VIRAL AND HOST FACTORS AFFECTING INFECTION, PATHOGENICITY AND TRANSMISSION OF INFLUENZA VIRUSES

DISSERTATION

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By

Smitha Pankajavally Somanathan Pillai, M.V.Sc.

Graduate program in Veterinary Preventive Medicine

The Ohio State University

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Dissertation Committee:

Dr. Chang Won Lee, Advisor
Dr. Yehia M. Saif
Dr. Daral J. Jackwood
Dr. Gireesh Rajashekara
ABSTRACT

Wild aquatic birds act as the global reservoirs of influenza viruses in nature. Though not natural hosts, different species of domestic birds can be infected with influenza viruses from wild bird sources or contaminated environment from these reservoirs. In addition, domestic poultry can serve as the source of influenza infections for other avian and mammalian species including humans. Studies on the biological properties of avian influenza viruses in domestic bird species is an important initial step towards understanding differing viral and host ecologies and their applications in studies on interspecies transmission, development of diagnostics and vaccines. In order to gain detailed understanding of the biological properties of low pathogenic influenza viruses in domestic birds, we undertook one of the most comprehensive studies on the replication and intraspecies transmission of these viruses in three different domestic bird species that included chickens, ducks and turkeys. Our study incorporated 20 low pathogenic influenza viruses of different origins and we studied viral shed titers from infected and contact control birds of the 3 bird species at 2 different time points, their serology as well as histopathology and immunohistochemistry for antigen detection from different tissues of infected birds. Our results indicated that most of these isolates can replicate and transmit within poultry without clinical disease. However, distinct virus and species
specific differences in transmission to contact control birds were noted emphasizing the importance of having contact control cage mates in biological characterization experiments. Ducks supported the replication of wild bird viruses in their respiratory and digestive tracts equally well, though in contact control birds, higher tendency for viral detection from cloacal swabs was observed. The wild bird viruses did not effectively transmit between chickens. However, the wild bird isolates as well as domestic bird viruses from live bird markets and commercial poultry operations tested, replicated and transmitted in turkeys to comparatively higher titers than in chickens or ducks. Further, determination of minimal infectious doses of 3 selected viruses for the three bird species revealed lower dose requirement for infection of turkeys suggesting that turkeys could be easily infected following a low dose exposure in comparison to chickens and ducks. Our data elucidate the important role of turkeys as being more susceptible hosts for wild and domestic bird low pathogenic avian influenza (LPAI) viruses in comparison to domestic ducks and chickens. The high susceptibility of turkeys also signify their role as bridging hosts for influenza viruses between different avian species and potentially mammals including humans.

Further, we were interested in looking at the host factors, especially the presence, distribution and type of receptors that influence susceptibility to influenza viral infections. It is commonly understood that avian influenza viruses preferentially bind to $\alpha_{2,3}$sialic acid(SA)-galactose (gal) linked receptors and correspondingly avian tissues predominantly express these receptors. To get a better understanding of the receptor profile in different avian species, we undertook detailed studies on the distribution of $\alpha_{2,3}$SA-gal (avian type) and $\alpha_{2,6}$SA-gal (human type) receptors on different tissues of
chickens, ducks and turkeys in day old, 2-4 week old and layer birds using specific plant lectins, MAA and SNA respectively. Our studies showed that birds of the 3 species expressed both $\alpha_{2,3}$SA-gal and $\alpha_{2,6}$SA-gal receptors on tissues to varying degrees. On the tracheal epithelium, the 3 bird species expressed almost 80-100% high intensity positive staining for the $\alpha_{2,3}$SA-gal receptors in 3 different age groups. However, the distribution of the $\alpha_{2,6}$SA-gal receptors varied with species and age. Ducks expressed high positive staining (more than 80%) for human type receptors while turkeys and chickens showed approximately 30% and 60% positive staining, respectively, with slight differences in distribution with age. The bronchial epithelium showed similar trends as in trachea with some exceptions being noticed in layer chickens and turkeys, where lower percent of positive staining was observed with MAA (50-60%) and SNA ($\leq 10\%$). The bronchioles and the alveoli of the lungs did not show positive staining for receptors in the 3 species. The small intestinal sections from turkeys and ducks showed almost negligible staining for the avian and human receptors in 3 different age groups whereas sections of large intestine consistently showed 40-50% positive staining for the avian receptors with low (less than 5%) or no staining for the human receptors. However, small and large intestinal sections from chickens showed positive staining for avian (60-80%) and human (20-50%) receptors. Kidney sections from the 3 species of birds expressed approximately 50-60% avian receptors and 20-30% human receptors in the different age groups. This is one of the most extensive studies undertaken to understand the receptor type and distribution from different tissues of domestic poultry of different ages using plant lectins. We also demonstrated viral binding to the receptors observed using tracheal membrane preparations from chickens and turkeys. Our results indicated that like other
terrestrial domestic poultry, like chickens and quail that have been implicated as potential intermediate hosts for influenza viruses, turkeys could also support infection, replication of influenza viruses from avian and mammalian sources.

In biological characterization studies with avian origin H5 viruses, we had demonstrated that turkeys are highly susceptible to wild and domestic bird origin influenza viruses. Further, the presence of \(\alpha_2,3\text{SA-gal}\) and \(\alpha_2,6\text{SA-gal}\) receptors indicated their suitability as potential intermediate hosts for influenza viruses. Hence, we expanded our studies to determine whether turkeys supported infection, replication and transmission of swine lineage viruses equally well. We employed swine lineage triple reassortant (TR) H3N2 influenza viruses that derive their gene segments from avian, human and swine lineage viruses. TR H3N2 viral infections have been found to result in huge economic losses due to clinical disease as well as low egg production in breeder turkeys. In our study, we conducted genetic, antigenic and biologic characterization of selected H3N2 viruses. Phylogenetically, all turkey origin TR H3N2 viruses isolated from different geographical locations as well as different time points shared high genetic similarity and grouped together with the swine origin isolates. Antigenically, all turkey isolates were similar, showed lesser cross-reactivity to swine origin viruses, and no cross reactivity to avian origin H3 subtype viruses that are not triple reassortants. Biologically, turkey origin TR H3N2 viruses replicated efficiently in 3-week-old turkeys and transmitted to the contact control cage mates. In 4-week-old chickens and 2-week-old ducks, these viruses replicated poorly and did not transmit. In 26-week-old layer turkeys, one of the TR H3N2 strains tested caused complete cessation of egg production within 13 days post infection. We confirmed high levels of virus replication and abundant
distribution of avian specific α2,3 sialic acid-galactose receptors in the oviduct of these turkeys. Our studies indicated that influenza infections alone, without any concurrent infections, can result in drastic declines in egg production in breeder turkeys. In addition, antigenic variation among the vaccine strains and circulating viruses indicated that vaccines for turkeys should be updated or new vaccines developed using antigenically related strains.

Further, our interests were to determine if the high viral titers observed in the oviduct of breeder turkeys could be passed into eggs laid by infected turkeys. Following experimental inoculation of the turkey origin virus, A/turkey/Ohio/313053/04, in breeder turkeys, we detected the virus in albumin of eggs using real time RT-PCR and virus isolation in embryonated chicken eggs. Swabs from egg shells were also found positive by real time RT-PCR. This is the first report of detection of low pathogenic influenza viruses from internal egg contents following experimental infection. Although, there is no available evidence for vertical transmission of influenza viruses, the possibility of hatchery contamination by egg-borne influenza viruses and spread of virus during movement of contaminated cracked eggs and egg flats pose serious food safety and public health concerns.

In summary, our biological characterization experiments indicate that turkeys support infection with influenza viruses from different sources efficiently. Also, the receptor profile, especially in the tracheal epithelium, indicates the suitability of turkeys as potential intermediate hosts for influenza viruses. The ability of influenza viruses to cause drastic declines in egg production and the transmission of influenza viruses into egg contents of infected turkeys raise serious economic and public health concerns.
Dedicated to my family

for their

unconditional love,

unwavering support,

endless encouragement

and

prayers
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February 10, 1979……………………Born – Kundara, India

1997 – 2003……………………Bachelor of Veterinary science
......................................................and animal husbandry (B.V.Sc.)
......................................................Kerala Agricultural University, India

2003 – 2005……………………Master of Veterinary Science (M.V.Sc.)
......................................................Department of Veterinary Preventive Medicine
......................................................Kerala Agricultural University, India

2005 – 2006……………………Graduate Research Associate
......................................................Department of Animal Sciences
......................................................The Ohio State University, Columbus, Ohio

2006 – present……………………Graduate Research Associate
......................................................Food Animal Health Research Program
......................................................Department of Veterinary Preventive Medicine
......................................................Ohio Agricultural Research and Development Center
......................................................The Ohio State University, Wooster, Ohio
PUBLICATIONS


FIELDS OF STUDY

Major Field: Veterinary Preventive Medicine

Studies in influenza virology and molecular biology
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CHAPTER 1
LITERATURE REVIEW

1.1 Influenza A viruses

Influenza is a highly contagious disease that is associated with serious economic and health concerns for humans and animals worldwide. Of the 3 types, type A influenza viruses cause severe fatal and acute respiratory illness and mortality in millions of people worldwide every year. The severity of infection depends on the attack rate, virulence of the circulating strain and degree of immunity of individuals in the population (4). Influenza A viruses can infect a wide range of mammalian and avian species. Although only limited numbers of subtypes of influenza viruses have been associated with disease in mammals, all the 16 hemagglutinin and 9 neuraminidase subtypes have been found in aquatic bird reservoirs (4, 188, 193). The plasticity of influenza A viral genome and its wide host range accounts for its pandemic potential (196). Since influenza viruses have segmented genomes, reassortment is an important mechanism for rapid generation of genetic diversity. Influenza viruses continue to evolve to produce new antigenic variants by mechanisms of antigenic shift or antigenic drift. These antigenic variants are cause of
yearly epidemics. Also, strains to which most humans have no immunity can also appear suddenly resulting in pandemics (191).

### 1.1.1 Structure of influenza A viruses

Influenza A viruses are enveloped and have single stranded, segmented RNA genome, consisting of 8 RNA segments of negative polarity. At the 3’ and 5’ termini of the 8 gene segments, there are conserved sequences that can base pair to form panhandle structure because they share partial sequence complimentarity (30, 71, 137, 160). Influenza viruses possess host cell derived lipid envelope, and are 80-120 nm in diameter and have a spherical shape when propagated in eggs or cell culture. However, they appear pleomorphic when they are initially isolated from animal or bird species. The major surface glycoproteins of influenza A viruses are hemagglutinin (HA) and neuraminidase (NA) that project from the lipid envelope. The integral membrane protein, M2 is also embedded in the lipid envelope which acts as the ion channel. The M1 protein, which is the most abundant protein of influenza A viruses, lies beneath the lipid envelope and provides rigidity to the membrane that surrounds the 8 ribonucleoprotein complexes (RNPs) (88). The viral RNA segments are encapsidated by nucleoprotein and the polymerase proteins to form the RNPs. Influenza viruses also encode two non structural proteins, NS1 and NS2. NS2 or the nuclear export protein is a part of the influenza virion, while NS1 is found only in infected cells during viral infection, and is not incorporated into progeny virions (136, 203). The RNPs acts as the basic replication and transcription machinery of the virus. The viral polymerase complex is a heterotrimer composed of two
basic proteins, PB1 and PB2, and the more acidic PA. PB1 acts as the backbone of polymerase complex and contain independent binding sites for PB2 and PA (32). PB1 is supposed to be responsible for nucleotide addition during RNA synthesis (8, 142). PB1 also has RNA endonuclease activity (92). PB2 binds to 5’ cap structures on host cell mRNAs which act as primers for viral RNA synthesis and hence plays a vital role in transcription of messenger RNA (mRNA). The function of PA in influenza viral replication or transcription is not fully understood (31, 68). PA has been found to have protease activity and it is believed that its protease activity may be involved in the release of the RNPs from the nuclear matrix and their transport into the cytoplasm (63, 69). Nucleoprotein (NP) plays an essential role in structural organization of the RNP and is required for the transcription and replication of the viral genome. NP has also been implicated as a possible regulator of the switch between replication and transcription of the viral genome (69).

1.1.2 Classification of influenza viruses

Influenza viruses are classified under the Orthomyxoviridae family. The family comprises of five genera; influenza A, B and C viruses, thogoto virus genus of tick borne viruses and aquatic infectious salmon anemia virus (200). These viruses share the common properties of possessing a segmented single stranded RNA genome of negative polarity. Influenza viruses are classified into 3 types, A, B and C, based on the antigenicity of the major internal proteins, the NP and matrix (M) proteins.
1.1.3 Host range of influenza viruses

Of the 3 types of influenza viruses, type A influenza viruses are the most virulent and cause severe and fatal disease that can result in epidemics and even cause worldwide pandemics (170). Influenza A viruses have been isolated from humans, and various animals including pigs, horses, sea mammals such as seals and whales, poultry and wild waterfowl, including ducks (193). Influenza B and C viruses have been mainly isolated from humans, although B virus was isolated from seals in Netherlands in 2000 (126). Influenza B and C viruses cause localized epidemics in human. Influenza C viruses have also been found to infect pigs and dogs (98, 120).

1.1.3.1 The avian reservoir

Wild waterfowl are considered to be the primary natural reservoirs of influenza viruses. They are believed to play important roles in perpetuation of influenza viruses in nature (193). All the 16 HA and 9 NA subtypes of influenza viruses can be found in them (2, 70, 193). The gene pool of influenza A viruses in aquatic birds provides enough diversity for infection of other species of birds and mammals and all known lineages of influenza viruses in land based birds and mammals originated from aquatic birds. This finding is supported by the phylogenetic analyses of gene sequences of influenza viruses from different hosts, geographic regions and virus subtypes (48, 54, 191).

In ducks, the viruses preferentially replicate in the digestive tract and are excreted in high concentration in the feces (up to $10^{8.7}$ 50% egg infectious dose/g) and are transmitted mainly by fecal-oral route. Avian influenza viruses have been isolated from
freshly deposited fecal material and unconcentrated lake water, and hence water supply contaminated with fecal material can be very efficient mode of transmission (191). Also, contaminated fecal material can be source of infection for newly hatched ducks and for wild and domestic birds during migration (61, 191). In wild ducks, influenza viruses generally cause no major disease signs or symptoms and it is believed that this phenomenon is due to the adaptation of the virus to ducks over many centuries creating a reservoir that maintains the virus in equilibrium with the hosts without endangering them (192, 193). Phylogenetic analyses indicate that avian influenza viruses unlike their mammalian counterparts have low evolutionary rates. Although nucleotide changes have been found in avian viruses within their reservoirs, they do not correspond to amino acid changes, however, mammalian viruses tend to accumulate changes in amino acids which indicate that there is no selective advantage for amino acid changes in the avian viruses within their reservoirs. Hence the avian viruses are maintained phenotypically unchanged in their reservoirs to infect other bird and mammalian species (54, 191). Although barriers exist that limit the transmission of influenza viruses from their aquatic bird reservoir to other species of birds and mammals, several reports indicate that cross species transmission can occur (190, 193). One of the significant examples of interspecies transmission is the transmission of highly pathogenic avian influenza virus to humans in Hong Kong in 1997 (29, 194). After interspecies transmission, the viruses in general do not persist or establish themselves in new hosts. However, on some occasions, they adapt well and circulate for long time establishing stable lineages in the new hosts (171).
1.2 Determinants of infection, pathogenicity and transmission

In general, the ability of influenza viruses to infect, replicate and cause disease in a particular host as well as shed the virus to transmit efficiently to susceptible hosts depend on viral, host and epidemiological factors.

1.2.1 Viral determinants

The lack of proof reading during influenza viral replication results in replication errors of one per $1.5 \times 10^5$ nucleotides. Therefore in each round of replication, a mixed population of viruses is produced known as quasispecies. Most of the variants may not confer any distinct advantage over the already existing dominant genotype. However, on introduction into a new host, the best fit variant survives and expands in adaptation to the new environment (33).

1.2.1.1 The surface glycoproteins

Hemagglutinin (HA) is the major surface glycoprotein of influenza A and B viruses responsible for virus attachment to susceptible cells and for mediating fusion between viral and endosomal membranes. HA is also the major surface protein against which neutralizing antibodies are produced. HA is a type I integral membrane protein consisting of N-terminal signal sequence, membrane anchor domain and a short cytoplasmic tail at C-terminus. The protein is post translationally modified by addition of N-linked glycans, covalent attachment of palmitic acid to cysteine residues located in the cytoplasmic tail and cleavage signal peptide. The monomeric HA units associate to form
homotrimers during maturation. The HA is cleaved into 2 HA subunits, HA1 and HA2, connected by a disulphide bond during final processing. The fibrous stem of HA is formed by amino acids belonging to HA1 and HA2 subunits and a globular head comprises of amino acid residues from HA1 (79, 80).

The receptor binding site (RBS) of the HA is a shallow pocket on the globular head and the amino acids at the RBS are conserved among the different virus strains (198, 199). The amino acids at the RBS (Gly134, Ser/Thr136, Try153) of HA contacting the carboxylic and acyl groups at N5 of sialic acid are highly conserved in all influenza viruses. Differences in the amino acids at the RBS have been found for avian and human viruses. Avian viruses have been found to possess Glu 190, Gly 225, Gln 226 and Gly 228, whereas human viruses typically have Asp 190, Gln190, Asp 225, Leu/Ile/Val in 226 and Ser 228 (110, 147, 166).

The presence of charged amino acids at the RBS of HA can also affect the specificity and affinity of binding to receptors. Increase in binding affinity was seen with substitutions that decreased the negative charge (Glu to Gly), created a positive charge (Asn to Lys) or that reversed the charge from negative to positive (Glu to Lys). This is believed to result from modulation of the electrostatic interactions between the virus and the negatively charged sialic acid molecules on the cell surfaces (41, 44, 78).

The receptor binding properties of HA should be compatible with cleavage specificity of NA and its stalk length for viral virulence. A short NA stalk length is inefficient in causing viral budding because the active site cannot access its substrate efficiently. A shortened NA stalk has been found to reduce ability of virus to elute from
erythrocytes (10, 34), can decrease virus growth in MDCK cells and eggs and can decrease virulence in mice (98). NA can cleave the sialic acid (SA) moieties attached to galactose(gal) by $\alpha$2,3 or $\alpha$2,6 linkages. During the evolution of N2 avian influenza viruses in human, $\alpha$2,3 cleavage activity of NA has been maintained and $\alpha$2,6 activity increased, possibly conferring a selective advantage to human viruses where the HA has $\alpha$2,6 receptor specificity (13). The NA stalk, which holds the active site of the protein, varies in sequence and length (15). NA has been found to have roles in enterotropism of avian influenza viruses. Avian virus NAs function better at higher temperature and lower pH than do their mammalian counterparts and hence function well at the low pH of digestive tracts of avian, however is restricted in activity in the human intestinal tract (36).

The combination of HA glycosylation and NA stalk length influences host range and virulence. Human H1 influenza viruses show additional glycosylation of its HA compared with those from aquatic birds, or pigs (72) during the adaptation of an avian virus to human hosts. It has been suggested that hyperglycosylation of HA, combined with a compensating deletion in the NA stalk, modifies the virulence of progenitor viruses during virus evolution in chickens (106). Hyperglycosylation of HA head has been associated with increased virulence of H7 influenza viruses in chickens (12).

1.2.1.2 Nucleoprotein and polymerase complex

Influenza virus segment 5 encodes nucleoprotein (NP) (96, 119) and it is relatively well conserved, with a maximum amino acid difference of less than 11% (158).
NP binds single stranded RNA with high affinity. NP also interacts with PB1 and PB2, but not with PA in virus infected cells (14, 115). NP also interacts with M1 (204). One of the most important roles of NP is to maintain the RNA template in an ordered conformation suitable for transcription by the polymerase and/or packaging into virions (75). NP also plays a potential role in the switch from mRNA transcription to genome replication (155, 161). NP is also found to have a role in RNP transport. Nuclear import of NP and NP–RNA complexes results from the interaction of NP with host cell importin α (123, 185). This trafficking event operates during the early period of the infectious cycle to direct nuclear import of the infecting RNPs and of newly synthesized NP to support the process of genome replication. However, later in infection, RNP export becomes dominant and interactions of M1 with the RNPs are an essential component of the process (19, 102, 197). It has been determined that NP is a significant player in determining species specificity. Sequence analysis suggests that NP has evolved into 5 host specific lineages: 2 equine, 1 pig and human lineage, 1 aquatic bird lineage and 1 lineage in other avian species (195). During viral replication, NP is phosphorylated by host cell derived phosphokinases and the phosphorylation pattern of NP determines the extent to which a particular cell line supports virus growth (1, 83, 177).

Regarding polymerase complex, the PB2 protein has been shown to have important role in mediating species specificity. It was demonstrated that an avian human reassortant virus that contained only the avian PB2 gene replicated efficiently in avian cells, but inefficiently in mammalian cells (27). Further research revealed that the species specificity depended on amino acid residue at position 627 (Glu in avian viruses, Lys in
human viruses) (168). H7N7 highly pathogenic avian influenza (HPAI) virus isolated from patient in Netherlands and H5N1 viruses isolated from humans in Asia contained Lysine at position 627 (37, 134). The interaction of viral polymerase with RNA depends on whether the 3’ or 5’ termini of viral template are in base paired or single stranded confirmation (91) and hence temperature at site of replication has been found to have a role in host tropism (11, 114). Viral polymerase complexes from avian viruses have been shown to exhibit cold sensitivity in mammalian cells, which was found to be controlled by amino acid 627 of PB2 (104).

