculation route to the choanal cleft was done to facilitate a more natural inoculation route which led to increased consistency of infection and shedding by mallards. Cloacal swabs were collected daily in 2ml BA-1 medium (MEM, 1% BSA, 350 mg/L sodium bicarbonate, 50ml/L 1M Tris, pH7.6, 5mg/L phenol red) supplemented with antibiotics (gentamicin 50 ug/ml, polymyxin B 100 U/ml, nyastatin 50 U/ml, penicillin, 100 U/ml and streptomycin 50 ug/ml) from days 0 through days 7 or 10. Swab samples were split into duplicate 1ml volumes and stored at -80° C until tested by plaque assay. In the first two groups (group 1 and 2), cloacal swabs were tested for viral titer by plaque assay, and individual plaques were picked, grown once in eggs, and tested in the NAI assay for drug resistance. Any sample that had an increased IC_{50} was then sequenced by the traditional Sanger sequencing method. For the last two groups (group 3 and 4), Sanger sequencing was performed on RNA extracted directly from cloacal swabs. The direct sequencing from cloacal swabs was performed to help eliminate the possibility that the growth of picked plaques in eggs contributed to the emergence of resistance.

Oseltamivir Phosphate Treatment of Mallards and AIV Infection

This experiment was designed to mimic treatment of ducks with a single dose of OP shortly after exposure to AIV, a practice discussed to treat ducks in live markets where AIV infection frequently occurs. Nine 5 week old mallard ducks were inoculated with 10^6 PFU of 182742WT virus via the choanal cleft. Six hours post inoculation, 4

inoculated ducks were gavaged with 5mg oseltamivir diluted in 2.5ml distilled water, with the other 5 inoculated ducks getting 2.5ml of distilled water. Cloacal swabs were collected on days 0 through day 7 and stored at -80° C until tested by plaque assay.

Transmissibility of Mutant AIV in the Mallards

To evaluate differences in direct contact or environmental contamination transmissibility of wild-type versus drug resistant AIV, we infected 5 week-old mallards with each virus, exposed them to non-infected ducks and monitored the rate of infection in the exposed ducks. Two ducks from group 1 were inoculated with 10^6 PFU of 182742WT virus and 2 hours later, those inoculated ducks were introduced to mingle freely with 6 naïve ducks. Similarly, two ducks from group 2 were inoculated with 10^6 PFU of 182742-MUT-E119V virus, 2 hours later they were then introduced to 6 naïve ducks. Group 3 was divided into 3 groups of 4 ducks. One duck in each group of 4 was inoculated with a mixture containing 10^6 PFU of 182742WT virus and 10^6 PFU of 182742-MUT-E119V virus, then introduced 2 hours later to the other three naïve ducks in that group. Blood was collected from all ducks prior to day 0 and again at day 14 to detect seroconversion utilizing the cELISA test. Cloacal swabs were collected as previously described from days 0 through day 14 from all 28 ducks. Cloacal swabs were divided into two separate 1 ml aliquots and stored at -80° C until testing for viral titer by plaque assay. RNA was also extracted directly from cloacal swabs for sequencing.

Results

Pharmacokinetics of Oseltamivir in Mallards

Mean values of the plasma concentrations of OP and OC following oral dosing of ducks with OP are listed in Table 3.3, and the pharmacokinetic parameters are listed in Table 3.4. Plasma concentrations of oseltamivir were detectable at 48 h post-dose while the carboxylate metabolite was detectable only to 24 h (Table 3.3). The ratio of OC metabolite to parent OP concentrations was maximal at 0.5 h and declined from 0.908 to 0.049 at 24 h. Based on the values of $AUC_{0-\infty}$, the ratio of metabolite to parent was 0.16 ± 011. In duck 3, the concentration of the metabolite was greater than the parent in the first 4 samples for up to 2 hours, following a more human-like pattern. The other 5 ducks had consistently higher concentrations of parent than metabolite.

Table 3.3. Mean (SD) Plasma Concentrations of Oseltamivir and Oseltamivir Carboxylate in Mallard Ducks Administered a Single Oral Dose of 5 mg Oseltamivir Phosphate

Hour	Oseltamivir	Oseltamivir Carboxylate	Carboxylate/Oseltamivir Ratio
0.25	1170 (585)	441 (200)	0.578 (0.587)
0.5	886 (463)	481 (174)	0.908 (1.02)
1	770 (405)	320 (134)	0.745 (0.948)
2	637 (347)	165 (68.4)	0.415 (0.454)
3	552 (273)	89.4 (39.0)	0.236 (0.209)
4	459 (217)	64.0 (29.5)	0.195 (0.155)
6	383 (180)	46.4 (19.3)	0.170 (0.122)
12	292 (99.2)	24.3 (13.5)	0.105 (0.713)
24	166 (72.3)	4.83 (7.90)	0.049 (0.085)
48	26.5 (18.9)	0.00 (0.00)	0.00 (0.00)

N = 6

Table 3.4. Mean (SD) Pharmacokinetic Parameters of Oseltamivir and Oseltamivir Carboxylate in Mallard Ducks Administered a Single Oral Dose of 5 mg Oseltamivir Phosphate

Parameter (units)	Oseltamivir	Oseltamivir Carboxylate
C_{max} (ng/mL)	1190 (575)	487 (188)
T_{max} (h)	0.54 (0.71)	0.46 (0.10)
$AUC_{0\text{-}12h}$ (ng·h/mL)	5490 (2500)	1140 (382)
$AUC_{0\text{-}t}$ (ng·h/mL)	10500 (4030)	1240 (528)
$AUC_{0\text{-}\infty}$ (ng·h/mL)	11000 (4240)	1390 (548)
$AUC_{0\text{-}\infty}$ (ng·h/mL/mg)	2200 (847)	303 (119)
t½ (h)	10.5 (3.00)	6.48 (1.61)
CL/F (L/h)	0.517 (0.206)	NC
CL/F (L/h/kg)	0.468 (0.191)	NC
Vz/F (L)	7.67 (3.60)	NC
Vz/F (L/kg)	6.81 (2.91)	NC

N = 6

The mean C_{max} values for OP and OC were 1190 and 487 ng/mL, respectively (Table 3.4). T_{max} was approximately 0.5 h for both the parent and metabolite. The mean t½ for OP was 10.5 h, which was longer than that for OC, which was 6.48 h. CL/F of oseltamivir was 0.468 L/h/kg and Vz/F was 6.81 L/kg. Comparisons of duck PK parameters and human parameters are outlined in Table 5. There are some distinct differences between ducks and humans. In humans, their exposure to OC as determined by $AUC_{0\text{-}\infty}$ is 22 times greater than exposure to OP, showing more efficient liver conversion to the active metabolite OC, which will interfere with NA in humans. In ducks, the ratio is only 0.16, suggesting the possibility of conversion to OC in the gut lumen, where it is likely to directly interact with virus replication in the gut of the duck playing a more direct role. Also, OP has a longer half-life in ducks compared to humans, but OC has a similar half-life in both species, suggesting they may share the same mechanism of clearance.

Table 3.5. Comparison of Mean Pharmacokinetic Parameters of Oseltamivir and Oseltamivir Carboxylate in Mallard Ducks and Humans Following a Single Oral Dose of Oseltamivir

Parameter (unit)	Oseltamivir		Oseltamivir Carboxylate	
	Duck[a]	Human[b]	Duck	Human
C_{max} (ng/mL)	1190	84	487	215
T_{max} (h)	0.54	1.00	0.46	5.00
$AUC_{0-\infty}$ (ng·h/mL/mg)	2200	1.87	303	40.8
t½ (h)	10.5	1.70	6.48	7.7
CL/F (L/h/kg)	0.47	7.1	NC	NC
Vz/F (L/kg)	6.81	17.6	NC	NC

a: Dose = 5 mg p.o.
b: Dose = 75 mg p.o.; mean wt = 75 kg; PK parameters from Clinical Study WP20727
NC = Not calculated

OC Treatment of Mallards and AIV Infection

Two strategies were used in an attempt to minimize the number of viruses tested in evaluating the shedding of oseltamivir-resistant virus from ducks treated with OC in drinking water. First, we tested viruses shed only from ducks receiving the highest dose of OC that did not completely inhibit virus shedding. Second, initial testing was restricted to viruses isolated toward the end of the shedding period, assuming that if resistant viruses evolved, they would be present at the highest frequency at that time due to the selective pressure of continued presence of OC.

Regarding the first group involving ducks administered OC in their drinking water and inoculated with A/mallard/MN/346250/00 virus, none of the shed viruses tested showed an increased IC_{50}. All samples were compared to control sensitive and resistant viruses in the NAI assay, and none of the picked plaques viruses from challenged ducks receiving OC or no OC showed an increased IC_{50}. Interestingly,

mallards that received 10,000 ng/L of OC in their drinking water failed to shed detectable virus on any day (Table 3.6).

Table 3.6. Group 1, 10 individual ducks receiving differing concentrations of OC or no OC in their drinking water, and inoculated with A/Mal/MN/346250/00 (H5N2) with viral titers represented in log10 (PFU/ml).

Ducks		D1*	D2	D3	D4	D5	D6	D7
1	10000ng/L	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	10000ng/L	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	1000ng/L	0.00	3.30	4.43	4.30	0.00	0.00	0.00
4	1000ng/L	0.00	2.60	4.36	2.00	0.00	0.00	0.00
5	100ng/L	0.00	0.00	4.58	2.00	0.00	0.00	0.00
6	100ng/L	0.00	0.00	0.00	0.00	0.00	0.00	0.00
7	10ng/L	0.00	0.00	0.00	3.78	0.00	0.00	0.00
8	10ng/L	0.00	0.00	4.78	4.40	0.00	0.00	0.00
9	No OC	4.00	3.95	4.48	3.30	0.00	0.00	0.00
10	No OC	0.00	0.00	4.88	2.85	0.00	0.00	0.00

*Days post inoculation

Titers of virus shed from the second group of ducks that received varying concentrations of OC in water followed by inoculation with A/mallard/MN/182742/98 virus are presented in Table 3.7. In these ducks, increased IC_{50}s were detected in 35 picked plaques, from days 5 and 6, of 1 (duck 3) of 2 ducks receiving 1,000ng/L OC in drinking water. None of 20 plaques picked from the sample collected from duck 3 on day 3 had an elevated IC_{50}. We utilized the method from Sheu et al, 2008 that was designed to minimize testing outliers and followed a cutoff of the mean IC_{50} + 3SD for which the 182742WT virus was 5.46nm. The mean IC_{50} for plaques from duck 3 were 17.43 ± 4.85 nM which was an 8.8 fold increase over the IC_{50} for the WT strain. The individual plaques that had a > 5.46nm fold increase were subjected to Sanger sequencing which confirmed an A to T nucleotide change at position 356 which causes the amino acid (aa) to switch from glutamic acid (E) to valine (V) at position 119 (H3 numbering) mutation in the NA sequence. There was also a mutation from A to G at nucleotide

position 510, but this did not affect the aa sequence. Five different plaques from duck 3 on day 3 were also sequenced, and no mutation was detected. We also evaluated a set of serial NA sequences from duck 3 from RNA extracted from cloacal swabs for days 2, 3, 4, 5, and 6 to determine which day post-inoculation the mutation is first detected. The A to T mutation was first detected at day 5 as a mixed A/T population, with the T mutation becoming the dominant nucleotide on day 6. This matches the results from the NAI assay that showed no increase in IC_{50} on day 3 but did detect an increase in IC_{50} on day 5. These results are also consistent with the A/Wuhan/395/95-like (H3N2) sensitive to resistant (E119V) control that also shows an 8-fold increase from sensitive to resistant change (Table 3.8). No increased IC_{50}s were seen in the 2 ducks getting no OC and challenge only. Ducks that received 10,000ng/L OC in their drinking water did not shed detectable virus on any days post challenge. This concentration appears to cut off detectable replication in the ducks, which is consistent with the IC_{50} determined from the mutant virus.