PA and PB1, the other two proteins of the polymerase complex have also been shown to have roles in determination of host range. Introduction of avian PB1 into human virus has been shown to decrease its replication efficiency in MDCK cells, but not affect viral replication in chicken kidney cells (162). Amino acid substitutions in polymerase proteins could affect its interactions with host cell factors and modulate host range (67).

1.2.1.3 Nonstructural and matrix proteins

Nonstructural proteins, especially NS1 of influenza viruses coded by segment 8 helps the virus to evade host immune responses by counteracting the cellular interferon responses and play significant roles in pathogenicity. NS1 protein acts by binding to double-stranded RNA and suppressing the activation of double-stranded RNA-activated protein kinase, a known stimulator of type I interferon, and by preventing the activation of transcription factors that stimulate interferon production (49, 84). The NS genes of highly pathogenic viruses have been found to be more efficient suppressors of interferon
responses than the low pathogenic ones. For example, the NS gene of 1918 Spanish flu virus suppressed the expression of interferon regulated genes in human cells more efficiently than the NS gene of the A/PR/8/34 virus (52). Similarly, the NS genes of 1997 and 2005 H5N1 viruses are believed to be potent inducers of proinflammatory cytokines in primary human macrophages (23, 56). The cytokine responses induce the unusual clinical signs associated with highly pathogenic influenza infections of humans.

RNA segment 7 encodes 2 polypeptides: M1 matrix protein and M2, an ion channel protein. There are four lineages of M1 – human, swine, Eurasian avian, and equine plus north American avian and three lineages of M2 – human, swine, and avian plus equine. Matrix protein has been found to be associated with restriction of replication of an H2N2 avian virus in squirrel monkeys and an H3N8 avian virus in pigs. Functional compatibility between the pH at which HA undergoes conformational change and the pH at which M2 ion channel opens ensures that intracellularly cleaved HAs of HPAI viruses can pass through the Golgi without undergoing a premature irreversible conformational change during virus budding (11). Compared with M proteins of early human viruses, M proteins of late human viruses have gradually lost the ability to cooperate with avian virus HAs, being unable to form replication – efficient reassortants in vitro (151).

1.2.2 Host determinants

1.2.2.1 Receptors

Influenza viruses are believed to bind to sialic acid molecules that occupy terminal positions on oligosaccharide chains of glycoproteins and glycolipids. Sialic
acids are 9-carbon sugars and are commonly found at the outermost ends of N-glycans, O-glycans and glycosingolipids (148, 181). The 5-carbon position of these molecules has an acetyl (Neu5Ac) or hydroxyl group. The 5-N-acetyl group can be hydroxylated, giving 5-N-glycoylneuraminic acid (Neu5Gc). Sialic acids can be attached to underlying sugar chain by α-glycosidic linkages. D-galactose (gal), N-acetyl-D-galactosamine (GalNac) and sialic acids often function as the penultimate sugars of oligosaccharide chains to which sialic acids are attached. Sialic acids are commonly attached to gal and GalNAc through α2,3 or α2,6 linkages (149, 180).

Influenza viruses attach to target cells via multivalent interactions of the viral hemagglutinin with terminal sialic acid molecules on cellular glycoproteins and glycolipids. The interactions between the virus and the cellular receptors are thought to play important roles in determining host range of influenza viruses (106). Human and avian influenza viruses are known to have distinct preferences for specific receptors. Different cellular tropism of viruses could account for differences in replication and pathogenicity of influenza viruses. Differentiated cultures of human airway epithelial cells showed that human viruses preferentially infect non-ciliated cells, whereas avian viruses mainly infect ciliated cells. These results correlated with the predominant presence of α2,6 linked sialic acids on non-ciliated cells and α2,3 linked sialic acids on ciliated cells (112).

Initially H3 viruses were found to differentiate between types of SA-gal glycosidic linkages (140). Further studies with other HA subtypes revealed that avian viruses bind to α2,3 linked sialic acids much more strongly than α2,6 linked sialic acids...
α2,3 or α2,6 linked sialic acid oligosaccharide molecules have different molecular shapes and influenza viruses can discriminate between them. Human and avian viruses were found to preferentially bind to α2,6SA-gal and α2,3SA-gal terminated receptors. The attachment of the avian viruses to the α2,3SA-gal linked receptors gives rise to the lowest energy trans conformation of the 2,3SA-gal glycosidic linkage. Hence, receptor preference of avian viruses is based on the energetically optimal fit of the trans conformer when virus binds to α2,3SA-gal linkage to the poor energy fit of cis conformer when virus binds to α2,6SA-gal (16, 132). Also, unfavorable polar- non polar interactions of Gln 226 at the RBS of avian viruses and α2,6 SA-gal linkage limits the binding of avian viruses to the α2,6SA-gal receptors (60). However, human viruses have Leucine at position 226 and the interactions of Leucine with 2,6SA-gal linkages are energetically favorable. This might account for the greater affinity of binding of human viruses to 2,6SA-gal linkages than avian viruses (59, 109, 110). The binding of viruses can be affected by structure of more distant parts of the oligosaccharide chain other than the sialic acid glycosidic linkages to the penultimate sugars (43, 169). The inner fragments of the glycoprotein or glycolipid can contribute to receptor binding preference of the viruses to α2,3 or α2,6 sialic acid linked galactose residues. These distant moieties can either stabilize the binding in the presence of energetically favorable bonds or decrease the strength of binding because of steric conflicts (109).

Influenza viruses possess approximately 400 to 500 spikes on the virus surface and hence the viral binding to receptors is mediated by several simultaneous interactions.
of the hemagglutinin of the virus with the receptors. This polyvalent binding is mediated by multiple low affinity contacts. Hence the attachment of the viruses to the receptors depends on the receptor density (64, 103, 108). Because of the participation of several hemagglutinin molecules in receptor binding, structures at the distal portions of the oligosaccharide chain can affect the binding. For example, the number, position and structure of N-linked carbohydrates located at the receptor binding site of the hemagglutinin globular head can affect the receptor binding property of influenza viruses. The presence of carbohydrates can affect the receptor binding by steric hindrance of the receptor binding site or steric conflicts between the carbohydrate chains and remote domains of the receptor molecules that interfere with polyvalent virus binding. Most researches indicate that presence of carbohydrates on hemagglutinin head decreases the binding affinity (41, 44, 55, 109, 183). Also, it has been shown that effects of carbohydrates on virus binding affinity depend on the nature of receptor to which the virus binds (9, 57). The presence of carbohydrate at CHO$_{131}$ on the affinity of H1N1 virus to bind to several receptor analogues showed that the carbohydrate interfered with the binding of virus to Neu5Ac(α2-6)Gal containing receptors but had no effect on binding to Neu5Ac(α2-3)Gal.

Acylation of the sialic acid terminated glycoconjugates at C$_7$-C$_9$ positions was not found to be favourable for binding to RBS of HA (65), however 4-O-acetylation of the sialyloligosaccharides did not affect receptor binding of H3 influenza viruses (107, 133). The ability of H3 viruses to bind N-glycoyl neuraminic acid has been found to be influenced by the amino acid at position 155 of HA (6, 65, 113).
Similarly, sulphation and fucosylation of subterminal oligosaccharide chains have been found to affect viral binding. Chicken viruses have been found to have higher affinity for $\alpha_2,3\text{SA-gal}\beta_1-4\text{GlcNAc}$ sulphated on the 6 position of GlcNAc (46). Sulphated sialyloligosaccharides are also expressed in human airway cells (89). Fucosylation at the 3 position of GlcNAc has been found to be bound by some gull influenza and H7N1 influenza viruses (40). Such changes in the subterminal residues appear to have more effect on $\alpha_2,6\text{SA-gal}$ than $\alpha_2,3\text{SA-gal}$ terminated receptors (143).

1.2.2.2 Immunohistochemistry for detection of receptors

Immunohistochemical studies using plant lectins revealed the presence of Neu5Ac($\alpha_2$-$3$)gal residues and no detectable expression of $\alpha_2,6$sa-gal receptors in duck intestinal epithelial cells (73). Also human viruses were not found to bind to plasma membranes isolated from duck intestinal cells thereby confirming the absence of $\alpha_2,6$SA-gal sialyloligosaccharides on duck intestinal epithelial cells (39).

Avian influenza viruses bind strongly to Neu5Ac receptors than to Neu5Gc containing receptors (65, 74, 109). The Neu5Gc were found in epithelial cells of duck colon (74). Duck viruses have been found to bind with greater affinity to Neu5Ac($\alpha_2$-$3$)Gal($\beta_1$-$3$)GalNAc containing receptors than Neu5Ac($\alpha_2$-$3$)Gal($\beta_1$-$4$)GlcNAc containing receptors indicating that distant part of the oligosaccharide chains affect binding of the duck viruses (45). Viruses from gulls and shore birds preferentially bind to Neu2Ac($\alpha_2$-$3$)gal receptors similar to ducks. However H13 viruses were found to weakly bind to $\alpha_2$-$3$sialic acid linkages and do not discriminate between $\beta_1$-$3$ and $\beta_1$-$4$ linkages.
The substitution of Leu226 to Gln226 in a reassortant virus harboring HA of human origin allowed its binding to α2-3 gal receptors of duck intestine and Ser228 to Gly228 substitution increased the binding affinity to duck intestinal cells. These two substitutions allowed the replication of the virus in the duck intestinal epithelial cells (105, 118, 182).

1.2.2.3 Detection of receptor binding specificity of influenza viruses

1.2.2.3.1 Using sialyltransferases

Erythrocytes are desialylated using *V. cholera* sialidase to abolish virus binding. The erythrocytes are then incubated with specific sialyltransferases and sialic acids to generate sialyloligosaccharides of defined sequence and sialic acid of defined glycosidic linkage on the surface of erythrocytes. These erythrocytes were used for hemagglutination and virus adsorption studies (128, 140). These experiments showed for the first time that influenza viruses attach to specific sequences on the cell surfaces and that receptor binding specificity depend on the species of virus origin (22, 140, 141).

1.2.2.3.2 Binding assays using natural and synthetic sialyloligosaccharides

Structurally defined artificial sialyloligosaccharides can be used to assess the receptor specificity of viruses from different origins (53, 81, 110, 146, 178). Also plasma membranes separated from epithelial cells coated on plates can be used in solid phase binding assays to study viral binding using fetuin peroxidase system (42, 47, 110). Such
assays are quantitative and can measure the association constants for virus receptor complexes.

Few natural glycoproteins have been shown to study receptor binding specificity of influenza viruses. Equine and pig α2-macroglobulin contain α2,6 gal terminated residues which bind with greater affinity to human influenza viruses than avian viruses. Hen ovomucin contains predominantly α2,3SA-gal sialyloligosaccharides which bind with greater affinity to avian viruses than human viruses (106, 111).

### 1.3 Role of intermediate hosts in influenza viral infections

The molecular determinants of influenza viral host range restriction are not fully understood. Because avian influenza viruses usually do not cross the species barrier to infect humans, it was believed that pre-adaptation of the avian viruses or their genes to a mammalian host like pig might be necessary (193).

The limited distribution of α2,3SA-gal in human trachea as well as the inefficient replication of avian viruses in humans led to the hypothesis that avian viruses had to adapt in an intermediate host that had both types of receptors on their tracheal epithelial surface before transmitting to humans. Swine have been proposed to be potential intermediate hosts or mixing vessels for influenza viruses for a long period of time. Because swine are susceptible to infection with both avian and human influenza viruses, genetic reassortment between human and avian influenza viruses can occur when these viruses co-infect an individual pig (150). The double (avian/human;human/swine) and triple (human/avian/swine) reassortant influenza A viruses isolated from pigs in the U.S.
or China provide supportive evidence for the “mixing vessel” theory. Most avian and human influenza viruses preferentially bind to specific receptor types having $\alpha_{2,3}$SA-gal (avian receptor)- or $\alpha_{2,6}$SA-gal (mammalian receptor)-terminated saccharides, respectively (139, 140). Both receptors have been found in the respiratory tract of swine (72), providing solid molecular evidence for pigs as “mixing vessels” for human and avian influenza viruses. Numerous laboratories have isolated AIVs with all 8 gene segments derived from avian sources from swine. In addition, pigs have been experimentally infected with the H1-H13 subtypes of AIVs (82) and may be susceptible to H14-H16 subtypes as well. The infection of pigs with human viruses has also been documented (17, 86, 205). Swine susceptibility to avian and human influenza A viruses has and continues to provide opportunities for the introduction of new influenza genes into swine influenza viruses within swine (100). Genetic reassortment among avian, human and/or swine viruses has been documented in pigs. Before 1998 only classical H1N1 swine influenza viruses were isolated from the U.S. pigs. Since 1998 the double (human/swine, subtype H3N2) and triple (avian/swine/human, subtypes H3N2, H1N2, H1N1 and H3N1) reassortant swine influenza viruses have emerged in the U.S. and Canada. Presently, triple but not double reassortant swine influenza viruses have spread widely within swine across the U.S. and Canadian swine herds (25, 77, 99, 125, 186, 207). These facts provide compelling evidence that pigs could serve as a “mixing vessel” for influenza A viruses. Also, the transmission of influenza viruses from swine to humans is not a rare event (138).
Recent evidence suggest that like pigs, land-based poultry like quail, chickens and turkeys might play critical roles as intermediate hosts in the emergence of pandemic influenza viruses (90). Land based birds have been found to support a variety of influenza virus subtypes (94, 129). Compared to H5N1 viruses isolated from aquatic birds, those viruses isolated from chickens have been found to have significantly lower affinity for NeuAcα2,3gal, similar to human virus isolates. H5N1 chicken isolates with reduced avian receptor specificity were found to share 2 characteristic features of human viruses, an additional glycosylation site in the globular head region of HA and a deletion in the NA stalk (106, 111). Similarly, the receptor specificity of H9N2 viruses isolated from land-based poultry, but not of those isolated from aquatic birds, were found to be similar to that of human isolates (144). These findings are further strengthened by the presence of avian and mammalian type receptors in the tracheal epithelium of these birds (39, 184). Hence, land-based poultry may serve as an adaptation host that facilitates the conversion of receptor preference from avian to human-type receptors. Studies show that H7N3 viruses circulating since 2002 in turkey population in northern Italy were closely related to H7N3 viruses isolated from wild ducks in 2007 and serological evidence indicated that transmission of these viruses also occurred to human beings (20, 21, 135).

1.4 Potential role of turkeys as intermediate hosts

1.4.1 Turkeys are highly susceptible to wild aquatic and shore bird influenza viruses

It has been documented that multiple subtypes of influenza viruses can establish stable lineages in domestic poultry (95, 165, 189). Though the source of influenza
infections for the domestic bird species are believed to be wild bird reservoirs, studies indicate that domestic poultry can be infected with influenza viruses from different sources, for example, swine influenza infections of turkeys. Avian influenza infections have been recorded from turkeys worldwide (3, 5, 20, 116, 176). Similarly, multiple subtypes of influenza viruses have been isolated from healthy and diseased chickens, however, it is not known whether the influenza virus was transmitted directly to the chickens from an aquatic bird, or if the virus acquired expanded host-range capabilities by replication in other avian species, prior to infection of the chicken. Several observations show that waterfowl origin influenza virus can be more easily transmitted to domestic turkeys than chickens. In the United States, since mid-1960s, influenza virus infections have been seen consistently in turkeys in California and Minnesota, where turkeys were range-reared and farms are heavily concentrated, and are situated on migratory waterfowl flyways (61, 62). Campitelli et al. (2004) (20) identified for the first time that the turkey H7N3 viruses were derived directly from influenza strains circulating in wild waterfowl (20). Several previous studies and field data also document that turkeys may be more susceptible to LPAI wild bird viruses than chickens (61, 130, 164).

Studies on replication and intraspecies transmission characteristics of H5 subtype wild bird viruses in our lab also indicated the higher susceptibility of turkeys to mallards and ruddy turnstone viruses in comparison to chickens (unpublished data). In turkeys, the viral shed titers and hemagglutinin inhibition (HI) antibody titers were comparable among infected and contact control cage mates indicating efficient intraspecies transmission of the wild bird viruses. On the contrary, with contact control chickens, the
virus detection was low and majority of the birds did not seroconvert indicating inefficient replication of the wild bird viruses and failure to reach the threshold viral titer required to initiate a humoral immune response. Hence, chickens in comparison to turkeys might not be good initial hosts in terms of viral amplification and transmission. It might take multiple passages for successful viral adaptation and transmission of wild bird influenza viruses in chickens. Along with data on biological characterization, available data on molecular determinants of interspecies transmission of influenza viruses also support the potential role of turkeys as bridging hosts for wild aquatic bird viruses to domestic poultry. While data from H5 subtype viruses are lacking, no species specific molecular changes in duck H7 HA were found to be required for influenza infections of turkeys, however, host species related mutations in duck H9 HA were found to be required for infection of chickens (130). The results from biological and genetic characterization of the viruses imply the importance of turkeys as good initial hosts for wild bird viruses that have no previous adaptation for terrestrial domestic poultry.

Studies on the minimum infectious dose of wild bird viruses for different domestic bird species are limited. Determination of 50% infectious dose titers of 2 wild bird viruses of H5 subtype indicated that a lower dose was needed to infect turkeys in comparison to chickens (159, unpublished data). The low minimum infectious dose suggests that turkeys can be easily infected following a low dose exposure and confirms the higher susceptibility of turkeys to influenza viruses of wild bird origin. Their high susceptibility also indicate that turkeys could be good sentinel hosts in influenza surveillance studies and/or better model hosts for studies on adaptation of wild bird origin
influenza viruses for domestic poultry. Once infected, the influenza viruses in domestic birds undergo rapid evolution (50, 207). It is possible that wild bird viruses circulating in turkeys could undergo favorable changes that permit their adaptation to chickens and other domestic poultry. Hence, the potential role of turkeys as bridging hosts for introduction of low pathogenic waterfowl origin influenza viruses into other domestic poultry cannot be ruled out. Although confinement rearing has become the norm in the U.S. and other developed countries, turkeys could be significant players in supporting and transmitting influenza infections to other land based poultry where wild birds have free access to domestic poultry.

1.4.2 Turkeys support replication and intraspecies transmission of influenza viruses showing different degrees of adaptation for domestic poultry

Though detailed experimental studies on the replication and transmission characteristics of domestic bird origin influenza viruses in turkeys are lacking, available evidence indicate susceptibility of turkeys to LPAI viruses isolated from commercial poultry operations and live bird markets (87, 175, 179). Among commercial poultry operations, more LPAI outbreaks have been reported in turkeys in comparison to chickens (172). Experimental studies with LPAI H7N2 isolate that caused an outbreak among commercial poultry primarily infecting turkeys indicated a lower 50% infectious dose titer for turkeys than chickens. Also, 20-158 fold higher infectious virus was recovered from turkeys in comparison to chickens (179). A similar outbreak with H7 subtype viruses in poultry in Italy in 1999 primarily affected turkey flocks (202). The
high susceptibility of turkeys may be explained by the low minimum infectious dose titers required for infection. Our studies using a highly poultry adapted strain of LPAI also showed a lower 50% infectious dose titers for turkeys in comparison to chickens (unpublished data). However, it should be noted that infectious dose titers vary with virus strains as well as the genetic constitution of the birds used in the studies. Also, under field conditions where secondary infections or other factors can reduce immune responses, susceptibility and clinical disease may be exacerbated. The higher susceptibility in an outbreak area may also be an indication of the higher population of turkey flocks in the outbreak area. In spite of the limitations of experimental infection studies in turkeys, it is important to understand the biological properties of viruses in this host and host ecologies. In addition to wild bird isolates as previously described, we tested 12 low pathogenic influenza virus isolates from live bird markets as well as chicken and turkey isolates from commercial poultry operations for their replication and transmission among turkeys and chickens. Viral shed titers, HI titers and histopathology revealed turkeys to be highly susceptible hosts for influenza viruses of different degrees of adaptation for domestic poultry. All the domestic bird isolates infected chickens, ducks and turkeys to comparable titers and percent of birds infected was also similar with most isolates. However serological data showed higher seroconversion with these isolates in contact control turkeys than chickens. Also, viruses isolated from different bird species from live bird markets (LBMs) that included ducks, chicken, emu and pheasants, replicated and transmitted to higher titers in turkeys indicating again that turkeys are better hosts for even chicken and duck adapted LPAI viruses (unpublished data). Influenza isolates from
LBMs are believed to be highly poultry adapted as they provide favorable environment for adaptation of influenza viruses for a variety of hosts including chickens, turkeys, pheasants, quails and ducks and have been implicated as the source of influenza outbreaks in commercial poultry operations as well as humans. LBMs in New York and New Jersey have been implicated as significant sources of influenza viruses for commercial chicken and turkey operations in the U. S. (127, 154). The high susceptibility of turkeys to domestic bird isolates again underscores their importance as potential bridging hosts for influenza viruses from different origins and domestic poultry.