Table 3.7. Group 2, 8 individual ducks receiving differing concentrations of OC or no OC in their drinking water, and inoculated with A/Mal/MN/182742/98 (H5N2) with viral titers represented in log10 (PFU/ml).

Ducks		D1*	D2	D3	D4	D5	D6	D7
1	10000ng/L	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	10000ng/L	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	1000ng/L	0.00	3.00	7.08	4.00	3.48	3.00	1.60
4	1000ng/L	0.00	3.30	0.00	3.40	4.00	2.30	0.00
5	100ng/L	0.00	2.78	0.00	0.00	0.00	0.00	0.00
6	100ng/L	0.00	2.70	1.00	2.70	3.48	0.00	0.00
7	No OC	0.00	3.78	3.70	0.00	0.00	0.00	0.00
8	No OC	0.00	3.30	0.00	0.00	0.00	1.00	0.00

*Days post inoculation

Table 3.8. IC50 comparisons between control sensitive and resistant strains and 182742WT and 182742-MUT-E119V.

Virus strain	Virus NA type	NA mutation	Mean IC50±SD(nm)(fold inc)
A/TX/36/91	A/N1		3.20 ± 1.01
A/TX/36/91 mut	A/N1	H274Y	262.3 ± 204.4 (81.96)
A/Wuhan/395/95-like	A/N2		3.215 ± 0.73
A/Wuhan/395/95-like	A/N2	E119V	26.25 ± 4.25 (8.16)
A/Mal/182742/98	A/mal/N2		1.97 ± 1.16
A/Mal/182742/98 mut	A/mal/N2	E119V	17.43 ± 4.85 (8.84)

Group 3 was a partial replicate of study group 2 in which 4 ducks received 1,000ng/L OC in their drinking water and 4 control ducks received no drug, with all 8 ducks being inoculated via the choanal cleft with the 182742 virus. This change in inoculation route shows a much better consistency of infection and viral shedding between ducks receiving drug or no drug as compared to groups 1 and 2. We did not repeat the 10,000ng/L due to lack of viral replication in the previous two experiments. Viral titers were determined by plaque assay on MDCK cells (Table 3.9) and RNA was extracted directly from original cloacal swabs on the latest time point that virus was detected by plaque assay. RNA was used in an RT-PCR reaction followed by Sanger sequencing of the NA gene. Three of 4 ducks who received OC in their drinking water showed an amino acid change of E119V mutation in the NA gene, along with one other nucleotide change of A to G at position 510 that caused no amino acid change. This is consistent with the results from group 2 that were inoculated with the same 182742 virus.

Group 4 was a replicate experiment for group 1 which included 5 ducks getting 1,000ng/L OC in their drinking water and 5 ducks getting no OC in their drinking water. All 10 were inoculated with the 346250 virus via the choanal cleft. Viral titers were determined by plaque assay (Table 3.10) and RNA was extracted from cloacal swabs and

subjected to RT-PCR and Sanger sequencing. None of the samples tested from the 10 ducks showed nucleotide or amino acid changes in the NA gene sequence.

Table 3.9. Group 3, 8 individual ducks were given either 1000ng/L (4 ducks) of OC or no OC (4 ducks) followed by inoculation with A/Mal/MN/182742/98 (H5N2) with viral titers represented in log10 (PFU/ml).

Ducks		D1*	D2	D3	D4	D5	D6	D7	D8	D9
1	1000ng/L	0.00	0.00	4.37	3.78	4.36	3.88	4.48	3.08	2.00
2	1000ng/L	0.00	0.00	4.90	3.11	2.70	3.18	1.78	1.85	1.48
3	1000ng/L	0.00	0.00	4.18	3.90	4.30	4.67	2.30	2.00	2.00
4	1000ng/L	0.00	5.24	6.08	5.29	6.26	0.00	2.00	2.60	0.00
5	no OC	0.00	5.71	3.68	4.30	4.70	3.04	0.00	0.00	0.00
6	no OC	0.00	6.76	4.53	3.78	2.95	1.78	0.00	0.00	0.00
7	no OC	0.00	0.00	2.95	5.22	2.40	2.60	3.85	0.00	0.00
8	no OC	0.00	8.40	6.20	4.18	2.23	3.11	0.00	0.00	0.00

*Days post inoculation

Table 3.10. Group 4, 10 ducks receiving 1000ng/L of OC (5 ducks) or no OC (5 ducks) followed by inoculation with A/Mal/MN/346250/00 (H5N2) with viral titers represented in log10 (PFU/ml).

Ducks		D1*	D2	D3	D4	D5	D6	D7	D8	D9
1	1000ng/L	0.00	4.48	3.48	6.00	3.60	2.60	2.48	0.00	0.00
2	1000ng/L	1.30	3.78	3.48	4.30	3.18	2.30	2.30	2.95	2.30
3	1000ng/L	0.00	3.20	3.78	3.48	2.48	0.00	0.00	0.00	0.00
4	1000ng/L	0.00	0.00	2.78	3.79	4.03	1.95	3.00	0.00	0.00
5	1000ng/L	0.00	3.30	2.60	5.00	4.02	2.04	0.00	1.95	0.00
6	no OC	4.30	5.15	4.85	3.30	0.00	0.00	0.00	0.00	0.00
7	no OC	6.90	4.30	3.70	3.30	0.00	0.00	1.00	0.00	0.00
8	no OC	4.40	3.70	4.30	2.85	2.70	0.00	0.00	0.00	0.00
9	no OC	0.00	5.30	4.95	3.59	4.08	2.78	0.00	0.00	0.00
10	no OC	0.00	3.95	4.48	2.76	0.00	3.48	0.00	0.00	0.00

*Days post inoculation

Effect of a Single Dose of Oseltamivir Phosphate in Mallards

Administration of a single dose of OP shortly after challenge of mallards with the 182742 virus resulted in significant suppression of virus replication in the host. Four of the 5 ducks that received a single dose of OP did not shed any detectable virus on any days 0 through day 7 (Table 3.11). One duck did shed virus on days 6 and 7 post challenge and drug treatment (Table 3.11). RNA was extracted from cloacal swabs from this duck and was subjected to RT-PCR and Sanger sequencing. Even though the plaque sizes were larger than the parental virus in the plaque assay, mutations in the NA gene were not detected by sequencing.

Table 3.11. Nine individual ducks were challenged with A/Mal/MN/182742/98 followed with 4 ducks receiving a single 5 mg OP, and 5 ducks receiving a no OP (water only) dose with viral titers represented in log10 (PFU/ml).

Ducks		D1*	D2	D3	D4	D5	D6	D7	D8
1	5mg OP	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	5mg OP	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	5mg OP	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	5mg OP	0.00	0.00	0.00	0.00	0.00	2.30	3.85	0.00
5	no OP	0.00	6.61	5.08	4.43	4.08	2.00	2.00	0.00
6	no OP	0.00	4.08	3.95	0.00	2.18	2.30	0.00	0.00
7	no OP	0.00	6.53	0.00	0.00	5.36	nt	nt	0.00
8	no OP	0.00	4.36	4.61	0.00	0.00	0.00	0.00	0.00
9	no OP	0.00	4.69	4.04	4.20	3.15	2.48	2.00	0.00

*Days post inoculation

Replication Kinetics of Wild-Type and Resistant Virus

We assayed the replication of both the 182742WT virus and 182742-MUT-E119V virus, which is the plaque picked, egg grown isolate from duck 3, experiment 2, in both single-step and multistep growth curves in MDCK cells. In both growth curves,

the MUT-E119V virus appeared to have a slight advantage over the growth of 182742WT virus *in vitro,* reaching higher viral yields at 10 hours pi for the single-step growth curve and at all time points in the multistep growth curve, with a ≥ 1 $\log_{10}$ difference over all time points except the 72 hour endpoint where the two titers were the same (Figures 3.1 and 3.2). A comparison of the slopes of each virus growth curve over time was calculated utilizing linear regression ANOVA. There were no statistical differences in slope between wild-type and mutant virus over time.

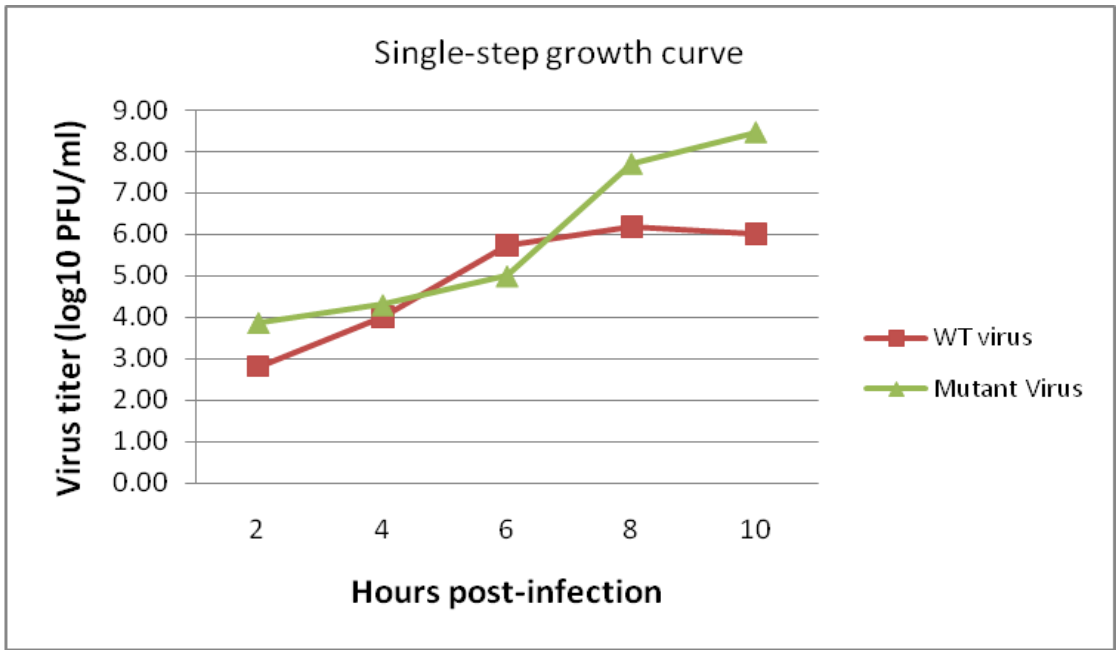

Figure 3.1. Single-step growth curve of 182742WT and 182742-MUT-E119V at an MOI of 2 PFU/cell