1.4.3 Interspecies transmission of swine influenza viruses to turkeys: a frequent event

Currently 3 subtypes of influenza viruses are commonly found in pigs worldwide, H1N1, H1N2 and H3N2. These viruses were found to be derived either from mammalian or avian viruses or their reassortants (18, 24). H1N1 influenza viruses were first isolated from pigs in the U.S. in 1930 (157) and till late 1990s, the classical H1N1 lineage was the predominant influenza subtype in pigs in the U.S. (124). The first reported isolation of swine influenza viruses in turkeys was in 1980-81 (66). Since then, transmission of swine H1N1 influenza viruses to turkeys has been documented several times (7, 35, 93, 97, 173, 201) and in majority of the cases, the turkey flocks were housed in close proximity to swine herds (167). In 1998, triple reassortant (TR) influenza viruses containing gene segments derived from recent human (HA, NA and PB1), swine (NS, NP and M) and avian (PB2, PA) viruses were first isolated from pigs and since then, have spread over
much of the U.S. swine population (187, 206). Since 2003, the swine origin TR H3N2 viruses have been isolated from U.S turkey populations (26, 176). The early reports of swine TR H3N2 viruses crossing the species barrier to infect turkeys were from farms in Minnesota and North Carolina in 2003 (26). Later, the TR H3N2 viruses have been isolated from turkey flocks in different parts of the U.S. (131, 176). In 2002, Suarez et al. reported the isolation of TR H1N2 influenza virus with gene segments derived from swine, human and avian lineages from turkey breeder hens associated with sudden drops in egg production (167). This virus was also believed to have a swine source of infection and provides additional evidence of cross species transmission of swine influenza viruses to turkeys. Among the different domestic bird species that are susceptible to swine influenza viruses including chickens, more number of field cases have been reported with turkeys, indicating that turkeys may be naturally more susceptible than chickens and other domestic poultry (152, 153). Experimental infections also supported the findings that turkeys are more susceptible to influenza infections than chickens. Studies with the four triple reassortant viruses isolated from turkeys in 2003 and 2005 revealed that all of these viruses could replicate and transmit within turkeys, however, only 2 of them could transmit among chickens based on serology, though viral shed titers were negative in the contact control chickens (131)unpublished data). The ability of the turkey triple reassortants to replicate in quail, but not the swine triple reassortants also indicate the potential role of turkeys as bridging hosts for influenza viruses to other land based poultry, mammals or even humans (26). The ability of these swine origin viruses to infect turkeys is not limited to birds of the younger age group that are usually used for
experimental infection studies. Several previous reports document drops in egg production in turkeys due to H1N1 (97, 159) reassortant H1N2 (167), and H3N2 viruses (26, 125, 176). Hence, along with the concern of introducing and establishing new influenza lineages in turkeys, interspecies transmission of swine influenza viruses to turkeys can be economically significant. The strong staining for avian receptors and high viral titers in the oviduct and drastic declines in egg production following infection are indicators that influenza viral infections alone without any concurrent infections can result in economic losses under field conditions (131). A recent study reported that turkey embryos are susceptible to swine TR H3N2 influenza viruses and continuous passage of the swine viruses have been found to result in mutations in HA similar to those found in turkey TR H3N2 viruses (163). However, although embryonated turkey eggs may serve as inexpensive tools to study the adaptation and interspecies transmission of swine influenza viruses, turkey embryos may not mimic the biology of a bird especially in terms of immune responses and several issues including the reproducibility of the HA mutations with other swine viruses and the changes in other gene segments should be considered in the study.

1.4.4 The receptor profile in turkeys support their role as intermediate hosts

Different sialic acid types and linkages in different species of birds and mammals are believed to be barriers for efficient interspecies transmission of influenza viruses. Influenza virus receptors on host cells are believed to be terminal sialic acid (SA) residues which are bound to glycans through an $\alpha 2,3$ or $\alpha 2,6$ linkage, mediated by
sialyltransferases that are expressed in a cell and species specific manner (38). It is believed that duck intestinal epithelial cells express α2,3SA-galactose (gal) receptors, while tracheal epithelial cells of humans mainly express α2,6SA-gal receptors. Avian viruses are believed to preferentially bind α2,3SA-gal linked receptors and influenza viruses from humans preferentially recognize human type receptors (109). Apart from the sialic acid-glycosidic linkages to the penultimate sugars, the binding of viruses can be affected by structure of more distant parts of the oligosaccharide chain (43, 65, 169). Also, the polyvalent binding of HA to receptors is mediated by multiple low affinity contacts. Hence the attachment of the viruses to the receptors depends on receptor density (64, 101).

Studies to elucidate the type and distribution of receptors in different tissues of domestic poultry as well as viral binding to the receptors observed are still incomplete. Plant lectins, Maackia amurensis agglutinin (MAA) and Sambucus nigra agglutinin (SNA), that are specific to α2,3SA-gal and α2,6SA-gal terminated sugars are usually used to identify the type of receptors present in different tissue sections. The earliest studies to identify receptor profile among birds were undertaken with duck intestinal cells. Lectin immunohistochemical studies revealed the presence of Neu5Ac(α2,3)gal residues and no detectable expression of α2,6SA-gal receptors in duck intestinal cells (73). Also human viruses were not found to bind to plasma membranes isolated from duck intestinal cells thereby confirming the absence of α2,6 sialyloligosaccharides on duck intestinal epithelial cells (45).
Epithelial cells from trachea and intestines of chickens and quail have been found to have moieties that can bind SNA and MAA and to bind viruses showing human and avian type receptor specificity (45, 58, 184). Studies by Wan and Perez (2006) reported that 62% and 30% of the quail tracheal epithelial cells were positive for α2,6SA-gal and α2,3SA-gal staining respectively (184). In quail colon, approximately 75% of the epithelial cells were positive for α2,3SA-gal staining, while 36% were found positive for α2,6SA-gal staining. In chicken trachea, approximately 85% and 10% of the epithelial cells were positive for α2,3SA-gal and α2,6SA-gal receptor staining respectively. Chicken intestine epithelial cells were negative for α2,6SA-gal linked receptor staining (184). However, a recent study by Kuchipudi et al (2009) reports that lectin staining revealed α2,6SA-gal to be the dominant receptor type in chicken tracheal epithelium (85). Using the two isoforms of MAA, MAA1 and MAA2, chicken tracheal and intestinal epithelial cells were also found to express both types of α2,3SA-gal receptors both β(1-4)GlcNAc and β(1-3)GalNAc subtypes. Their studies using duck tracheal epithelial cells also reported both α2,6SA-gal and α2,3SA-gal receptors, with a predominance for α2,3SA-gal receptors. However, studies with duck tracheal epithelial cells in our lab showed similar intensity of positive staining for α2,3SA-gal and α2,6SA-gal receptors. These discrepancies in results, especially with MAA staining can be explained by the use of either of the two isoforms or a combination of the two in lectin studies. The MAA1 isoform has been found to have the greatest affinity for α2,3SA-gal β(1-4)GlcNAc and even binds non-sialic acid residues. MAA2 preferentially recognizes α2,3SA-gal β(1-3)GalNAc. Hence results of lectin immunostaining will depend on the isoform of MAA.
used as well as subterminal structures of the glycan chain (121). The use of different
breeds of birds within the same species as well as differences in tissue processing
techniques may also account for the different staining results observed. Our studies using
MAA which shows similar binding pattern to MAA1 in turkey tracheal sections indicated
that like quail and chickens, turkeys also possess α2,3SA-gal and α2,6SA-gal terminated
sialyloligosaccharide residues with 80-90% and 70% of the tracheal epithelial cells
respectively, showing positive staining (unpublished data). The sections from colon
showed only the presence of α2,3SA-gal receptors. The presence of avian and human
type receptors in turkeys along with their higher susceptibility to wild and domestic bird
origin and swine viruses strengthens the argument that turkeys can be potential
intermediate hosts for interspecies transmission and spread of reassortant viruses between
birds and humans.

The actual viral binding to the receptors observed have been found to be affected
by different factors other than receptor specificity. Avian influenza viruses have been
found to bind strongly to Neu5Ac receptors than to Neu5Gc containing receptors (65, 74,
109). Also, duck viruses have been found to bind with greater affinity to Neu5Ac(α2-
3)Gal(β1-3)GalNAc containing receptors than Neu5Ac(α2-3)Gal(β1-4)GlcNAc
containing receptors indicating that distant part of the oligosaccharide chains affect
binding of the duck viruses (45). Viruses from gulls and shore birds preferentially bind to
Neu2Acα2-3gal receptors similar to ducks. However H13 viruses were found to weakly
bind to 2-3sialic acid linkages and do not discriminate between β1-3 and β1-4 linkages.
The length of the gangliosides was also found to have an effect on viral binding. Duck
viruses bound to gangliosides with short sugar chains that were found to be abundant in duck intestines, whereas chicken viruses bound more strongly to gangliosides with long sugar chains that were abundant in chicken intestinal tissues (45). As expected, in accordance with the detection of SAα2,3-gal and SAα2,6-gal receptors, epithelial cells from quail and chicken intestines were found to bind both avian and human type viruses (58). Similar studies on epithelial cells from trachea and intestines of turkeys are necessary to confirm that they play important roles as intermediate hosts where influenza viruses of avian and human origin can be amplified and spread to susceptible hosts. Also, highly pathogenic avian influenza virus infections are fewer in turkeys in comparison to chickens, which may also strengthen the role of turkeys as suitable intermediate hosts between wild bird and other land based poultry or even mammals.

The inherent susceptibility of turkeys to wild bird, domestic bird and swine origin low pathogenic influenza viruses signify the important role of turkeys as the domestic bird species that could act as the potential entry points and the bridging species for influenza viral entry into agricultural and commercial poultry systems. Also, the fact that turkeys are easily infected with aquatic bird and swine viruses and the ability of these viruses to reassort increases the probability that further reassortments and evolution of these viruses can take place in turkey hosts. Lectin histochemical studies also indicate that tracheal epithelium of turkeys can support infections with avian and human influenza viruses. Mixing of poultry and outdoor rearing could favor adaptation of low pathogenic influenza viruses from different sources for domestic poultry and pose serious health risks for birds and mammals including humans.
1.5 Egg-borne transmission of influenza viruses

Type A influenza infections of domestic birds may be asymptomatic or cause clinical disease with symptoms ranging from varying degrees of depression, anorexia, mild to severe respiratory illness, diarrhea, sinusitis, edema of head and face, cyanosis, decline in egg production, egg shell abnormalities, decrease in egg hatchability and occasional mortality (167, 174, 176). These symptoms can manifest themselves alone or in any combination. In breeder turkeys, low pathogenic influenza A infections almost always result in decreased egg production. Several previous reports document drops in egg production in turkeys due to H1N1 (97, 159), reassortant H1N2 (167), and H3N2 viruses (26, 125, 176) from different parts of the world.

Our recent studies using swine lineage triple reassortant (TR) H3N2 influenza viruses demonstrated that influenza viruses preferentially replicate in the oviduct of breeder turkeys in comparison to respiratory and digestive tracts and result in drastic declines in egg production without any concurrent infections (131). This finding was strengthened by the detection of α2,3 SA-gal receptors in different sections of oviduct of breeder turkeys and the exact match between the presence of the avian type influenza receptors and viral antigen in the oviduct (131). α2,3 SA-gal receptors have also been reported from turkey and chicken ovarian epithelial cells (76). The presence of avian type receptors in the ovaries and oviduct and the high titers of viral replication in the reproductive tract suggested that there is possibility of eggs being contaminated with virus before or after oviposition, hence chance for internal or external contamination of eggs or both. In addition, eggs could be externally contaminated by high viral loads in the
feces of infected birds. Avian influenza viruses have been detected from shell surfaces and the yolk and albumen of eggs from chickens and turkeys experimentally infected with highly pathogenic influenza viruses (120). Influenza viruses have also been recovered from day old turkey poults hatched from eggs incubated with virus on day 18 of incubation (145). However, similar studies on the egg – borne transmission of low pathogenic influenza viruses are limited. Considering the virulence of highly pathogenic strains, it is unlikely that infected eggs may hatch. However, with low pathogenic influenza viruses such as TR H3N2 viruses, which normally do not kill embryos in routine propagation of the virus in embryonating eggs, there is high possibility that the virus may be present in hatched poults. The possibility of egg borne transmission of influenza viruses could have serious implications in viral spread during movement of cracked eggs, hatchery contamination as well as contamination of food products and biologics where raw eggs are used. In recent decades, triple reassortant swine influenza viruses have occasionally been isolated from humans (117, 156). Also, since their first identification in April 2009, H1N1 viruses containing unique combination of gene segments from north American and Eurasian swine lineages (51) has emerged into a pandemic following efficient human to human transmission. The possibility that these swine origin TR H3N2 viruses can be transmitted through eggs of infected birds, even when the infections are asymptomatic, poses the need for additional biosecurity and containment measures while handling infected birds and their products.
1.6 REFERENCES


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CHAPTER 2

STUDIES ON PATHO-BIOLOGICAL CHARACTERIZATION OF LOW PATHOGENIC AVIAN INFLUENZA VIRUSES OF DIFFERENT ORIGINS IN DOMESTIC POULTRY HIGHLIGHT THE ROLE OF TURKEYS AS POTENTIAL INTERMEDIATE HOSTS

2.1 SUMMARY

We undertook one of the most comprehensive studies on the replication and intraspecies transmission characteristics of 20 low pathogenic influenza viruses of different origins, which included 8 isolates from wild aquatic birds, in chickens, ducks and turkeys. We studied viral shed titers from infected and contact control birds of the 3 bird species, their antibody response as well as histopathology and immunohistochemistry for antigen detection from different tissues of infected birds. Our results indicated that most of these isolates can replicate and transmit within poultry without clinical disease. However, distinct virus and species specific differences in transmission to contact control birds were noted emphasizing the importance of having
contact control cage mates in biological characterization experiments. Ducks supported the replication of wild aquatic bird origin viruses in their respiratory and digestive tracts equally well, though in contact control birds, higher tendency for viral detection from cloacal swabs was observed. The wild aquatic bird viruses did not effectively transmit between chickens. In contrast, the wild bird isolates as well as domestic bird viruses from live bird markets and commercial poultry operations tested, replicated and transmitted to comparatively higher titers in turkeys in comparison to chickens or ducks. We also found lower minimal infectious dose requirement for infection of turkeys compared to chickens and ducks suggesting that turkeys could be easily infected following a low dose exposure. Our data support the important role of turkeys as being more susceptible hosts for avian influenza viruses in general in comparison to domestic ducks and chickens. Our studies promote greater understanding of the infection, pathogenicity and transmission characteristics of low pathogenic influenza viruses of different origins in domestic poultry that could be utilized to better understand the genetic basis of these phenotypes. Such pathogenicity studies along with antigenic and genetic analysis are also good tools to identify future vaccine candidates.

2.2 INTRODUCTION

Wild aquatic and shore birds act as the global reservoirs of influenza viruses in nature in which all the 16 hemagglutinin and 9 neuraminidase subtypes have been found (35). Among wild birds, most frequent isolations have been made from order
Anseriformes, with the highest numbers from the Anatidae family. In wild bird reservoirs, infections are usually asymptomatic and follow the fecal-oral route of transmission (47). Most of the wild bird influenza viruses in their natural hosts have developed highly evolved relationships which depend on a multitude of interactions among viral components, intracellular machinery of the hosts, cell surfaces and their receptors and host innate and adaptive responses (22). Phylogenetic evidence suggests that all influenza A viruses in all other species of birds and mammals are derived from viruses circulating in aquatic bird species (46). For example, reports on H7N3 viruses from turkeys in northern Italy in 2002 showed that these viruses were closely related to H7N3 viruses that circulated in wild ducks in 2001 (2). These H7N3 viruses also seemed to have infected humans in close contact with the infected turkeys (27). However, the replication of most avian influenza viruses in mammalian species is inefficient and it was postulated that an intermediate host like swine may help overcome this species barrier (13, 28). However, recent human infections caused by H5N1 and H9N2 viruses indicated that these avian influenza viruses could directly transmitted to humans from their precursors in terrestrial poultry (1, 8, 31). These findings have led to the hypothesis that domestic poultry like swine can act as potential intermediate hosts for the transmission of influenza viruses from wild aquatic birds to humans. Many land based poultry like chickens, turkeys and quails, have been found to support the replication and transmission of a variety of influenza viruses of different subtypes (18, 24). Also, the detection of α2,3 (avian type) and α2,6 (mammalian type) sialic acid linked receptors in the tracheal epithelium of
domestic poultry (6) indicate that they can be infected with avian and mammalian viruses and serve as adaptation hosts for changing the binding preference from avian to mammalian receptors. Reports indicate that the transmission of influenza viruses from their wild bird reservoirs to domestic poultry might be a frequent event (3, 12, 46). In most cases, low pathogenic influenza infections in domestic poultry result in mild respiratory disease, reduction in egg production and occasionally increased mortality (41). However, in some cases, following transmission from wild bird reservoirs, influenza viruses undergo rapid evolution (19) though changes in nucleotide bases or amino acids can occur in all gene segments, it is most apparent in the hemagglutinin (HA) and neuraminidase (NA) genes, where the changes can result in antigenic drift. In some H5 and H7 viruses additional insertions of basic amino acids at the cleavage site of the HA can contribute to development of highly pathogenic strains. Also, the availability of avian and mammalian type receptors as well as susceptibility to viruses from different sources make domestic poultry an ideal milieu for viral evolutions and potential entry points for introduction of viruses into agricultural and commercial poultry systems as well as source of infections for other mammals and humans. These findings highlight the importance of understanding the biological properties of avian influenza viruses from different sources, which may represent the different level of poultry adaptation, in domestic bird species. Further, studies on the pathogenic and transmission properties of wild aquatic bird and domestic bird viruses in different host systems are a good initial step towards understanding viral and host ecologies. Similarly, such studies are important in
identifying the susceptibility of different avian species to influenza viruses and their potential role as sentinels. The seasonal pattern of distribution of avian influenza viruses in wild waterfowl is often detected by means of sentinel domestic birds that are highly susceptible to avian influenza viruses (49).

With these objectives in mind and in order to get a detailed understanding of the biological properties of low pathogenic avian influenza (LPAI) viruses, we studied the replication, pathogenicity and transmission characteristics of avian influenza viruses of different origins in 3 different domestic bird species. To further explain the replication and transmission characteristics observed, we also determined the minimum infectious dose titers required for 3 selected isolates.

2.3 MATERIALS AND METHODS

Virus: The viruses used in this study were obtained from the repository of Southeast Poultry Research Laboratory (SEPRL), Athens, Georgia. The viruses were passaged once or twice in 10-day-old embryonated chicken eggs (ECE) to make working stocks of the virus.

Comparative pathogenicity, replication and intraspecies transmission of viruses in chickens, ducks and turkeys: Four week old SPF chickens (Charles River Laboratories, Inc. Wilmington, MA), two week old commercial Pekin ducks (Ridgeway Hatcheries, Inc. LaRue, OH) and three week old Eggline turkeys (flocks maintained by Animal Science
Department at OARDC) were used in the present study. Eleven to twelve birds of each species were inoculated with 0.2 ml of $10^6 \text{EID}_{50}$ of the virus through choanal route. Three or four birds were introduced as contact controls at one day post infection (DPI). Tracheal and cloacal swabs were collected from all the birds at 2, 4 and 7 DPI (1, 3, 6 DPI for contact controls). Individual swabs were placed in 2.0 ml of sterile phosphate buffered saline (PBS) containing gentamycin (1mg per 100ml). RNA was extracted from the tracheal and cloacal swabs with RNeasy Minikit or Viral RNA extraction kit (Qiagen, Inc., Valencia, CA) in accordance with the manufacturer’s instructions. The virus was quantitated by real-time RT-PCR (RRT-PCR) directed to the matrix (M) gene as previously reported (16). For quantitation, swab samples were run together with known amounts of control viral RNA. We used RNA extracted from $10^1$ to $10^5 \text{EID}_{50}$/0.2 ml dilutions of the stock viruses as standard controls. Standard curves were generated with those control viral RNAs and the amount of RNA in the swab samples was converted into EID$_{50}$/ml by interpolation. High correlation ($r^2 > 0.99$) between cycle number and dilution factors were observed with standard controls.

At 3 DPI, 3 infected chickens, turkeys and ducks were euthanized and tissues (trachea, lungs, kidney, bursa, cloaca, spleen, portions of small and large intestine, cecal tonsils) were collected for histopathology. All the infected birds were bled for serum collection at 7 and 14 DPI (6 and 13 DPI for contact controls). The hemagglutination inhibition (HI) titers were determined using one percent turkey erythrocytes as described (42).
Determination of 50% infectious dose of 3 selected viruses in chickens, ducks and turkeys: Four to five birds of each species were inoculated with virus dilutions ranging from $10^1$ to $10^5$ EID$_{50}$/0.2 ml. Tracheal and cloacal swabs were collected from the infected birds at 2 and 4 DPI. The virus was quantitated by real-time RRT-PCR directed to the matrix (M) gene as described earlier. The birds were bled at 14 DPI for sera collection to determine HI titers.

Histopathology and immunohistochemistry (IHC). Collected tissues were fixed by submersion in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Sections were made at 5 µm and were stained with hematoxylin and eosin (HE). A duplicate 4-µm section was immunohistochemically stained by first microwaving the sections in Antigen Retrieval Citra Solution (Biogenex, San Ramon, CA) for antigen exposure. A monoclonal antibody (P13C11) specific for a type A influenza virus nucleoprotein, developed at Southeast Poultry Research Laboratory, was used as the primary antibody for a streptavidin-biotin-alkaline phosphatase complex-based IHC method as previously described (38).

2.4 RESULTS

We biologically characterized H5 subtype LPAI viruses of different origins to study their replication and intraspecies transmission characteristics in 3 domestic bird species that included chickens, ducks and turkeys. The 8 wild bird isolates that were used in the study included 4 isolates from mallard ducks, 1 from mute-swan and 3
from ruddy turnstones. Along with the wild bird isolates, we included low pathogenic domestic bird viruses from live bird markets (LBMs) as well as chicken and turkey isolates from commercial poultry operations. All these isolates tested, belong to the North American lineage avian influenza viruses and within the lineage some isolates belong to LBM sublineage and a parrot isolate belong to the Mexican sublineage.