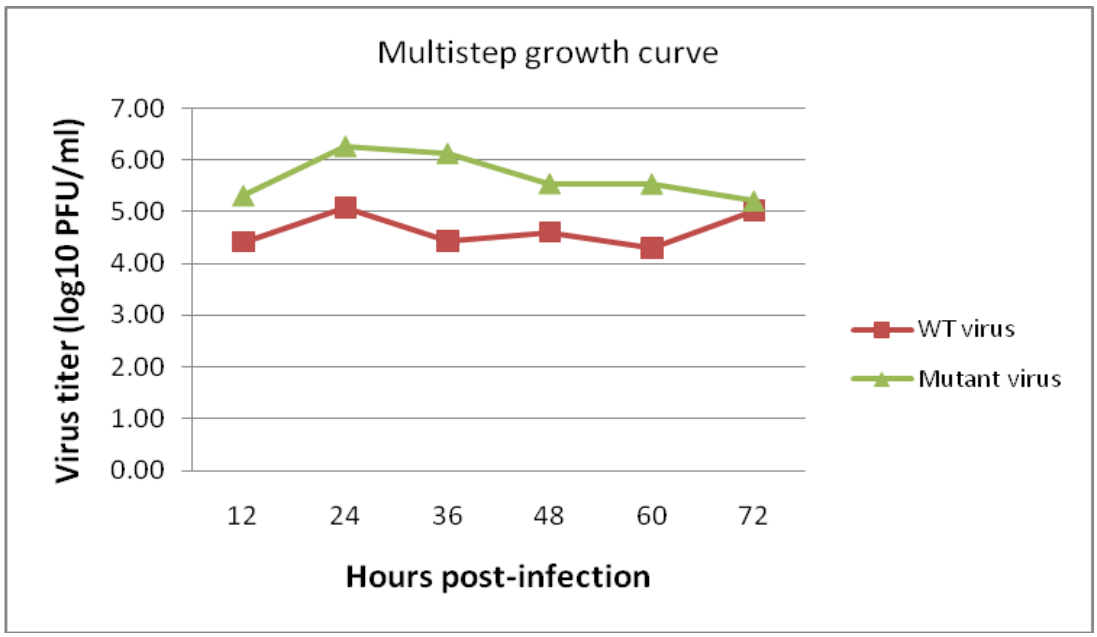

Figure 3.2. Multistep growth curve of 182742WT and 182742-MUT-E119V at an MOI of 0.01 PFU/cell

Plaque size also increased from 1mm in diameter in the 182742WT to 3-5mm in diameter for the 182742-MUT-E119V (Figure 3.3). While the increase in plaque size may play a role in the enzymatic activity of NA of the MUT-E119V virus it was not the only indication as mutations in the HA gene also need to be considered. An occasional large plaque was detected in the 182742WT virus stock, but following sequencing of three large plaques picked from 182742WT virus, it was determined to be wild-type. This suggests that the increase in plaque size may play a role *in vitro*, but there are other factors to be considered, with the remaining 7 genes all working together, that are important for successful replication.

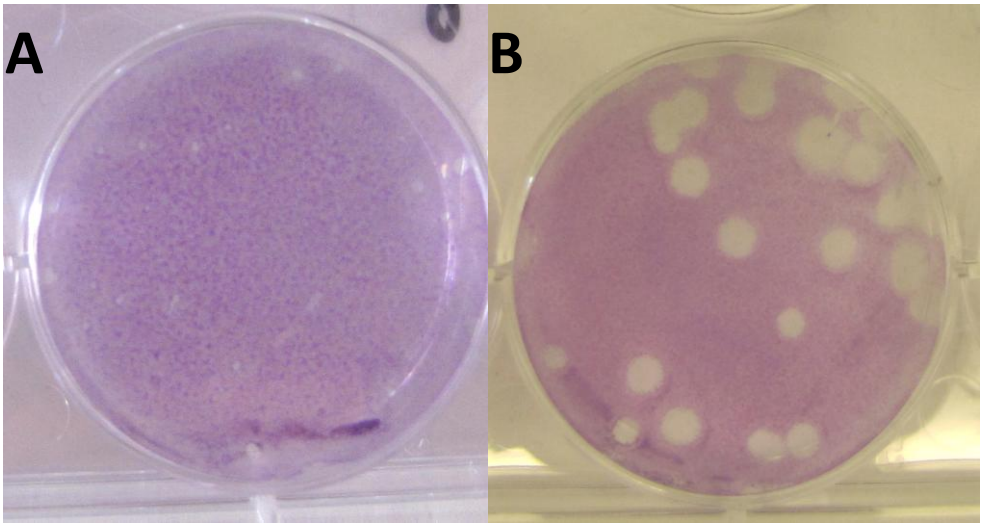

Figure 3.3. (A) 182743WT virus on MDCK cells, with plaque sizes of 1mm and **(B)** 182742-MUT-E119V virus on MDCK cells, with plaque sizes of 3-5mm.

Transmission of the E119V Mutant AIV in Mallards

We evaluated both the replicative ability and the transmissibility of the 182742WT virus, the 182742-MUT-E119V virus and a competitive 1:1 mixture of both viruses in mallard ducks. With the 182742WT virus we saw good replication in the 2 inoculated ducks at day 2 post-inoculation, and recorded detectable shedding on day 3 from all 6 naïve contact ducks. Viral shedding was detected through day 7 in three ducks with 1 duck still having detectable virus on day 8, the last day samples were collected (Table 3.12).

Shedding was not detected in the 2 ducks inoculated with the 182742-MUT-E119V virus until day 3, 1 day later than detectable shedding in ducks inoculated with 182742WT virus. The 6 naïve contact control ducks housed with inoculated ducks had detectable virus starting on day 4 and continuing through day 7, showing there was virus transmitted (Table 3.12).

Table 3.12. Results of viral titers of all 28 ducks in the transmission experiment separated by type of inoculated virus, and group. Ducks were either directly inoculated with virus or were contact naïve ducks placed in the same room as inoculated ducks. Viral titers are represented in log10 (PFU/ml).