In general, almost all the isolates tested, infected and transmitted to contact control birds of the three species of birds, although showing differing degrees of viral shedding and seroconversion. With the chicken/PA/13609/93 isolate, respiratory symptoms and swollen heads were observed in infected chickens and turkeys. The virus caused mortality in 1, 2 and 1 of the infected turkeys at 4, 5 and 6 DPI respectively and resulted in death of 2 infected chickens at 4 DPI. Infection with chicken/Texas/167280-4/02 virus resulted in respiratory symptoms in infected chickens and turkeys. The turkey/MN/10734-2/95 and turkey/CA/8651-C/02 isolates also caused respiratory symptoms in infected turkeys. No clinical signs or deaths were noticed in any of the species of birds with the other isolates.

**Replication, pathogenicity and intraspecies transmission of wild aquatic bird origin H5 low pathogenic avian influenza viruses in chickens, ducks and turkeys**

The wild bird viruses used in the study showed low viral shed titers from the respiratory and digestive tracts of the infected and contact control ducks. Except for a mute swan isolate and two mallard isolates, the average Ct values from tracheal and
cloacal swab samples from the infected and contact control ducks as determined by RRT-PCR were above 30.0 (EID₅₀ titers of < 10³ EID₅₀/0.2 ml). All the infected as well as contact control ducks seroconverted indicating the efficient intraspecies transmission of the viruses. The average HI titers from treatment groups of ducks infected with ruddy turnstone viruses (5.6 log₂) were 1.3 log₂ higher than the average HI titers from the groups inoculated with mallard viruses (4.3 log₂). Though we did not observe a preferential tropism of the wild bird viruses for the digestive or respiratory tract of infected ducks, we observed a higher tendency for virus detection from the digestive tract of contact control ducks from the groups infected with mallard viruses. Of the 8 wild bird isolates that we tested, the mute swan isolate replicated and transmitted to the highest titers in ducks (6.4 log₂) based on the viral shed titers as well as average HI titers from the infected and contact control ducks. Our results indicated that wild aquatic bird origin influenza viruses can efficiently infect and transmit among domestic ducks although the viral shedding was relatively low and differed with the isolates tested.

In comparison to ducks, in chickens, the intraspecies transmission of the wild bird viruses was poor or none. Positive viral shed titers (< 10³ EID₅₀/0.2 ml) were detected from the infected chickens and they seroconverted. However, except for the contact control chickens of the muteswan/MI/06 and mallard/WI/75 groups, contact control chickens from other infected groups showed no seroconversion or low HI titers (≈ 2.0 log₂). Similar to the observation for infected ducks, no preferential tropism of these viruses was detected for the respiratory or the digestive tracts of chickens.
In turkeys, on the other hand, efficient replication and intraspecies transmission of the wild aquatic bird viruses were observed. An only exception was mallard/MN/00 isolate that did not transmit to contact control turkeys. Although these viruses did not show a distinct tropism for the digestive or respiratory tracts of turkeys, it was interesting to note that in the ruddy turnstone virus infected groups, comparatively higher viral titers and more number of positive birds were detected from the digestive tracts in comparison to the respiratory tracts, though such differences could not be observed with the mallard or the mute swan virus infected group of turkeys. As seen for ducks and chickens, the mute swan virus replicated and transmitted efficiently in turkeys. Based on the viral shed titers, the number of birds showing positive viral shed titers from tracheal and cloacal swabs and the average HI titers from the infected and contact control birds of the 3 species of birds, turkeys were found to support the replication and intraspecies transmission of wild bird viruses better in comparison to chickens and turkeys.

Replication, pathogenicity and intraspecies transmission of H5 low pathogenic avian influenza viruses in chickens, ducks and turkeys from other sources

Our study incorporated the biological characterization of 12 LPAI viruses from LBMs as well as commercial poultry operations and other sources. As observed with the wild bird isolates, we observed differing levels of viral replication and seroconversion with these isolates in the 3 bird species. A striking finding was the higher viral shed titers, higher no. of positive birds and higher average HI titers in infected and contact control turkeys in comparison to chickens and ducks. In contact control ducks, lower HI
titers or no seroconversion was observed indicating the poor transmissibility of these viruses for domestic ducks. Two exceptions noted were with the LBM origin viruses, pheasant/NJ/98 and avian/NY/00, that replicated and transmitted efficiently in ducks. Two of the 3 duck viruses from LBM also did not transmit to contact control ducks, however, all the 3 strains efficiently transmitted to turkeys and two of them to chickens indicating their higher adaptation for terrestrial poultry. Two chicken origin viruses, chicken/PA/93 and chicken/TX/02, which have been found to be poultry adapted viruses in previous studies (15), replicated to higher titers (>10^3 EID_{50}/0.2 ml) and induced higher HI antibody in infected and contact control chickens (7.0 and 6.6 log_{2} respectively) and turkeys (6.7 and 9.1 log_{2} respectively).

The pheasant isolates, pheasant/MD/93 and pheasant/NJ/98, showed poor intraspecies transmission in ducks similar to the other LBM isolates used in the study. Although their replication and seroconversion in infected chickens was higher than in turkeys, viral titer and serology indicated that they transmit better to contact control turkeys than to chickens. Unlike other poultry adapted LBM isolates, these isolates showed tropism for the digestive tracts of chickens. The two turkey isolates, turkey/MN/95 and turkey/CA/02, showed replication and transmissibility in turkeys and ducks, however, the turkey/CA/02 isolate did not transmit among chickens. The emu isolate replicated to similar titers in infected chickens, ducks and turkeys, however, did not transmit within ducks and chickens, but transmitted among turkeys.

The parrot isolate, parrot/CA/04, showed moderate (≥ 10^3 EID_{50}/0.2 ml) viral
shed titers in infected and contact control chickens and turkeys and seroconversion as indicated by HI titers were high in infected and contact control chickens (6.9 and 6.5 log$_2$ respectively) and turkeys (8.0 and 7.3 log$_2$ respectively). In ducks, although the viral shed titers from infected and contact control birds were comparable to chickens and turkeys, the seroconversion as indicated by average HI titers in contact control ducks were low. The high viral shed titers and efficient transmission in the 3 bird species indicate the parrot isolate to be highly poultry adapted (26). The parrot virus was isolated from a red-lored Amazon parrot in California in 2004 and is the first reported H5N2 virus of Mexican lineage in the U.S. and the first one in a psittacine bird (10).

Taken together, our data indicate that similar to the wild bird viruses, turkeys support initial infection as well as transmission of domestic bird viruses better than chickens or ducks.

**Histopathology and immunohistochemistry for viral antigen detection in different tissues of chickens, ducks and turkeys**

Lesions in the infected birds of the 3 species were mostly confined to the respiratory tracts. The most common lesions consisted of mild tracheitis and bronchitis consisting of mild hyperplasia of the epithelium and mild lymphoplasmacytic infiltration. Non-specific mild congestion of lungs, interstitial pneumonia and pulmonary edema was observed. Mild lymphoid hyperplasia and atrophy of lymphoid follicles of bursa, spleen and lymph nodes were observed in the 3 bird species. Infrequently, desquamation of the
intestinal epithelium, and lymphocytic infiltration of the intestinal submucosa and kidneys were observed. The antigen staining using immunohistochemistry was in most cases mild and infrequent. When observed, the staining was mostly found in the tracheal and bronchial epithelial cells and infiltrating macrophages. Infrequently, minimal antigen staining was also found in the lung, intestine, spleen and bursa. In general, we did not observe marked differences in the pathology of different organs from the wild bird versus domestic bird virus infected birds.

An unexpected finding was the detection of severe tracheitis in ducks infected with wild and domestic bird viruses. Though we did not see a strict correlation between the tracheal lesions and virus detection from the trachea of infected ducks, in infections with avian/NY/00, turkey/CA/04 and chicken/PA/93 viruses, the tracheal lesions matched with the viral shed titers from infected ducks. Of the different domestic bird origin viruses tested in ducks, avian/NY/00 was the only domestic bird virus that showed high average HI titers from the infected and contact control ducks than in chickens or turkeys. With the avian/NY/00 isolate, mild antigen staining was also observed in the intestinal tract. In general, no marked lesions were observed in the intestines of the ducks infected with wild or domestic bird influenza viruses. In some cases, increased number of lymphoid follicles in cecal tonsils and infrequently, desquamation of intestinal epithelium and increased lymphocytes in intestinal submucosa were observed. Immunohistochemistry revealed minimal or no antigen staining in the trachea, intestines and other organs from the infected ducks in most cases.
In turkeys, two of the wild bird viruses, mallard/MN/479/00 and ruddy turnstone/DE/01 caused moderate tracheitis. Of the domestic bird isolates, chicken/PA/93 and chicken/Texas/02 viruses caused severe trachietis and lymphocytic infiltration in infected turkeys. In the infected birds from the two groups, severe bronchitis, pneumonia, congestion of lungs and strong staining for influenza viral antigen was demonstrated in the tracheal and bronchial epithelium and macrophages. Infections with these two viruses resulted in clinical signs including respiratory distress, swollen head and mortality in infected turkeys. The parrot/CA/04 virus caused moderate tracheitis, and congestion of the lungs with moderate staining for influenza viral antigen. Similar to ducks, lesions in other organs and antigen staining were minimal with other wild and domestic bird viruses.

Chickens seemed to be more resistant to disease caused by low pathogenicity avian influenza viruses as they showed almost no lesions in the different organs examined. Of the wild bird viruses tested, only RT/DE/01 isolate caused moderate tracheitis, with the other isolates, the lesions observed in the trachea were minimal or none. Of the domestic bird isolates, only emu/NY/94 and avian/NY/00 viruses caused mild to moderate tracheitis and mild antigen staining in the tracheal epithelium and macrophages. Although chicken/TX/02, chicken/PA/93 and parrot/CA/04 viruses showed viral shed titers in chickens comparable to turkeys and produced respiratory signs in infected chickens, no antigen staining was detectable in the tracheal sections from the infected birds.
Minimum infectious dose titers of selected isolates for chickens, ducks and turkeys

The 50% minimum infectious dose titers (MID50) of 3 selected isolates confirmed our hypothesis that turkeys support initial infections with wild aquatic and domestic bird viruses better in comparison to chickens or ducks. Except for the mallard/MN/00 virus, the only virus that did not transmit to contact control turkeys, the infectious dose titer for the other two viruses was lower in turkeys. Even for the mallard/MN/00 isolate, the infectious dose was low for turkeys than chickens. It was interesting that mute swan virus, which is an aquatic bird isolate, that showed efficient replication and transmission in ducks also had lower minimum infectious dose for turkeys than for ducks. The 3 viruses tested showed comparatively higher infectious dose titers in chickens. Only the parrot isolate showed comparatively lower infectious dose for chickens than ducks. Requirement of lower 50% infectious dose, better replication and efficient intraspecies transmission in turkeys indicate the higher susceptibility of turkeys for low pathogenic influenza viruses in comparison to chickens and ducks.

2.5 DISCUSSION

Though several reports are available on the biological properties of LPAI viruses in domestic poultry (36),(33),(38),(25, 32), these studies were in general restricted to comparison of a few strains in single bird species. In order to gain a detailed understanding of the biological properties of LPAI viruses in domestic birds, we compared the replication and intraspecies transmission characteristics of 20 LPAI
viruses of wild and domestic bird origins in chickens, ducks and turkeys. To our knowledge this is one of the most comprehensive characterization studies that included viral shed titers from infected and contact control birds of 3 bird species at 2 different time points, their serology as well as histopathology and immunohistochemistry for antigen detection from different tissues of the infected birds. In addition, the minimum infectious dose titers of 3 selected viruses were determined in the 3 bird species. Though, our study focused on the replication and intraspecies transmission characteristics of H5 subtype wild bird origin viruses, as controls and comparisons, we also included low pathogenic isolates from commercial poultry operations and live bird markets that show differing degrees of adaptation to domestic poultry. The H5 subtype viruses gain significance considering their importance for human and animal health worldwide (17, 48). Also, domestic birds were found to be the direct precursor hosts for recent interspecies transmissions to humans (7, 23, 37). Furthermore, the susceptibility of different species of domestic birds to influenza viruses from different sources indicate their suitability as bridging species for influenza viral entry into agricultural and commercial poultry systems (43).

As observed in previous studies (4) the wild bird origin isolates that we used in our study did not produce clinical infection in the infected or contact control birds and the average RRT-PCR Ct values from their tracheal and cloacal swabs were in the range of 30.0-37.0 (EID\textsubscript{50} titers of < 10\textsuperscript{3.0} EID\textsubscript{50}/0.2 ml). Positive viral shed titers from the tracheal and cloacal swabs and the lack of pathological lesions and antigen detection
from other organs indicated localized infections of respiratory and digestive tracts of the infected birds. The trypsin like activity of epithelia and luminal contents of respiratory and digestive organs supposedly make them primary sites for replication of LPAI viruses (38).

Dabbling ducks of the genus Anas that include mallards are the most extensively studied species as influenza viral hosts and found to be more frequently infected with influenza viruses than other species (11, 35). Domestic ducks are genetically close to their wild bird counterparts and our results indicate that they could support influenza viruses from wild aquatic and shorebirds without any prior adaptation. Though the wild bird isolates did not preferentially replicate in the digestive tracts of infected ducks, among the contact control ducks, a higher tendency was noted for viral detection from cloacal swabs and more number of birds were detected positive by RRT-PCR from the cloacal swabs than the tracheal swabs. These discrepancies in results between the infected and contact control groups underscores the importance of having contact controls in biological characterization studies. In experimentally infected ducks, the high viral titers used ($10^6$EID$_{50}$) and the choanal route of inoculation might have favored a localized infection of the respiratory and digestive system as choana are known to connect the respiratory and digestive tracts. Our findings that the wild bird viruses can replicate equally well in the respiratory and digestive tracts of ducks question the use of cloacal swabs or fecal samples alone in influenza viral surveillance studies in wild aquatic birds. Also, a previous study indicated time lag in cloacal shedding
following inoculation of ducks (14). Though these results could vary with route of inoculation and viral strains, we believe that in surveillance studies for avian influenza viruses in ducks, the tracheal swab samples should also be included whenever possible. A recent surveillance study for AI viruses in mallard ducks in Sweden lends credence to this hypotheses as 3% of infections in their study were found positive only by oropharyngeal swabs and these infections went undetected when only cloacal swabs were used (5).

Among the wild bird isolates that we studied in ducks, our data indicated higher average HI titers in groups of ducks infected with ruddy turnstone isolates in comparison to mallard isolates (p<0.001). We do not know the intricate details of the differing ecologies of influenza viruses in two different host systems, the ruddy turnstones and mallards. Also, it is difficult at this point to conclude that the ruddy turnstone isolates possessed point mutations in the antigenic site of HA that correlated with their increased immunogenicity. Phylogenetic analyses of H5 gene segments from recent wild bird isolates grouped 2 of the 3 ruddy turnstone isolates in one group and another isolate grouped together with mallard and mute swan viruses (34). Though these genetic similarities or dissimilarities do not correlate with antigenic relatedness, similar studies combining antigenic, genetic and biological characterization could throw light on selection of candidate vaccine strains for poultry. Also, among the 8 wild bird viruses used in the study, the mute swan isolate showed comparatively higher viral titers and HI titers in infected ducks. This observation raises the possibility that replication of the virus in mute swan confers some advantage to low pathogenic influenza viruses that make
them better suited to domestic ducks. Mute swans are reported to be the most frequently affected wild bird species with HPAI virus in Europe (9, 20, 44). The differences in the biological properties of viruses from 3 different wild bird hosts, the mallards, ruddy turnstones and mute swan, in domestic ducks, a relatively close genetic counterpart, was an interesting observation and indicate the need for understanding the host ecologies for solving the complexities of influenza viral infections.

Our studies revealed that wild bird viruses did not effectively transmit within chickens. The HI titers in contact control chickens were low or none in comparison to the treatment groups (p<0.001), however, we could detect low and inconsistent viral replication in the respiratory and digestive tracts of the contact control birds. The low viral titers detected by RRT-PCR led us to speculate whether actual infection occurred in the contact control chickens or the titers observed were non-specific. The low HI titers in the contact control chickens might also indicate that infection did not result in efficient replication to reach the threshold viral titer required to initiate a humoral immune response. In the infected birds on the other hand, the high viral titers in the inoculum (10^6 EID_{50}) might have accounted for infection and seroconversion. In turkeys, the viral shed titers and HI titers were comparable among infected and contact control cage mates indicating efficient intraspecies transmission of the wild bird viruses in them. The poor intraspecies transmission in chickens in comparison to turkeys indicated that chickens might not be good initial hosts in terms of viral amplification and transmission. It might take multiple passages for successful viral adaptation and transmission of wild bird
influenza viruses in chickens. Along with data on biological characterization, available data on molecular determinants of interspecies transmission of influenza viruses also support the potential role of turkeys as bridging hosts for wild aquatic bird viruses to domestic poultry. While data from H5 subtype viruses are lacking, no species specific molecular changes in duck H7 HA were found to be required for influenza infections of turkeys, however, host species related mutations in duck H9 HA were found to be required for infection of chickens (24). The results from biological and genetic characterization of the viruses imply the importance of turkeys as good initial hosts for wild bird viruses that have no previous adaptation for terrestrial domestic poultry. Our findings also reiterate the importance of having control cage mates in biological characterization experiments. Use of inoculated birds alone would have revealed similar titers of viral shed in the 3 bird species and such striking differences in the biological properties of viruses would have been missed.

Along with the wild bird viruses, we studied the biological properties of low pathogenic influenza viruses showing differing degrees of adaptation to domestic poultry. We included isolates from different species of birds from live bird markets as well as chicken and turkey isolates from commercial poultry operations. Replication characteristics of few of the isolates including chicken/PA/93, chicken/Texas/02, duck/ME/02 and turkey/CA/02 had been studied in chickens previously (15). As reported, these isolates showed differing degrees of adaptation for chickens in our studies. With ducks, the transmission of most of these isolates was poor suggesting a higher
adaptation for terrestrial domestic poultry. An interesting observation was the comparatively higher viral titers and seroconversion in the infected and contact control turkeys with the domestic bird isolates irrespective of their species of isolation and degree of adaptation for domestic poultry. Though detailed experimental studies on the replication and available evidence indicate susceptibility of turkeys to LPAI viruses isolated from commercial poultry operations and live bird markets (39, 43, 45). Among commercial poultry operations, more LPAI outbreaks have been reported in turkeys in comparison to chickens (40). In addition, viruses isolated from different bird species from live bird markets (LBMs) that included ducks, chicken, emu and pheasants, replicated and transmitted to higher titers in turkeys indicating again that turkeys are better hosts for even chicken and duck adapted LPAI viruses (unpublished data). Influenza isolates from LBMs are believed to be highly poultry adapted as they provide favorable environment for adaptation of influenza viruses for a variety of hosts including chickens, turkeys, pheasants, quails and ducks and have been implicated as the source of influenza outbreaks in commercial poultry operations as well as humans (30). LBMs in New York and New Jersey have been implicated as significant sources of influenza viruses for commercial chicken and turkey operations in the U.S. (21, 29). The high susceptibility of turkeys to domestic bird isolates again underscores their importance as potential bridging hosts for influenza viruses from different origins and domestic poultry.

The high susceptibility of turkeys to influenza viruses might be accounted for by the low minimum infectious dose titers required for infection. Our studies using 2
wild bird viruses and a highly poultry adapted strain of LPAI showed a lower 50% infectious dose titers for turkeys in comparison to chickens. The low ID50 dose titers implied that turkeys can be easily infected following a low dose exposure and the low dose is sufficient for efficient viral replication in these hosts. Although the mallard/MN/00 virus replicated to low titers in turkeys and did not transmit efficiently to contact control turkeys, ID50 studies revealed the virus to have a comparatively lower viral dose requirement for infection (10^{2.5} EID_{50}). Similarly, the muteswan/MI/06 virus replicated and transmitted well within chickens, although it was found to require a higher dose (10^{6.3} EID_{50}) for infection. These findings further suggested that minimum dose of influenza virus required for infection might differ from the dose required for transmission. However, it should be noted that infectious dose titers vary with viral strains as well as the genetic constitution of the birds used in the studies. Conditions within the incubator like the temperature, humidity, airflow and the bird density are also found to have significant effects on the viral load required for infection and transmission (43). However, an important implication from our studies was the low infectious dose requirement for turkeys in comparison to chickens or ducks under similar experimental conditions that we employed and thereby supporting our findings that turkeys are more susceptible to influenza viruses from different sources.

Histopathology and immunohistochemistry revealed mild tracheitis to be one of the consistent findings in infected birds of the 3 species. This was expected as LPAI viruses mainly cause localized infections of the respiratory and digestive tracts.
Absence of pathological lesions and antigen staining in the intestines could be due to the very low titers of viral replication in the digestive tract. Our study did not reveal any pathology or positive antigen staining in kidney sections following infection. Several wild waterfowl origin influenza viruses have been found to be nephrotropic following intravenous exposure (33). It is possible that the choanal route of infection that we employed in our study resulted in this finding as it could have favored localized infections of the respiratory and digestive tracts. An interesting finding was the severe tracheitis in ducks infected with several wild and domestic bird isolates. However, antigen staining in the tracheal epithelial cells was minimal or infrequent, which could be due to the sloughing away of the infected dead cells. It is also possible that tracheitis observed was a result of physical injury while swabbing or it could be associated with respiratory illness unrelated to influenza infections. The ducks used in our studies were not specific pathogenic free and their eating habits resulted in accumulation of fecal matter and water below their cages which could have resulted in bacterial infections of the upper respiratory tract and consequent tracheitis. In turkeys, the comparatively higher viral replication titers seen with most isolates were not reflected in the histopathology results except for the chicken/PA/93 and chicken/TX/02 isolates. In chickens, no lesions were observed with most of the isolates and they appeared tolerant even to some of the highly poultry adapted strains that replicated relatively well in them. To summarize, results from histopathology and immunohistochemistry from the infected poultry tissues indicated that the higher replication titers need not necessarily match with pathology in
terms of host susceptibility or adaptation.

In conclusion, our extensive data on pathobiological characterization of low pathogenic influenza viruses of different origins in domestic birds elucidate the important role of turkeys as being more susceptible hosts for wild and domestic bird LPAI viruses in comparison to domestic ducks and chickens. Our results also signify the role of turkeys as intermediate or bridging hosts in the transmission of influenza viruses from wild birds to land based domestic poultry or among different land based bird species. This potential role of turkeys assumes greater risk in areas where turkeys, and or waterfowl and other birds share common pasturelands. The higher susceptibility of turkeys to influenza viruses also indicate that they could be better sentinel hosts than chickens in influenza surveillance studies and/or better model hosts in studies on adaptation of wild bird origin influenza viruses for domestic poultry. Our data also promote greater understanding of the infection, pathogenicity and transmission characteristics of low pathogenic influenza viruses of different origins in domestic poultry. The results from our study can be used as foundation to undertake further studies on the genetic basis of infection, pathogenicity and transmission of avian influenza viruses. Our studies also show that wild bird isolates of Anseriformes and Charadriiformes can replicate and transmit within domestic bird species without need for any prior adaptation. Surveillance and characterization of wild bird isolates could provide early warning signals for the introduction and emergence of potential high pathogenic viruses. Also, such characterization studies may accurately assess the threat that these viruses may pose for poultry or emergence of a potential
pandemic. Such pathogenicity studies along with antigenic and genetic analysis are also good tools to identify future vaccine candidates.
2.6 REFERENCES


Table 2.1: Replication and transmission of H5 subtype low pathogenic avian influenza viruses of different origins in ducks
Table 2.1

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<sup>a</sup>HI titer

<sup>b</sup>Virus detection

<sup>c</sup>Infected

<sup>d</sup>Virus detection

<sup>e</sup>2 DPI

<sup>f</sup>4 DPI

<sup>g</sup>14 DPI

<sup>h</sup>Contact control

<sup>i</sup>Virus detection

<sup>j</sup>14 DPI

<sup>k</sup>Tracheal

<sup>l</sup>Cloacal

<sup>m</sup>Tracheal

<sup>n</sup>Cloacal

<sup>o</sup>Tracheal

<sup>p</sup>Cloacal

79
a Ducks infected with $10^6$ 50% egg infectious dose (EID$_{50}$)/0.2 ml of virus through the choanal slit

b Contact control ducks introduced into the cage 1 day post infection

c Average viral titers expressed as Ct values determined by RRT-PCR/0.2 ml of swab fluid

d Log$_2$ hemagglutination inhibition (HI) titer of the sera ± standard deviation

e Number of days post infection

f Viral titers determined from the tracheal swab fluid

g Viral titers determined from cloacal swab fluid

h No. of positive samples/total number of samples tested
Table 2.2: Replication and transmission of H5 subtype low pathogenic avian influenza viruses of different origins in chickens
### Table 2.2

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a Chickens infected with $10^6$ 50% egg infectious dose (EID$_{50}$)/0.2 ml of virus through the choanal slit

b Contact control chickens introduced into the cage 1 day post infection

c Average viral titers expressed as Ct values determined by real time RT-PCR/ 0.2 ml of swab fluid

d $\log_2$ hemagglutination inhibition (HI) titer of the sera ± standard deviation

e Number of days post infection

f Viral titers determined from the tracheal swab fluid

\^g Viral titers determined from cloacal swab fluid

\^h No. of positive samples/ total number of samples tested
Table 2.3: Replication and transmission of H5 subtype low pathogenic avian influenza viruses of different origins in turkeys
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a Turkeys infected with $10^6$ 50% egg infectious dose (EID$_{50}$)/0.2 ml of virus through the choanal slit

b Contact control turkeys introduced into the cage 1 day post infection

c Average viral titers expressed as Ct values determined by real time RT-PCR/ 0.2 ml of swab fluid

d Log$_2$ hemagglutination inhibition (HI) titer of the sera ± standard deviation

e Number of days post infection

f Viral titers determined from the tracheal swab fluid

g Viral titers determined from cloacal swab fluid

h No. of positive samples/ total number of samples tested
CHAPTER 3

SPECIES AND AGE RELATED DIFFERENCES IN THE TYPE AND DISTRIBUTION OF INFLUENZA VIRUS RECEPTORS IN DIFFERENT TISSUES OF CHICKENS, DUCKS AND TURKEYS

3.1 SUMMARY

It is commonly understood that avian influenza viruses preferentially bind to α2,3sialic acid(SA)-galactose (gal) linked receptors and correspondingly avian tissues predominantly express these receptors. To get a better understanding of the receptor profile in different avian species, we undertook detailed studies on the distribution of α2,3SA-gal and α2,6SA-gal receptors on different tissues of chickens, ducks and turkeys in day old, 2-4 week old and adult birds. Our studies showed that birds of the 3 species expressed both α2,3SA-gal and α2,6SA-gal receptors on tissues to varying degrees. On the tracheal epithelium, the 3 bird species expressed almost 80-100% high intensity positive staining for the α2,3SA-gal receptors in 3 different age groups. However, the distribution of the α2,6SA-gal receptors varied with species and age. Ducks expressed high positive staining (more than 80%) for human receptors, while turkeys and chickens
showed approximately 30% and 60% positive staining, respectively, with slight differences in distribution with age. The bronchial epithelium showed similar trends as in trachea with some exceptions being noticed in layer chickens and turkeys, where lower percent of positive staining were observed with Maackia amurensis agglutinin (MAA) (50-60%) and Sambucus nigra agglutinin (SNA) (≤ 10%). The bronchioles and the alveoli of the lungs did not show positive staining for receptors in the 3 species. The small intestinal sections from turkeys and ducks showed almost negligible staining for the avian and human receptors in 3 different age groups whereas sections of large intestine consistently showed 40-50% positive staining for the avian receptors with low (less than 5%) or no staining for the human receptors. However, small and large intestinal sections from chickens showed positive staining for avian (60-80%) and human (20-50%) receptors. Kidney sections from the 3 species of birds expressed approximately 50-60% avian receptors and 20-30% human receptors in the different age groups. This is one of the most extensive studies undertaken to understand the receptor type and distribution from different tissues of domestic poultry of different ages using plant lectins. However, we understand that even though avian and human influenza viruses have been shown to differentiate between the two receptor types, recognition of these receptors and viral binding is not absolute. The distribution pattern of these receptors in different tissues might also not be as clear cut as has been observed (33) and demonstrates the involvement of different viral and host factors in influenza viral infections, replication and transmission.
3.2 INTRODUCTION

Wild aquatic birds are considered to be the natural reservoirs of influenza viruses. They have been implicated as the source of influenza viruses for all other species of birds and mammals (1, 46). In wild aquatic birds, influenza viruses are believed to have tropism for the digestive tract and follow fecal oral mode of transmission. Influenza viruses in wild aquatic birds are believed to possess strict binding preference for sialic acids (SA) linked to galactose (Gal) through α2,3 linkages (12). Immunohistochemical studies using plant lectins revealed the presence of α2,3SA-gal residues and no detectable expression of α2,6SA-gal receptors in duck intestinal cells (20). Similarly, human viruses were found not to bind to plasma membranes isolated from duck intestinal cells thereby confirming the absence of α2,6SA-gal linked sialyloligosaccharides on duck intestinal epithelial cells (13). Though not natural hosts, many land based poultry like chickens, turkeys and quails have been found to support the replication and transmission of a variety of influenza subtypes (26). Recent studies as well as the human infections caused by H5N1 and H9N2 viruses suggested that domestic poultry can be immediate precursors as well as potential intermediate hosts, like pigs, for influenza viruses. α2,3 and α2,6SA-gal linked receptors have been detected in the tracheal epithelium of chickens and quails (45) suggesting that they can be infected with avian and mammalian viruses and serve as adaptation hosts for changing the receptor preference of avian viruses from α2,3SA-gal to α2,6SA-gal. Though, turkeys are frequently infected with avian and swine influenza viruses reports on the receptor profile of tissues from turkeys are lacking. Influenza
viruses circulating in turkey and chicken populations are found to evolve much faster than the ones in aquatic birds and differ from isolates from duck by the presence of additional carbohydrates on hemagglutinin and deletions in the stalk of neuraminidases (7, 30). These findings can have implications for the receptor binding and destroying activities of the virus. Structural and functional distinctions between hemagglutinin and neuraminidase of viruses from aquatic birds and land based birds also suggest that the spectrum of sialic acid containing receptors on wild ducks and domestic poultry is not identical.

Studies on the type and distribution of receptors in different tissues of domestic poultry as well as viral binding to the receptors observed are still incomplete. In this study, we attempted to fill this gap by determining the presence and type of \( \alpha_{2,3} \)SA-gal and \( \alpha_{2,6} \)SA-gal receptors on different tissues of domestic poultry that included chickens, ducks and turkeys. We also looked at the age related differences in the distribution of receptors in these 3 bird species.

3.3 MATERIALS AND METHODS

3.3.1. Immunohistochemistry for the detection of receptors using plant lectins

We used different tissues that included trachea, lungs, spleen, bursa, portions of small and large intestines, and cecal tonsils from chickens, ducks and turkeys of day old
age, 2-4 weeks old and adult (layer) birds for studying the receptor profile by employing two specific lectins, Maackia amurensis agglutinin (MAA) for \( \alpha_2,3\text{SA-gal} \) receptors and Sambucus nigra agglutinin (SNA) for \( \alpha_2,6\text{SA-gal} \) receptors (DIG Glycan Differentiation Kit, Roche Applied Science, Mannheim, Germany). Paraffin embedded tissue sections were deparaffinized and immersed in 3% hydrogen peroxide to eliminate the endogenous peroxidase activity. The sections were treated with blocking agent to avoid nonspecific staining and then incubated with digoxigenin (DIG)-labelled MAA or SNA (1\( \mu \)g/\( \mu \)l) at 4\(^\circ\)C overnight. Negative control slides were incubated with PBS instead of lectin. After two washes in phosphate-buffered saline (PBS), the sections were incubated with peroxidase-labelled anti-DIG FAb fragments (Roche Applied Science) for 1.5 h at 37\(^\circ\)C. Lectin binding was visualized using DAB (3, 3’–diaminobenzidine-tetrahydrochloride) substrate (Roche diagnostics GmbH, Mannheim, Germany) and slides were counterstained with hematoxylin.

3.3.2. Determination of viral binding to receptors using solid phase binding assay

3.3.2.1. Preparation of cell membranes

Plasma membranes were prepared as described previously (14). Briefly, turkey tracheal epithelial cells were grown to confluence, washed with serum free media, scrapped from the glass support and washed three times with ice-cold TSE buffer (10mM Tris, 0.15M NaCl, 0.5mM EDTA, pH 7.2). For chicken tracheal epithelial cells, trachea from 6-week old birds were scrapped gently after thoroughly washing with ice cold TSE
buffer. The cells were suspended in ice cold lysis buffer (0.01 M Tris, pH 7.2, 1mM phenylmethylsulfonyl fluoride (PMSF) and incubated for 10 minutes at 4°C. They were disrupted in a standard dounce homogenizer. Nuclei and cellular debris were removed by centrifugation at 1000xg for 1 minute and cell membranes were pelleted at 40,000xg for 1.5 hours. Membrane pellets were suspended in TSE containing 1mM PMSF, sonicated for 2 minutes with cooling in ice bath and stored in aliquots at -20°C.

3.3.2.2. Rescue of viruses using reverse genetics

Hemagglutinin and neuraminidase genes of A/parrot/California/00406032/04 and A/muteswan/Michigan/451072-2/06 were cloned into pHH21 vector between the promoter and terminator sequences of RNA polymerase I. Plasmids expressing the remaining six influenza virus genes from influenza A/turkey/Ohio/313053/04 virus were kindly provided by Hadi M. Yassine, FAHRP, The Ohio state university. Recombinant viruses were generated by DNA transfection as previously described with minor modifications. Briefly, 293T cells were transfected with 1 μg of each of the eight viral RNA genes expressing plasmids and four expression plasmids for the influenza virus proteins NP PA, PB1, and PB2 with the use of Lipofectamine 2000 reagent (Invitrogen). Forty-eight hours after the transfection, the supernatants were collected and subsequently inoculated into 10-day-old ECE for virus propagation. At 72 h post-inoculation, allantoic fluids containing recombinant viruses were harvested and stored at −70 °C for additional experiments.
3.3.2.3. Purification of viruses

Freshly collected allantoic fluid was clarified by low speed centrifugation (1000xg, 20 minutes). The virus was pelleted by high speed centrifugation followed by centrifugation through 35% sucrose in TNC buffer (Tris hydrochloride 10mM, NaCl 140mM, CaCl₂ 10mM) at 100,000xg for 2 hours. The pellet was suspended in sterile PBS and stored as aliquots at -20°C.

3.3.2.4. Solid-phase binding assay

Viral binding to cell membranes on solid phase were assayed using method described by Matrosovich et al., 1996 (28) with slight modifications. Briefly, 100ul of membrane preparation suspended in PBS to a final concentration of 10ug/ml total protein were incubated in the wells of a 96-well plate overnight at 4°C. The plates were washed with PBS and blocked by incubating with 200ul of 0.2% BSA in PBS at 37°C for 1.5 hours. The BSA-PBS was discarded and 50ul of two fold serially diluted virus in BSA-PBS was incubated in the wells for 2 hours at 4°C. The plates were rinsed four times with ice cold washing solution (0.2XPBS-0.02% Tween 80) and incubated with 50ul/well of fetuin-HRP conjugate in PBS-0.02% Tween 80 for 1 hour at 4°C. After 6 washings with washing solution, the amount of bound virus was quantitated using Sure blue TMB substrate (Roche). The absorbance was measured at 450nm.
3.4 RESULTS

3.4.1. Differences in receptor distribution in the respiratory tracts of chickens, ducks and turkeys with age

The receptor distribution in different tissues were determined as the average percent of positive staining observed by visual examination of 3 different fields of the same tissue from 3 different birds as observed under 20X objective of light microscope. The staining intensity was relatively compared and assigned as mild (+), moderate (++) , strong (+++) or very strong (++++) . The percent positive staining matched approximately the average no. of cells stained in each field, while intensity of staining approximately matched the number of sialic acid residues on each cell being stained.

In the 3 bird species, the tracheal epithelium showed the predominance of $\alpha_{2,3}$SA-gal receptors (Fig. 3.1). Very strong positive staining (80-100%, ++++) for $\alpha_{2,3}$SA-gal receptors was visible throughout the tracheal epithelial lining in the 3 bird species (+++). Staining for $\alpha_{2,6}$SA-gal receptors was also noticed along the tracheal epithelium, although to a lesser intensity. In 1-day-old ducks, 80-100% and in 1-day-old chickens, 60% of the lining cells were positive for the $\alpha_{2,6}$SA-gal receptors. However, in 1-day-old turkeys, the distribution and intensity of staining for avian type receptors was comparatively lower (70-80%) and approximately, 20% of the tracheal epithelial cells showed moderate positive staining for $\alpha_{2,6}$SA-gal receptors (Table 3.1).

In day-old ducks and chickens, similar results as for trachea were observed for
bronchial epithelial cells, with almost 100% of the epithelial cells staining positive for α2,3SA-gal receptors and lesser intensity and fewer percent (60-80%) of cells showing positive staining for α2,6SA-gal receptors. In chickens, the intensity of staining for the avian and mammalian receptors were comparatively lower than in ducks. In turkey poults, intensity (++++) and percent of α2,3SA-gal staining along the bronchial epithelium was 80% as for the tracheal sections, however, there was an increase in the percent of mammalian receptors on the bronchial epithelium, though with a lower staining intensity.

Similar results as for 1-day-old birds were observed along the respiratory epithelium of 2-4-week-old birds of the 3 bird species. The tracheal epithelial cells showed predominance of α2,3SA-gal receptors along with positive staining for mammalian type receptors in the 3 bird species. Along duck trachea and bronchial epithelium, similar results were seen as for 1-day old birds. However, with turkey tracheal and bronchial epithelium, approximately 10% increase in percent of cells staining positive for the mammalian receptors were seen.

The receptor distribution in the respiratory system in layer ducks was similar to the distribution in 1-day-old as well as 2-4-week-old ducks. In chickens, an increase (upto 80% positive cells); and in turkeys, a decrease to upto 10% positive staining for mammalian receptors was observed along the tracheal epithelium. The bronchi of layer chickens showed did not show the presence of mammalian type receptors. Other than
bronchial epithelium, different sections of the lung were negative for the presence of avian or mammalian receptors in different age groups of the 3 bird species.

3.4.2. Differences in receptor distribution along the small and large intestinal epithelium of chickens, ducks and turkeys with age

In day old ducks, less than 5% of the small intestinal epithelial cells showed positive staining for the avian type receptors with no detectable presence of mammalian type receptors. In turkey poults, no mammalian or avian type receptors were detected in the small intestinal epithelial cells. However, in day-old chickens, approximately 60% positive staining was observed for $\alpha_{2,3}$SA-gal receptors, with fewer positive staining (10%) for $\alpha_{2,6}$SA-gal receptors. The large intestinal cells showed the presence of avian type receptors in the day old birds of the 3 species, with chickens also showing the presence of mammalian receptors (20%). The distribution of the avian receptors varied from 60-80% along most of the large intestinal sections to almost 100% along the lining epithelial cells of cecum in the 3 bird species (Table 3.2).

We did not observe the presence of either type of receptors in the small intestines of 2-week-old ducks. However with the 3-week-old turkeys, epithelial cells from jejunum and ileum showed positive staining for avian receptors ($\approx$10%). In 3 week old chickens, jejunum and ileum showed higher percent (40-60%) of positive staining for $\alpha_{2,3}$SA-gal receptors. The ileum from chickens also showed presence of $\alpha_{2,6}$SA-gal receptors ($\approx$20%). The large intestinal sections showed 30-50% staining for the presence of
α2,3SA-gal receptors in 2-3-week-old turkeys and ducks with no positive staining for mammalian type receptors. In 4-week-old chickens, along the large intestinal epithelium, a higher percent of positive staining (70%) was observed for avian receptors along with presence of mammalian receptors (20-50%) (Fig. 3.1).

The small intestines of layer chickens and ducks showed positive staining for avian receptors (30%), however, sections of small intestine from breeder turkeys were negative for the presence of avian type receptors. The small intestinal epithelia from the layer birds of the 3 species were negative for the presence of mammalian type receptors. Layer chicken showed the higher percent of positive staining for avian type receptors in the large intestines (≈80%) in comparison to ducks (40-50%) or turkeys (≈50%). Sections of large intestines from layer chickens were mildly positive for the presence of mammalian type receptors (≈10%), whereas sections from layer ducks and breeder turkeys were negative for the presence of mammalian receptors.

3.4.3. Differences in distribution of receptors related to age and species in other organ systems

In day old birds, the tubular cells of the kidney showed positive staining for α2,3SA-gal and α2,6SA-gal receptors in the 3 bird species. Approximately, 50-70% of the cells showed intense positive staining for the presence of avian type receptors. However, less than 30% of the cells were positive for α2,6SA-gal receptors and the staining intensity was moderate (++).
Like for the 1-day-old birds, the 2-4-week-old birds as well as layer birds of the 3 species showed intense staining in the tubular cells of the kidney (50-60%) for the avian type receptors. The tubular cells also showed positive staining for the mammalian type receptors, although only fewer percent of cells (10-30%) and lesser intensity of staining was observed.

Among the layer birds of the 3 species tested, all the sections of the oviduct including the infundibulum, magnum, isthmus and the uterus showed high intensity of positive staining for the avian type receptors. These sections did not give any positive staining for the mammalian receptors.

Different tissues including sections of brain, breast muscles, oesophagus, bursa, spleen, cecal tonsils were also tested for the presence of receptors in the different age groups of chickens, ducks and turkeys. In the brain, the positive staining for avian type receptors was restricted to the meningeal layer surrounding the brain and no positive staining was detected in the neurons. No positive staining was observed for human type receptors in the brain sections. In the breast muscle tissues examined, no positive staining for the avian or human type receptors were observed in the 3 bird species irrespective of age. The sections of the oesophagus gave intense positive staining for the avian type receptors along the mucosal epithelium with no positive staining for human type receptors. Sections of the lymphoid tissue including bursa, spleen and cecal tonsils
showed no detectable presence of avian and human type receptors in the different age
groups of the 3 bird species tested.