182742WT	D1	D2	D3	D4	D5	D6	D7	D8
Duck 1 inoculated	0.00	2.00	3.70	2.30	0.00	0.00	0.00	0.00
Duck 2 inoculated	0.00	1.90	3.30	2.00	1.00	1.90	1.85	0.00
Duck 3 naïve	0.00	0.00	3.75	3.30	4.04	0.00	2.60	0.00
Duck 4 naïve	0.00	0.00	3.58	1.85	3.48	2.00	1.78	0.00
Duck 5 naïve	0.00	0.00	5.15	0.00	0.00	1.70	0.00	0.00
Duck 6 naïve	0.00	0.00	0.00	3.30	3.30	2.00	0.00	0.00
Duck 7 naïve	0.00	0.00	4.78	2.90	3.18	0.00	0.00	2.30
Duck 8 naïve	0.00	0.00	0.00	4.60	3.48	1.90	0.00	0.00
182742-MUT-E119V								
Duck 9 inoculated	0.00	0.00	2.08	1.85	2.36	1.78	2.60	0.00
Duck 10 inoculated	0.00	0.00	1.85	2.54	3.70	1.70	1.85	0.00
Duck 11 naïve	0.00	0.00	0.00	1.60	2.18	3.70	3.30	0.00
Duck 12 naïve	0.00	0.00	0.00	0.00	1.70	3.48	2.60	0.00
Duck 13 naïve	0.00	0.00	0.00	1.78	2.00	3.48	2.30	0.00
Duck 14 naïve	0.00	0.00	0.00	0.00	3.70	2.85	3.00	0.00
Duck 15 naïve	0.00	0.00	0.00	3.30	2.04	1.78	0.00	0.00
Duck 16 naïve	0.00	0.00	0.00	0.00	3.18	4.30	0.00	0.00
1:1 mixture								
Duck 17 inoculated	0.00	1.70	7.69	3.60	0.00	2.49	0.00	0.00
Duck 18 naïve	0.00	0.00	0.00	3.18	2.23	3.30	2.60	0.00
Duck 19 naïve	0.00	0.00	4.30	3.79	4.00	3.30	1.70	0.00
Duck 20 naïve	0.00	0.00	0.00	3.30	3.48	3.90	2.60	0.00
Duck 21 inoculated	0.00	3.60	4.04	2.85	3.00	2.00	0.00	1.30
Duck 22 naïve	0.00	0.00	4.08	3.30	2.36	3.78	0.00	0.00
Duck 23 naïve	0.00	0.00	3.95	3.85	2.00	2.60	2.18	0.00
Duck 24 naïve	0.00	0.00	3.70	3.78	3.30	0.00	0.00	0.00
Duck 25 inoculated	0.00	5.60	4.00	4.48	3.58	3.30	2.78	1.85
Duck 26 naïve	0.00	0.00	0.00	4.34	3.00	2.08	2.70	0.00
Duck 27 naïve	0.00	0.00	4.15	6.00	5.70	3.71	1.48	0.00
Duck 28 naïve	0.00	0.00	3.49	4.20	4.34	2.78	2.60	0.00

For the 3 ducks inoculated with a 1:1 mixture of 182742WT and 182742-MUT-E119V viruses, there was detectable shedding on day 2 that continued through day 7 in group 1, and through day 8 in groups 2 and 3 (Table 3.12). Titers detected in both inoculated and contact naive ducks were analyzed utilizing ANOVA and comparing their area under the curve (AUC) values. There were some statistically significant differences in viral titers between the 3 different groups. Wild-type naïve ducks had significantly higher titers than mutant directly inoculated ducks ($P = 0.02$). Mutant naïve ducks had higher titers than dual infected naïve ducks ($P = 0.004$). Wild-type naïve ducks had higher titers than wild-type inoculated ducks ($P = 0.04$), than dual infected directly inoculated ($P = 0.03$), and dual infected naïve ducks ($P<0.0001$).

RNA was extracted from cloacal swabs of ducks on the latest day of detectable shedding for all 28 ducks that were inoculated with virus or infected by bird-to-bird transmission. Sanger sequencing was performed on RNA from all samples, and all 28 samples had the E119 wild-type phenotype. Earlier samples from the ducks that received 182742-MUT-E119V samples were also subjected to Sanger sequencing and were determined to not contain the E119V mutation. Based on the cELISA, all 28 ducks did seroconvert by day 14 detecting positive for antibody against avian influenza.

Detection of Mutant Virus in Wild-type Virus Stocks

The site-specific RT-PCR was designed to be more sensitive than Sanger sequencing and to provide another measure of confirmation of both the NAI assay and sequencing. We needed to determine if our original stock of 182742WT virus had the E119V mutation at a low level or to discern if the 182742-MUT-E119V stock had a low

level of E119 present in the stock. This was done through the optimization of the primers both by adjusting annealing temperature as well as adding the additional intentional mismatch at the 3′ end, in addition to the site specific mutation of the most 3′ base pair. We did not detect the E119V mutation in the 182742WT stock or the E119 in the 182742-MUT-E119V stock (Figure 3.7). Primer controls used to test the quality of RNA were the 2.1F and 2.3R as previously noted in our methods, and can be seen in lanes 4 and 7 as positive controls for the 182742WT stocks. There were no bands present in lanes 3 and 6 showing no detectable E119V in the WT stock. There was also no band in lane 8 showing that the E119 is undetectable in the MUT stock.

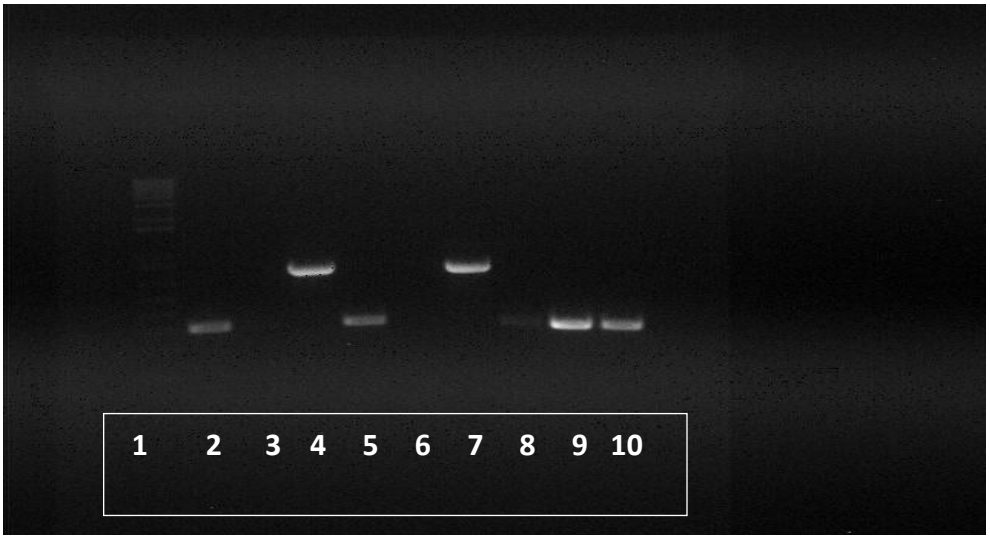

Figure 3.7. Site-specific RT-PCR with specific primers on 1% agarose gel stained with Ethidium bromide