3.4.4. Binding assay to demonstrate binding of viruses to the receptors observed

Binding assays was used to determine the actual viral binding to receptors observed in tracheal epithelium of chickens and turkeys. As expected, the viruses of avian origin bound well to the tracheal epithelial membrane preparations from chickens and turkeys (Figs. 3.3, 3.4). The A/mallard/Minnesota/35581/00 virus was found to bind well to both chicken and tracheal epithelium, especially at higher viral concentrations. A/turkey/Ohio/313053/04 as well as A/muteswan/Michigan/451072-2/06 viruses also bound well to the tracheal epithelium from the two species. We included human/Ohio/06 virus as a control, as human viruses are preferentially known to bind α2,6SA-gal receptors, which were found to be less extensively distributed in tracheal epithelium from chickens and turkeys in comparison to α2,3SA-gal receptors. As expected, the human virus bound to the tracheal epithelial membrane form the two bird species, though to a lesser extent in comparison to the wild bird as well as turkey isolates.

3.5 Discussion

Influenza viruses attach to host cells through interactions of the hemagglutinin of viruses with sialic acid terminated oligosaccharide residues on the host cells. These interactions determine to a large extent the host range and successful interspecies
transmission of influenza viruses (30). Sialic acids, a family of 9-carbon acid sugars were identified and are still believed to be major receptor determinants of influenza viruses (22). Avian viruses bind to α2,3-linked sialic acids much more strongly than α2,6 linked sialic acids (11, 18, 27, 38). α2,3 or α2,6 linked sialic acid oligosaccharide molecules have different molecular shapes and influenza viruses can discriminate between them. Human and avian viruses were found to preferentially bind to α2,6SA-gal and α2,3SA-gal terminated receptors, respectively, by generating specific receptor determinants using sialyltransferases (10, 39). The presence of the different types of receptors can be detected using immunohistochemistry employing plant lectins that differentiate between these linkages. The availability of receptors as well as type of receptors on different tissues of hosts can have effect on viral host ranges and their pathogenicities (31). For a better understanding towards this end, we undertook detailed studies on the type and distribution of receptors in different tissues of 3 species of domestic poultry, and studied the differences in receptor profile with age in these 3 bird species.

The presence of avian and human receptors on the tracheal epithelium of the 3 bird species even at day old stage indicate their susceptibility to infection with avian and human influenza viruses at an early age. The presence of avian receptors in the trachea and bronchial epithelium and their absence in other parts of lung might indicate that influenza viruses localize to the upper respiratory tracts in domestic birds. Chicken tracheal epithelial cells have been previously shown to posses both types of receptors (17) and they have been proposed to be intermediate hosts in the interspecies
transmission of influenza viruses. However, the equal intensity of strong positive staining for the avian and human type receptors in the trachea of ducks of the 3 age groups was an interesting finding especially considering the presence of only $\alpha_{2,3}$SA-gal receptors on the duck intestinal epithelium. The presence of receptors on the tracheal epithelium of ducks is supported by their susceptibility to low and highly pathogenic influenza viruses and successful oropharyngeal shedding has been demonstrated with them (9). Also surveillance studies report high rates of viral recovery from tracheal swabs similar to cloacal swabs from ducks (1-3, 21, 43). A recent study employing immunofluorescence also indicated the presence of $\alpha_{2,6}$SA-gal and $\alpha_{2,6}$SA-gal receptors on duck tracheal epithelial cells (24). With turkeys, studies on the receptor distribution profile from the tracheal epithelium are lacking. Our results indicate that like chickens and ducks, turkey tracheal epithelium can also support infection with avian and human influenza viruses. Turkeys have also been found to be naturally and experimentally infected with influenza viruses of avian and mammalian origins (2, 4, 6, 25, 42). An increase in percent of human receptors was seen with age in turkey tracheal epithelial cells up to 3-week old stage followed by a decline in the percent in layer turkeys, whereas in 2-4-week old chickens and ducks, the distribution of mammalian influenza receptors were similar to 1-day old birds. We do not know if such percentages have an effect on the infection with viruses from different sources at these time points or if a minimum percent of receptors are enough to initiate infections. Using tracheal epithelial membranes from chickens and turkeys in a solid phase binding assay, we demonstrated that avian and human viruses
indeed utilize these receptors for binding. The human virus that we used in our study bound to a lesser extent even at higher concentrations in comparison to the avian isolates, which is in accordance with our findings of fewer $\alpha_2,6\text{SA-gal}$ receptors in the tracheal epithelium from chickens and turkeys in comparison to $\alpha_2,3\text{SA-gal}$ receptors.

The distribution and intensity of receptors in the bronchial epithelium of the 3 bird species was similar to the results observed for tracheal epithelium indicating that avian and human viruses can utilize these receptors for binding and cause infections of the upper respiratory tracts. Lung tissues from the 3 bird species were negative for the presence of receptors. Failure to detect receptors in lung tissues does not indicate absence of influenza virus replication in lung tissues of domestic birds. Many high and low pathogenic influenza infections of birds have been found to infect lungs of domestic birds and influenza antigen has been demonstrated in them. Human lung tissues have shown the presence of avian type receptors in different cell types including pneumocytes, alveolar macrophages and bronchial epithelial cells by lectin histochemistry employing MAA (8). Such detailed observations on different cell types of poultry lung sections have not been undertaken to date. The presence of lung infection in conjunction with failure to detect receptors might indicate that the distribution of receptors in the respiratory tract might not be as clear cut as we observe using lectin histochemistry. Our observations as well as previous studies indicate that not all cells that bind viruses cause productive viral replication and vice versa (33). Apart from influenza viruses similar observations were made for parainfluenza as well as influenza B viruses (5, 15). Apart from the receptors,
other viral components might play a role. Also, apart from the terminal lectins, other viral and receptor components might influence viral binding and infections.

Among the receptor components, the length and structure of inner fragment of the carbohydrate chain has been found to affect viral binding. Duck viruses have been found to bind gangliosides with short sugar chains that were abundant in duck intestines. Human and chicken viruses bound more strongly to gangliosides with long sugar chains that were found in chicken intestinal and monkey lung tissues (16). It has been postulated that additional carbohydrate moieties on the HA globular head and deletions in NA stalk deepen the sialic acid binding sites of both glycoprotein with respect to the surface of the virion. This may facilitate the recognition of longer receptor moieties by chicken viruses. Turkey and chicken viruses have been shown to evolve much faster than aquatic bird isolates and differ from duck viruses in the presence of additional carbohydrates on hemagglutinin and deletions in the stalk of neuraminidases (7, 30). Further studies with turkey viruses are important to understand whether turkey viruses also retain their specificity for long gangliosides similar to chicken viruses. Chicken and duck viruses also differed by their ability to recognize the third sugar moiety in α2,3SA-gal terminated receptors (16) and the different isoforms of MAA can distinguish between these linkages. The MAA1 isoform, that we used in our studies, has been found to have the greatest affinity for α2,3SA-gal β(1-4)GlcNAc and even binds non-sialic acid residues. MAA2 preferentially recognizes α2,3SA-gal β(1-3)GalNAc. Hence results of lectin immunostaining will depend on the isoform of MAA used as well as subterminal
structures of the glycan chain (27). Results from our study also indicate that age group of
different bird species does not significantly affect the distribution or intensity of receptors
in different tissues. The use of different breeds of birds within the same species as well as
differences in tissue processing techniques may also account for the different staining
results observed.

With the intestinal sections, only chicken intestinal epithelial cells detected
positive for the presence of avian and human receptors among the 3 bird species tested.
Also, chicken small intestines were positive for receptors at an early age, indicating
possible cloacal shedding of viruses when infected at an early age. With turkeys and
ducks, only avian receptors were predominant and were mostly restricted to the large
intestines. Many studies on influenza viral replication and shedding from avian species
concentrate on the viral shedding in the feces. The localization of receptors and
replication of viruses in different parts of the intestines were not extensively studied
previously. Few previous reports indicate high frequency of viral isolation from cloaca,
jejunum and ileum following experimental inoculation of wild waterfowl origin viruses
in chickens (29). Lectin histochemical studies of intestinal sections indicate the
localization of receptors to large intestines, especially in ducks and turkeys. Among 1-
day-old birds, only chicken small and large intestinal epithelial cells were positive for
avian and human type receptors indicating their suitability as intermediate hosts as
indicated in previous studies. Our results are in agreement with previous studies that
reported the presence of $\alpha_{2,3}$ and $\alpha_{2,6}$ linked sialic acid residues on chicken colon (19)
and absence of SNA staining in duck intestinal cells (20). Also, chicken duodenum was not found to express α2,6SA-gal receptors as previously reported (32). However, studies by Wan and Perez, 2006 (32) reported large amounts of positive cells for α2,3SA-gal residues along the chicken duodenal sections, especially in crypts. However, our studies revealed positive staining for α2,3 receptors along the jejunum and ileum and α2,6 receptors in ileal sections of chicken intestines, with no positive staining for either type of receptors along the duodenal sections of chickens. We do not know if such discrepancies in results were due to the different MAA isoforms that were employed in these studies or due to differences in tissue processing techniques.

Kidney sections from the 3 bird species were found to be positive for the presence of avian and human type receptors. Many influenza viruses have been found to be nephrotropic following infection (37, 40, 41). Also MDCK, which is a canine kidney cell line, has been found to support the replication and transmission of influenza viruses. Our data might indicate kidney cell lines from avian species could also support replication of avian and mammalian influenza viruses.

Oviduct from all species of birds showed the presence of α2,3 sialic acid linked receptors. Influenza infections have been associated with lowered egg production in layer chickens, and breeder turkeys. It is possible that the viruses utilize the α2,3 linked sialic acid receptors in the oviduct for binding and subsequent infections. Our previous studies in breeder turkeys using a triple reassortant turkey virus, A/turkey/Ohio/04
showed that the virus preferentially replicates in the oviduct of breeder turkeys in comparison to the respiratory or digestive tracts and result in drastic declines in egg production in breeder turkeys. Our studies showed exact match between the presence of α2,3SA-gal receptors and viral antigen in duplicate sections of the oviduct indicating that the viruses might utilize these receptors for virus-cell interactions (37). Viral binding assay using plasma membranes from the oviduct epithelial cells from different bird species will confirm our hypothesis that the different viruses that replicate in the oviduct utilize these receptors for binding.

Also different tissues like bursa, spleen, brain, cecal tonsils have been studied. The absence of receptors in these organs studied does not indicate absence of infection with influenza viruses especially following infection with high pathogenic isolates. Avian species susceptible by natural or experimental inoculation to highly pathogenic isolates, the virus is found to consistently localize to brain and pancreas (23, 35, 36). Viral antigen has also been demonstrated from muscle tissues of ducks experimentally infected (44). High frequency of viral recovery has been demonstrated from bursa of chickens following experimental inoculation of waterfowl origin influenza viruses (32) as well as experimental inoculation of high pathogenic viruses had revealed histological lesions of necrosis and inflammation in cloacal bursa, thymus, spleen, heart muscle, brain along with lesions in pancreas and lung tissues (32). Highly pathogenic avian influenza viruses have also been isolated from duck meat following infection (43).
Perez et al (2003) (34) suggested that land based birds act as potential disseminators of avian/ mammalian reassortants. The presence of both receptors might indicate that the adaptation of wild bird viruses in turkeys and chickens could cause occasional emergence of viruses with an increased affinity to bind to α2,6 receptors and an enhanced propensity for transmission to humans. The natural reservoirs of avian influenza viruses are wild aquatic birds. Most avian influenza viruses are thought to have strict preference for α2,3 sialic acid linked receptors. These along with many other factors limit the replication of influenza viruses in humans in which α2,6 gal terminated residues predominate. Alteration of receptor specificity of aquatic birds in some intermediate host may facilitate their transmission to humans (29). Domestic bird isolates, especially, turkey and chicken viruses have been shown to evolve much faster than viruses from aquatic bird sources (7, 27). The higher rate of evolution along with the presence of avian and human receptors on turkey tracheal epithelial cells indicate that like other terrestrial poultry, chickens and quail, turkeys can be potential intermediate hosts in the transmission of influenza viruses.
3.6 REFERENCES


### Table 3.1: Receptor type, intensity and distribution from different tissues of chickens, ducks and turkeys

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*a:* Distribution of α2,3SA-gal and α2,6SA-gal receptors detected by MAA and SNA respectively. The percent positive staining was determined by visual examination of 3 different fields of the same tissue from 3 different birds as observed under 20X objective of light microscope.

*b:* Intensity of staining observed expressed as +(mild), ++(moderate), +++(strong) and ++++(very strong)

*c:* No staining observed

*d:* Not applicable
Receptor distribution along the tracheal epithelium of 4-week-old chickens (Figs. 1.A, 1.B), 2-week-old ducks (Figs. 2.A, 2.B) and 3-week-old turkeys (Figs. 3.A, 3.B) stained with MAA and SNA respectively. The brown color indicates the presence of receptors.

Figure 3.1: Receptors along the tracheal epithelia of chickens, ducks and turkeys
Receptor distribution along the large intestinal epithelium of 4-week-old chickens (Figs. 1.A, 1.B), 2-week-old ducks (Figs. 2.A, 2.B) and 3-week-old turkeys (Figs. 3.A, 3.B) stained with MAA and SNA respectively. The brown color indicates the presence of receptors along the intestinal epithelia.

Figure 3.2: Receptor distribution along the large intestinal epithelia of 2-4-week old chickens, ducks and turkeys
Binding assay using chicken tracheal epithelial cell membrane using A/turkey/Ohio/313053/04 (TK/OH/04), A/mallard/Minnesota/35581/00 (Mallard/MN/00), A/muteswan/Michigan/451072-2/06 (MS/MI/06), human/Ohio/06(human/OH/06) and viruses created by reverse genetics containing the backbone of TK/OH/04 and hemagglutinin and neuraminidase genes from MS/MI/06 (RG-MS/MI/06) and A/parrot/California/406032/04 (RG-PS/CA/04).

Figure 3.3: Binding assay using chicken tracheal epithelial cell membrane
Binding assay using turkey tracheal epithelial cell membrane using
A/turkey/Ohio/313053/04 (TK/OH/04), A/mallard/Minnesota/35581/00
(Mallard/MN/00), A/muteswan/Michigan/451072-2/06 (MS/MI/06),
human/Ohio/06(human/OH/06) and viruses created by reverse genetics containing the
backbone of TK/OH/04 and hemagglutinin and neuraminidase genes from MS/MI/06
(RG-MS/MI/06) and A/parrot/California/406032/04 (RG-PS/CA/04).

Figure 3.4: Binding assay using turkey tracheal epithelial cell membrane
CHAPTER 4

PATHOGENIC, ANTIGENIC AND GENETIC CHARACTERIZATION OF TRIPLE REASSORTANT H3N2 INFLUENZA VIRUSES ISOLATED FROM TURKEYS

4.1 SUMMARY

Triple reassortant (TR) H3N2 viruses are now endemic in the U.S. turkey population. These viral infections result in huge economic losses due to clinical disease as well as low egg production. In this study, we conducted genetic, antigenic and biologic characterization of selected H3N2 viruses. Phylogenetically, all turkey origin TR H3N2 viruses isolated from different geographical locations as well as different years shared high genetic similarity and grouped together with the swine origin isolates. Antigenically, all turkey isolates were similar, showed lesser cross-reactivity to swine origin viruses, and no cross reactivity to avian origin H3 subtype viruses that are not triple reassortants. Biologically, turkey origin TR H3N2 viruses replicated efficiently in 3-week-old turkeys and transmitted to the contact control cage mates. In 26-week-old breeder turkeys, one of the TR H3N2 strains tested caused complete cessation of egg production within 13 days post infection. We confirmed high levels of virus replication and abundant distribution of
avian specific $\alpha$2,3 sialic acid-galactose receptors in the oviduct of these turkeys. In 4-week-old chickens and 2-week-old ducks, these viruses replicated poorly and did not transmit. The endemicity of the TR H3N2 viruses in turkeys as well as their economic importance calls for their increased monitoring and surveillance. In addition, vaccines for turkeys should be updated or new vaccines developed using antigenically related strains.

4.2 INTRODUCTION

The genome of influenza viruses consist of eight gene segments of negative polarity. The segmented nature of the genome makes reassortments possible when different viruses infect the same host (23). Wild aquatic birds act as the primary natural reservoirs of influenza viruses (21, 22). However influenza viruses can affect a wide range of mammals and birds resulting in infections of varying severities. In most cases, wild bird isolates replicate poorly or die out in their new or aberrant hosts. However, in some cases, these viruses can adapt well and replicate efficiently for a prolonged period of time in their new hosts and establish a stable lineage (10). One of the major selective pressures for influenza viral infections is the availability of receptors on the cell surfaces of the hosts (9, 13). Mammalian influenza viruses preferentially bind to $\alpha$2,6 sialic acid (SA)-galactose (gal) terminated sialyloligosaccharides, whereas avian viruses prefer $\alpha$2,3SA-gal terminated residues (2). Pigs, however, express both $\alpha$2,3SA-gal and $\alpha$2,6SA-gal receptors on their tracheal epithelium and are implicated as potential mixing vessels of avian and mammalian influenza viruses (5, 14).
H1N1 influenza viruses were first isolated from pigs in the U.S. in 1930 (15) and till late 1990s, H1N1 was the predominant influenza subtype in pigs in the U.S. Also, transmission of these swine H1N1 influenza viruses to turkeys has been documented several times (3, 11). The first reported isolation of swine influenza viruses in turkeys was in 1980-81 (4). Since then, several swine influenza virus related outbreaks have been reported in turkeys (7, 8, 24) and in majority of the cases, the turkey flocks were housed in close proximity to swine herds (17).

Since their first isolation in 1998, triple reassortant (TR) H3N2 influenza viruses have spread over much of the U.S. swine population (20). These TR viruses contain gene segments derived from recent human (HA, NA and PB1), swine (NS, NP and M) and avian (PB2, PA) influenza viruses (20, 27). And since 2003, the swine origin TR H3N2 viruses have been isolated from U.S turkey populations (2, 19). The early reports of swine TR H3N2 viruses crossing the species barrier to infect turkeys were from farms in Minnesota and North Carolina in 2003 (2). Later, the TR H3N2 viruses have been isolated from turkey flocks in different parts of the U.S. Some of these infected flocks had a history of vaccination with H1N1 viruses or swine TR H3N2 viruses (19, 26), however these vaccines were not effective in preventing infections with turkey TR H3N2 viruses. Previous studies reported antigenic differences among the turkey and swine origin TR H3N2 viruses and address the need for a vaccine based on turkey TR H3N2 (26).

In this study, to evaluate the relationship among different isolates and the evolution of this new lineage of viruses, we undertook genetic and antigenic
characterization of recent turkey origin TR H3N2 viruses. Furthermore, to assess the ability of these TR H3N2 viruses to infect and transmit among domestic birds, infection and transmission studies were conducted in 2- to 4-week-old chickens, ducks and turkeys.

### 4.3 MATERIALS AND METHODS

**Viruses:** Two of the viruses used in this study, A/turkey/Illinois/04 (TK/IL/04) and A/turkey/Ohio/313053/04 (TK/OH/04), were isolated at Food Animal Health Research Program, Wooster, Ohio. The two recent turkey TR H3N2 isolates, A/turkey/Minnesota/366767/05 (TK/MN/05) and A/turkey/North Carolina/353568/05 (TK/NC/05) and the chicken isolate, A/chicken/New York/11602-12/98 were obtained from the repository of Southeast Poultry Research Laboratory (SEPRL), Athens, Georgia. The viruses, A/turkey/North Carolina (TK/NC/03) and swine vaccine strain virus, A/swine/North Carolina/03 (SW/NC/03) were kindly provided by Dr. Eric Gonder (Goldsboro Milling Co. Goldsboro, NC). The duck vaccine strain virus, A/mallard duck/Minnesota/79 (DK/H3N4) was obtained from Lohmann Animal Health (Lohmann Animal Health, Winslow, Maine). All viruses were passaged either in Madin-Darby Canine kidney cells (MDCK) or 10-day-old embryonated chicken eggs (ECE) to make working stocks.

**Pathogenicity of TK/IL/04 and TK/OH/04 viruses in breeder turkeys:** Pathogenicity studies were undertaken in 26-week-old breeder turkeys by infecting two groups of 18 birds each with $10^{6.5} \text{TCID}_{50}$/0.5 ml of virus through choanal route. One additional group
of 12 turkeys were inoculated with sterile phosphate buffered saline (PBS) and served as negative controls. Tracheal and cloacal swabs were collected from the infected and control birds at 2 and 4 days post-infection (DPI). Individual swabs were placed in 2.0 ml of PBS containing gentamycin (1mg per 100 ml). RNA was extracted from the tracheal and cloacal swabs with RNeasy Minikit (Qiagen, Inc., Valencia, CA) in accordance with the manufacturer’s instructions. The virus shedding/replication were quantitated by real-time RT-PCR (RRT-PCR) directed to the matrix (M) gene as previously reported (6). At 7 DPI, two infected turkeys from each group were euthanized and tissues (trachea, lungs, kidney, spleen, portions of small and large intestine, cecal tonsils and different parts of the oviduct) were collected and preserved in 10% buffered formalin for histopathology. Virus replication in reproductive tract of turkeys was assessed by collecting 1g each of infundibulum, magnum, isthmus and uterus at 7 DPI. Egg production data were monitored twice daily in the treatment and control birds for 1 week prior to and three weeks post-infection. All the birds were bled at 21 DPI and HI titers were determined as described (1). The endpoint HI titer was defined as the last dilution of serum that completely inhibited hemagglutination.