Lanes

1 – 1kb plus DNA ladder
2 – 182742WT with WT primers
3 – 182742WT with MUT primers
4 – 182742WT NA control
5 – 182742WT (P2) with WT primers
6 – 182742WT (P2) with MUT primers
7 – 182742WT (P2) NA control
8 – 182742-MUT with WT primers
9 – 182742_MUT with MUT primers
10 – 182742WT (picked plaque) with WT primers

Discussion

Ducks are the primary reservoir host of all influenza A virus subtypes and thus are the source of all influenza A viruses that affect mammals, including humans. This project was founded on the hypothesis that consumption by ducks of low levels of OC, similar to what has been reported to occur naturally, will promote selection of mutant viruses that are resistant to OC. To test this hypothesis, we infected ducks with 2 different LP H5N2 AIVs and attempted to detect OC-resistant virus in cloacal secretions. Detection of resistant viruses from swab samples was performed either by assaying individually plaque-picked virus for neuraminidase enzymatic activity in the presence of OC, followed by nucleotide sequencing, or by direct sequencing of virus from cloacal swab samples.

We found that oseltamivir-resistant viruses arose on several occasions in mallard ducks that received water containing a low concentration of OC and that were infected with AIV. Furthermore, those OC-resistant viruses rapidly came to be the dominant population of virus in treated ducks, likely the result of continued selective pressure. The day of detection of resistant virus was consistent with previous studies of children treated with oseltamivir during influenza virus infection, in which resistant strains were first detected on day 4 (Kiso et al, 2004) and on days 4 and 6 post treatment (Whitley et al, 2001). Resistant virus was not detected in control ducks that were not exposed to OC.

Previous investigations indicate that a considerable amount of OC is not broken down during normal or chemical processes, which can lead to the buildup of OC in the environment. Proposed calculations suggest that OC can remain in the environment for up to 18 days at levels of 0.08-0.23 μg/L (Singer et al, 2007, Bartels and von Tumpling jr

2008). An indication of the highest predicted environmental concentration was proposed for Japan, a country which currently prescribes a large amount of oseltamivir, to be 0.028 μg/L (Bartels and von Tumpling jr 2008). These are projected values, as different issues such as water size, prescribed amounts of oseltamivir, and the actual level of a pandemic would all play a role in environmental concentrations.

Recently, actual levels of OC were measured in regional waters of Japan. Prior to the beginning of the 2007-2008 influenza season OC was not detected in the Yodo river system, but concentrations of 2-58 ng/L were detected during the influenza season (Söderström et al, 2009). During the 2008-2009 influenza season, levels of OC rose to peak concentrations of 293.3 ng/L of OC in sewage treatment plant discharge with river water levels ranging from 6.6-190.2 ng/L (Ghosh et al, 2010). Both of these proposed and detected values guided our choice of concentrations of OC in our mallard studies.

We analyzed the effects of AIV infection in mallards during consumption of OC contaminated water and concluded that drug induced resistant viruses can emerge. This is concerning in that OC resistant viruses are already on the rise in humans (Sheu et al, 2008, Dharan et al, 2009, Meijer et al, 2009, Hurt et al, 2009), even in areas of low oseltamivir drug use (Hauge et al, 2009). Since waterfowl are the natural reservoir for all influenza virus subtypes, there is a need to understand all possible effects of mallards ingesting OC, and the outcomes in waterfowl with different AIV strains.

Our PK studies indicated that ducks vary considerably from humans in their uptake and metabolism of OP and its metabolite OC. Based on plasma levels, ducks appear to absorb OP adequately after oral gavage, but may convert OP to OC in the gut instead of trafficking through the liver. This conclusion was based on low clearance and

short T_{max} values. This potential conversion in the gut may play a larger role when the duck is infected with LPAI, since replication occurs in the digestive tract. Additional research to look at the actual concentrations of OC present in the duck digestive tract may better elucidate the actual interaction of drug and virus.

Analyzing the replication kinetics of both viruses indicated that the E119V mutation from the 182742 mallard virus appears to have a selective advantage in the presence of OC, and has similar growth kinetics in MDCK cells compared to the wild-type parental virus. While we did not evaluate the HA gene in this study, the interactions of HA and NA interplay with compensations in the HA or differences in NA enzymatic activity may both be responsible for the increased plaque size relative to wild-type and warrants further evaluation (Kaverin et al, 1998, Mitnaul et al, 2000, Wagner et al, 2002, Gerloff et al, 2009, Rameix-Welti et al, 2008). HA mutations can also lead to reduced ability of binding of virus to sialic acids that can lead to a reduced dependence of virus on functions provided by NA (Staschke et al, 1995, McKimm-Breschkin et al, 1996, Gubareva et al, 1996). We were not able to assay NA activity accurately due to a loss of stock viruses due to a freezer failure.

Within the neuraminidase gene there are 8 amino acids called catalytic residues (R-118, D-151, R-152, R-224, E-276, R-292, R-371, and Y-406) so named because they have direct contact with the substrate, and 11 framework residues (E-119, R-156, W-178, S-179, D-198, I-222, E-227, E-277, N-294, and E-425) which play a role in the stabilization of the active site (Coleman et al, 1983). The E119V mutation has been detected in virus from patients treated with oseltamivir (Baz et al, 2006, Ison et al, 2006, Kiso et al, 2004, Okomo-Adhiambo et al, 2010) and *in vitro* and *in vivo* models have

shown that viruses with this mutation replicate and transmit as efficiently as its parent wild-type virus (Herlocher et al, 2004, Yen et al, 2005).

We observed that mallards inoculated with the E119V mutant virus, alone or as a mixture with wild-type virus, failed to shed detectable quantities of the E119V virus or to transmit that virus to contact naive ducks. Only the wild-type virus was transmitted in all three groups. This observation suggests that that the neuraminidase mutation of E119V is not as stable within mallards as it is in cell culture. While *in vitro*, the 182742-MUT-E119V had similiar replication efficiencies and larger plaque sizes compared to wild-type virus, but, *in vivo*, the E119V mutation was not detected either through Sanger sequencing or site-specific RT-PCR following transmission from infected to contact ducks. There is the possibility that this mutation was unstable upon infection, and that the 182742-MUT-E119V virus reverted to wild-type within the mallard host. It is also the possible that the wild-type was present below the level of detection of our assays in the E119V resistant viral stock used to inoculate the mallards, or that the wild-type strain out-competed the E119V mutant virus within the mallard. Indeed, because of the poor fidelity of the influenza virus RNA polymerase, it is likely that some very low level contamination of the wild-type and mutant stock viruses with each other was present simply because the viruses are essentially a quasispecies.

While Sanger sequencing has been shown to be very accurate in detecting mutations, studies have shown that customized pyrosequencing is more sensitive in detecting very low levels of point mutations (Levine et al, 2011). However, pyrosequencing is very expensive and was not conducted as part of these experiments. Other recent research has utilized quantitative real-time or real-time RT-PCR coupled

with mathematical models based on differences in Ct values between wild-type and resistant strains for comparison. This can be extremely sensitive for detecting very low levels of a specific mutation (Chidlow et al, 2010, Hurt et al, 2010, Liu et al, 2010, Renaud et al, 2010, van der Vries et al, 2010). While we did base our site-specific RT-PCR on these models, which correlated with our Sanger sequencing results (Figure 3.8), we recognize that there is a different level of sensitivity in these methods.

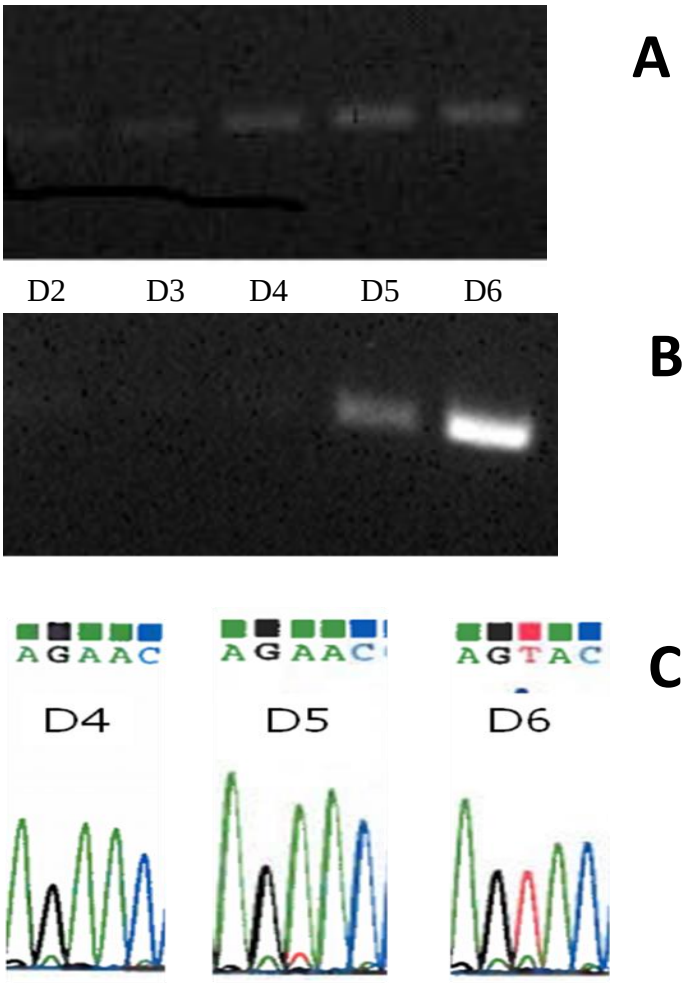

Figure 3.8. (A), site-specific RT-PCR on RNA isolated from cloacal swabs of duck 3 in experiment two on days 2-6 post inoculation using WT primers (B), site-specific RT-PCR on the same RNA from fig A using MUT primers (C) chromatograms of DNA sequenced from same duck as in fig A and B on days 4-6 post inoculation showing the visible mutation on day 5 as a small subpopulation and as a larger population on day 6.

We did not detect the E119 wild-type in the E119V mutant strain or the E119V mutant strain in the wild-type strain using either Sanger sequencing or site-specific RT-PCR assays. We also utilized both methods in the mallard transmission studies and did not detect the E119V mutation in cloacal swabs from any ducks in all 3 groups. While traditional ferret transmission studies look at aerosol or direct contact transmission once the animal is already shedding virus, we choose a different route for these mallard studies. Since there is repeated and close interaction of mallards directly in the wild, we allowed this same close contact with the mallard ducks following inoculation of virus to time of interaction with contact naïve ducks. This would be a more likely representation of what happens in nature and would include the possibility of either direct transmission or environmental contamination transmission. More avian viruses with neuraminidase mutations should be tested in mallards both in the presence and absence of OC, to better evaluate the potential role this could play in nature.

We have utilized a novel experimental procedure to evaluate the effects of LPAI in mallard ducks within an environment contaminated with OC. As resistant viruses continue to emerge and evolve, we need to evaluate their ability to transmit and persist with or without selective pressure. While ferrets are the acceptable model to evaluate oseltamivir-resistant strains that could affect humans, we need to consider the natural reservoir of influenza viruses and the likelihood that these viruses will survive in nature. The results of this study can now be used with other models to continue to understand the role that wild water fowl may play in the next pandemic.

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