**Lectin immunostaining for receptors:** We used different parts of the oviduct (magnum, isthmus, uterus) for studying the receptor profile by employing two specific lectins, Maackia amurensis agglutinin (MAA) for α2,3SA-gal receptors and Sambucus nigra agglutinin (SNA) for α2,6SA-gal receptors (DIG Glycan Differentiation Kit, Roche Applied Science, Mannheim, Germany). Due to the practical difficulties in getting tissue
sections, we did not include infundibulum for receptor studies. Paraffin embedded sections of the oviduct were deparaffinized and immersed in 3% hydrogen peroxide to eliminate the endogenous peroxidase activity. The sections were treated with blocking agent (DIG Glycan Kit, Roche) to avoid nonspecific staining and then incubated with digoxigenin (DIG)-labelled MAA or SNA (1μg/μl) at 4°C overnight. After two washes in PBS, the sections were incubated with peroxidase-labelled anti-DIG FAb fragments (Roche Applied Science) for 1.5 h at 37°C. Lectin binding was visualized using DAB (3, 3’-diaminobenzidine-tetrahydrochloride) substrate (Roche diagnostics GmbH, Mannheim, Germany) and slides were counterstained with hematoxylin. Negative controls were incubated with PBS instead of lectin.

**Sequencing and phylogenetic analysis:** The eight gene segments of A/turkey/Minnesota/366767/05 (TK/MN/05) and A/turkey/North Carolina/353568/05 (TK/NC/05) were amplified and sequenced at the SEPRL sequencing facility. The sequences have been deposited in the GenBank database (Accession Nos. EU697204-EU697213, EU735831-EU735833, EU743215-EU743217). Sequence comparisons of the genes were conducted using the Megalign program using the Clustal V alignment algorithm (DNASTAR, Madison, Wisc.), and phylogenetic relationships were estimated by the method of maximum parsimony (PAUP software, version 4.0b10; Sinauer Associates, Inc, Sunderland, Mass.) using a bootstrap resampling method with a heuristic search algorithm.
**Hyperimmune sera production:** For raising antisera, viruses were grown in 10-day-old ECE, the pooled infectious allantoic fluid was inactivated with 0.1 percent beta-propionolactone (Sigma, St.Louis, Mo.) and the inactivated virus was used to prepare an oil-emulsion vaccine as previously described (16). The vaccine was inoculated subcutaneously into three week old specific pathogen free (SPF) chickens or turkeys. The birds were bled on the third week after vaccination to determine the HI titer of the sera and booster vaccinated in the same way with the same amount of inoculum. Two weeks after booster vaccination, the birds were bled and the sera were tested for antibodies utilizing the HI test.

**Comparative pathogenicity, replication and transmission of TK/MN/05 and TK/NC/05 viruses in chickens, ducks and turkeys:** Four week old SPF chickens (Charles River Laboratories, Inc. Wilmington, MA), two week old commercial Pekin ducks (Ridgeway Hatcheries, Inc. LaRue, Ohio) and three week old Eggline turkeys (OARDC flock) were used in the present study. Twelve chickens and ducks and nine turkeys were included in each group. These were inoculated with 0.2 ml of 10^6 EID_{50} of the virus through intrachoanal route. Three birds were introduced as contact controls at one DPI. Tracheal and cloacal swabs were collected from all the birds at 2, 4 and 7 DPI (1, 3, and 6 DPI for contact controls) and viral titers were determined as described above.

At 3 DPI, three infected chickens, turkeys and ducks were euthanized and tissues (trachea, lungs, kidney, bursa, cloaca, spleen, portions of small and large intestine, cecal tonsils) were collected for histopathology. All the infected birds were bled for serum
collection on 7 and 14 DPI (6 and 13 DPI for contact controls). The HI tests were performed with one percent chicken erythrocytes as described above.

**Histopathology and immunohistochemistry (IHC) for viral antigen:** Collected tissues were fixed by submersion in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Sections were made at 5 µm and were stained with hematoxylin and eosin (HE). A duplicate 4-µm section was immunohistochemically stained by first microwaving the sections in Antigen Retrieval Citra Solution (Biogenex, San Ramon, CA) for antigen exposure. A monoclonal antibody (P13C11) specific for a type A influenza virus nucleoprotein, developed at Southeast Poultry Research Laboratory, was used as the primary antibody for a streptavidin-biotin-alkaline phosphatase complex-based IHC method as previously described (18).

**4.4 RESULTS**

**Replication and pathogenicity of TK/OH/04 and TK/IL/04 viruses in breeder turkeys:** Pathogenicity studies were undertaken by infecting two groups of breeder turkeys with 10^6.5 TCID50/0.5ml of either virus through choanal route. In turkeys inoculated with the TK/IL/04 virus, only 3 out of the total 18 birds tested positive for viral RNA from tracheal and cloacal swabs at 2 DPI with the mean interpolated viral titers of 0.37 and 0.23 log10 EID50/ 0.2 ml, respectively (Table 4.1). In the group inoculated with TK/OH/04 virus, we observed comparatively higher titers of 1.05 and 1.47 log10 EID50 / 0.2 ml in the tracheal (4/18 positive birds) and cloacal swabs (7/18
positive birds), respectively, at 2 DPI. Similar trends were observed with swab samples collected at 4 DPI with slightly higher viral titers observed in TK/OH/04 infected birds.

Viral titers in different parts of the oviduct were also determined. In the TK/IL/04 group, only infundibulum and magnum showed positive results with average titers of 1.07 and 0.19 log_{10} EID_{50}/g, respectively. The turkeys belonging to the TK/OH/04 group showed comparatively higher viral titers in all four parts of the oviduct. The average viral titers in infundibulum was 3.76, magnum showed the highest titers of 5.84, and isthmus and uterus had titers of 4.96 and 5.23 log_{10} EID_{50}/g of the tissue, respectively. All the infected birds in both groups seroconverted as shown by the HI titers observed at 21 DPI (Table 4.1). The HI titers were higher in the TK/OH/04 group in comparison to the TK/IL/04 group (average titers of 11.9 log_{2} and 4.8 log_{2}, respectively).

The TK/IL/04 group maintained a steady egg production similar to the control group. However, the TK/OH/04 group showed a drastic decline in egg production at around 7 DPI, finally leading to a complete cessation of production at about 13 DPI (Fig. 4.1). Apart from the lowered egg production, we did not observe any other clinical signs in the infected birds from either treatment groups.

We collected tissues for microscopic examination from infected birds at 7 DPI. In both groups of birds, we observed only mild lesions in the trachea and lungs consisting of mild hyperplasia of the tracheal epithelium with mild lymphoplasmacytic infiltration, mild bronchitis and mild congestion of the lungs. Viral antigen was not detectable in the trachea and lungs of turkeys from either group. Mild lymphoid hyperplasia in the cecal tonsils and lymphoid atrophy in the spleen was observed, however, viral antigen was not
demonstrable. In the intestines, mild infiltration of lymphocytes in the lamina propria was present mostly in the jejunum with no or infrequent viral antigen staining. In kidneys, mild to moderate multifocal tubule necrosis and associated lymphocytic interstitial nephritis was observed in birds from both groups, with viral antigen staining present in the tubule cells. The oviduct presented marked lesions in the TK/OH/04 infected birds. In these birds, degenerative and necrotic changes were found throughout the oviduct, with loss of cilia and degeneration and necrosis of the oviduct surface epithelium, and atrophy of the glandular epithelium. Viral antigen staining was demonstrated in the lining cells of all sections of the oviduct and was less evident in the glandular epithelium (Fig. 4.2 A, B and D). Degenerative and diffuse necrotic changes were observed in the glandular epithelial cells of isthmus and uterus with accumulation of cellular debris between folds and within the lumen. Multifocal lymphocytic infiltration of the glands was also observed. In the TK/IL/04 infected group, mild to moderate degeneration of epithelial cells, mild atrophy of oviduct glands, and edema of the submucosa were observed with mild staining for viral antigen.

We also studied the type and distribution of receptors in the oviduct of the breeder turkeys using immunostaining employing specific lectins. We observed an abundance of \( \alpha_{2,3}\text{SA-gal} \) terminated sialyloligosaccharide receptors in different parts of the oviduct (Fig. 4.2 G, H). Staining for these receptors was very marked on the surface epithelium of all sections of the oviduct and less common in the glandular epithelium. We observed little or no specific staining for the \( \alpha_{2,6}\text{SA-gal} \) receptors (Fig. 4.2F). In duplicate sections of isthmus that were stained for receptors as well as viral antigen, we observed that the
lining epithelial cells of isthmus that stained positive for MAA gave positive results for viral antigen staining (Fig. 4.2 D and E). Due to extensive loss of epithelia in other sections of the oviduct, we could not duplicate this result for other oviduct sections.

**Phylogenetic analysis:** We sequenced all eight gene segments of the two recent turkey TR H3N2 viruses, TK/MN/05 and TK/NC/05. These two viruses shared high sequence homology in all eight gene segments (98.4-99.5%). Both the viruses are triple reassortant H3N2 viruses and the genetic relationship of TK/NC/05 virus to other influenza viruses isolated in the U.S is shown in table 4.3. The two viruses we sequenced showed at least 97% sequence similarity in all the eight genes with one of the turkey or swine-origin TR viruses that are circulating in the U.S.

Phylogenetic analysis of the H3 hemagglutinin gene sequences of these two recent isolates along with other turkey, swine, human and avian H3 viruses is shown in Fig. 4.3. The turkey isolates from 2005 grouped together with other recent TR H3N2 viruses from turkeys and swine. Hemagglutinin gene phylogenetic analysis specifically grouped the recent turkey H3 isolates in the phylogenetic cluster IV of recent human lineage viruses that included the recent TR H3N2 swine and turkey isolates from the U.S. and Canada (12). The swine vaccine strain virus, though grouped together with the turkey and swine TR H3N2 viruses, was phylogenetically distinct from other members of the group. The duck virus isolate (DK/H3N4) which has been in use as a vaccine strain for poultry against the TR H3N2 viruses including avian lineage H3N2 chicken isolates, belonged to a lineage distinct from that of swine and turkey origin H3N2 viruses.
Antigenic analysis: We conducted cross-HI tests to evaluate the antigenic relatedness among the different H3N2 isolates. Our studies revealed that the two recent TR H3N2 viruses, TK/MN/05 and TK/NC/05, are antigenically identical (Table 4.2). Also, these two viruses were antigenically similar to other turkey origin TR H3N2 viruses. All turkey TR H3N2 viruses that we tested were antigenically similar; the differences observed between TK/NC/03 and TK/OH/04 was within four-fold range. The HI results revealed that all the TR H3N2 viruses from turkeys including the recent isolates fit into a single antigenic group. However, these turkey isolates were antigenically different from H3N2 viruses of non-turkey origins as observed in previous studies (26). Hyperimmune sera raised against the swine vaccine strain virus, SW/NC/03, showed 32-64 fold differences in HI titers against the recent TR H3N2 viruses, TK/MN/05 and TK/NC/05. Also, we observed 8-16 fold differences among swine vaccine strain virus and the turkey TR H3N2 viruses isolated in 2003 and 2004. Our results also showed substantially reduced cross-reactivity between the recent turkey TR H3N2 isolates and the chicken-origin H3N2 virus. The duck origin H3N4 virus isolate being used as a vaccine seed strain (DK/H3N4) surprisingly exhibited very poor antigenic reactivity with recent TR H3N2 viruses from swine and turkeys (Table 4.2). We employed polyclonal hyperimmune sera raised in both chickens and turkeys against the TK/MN/05 and TK/NC/05 viruses to assess if the turkey and chicken sera perform similarly in antigenic analysis HI test. The HI titers obtained with sera from both sources were identical in most reactions except for a two to four fold difference in a few cases (Table 4.2). This indicates that antisera
against influenza viruses produced either in chickens or turkeys could be used in HI tests without significantly affecting the interpretation of antigenic analysis.

**Pathogenicity and transmission studies of TK/MN/05 and TK/NC/05 in chickens, ducks and turkeys:** Comparative pathogenicity and transmission studies were undertaken in chickens, ducks and turkeys using the two recent turkey TR H3N2 viruses, TK/MN/05 and TK/NC/05. None of the infected or contact control birds of the three species tested showed any clinical signs of disease after virus inoculation. We observed differences in the replication and transmission characteristics of these two viruses in the three bird species (Table 4.4). The TK/NC/05 virus replicated to higher titers in turkeys in comparison to the TK/MN/05 virus. The two viruses were also transmitted to contact control turkeys as shown by the positive viral titers in the tracheal and cloacal swabs at 2 and 4 DPI. However, seroconversion as well as viral detection by RRT-PCR was lower in the contact control turkeys of TK/MN/05 infected group in comparison to the TK/NC/05 infected group. In ducks, TK/MN/05 established a mild, transient infection. The tracheal and cloacal swabs in infected ducks were positive only at 2 DPI with low titers of 0.7 and 0.8 log_{10} EID_{50}/0.2 ml, respectively. Among the contact control birds tested, only three birds were positive at 2 DPI with an average titer of 0.7 log_{10} EID_{50}/0.2 ml. In ducks infected with TK/NC/05, we did not detect virus from the infected or contact control birds by RRT-PCR. However, seroconversion as revealed by HI titers at 14 DPI was comparable in infected and contact control ducks infected with either virus. Neither of these viruses, TK/MN/05 or TK/NC/05, replicated or transmitted among chickens as
shown by RRT-PCR titers from tracheal and cloacal swabs. However, HI titers though low, showed that TK/MN/05 replicated in infected chickens, but did not transmit; however, TK/NC/05 replicated and transmitted within chickens. Taken together, TR H3N2 viruses of turkey origins replicate and transmit among turkeys to comparatively higher titers than in chickens and ducks. Among chickens and ducks, the viral replication and transmission was poor and ducks seemed to support the viruses better than chickens.

4.5 DISCUSSION

H3N2 influenza infections in turkeys may be asymptomatic or cause clinical disease with symptoms ranging from varying degrees of depression, anorexia, mild to severe respiratory illness, diarrhea, sinusitis, edema of head and face, cyanosis, decline in egg production, egg shell abnormalities, decrease in egg hatchability and occasional mortality (17, 19). These symptoms can manifest themselves alone or in any combination. In breeder turkeys, low pathogenic influenza A infections almost always result in decreased egg production. Several previous reports document drops in egg production in turkeys due to H1N1 (8, 16), reassortant H1N2 (17), and H3N2 viruses (2, 12, 19) from different parts of the world. TR H3N2 viruses have been associated with sudden declines in egg production in breeder turkeys within the U.S.(2, 19). However, although influenza viruses were isolated or serological evidence of influenza infections were reported in those flocks, it is possible that co-infection with multiple agents could be responsible for the observed reduced or complete cessation of egg production. To date, there are no reports of pathogenicity studies in breeder turkeys using TR H3N2 viruses.
In this study, we used two turkey TR H3N2 viruses, TK/OH/04 and TK/IL/04, to assess their replication and pathogenicity for breeder turkeys. We observed that TK/OH/04 was better adapted to turkeys based on the higher virus replication observed by RRT-PCR in tracheal and cloacal swabs and oviduct samples. Previous studies showed that among TR H3N2 viruses (TK/OH/04 and TK/IL/04 were included) tested for interspecies transmission from turkeys to pigs and vice-versa, only TK/OH/04 virus was capable of transmission in both directions (25). Thus, though these two viruses are antigenically and genetically similar, individual viruses vary in their replication and transmission characteristics. It is also possible that multiple egg passages to prepare viral stocks might have led to attenuation of TK/IL/04 virus that might have accounted for its lower replication in breeder turkeys.

We observed that TK/IL/04 and TK/OH/04 viruses efficiently replicated in the oviduct of the breeder turkeys as compared to the respiratory or digestive tract. The TK/OH/04 infected turkey group showed high viral titers in all four parts of the oviduct with the lowest titers in the infundibulum. The extensive replication of the TK/OH/04 virus in different parts of the oviduct altering its physiology and anatomical architecture appears to be responsible for the drastic decline in egg production. Microscopic examination of the TK/OH/04 virus infected breeder turkey tissues revealed degenerative and necrotic changes of the surface epithelia of the oviduct as well as necrosis and severe atrophy of the glandular epithelium. In contrast, the TK/IL/04 group, the virus replication was noticeable only in the infundibulum, the portion of the oviduct that is involved in capture of the released ova from the ovary. Histopathology also revealed milder lesions in
the oviduct of these birds. This study alone does not provide the answers for preferential multiplication of these viruses in the oviduct and severe lesions observed with TK/OH/04 virus infection, however it confirms that TR H3N2 influenza virus alone, without any concurrent infections, can lead to drastic decline in egg productions in breeder turkeys.

We demonstrated the predominance of $\alpha_2,3$SA-gal receptors in the magnum, isthmus and uterus of the oviduct of the breeder turkeys (Fig. 4.2). In these oviduct sections, the staining for $\alpha_2,6$SA-gal receptors were very few or none. The direct correlation between the presence of $\alpha\,2,3$SA-gal receptors and viral antigen in duplicate sections of the oviduct indicates that the viruses indeed utilized these receptors for virus-cell interactions. Further binding assay studies using epithelial lining of the oviduct from breeder turkeys are necessary to validate these findings. It has been reported that the swine TR H3N2 viruses are the precursors of currently circulating turkey TR H3N2 viruses (2). Pigs have been demonstrated to have $\alpha_2,3$SA-gal and $\alpha_2,6$SA-gal terminated sialyloligosaccharide receptors in their tracheal epithelium (5). It is possible that as these viruses evolved in pigs and further transmitted to turkeys, they may have adapted to preferentially bind to $\alpha_2,3$SA-gal receptors although their HA gene is of human lineage which preferentially binds $\alpha_2,6$SA-gal linked receptors.

In the U.S., only breeder turkeys are routinely vaccinated against avian influenza to offset economic losses due to lowered egg production or fertility. Swine H1N1 viruses, swine H3N2 and duck origin H3N4 vaccine strains have been commonly employed to vaccinate turkeys against influenza. However, the TR H3N2 viruses have been isolated from vaccinated turkey flocks. Genetic and antigenic analysis of the turkey TR H3N2
viruses had previously shown that though these viruses are genetically similar to swine H3N2 viruses, antigenically they are distantly related to them as well as to swine H1N1 and duck H3N4 vaccine strains (25). Our current study using the two recent turkey TR H3N2 isolates, TK/MN/05 and TK/NC/05, showed that these viruses belong antigenically and genetically to the turkey TR H3N2 group. This indicates that these turkey influenza viruses can be suitable vaccine candidates against the TR H3N2 viruses that are endemic in the U.S. turkey populations.

To further investigate the potential of these viruses to transmit to other species of domestic birds, we conducted replication and transmission studies with the two recent isolates, TK/NC/05 and TK/MN/05 in chickens, ducks and turkeys. Our results support the previous findings (25) that the TR H3N2 viruses can replicate and transmit among turkeys. These viruses were not found to transmit among ducks and did not replicate or transmit among chickens. These results reiterate that influenza viral infections depend on interactions of multiple variables of the host and virus.

In summary, we experimentally confirmed that TR H3N2 influenza viruses are directly involved in drastic reduction/complete cessation of egg production and pathology of the reproductive tract in breeder turkeys. Currently turkey TR H3N2 viruses have been isolated from flocks immunized with antigenically distinct duck origin H3N4 or swine H3N2 vaccine strains. We propose that there is a need to update H3N2 vaccine strain for turkeys using the recent turkey origin TR H3N2 viruses.
4.6 REFERENCES


### Table 4.1: Replication of A/turkey/Illinois/04 (TK/IL/04) and A/turkey/Ohio/04 (TK/OH/04) in 26-week old breeder turkeys

<table>
<thead>
<tr>
<th>VIRUS</th>
<th>SAMPLES</th>
<th>VIRUS ISOLATION(^a)</th>
<th>HI TITER(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 DPI(^d)</td>
<td>4 DPI</td>
</tr>
<tr>
<td>TK/IL/04</td>
<td>Tracheal swabs</td>
<td>0.37±0.33(3/18)(^b)</td>
<td>0.21±0.19(3/18)</td>
</tr>
<tr>
<td></td>
<td>Cloacal swabs</td>
<td>0.23±0.18(3/18)</td>
<td>0.22±0.2(4/18)</td>
</tr>
<tr>
<td></td>
<td>Oviduct</td>
<td>Infundibulum</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Magnum</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isthmus</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Uterus</td>
<td>ns</td>
</tr>
<tr>
<td>TK/OH/04</td>
<td>Tracheal swabs</td>
<td>1.05±0.34(4/18)</td>
<td>1.95±0.58(16/18)</td>
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<td>Cloacal swabs</td>
<td>1.47±0.65(7/18)</td>
<td>3.45±1.01(6/18)</td>
</tr>
<tr>
<td></td>
<td>Oviduct</td>
<td>Infundibulum</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Magnum</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isthmus</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Uterus</td>
<td>ns</td>
</tr>
</tbody>
</table>

\(^a\) Log\(_{10}\) 50% egg infectious dose (EID\(_{50}\))/0.2 ml of swab fluid + standard deviation determined by RRT-PCR.

\(^b\) Number of positives/ Total numbers tested

\(^c\) Log\(_2\) hemagglutination inhibition (HI) titer of the antisera + standard deviation

\(^d\) Days post infection

ns: Not sampled

---

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Table 4.2: Antigenic analysis of H3N2 viruses using cross hemagglutination-inhibition test

<table>
<thead>
<tr>
<th>Antigen</th>
<th>SW/NC/03</th>
<th>TK/NC/03</th>
<th>TK/OH/04</th>
<th>TK/IL/04</th>
<th>TK/NC/05 (Turkeys)</th>
<th>TK/NC/05 (Turkeys)</th>
<th>CK/NY/98</th>
<th>DK/H3N4</th>
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</thead>
<tbody>
<tr>
<td>SW/NC/03</td>
<td>256a</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>128</td>
<td>512</td>
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<td>64</td>
</tr>
<tr>
<td>TK/NC/03</td>
<td>16</td>
<td>128</td>
<td>64</td>
<td>256</td>
<td>1024</td>
<td>1024</td>
<td>4096</td>
<td>4096</td>
</tr>
<tr>
<td>TK/OH/04</td>
<td>32</td>
<td>8</td>
<td>512</td>
<td>512</td>
<td>2048</td>
<td>4096</td>
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<td>4096</td>
</tr>
<tr>
<td>TK/IL/04</td>
<td>16</td>
<td>128</td>
<td>512</td>
<td>512</td>
<td>4096</td>
<td>4096</td>
<td>4096</td>
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<td>4096</td>
</tr>
<tr>
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<td>0</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>8</td>
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</tbody>
</table>

\( ^a \) HI titers using antisera raised in chickens, unless indicated otherwise, with homologous titers in bold characters

\( ^b \) Antisera raised in turkeys

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<table>
<thead>
<tr>
<th>Gene</th>
<th>Virus</th>
<th>% Identity</th>
<th>Lineage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic polymerase 2 (PB2)</td>
<td>A/turkey/Minnesota/2005</td>
<td>99.5</td>
<td>avian</td>
</tr>
<tr>
<td></td>
<td>A/swine/North Carolina/2003</td>
<td>96.7</td>
<td></td>
</tr>
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<td>Basic polymerase 1 (PB1)</td>
<td>A/turkey/Minnesota/2005</td>
<td>99.4</td>
<td>human</td>
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<tr>
<td></td>
<td>A/swine/North Carolina/2003</td>
<td>95.8</td>
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</tr>
<tr>
<td>Acidic polymerase (PA)</td>
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<td>98.5</td>
<td>avian</td>
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<tr>
<td></td>
<td>A/swine/North Carolina/2003</td>
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<td>Hemagglutinin (H3 HA)</td>
<td>A/turkey/Minnesota/2005</td>
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<td>human</td>
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<td>A/swine/North Carolina/2003</td>
<td>95.7</td>
<td></td>
</tr>
<tr>
<td>Neuraminidase (NA)</td>
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<td>human</td>
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<td></td>
<td>A/swine/North Carolina/2003</td>
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<td></td>
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<td>Nucleoprotein (NP)</td>
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<td>swine</td>
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<td></td>
<td>A/swine/North Carolina/2003</td>
<td>99.2</td>
<td></td>
</tr>
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<td>Matrix (M)</td>
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<td>swine</td>
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<td></td>
<td>A/swine/North Carolina/2003</td>
<td>99.4</td>
<td></td>
</tr>
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<td>Non-structural (NS)</td>
<td>A/turkey/Minnesota/2005</td>
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<td>swine</td>
</tr>
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<td></td>
<td>A/swine/Minnesota/593/1999</td>
<td>97.6</td>
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</table>

Table 4.3: Genetic homology of A/turkey/North Carolina/353568/2005 genes with influenza A viruses isolated in the US.
<table>
<thead>
<tr>
<th>VIRUS SPECIES GROUP SWABS</th>
<th>VIRUS ISOLATION&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HI TITERS&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 DPI&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4 DPI</td>
</tr>
<tr>
<td><strong>chickens</strong></td>
<td>TREATMENT</td>
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</tr>
<tr>
<td></td>
<td>TRACHEAL</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CLOACAL</td>
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</tr>
<tr>
<td></td>
<td>CONTACT</td>
<td>TRACHEAL</td>
</tr>
<tr>
<td></td>
<td>CONTROL</td>
<td>CLOACAL</td>
</tr>
<tr>
<td><strong>TK/MN/366767/05</strong></td>
<td><strong>ducks</strong></td>
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</tr>
<tr>
<td></td>
<td>CLOACAL</td>
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</tr>
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<td></td>
<td>CONTACT</td>
<td>TRACHEAL</td>
</tr>
<tr>
<td></td>
<td>CONTROL</td>
<td>CLOACAL</td>
</tr>
<tr>
<td><strong>turkeys</strong></td>
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<td>TRACHEAL</td>
</tr>
<tr>
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<td>CLOACAL</td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>CONTROL</td>
<td>CLOACAL</td>
</tr>
<tr>
<td><strong>chickens</strong></td>
<td>TREATMENT</td>
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<tr>
<td></td>
<td>CLOACAL</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CONTACT</td>
<td>TRACHEAL</td>
</tr>
<tr>
<td></td>
<td>CONTROL</td>
<td>CLOACAL</td>
</tr>
<tr>
<td><strong>TK/NC/353568/05</strong></td>
<td><strong>ducks</strong></td>
<td>TREATMENT</td>
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<td></td>
<td>CLOACAL</td>
<td>0</td>
</tr>
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<td></td>
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<td>TRACHEAL</td>
</tr>
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<td></td>
<td>CONTROL</td>
<td>CLOACAL</td>
</tr>
<tr>
<td><strong>turkeys</strong></td>
<td>TREATMENT</td>
<td>TRACHEAL</td>
</tr>
<tr>
<td></td>
<td>CLOACAL</td>
<td>2.6+0.3(11/11)</td>
</tr>
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<td></td>
<td>CONTACT</td>
<td>TRACHEAL</td>
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<tr>
<td></td>
<td>CONTROL</td>
<td>CLOACAL</td>
</tr>
</tbody>
</table>

<sup>a</sup> Log<sub>10</sub> 50% egg infectious dose (EID<sub>50</sub>) / 0.2 ml of swab fluid + standard deviation determined by RRT-PCR.

<sup>b</sup> Number of positives / Total numbers tested

<sup>c</sup> Log<sub>2</sub> hemagglutination inhibition (HI) titer of the antisera + standard deviation

<sup>d</sup> Days post infection

Table 4.4: Replication and transmission studies with TK/MN/05 and TK/NC/05 in chickens, ducks and turkeys

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Egg production data from infected and control breeder turkeys for a period of 28 days (from 1 week prior to and 3 weeks post infection). The X-axis represents the average days in production, 2 days being represented as one unit. The Y-axis represents the average egg production during the 28 day-period, average no. of eggs produced by each bird in two days being represented as one unit. The blue and red lines represent the egg production data from the A/turkey/Ohio/313053/04 (TK/OH/04) and A/turkey/Illinois/04 (TK/IL/04) infected birds respectively. The black line represents the control birds that were inoculated with phosphate buffered saline (PBS).

Figure 4.1: Egg production data from breeder turkeys following infection
Sections A, B and D stained by IHC methods to detect viral antigen. Sections E, G and H stained with Maackia amurensis agglutinin (MAA) to demonstrate $\alpha_{2,3}$SA-gal receptors. Positive staining for both virus and receptors is observed on the lining epithelium of the magnum (A and G), uterus (B) and isthmus (H) (magnification 200X). Magnum showing correlation between Maackia amurensis agglutinin (MAA) staining for $\alpha_{2,3}$SA-gal receptors (E) and IHC staining for viral antigen (D). Hematoxylin and eosin stained section of magnum demonstrating histological lesions (C). Magnum section stained with Sambucus nigra agglutinin (SNA) to demonstrate $\alpha_{2,6}$SA-gal receptors (F).

Figure 4.2: Oviduct sections from turkeys infected with A/turkey/Ohio/313053/04 virus
Figure 4.3: Phylogenetic tree based on nucleotide sequence of the HA genes from selected H3 isolates.
The phylogenetic tree was generated by the maximum parsimony method with the PAUP4.0b10 program with bootstrap replication (1000 bootstraps) and a heuristic search method. Numbers at the nodes of the phylogram indicate the bootstrap confidence levels. The designations I, II and III represent antigenic clusters within the triple reassortant viruses as previously described [28] whereas, cluster IV represents viruses that are genetically distinct from the clusters I, II and III viruses based on HA1 gene sequences as described for triple reassortant swine and turkey viruses from the U.S. and Canada [26]. The viruses used for antigenic analysis are indicated in bold letters.
CHAPTER 5

THE POTENTIAL FOR EGG-BORNE DISSEMINATION
OF INFLUENZA A VIRUSES

5.1 SUMMARY

Studies on evaluating the potential of influenza viruses for egg borne transmission are limited. In our previous studies, experimental infection of breeder turkeys with A/turkey/Ohio/313053/04, resulted in drastic decline in egg production and we confirmed high levels of virus replication and abundant distribution of avian specific α2,3 sialic acid-galactose receptors in oviduct that matched with viral antigen staining. In the present study, following experimental inoculation of A/turkey/Ohio/313053/04 in breeder turkeys, we detected these viruses in albumin of eggs using real time RT-PCR and virus isolation in embryonated chicken eggs. Swabs from egg shells were also found positive by real time RT-PCR. This is the first report of detection of low pathogenic influenza viruses from internal egg contents following experimental infection. The possibility of hatchery contamination by egg-borne influenza viruses and spread of virus during movement of contaminated cracked eggs and egg flats pose serious food safety concerns.
5.2 INTRODUCTION

Type A influenza infections of domestic birds may be asymptomatic or cause clinical disease with symptoms ranging from varying degrees of depression, anorexia, mild to severe respiratory illness, diarrhea, sinusitis, edema of head and face, cyanosis, decline in egg production, egg shell abnormalities, decrease in egg hatchability and occasional mortality (19, 21, 22). These symptoms can manifest themselves alone or in any combination. In breeder turkeys, low pathogenic influenza A infections almost always result in decreased egg production. Several previous reports document drops in egg production in turkeys due to H1N1 (7, 18), reassortant H1N2 (19), and H3N2 viruses (3, 12, 22) from different parts of the world. However, although influenza virus infections were reported in different field outbreaks either by serology or virus isolation, it is possible that co-infection with multiple agents could be responsible for the observed reduced or complete cessation of egg production. Our recent studies using swine lineage triple reassortant (TR) H3N2 influenza viruses demonstrated that influenza viruses preferentially replicate in the oviduct of breeder turkeys in comparison to respiratory and digestive tracts and result in drastic declines in egg production without any concurrent infections (13). This finding was strengthened by the detection of α2,3 sialic acid (SA) linked to galactose (gal) receptors in different sections of oviduct of breeder turkeys and the exact match between the presence of the avian type influenza receptors and viral antigen in the oviduct (13). α2,3 SA-gal receptors have also been reported from turkey and chicken ovarian epithelial cells (5). The presence of avian type receptors in the
ovaries and oviduct and the high titers of viral replication in the reproductive tract suggested that there is possibility of eggs being contaminated with virus before or after oviposition, hence chance for internal or external contamination of eggs or both. In addition, eggs could be externally contaminated by high viral loads in the feces of infected birds. Avian influenza viruses have been detected from shell surfaces and the yolk and albumen of eggs from chickens and turkeys experimentally infected with highly pathogenic influenza viruses (11). Influenza viruses have also been recovered from day old turkey poults hatched from eggs incubated with virus on day 18 of incubation (14). However, similar studies on the egg–borne transmission of low pathogenic influenza viruses are limited. Considering the virulence of highly pathogenic strains, it is unlikely that infected eggs may hatch. However, with low pathogenic influenza viruses such as TR H3N2 viruses, which normally do not kill embryos in routine propagation of the virus in embryonating eggs, there is high possibility that the virus may be present in hatched poults. The possibility of egg borne transmission of influenza viruses could have serious implications in viral spread during movement of cracked eggs, hatchery contamination as well as contamination of food products and biologics where raw eggs are used. In recent decades, triple reassortant swine influenza viruses have occasionally been isolated from humans (9, 17). Also, since their first identification in April 2009, H1N1 viruses containing unique combination of gene segments from north American and Eurasian swine lineages (4) has emerged into a pandemic following efficient human to human transmission. The possibility that these swine origin TR H3N2 viruses can be transmitted through eggs of infected birds, even when the infections are asymptomatic, poses the
need for additional biosecurity and containment measures while handling infected birds and their products. In the present study, our interest was to determine whether viruses could be detected from the internal egg contents and egg surfaces following experimental infection of breeder turkeys with low pathogenic H3N2 influenza virus.

5.3 MATERIALS AND METHODS

Virus: The H3N2 subtype influenza virus used in the present study, A/turkey/Ohio/313053/04 (TK/OH/04), was isolated at Food Animal Health Research Program, Wooster, Ohio (22). The virus stock was passaged initially in Madin-Darby Canine kidney cells (MDCK) followed by passage in 10-day-old embryonated chicken eggs (ECE) to make working stocks.

Infection studies in breeder turkeys:

Experiment 1. We conducted preliminary studies using 12 26-week-old breeder turkeys that were inoculated with $10^{5.13} \text{TCID}_{50}$ /0.2 ml of TK/OH/04 virus through the choanal route. The choana connect the oral and nasal passages in poultry and we wanted to mimic the natural route of infection in birds that usually occur oronasally under field conditions. Egg production data were monitored daily. Tracheal and cloacal swabs were collected from the infected birds at 3 days post inoculation (DPI). Individual swabs were placed in 2.0 ml of PBS containing gentamycin (1mg per 100 ml). The swab samples were vortexed for 3-5 seconds and centrifuged for 5 minutes at 1200 rpm to pellet debris.
100 µl of the supernatant from the swab samples was used for RNA extraction using Viral RNA extraction kit (Qiagen, Inc., Valencia, CA) in accordance with the manufacturer’s instructions. The RNA was eluted in 50µl of nuclease free water and 8 µl of it was used for quantitating the virus shedding/replication by real-time RT-PCR (RRT-PCR) directed to the matrix (M) gene as previously reported (6). For quantitation, swab samples were run together with known amounts of control viral RNA. We used RNA extracted from $10^1$ to $10^5$ EID$_{50}$/0.2 ml dilutions of the TK/OH/04 stock virus as standard controls. Standard curves were generated with those control viral RNAs and the amount of RNA in the swab samples was converted into EID$_{50}$/ml by interpolation. High correlation ($r^2 > 0.99$) between cycle number and dilution factors were observed with standard controls. Three birds were euthanized at 3, 5, 7 and 9 DPI and different parts of the oviduct including infundibulum, magnum, isthmus and uterus were collected and used for viral titration using RRT-PCR. Virus replication in the reproductive tract of turkeys was assessed by collecting each of infundibulum, magnum, isthmus and uterus in sterile PBS (1g/5ml ratio). RNA extractions and viral titrations by RRT-PCR were done as for tracheal and cloacal swabs using 100µl of tissue supernatant. The RNA was eluted in 50µl of nuclease free water and 8µl of eluted viral RNA was used for quantitation using RRT-PCR. The amount of RNA in the tissue samples was converted into EID$_{50}$/g by interpolation. The eggs collected were used for viral detection from the shell surfaces and albumin as described below.

Experiment 2. Following detection of virus from the albumin and egg shell surfaces from eggs of infected turkeys in Experiment 1, we further extended our study using 24 26-
week-old breeder turkeys that were inoculated with $10^{5.13}$ TCID$_{50}$/0.2 ml of TK/OH/04 virus through intranasal (choanal) route. We followed a similar procedure as described in Experiment 1 with minor modifications to maximize egg collection for virus detection. Egg production data were monitored twice daily. Tracheal and cloacal swabs were collected at 5 DPI and viral titers were determined by RRT-PCR as described. At 5 DPI, 4 turkeys each were euthanized and different parts of the oviduct were collected for viral titration using real time RT-PCR (RRT-PCR). At 14 DPI, all turkeys were bled and hemagglutination inhibition titers were determined (1). The endpoint HI titer was defined as the last dilution of serum that completely inhibited hemagglutination.

**Detection of virus from egg shell surfaces and internal egg contents:** Eggs from the infected turkeys were collected twice daily in egg crates and transported to the laboratory in sterile biohazard bags. For viral detection from egg shell surfaces, the surfaces of egg shells were swabbed with sterile swabs dipped in PBS and suspended in 2ml of sterile PBS supplemented with antibiotics. After one freeze thaw cycle, the tubes were vortexed briefly, and 100μl of the swab supernatant was used for RNA extraction using the viral RNA extraction kit (Qiagen, Inc., Valencia, CA) as per the manufacturer’s instructions.

For detection of virus from albumin, the surface of the eggs was decontaminated using 70% ethanol and the air cell area was disinfected using iodine. After cracking open the egg at its air cell area using a sterile forceps, the albumin was carefully dispensed into a sterile Petri dish. It was then transferred into a sterile stomacher bag (16 oz, VWR
International, West Chester, PA) and homogenized for 5 minutes in a stomacher (STO-400, Tekmar Company, Cincinnati, OH). A 1:5 dilution of a small aliquot of albumin in sterile PBS was briefly vortexed and then centrifuged at 10000xg for 5 minutes. A 100µl volume of the supernatant was used for RNA extraction using a viral RNA extraction kit (Qiagen, Inc., Valencia, CA). We repeated the procedure twice using two independent albumin samples to avoid errors due to sampling. Samples found positive by RRT-PCR were inoculated into embryonated chicken eggs for virus isolation. For virus isolation, undiluted albumin was briefly centrifuged (10,000xg for 5 minutes). A 300µl of the supernatant from each egg was used to inoculate four 10-day old embryonated chicken eggs. The eggs were checked daily and at 3 DPI allantoic fluids were harvested to test the presence of virus using hemagglutination test employing 1% turkey erythrocytes.

5.4 RESULTS

In our initial study with a group of 12 breeder turkeys, a gradual decline in egg production was noticed in the infected birds starting at 4DPI followed by a complete cessation of production by 7 DPI. To determine the time point for maximum virus replication and duration in the oviduct, 3 birds were euthanized at 3, 5, 7 and 9 DPI and viral titers from different parts of the oviduct were determined by RRT-PCR. At 3 DPI, viral titers from different parts of the oviduct were low and inconsistent. Of the 3 birds tested, only 1 bird had positive viral titers from the infundibulum, isthmus and uterus. Our results indicated higher average viral titers at 5 DPI in the four parts of the oviduct tested. At 5 DPI, the average viral titers in the infundibulum, magnum, isthmus and
uterus were 6.34, 6.24, 7.10 and 7.3 log EID50/g of tissues respectively (Table 5.1). By 7 and 9 DPI, the average viral titers from the different parts of the oviduct were found to be lower in comparison to the titers observed at 5 DPI. Infundibulum and magnum, the initial parts of the oviduct were especially found to have lower average viral titers in comparison to the distal parts, the isthmus and the uterus. We were unable to detect virus from the egg shell surface as well as albumin of eggs laid by infected turkeys until 3DPI. However, eggs laid from 4 DPI onwards tested positive for the presence of the virus on the egg shell surface as well as from the albumin. A total of 5 eggs were laid by the infected birds from 4 to 6 DPI, before complete cessation of production at 7 DPI. The shell surfaces of all 5 eggs were found to be contaminated with the virus with an average viral titer of 4.04 log EID50/ml of swab fluid. Albumin samples from 3 out of 5 eggs tested were found to be positive for the presence of virus using RRT-PCR with an average viral titer of 3.7 log EID50/ml.

Based on experiment 1, birds were euthanized only at 5 DPI as the time point for collection of oviduct tissues for determination of viral titers in the subsequent study using 24 breeder turkeys. The average viral titers as determined by RRT-PCR from the infundibulum, magnum, isthmus and uterus were 5.64, 5.48, 6.09 and 5.51 log EID50/g of tissue, respectively (Table 5.2). These values were higher (p = 0.073) than the average viral titers from the tracheal and cloacal swabs of the infected birds at 5 DPI. No clinical signs were observed in the infected birds except for a decline in egg production. A gradual decline in egg production was observed from 3 DPI finally leading to complete cessation of egg production by 7 DPI. Virus could be detected from the egg shell swabs.
as well as albumin samples from eggs laid by infected turkeys as early as 2 DPI. Also, shell surfaces and albumin samples from eggs were positive for viruses during a 2-6 DPI period before complete cessation of production at 7 DPI (Table 5.3). The average viral titers from the albumin samples from eggs collected showed an increase from 2 to 4 DPI and started declining thereafter. RRT-PCR titers from duplicate albumin samples from the same egg were found to be consistent. At 3 DPI, 3 out of the 5 albumin samples and at 4 DPI, 1 out of the 4 albumin samples that tested positive by RRT-PCR, were from eggs that were found to be malformed and shell less respectively, at the time of collection. The viral titers from the albumin of the shell-less eggs were comparatively high and could be due to contamination of the internal contents from viral load in the cage or feces after being laid. Of the 17 albumin samples that were positive by RRT-PCR, we could isolate the virus from 3 samples following inoculation into embryonated chicken eggs. The highest average viral titers from the egg shell surfaces were observed at 2-3 DPI followed by 5 DPI. With the viral titers from the albumin of shell-less eggs were excluded from statistical analysis, the egg shell surfaces showed significantly higher (p < 0.005) percentages of viral detection compared to the albumin samples.

5.5 DISCUSSION

Ours is one of the first studies that demonstrate that low pathogenic influenza viruses can be transmitted to egg shell surfaces and internal egg contents following experimental infection of birds with low pathogenic influenza viruses. We chose the
swine lineage triple reassortant H3N2 virus, TK/OH/04 in our study to inoculate breeder turkeys as a model to elucidate egg borne transmission of influenza viruses because TR H3N2 viruses have been associated with sudden declines in egg production in breeder turkeys in the U.S. (12, 22). Also, the swine origin reassortant viruses gain public health significance following the recent human cases of H1N1 viruses containing a combination of genes from North American and Eurasian swine lineages (4).

Our previous studies with TK/OH/04 virus in breeder turkeys also confirmed high titers of virus in different parts of the oviduct that resulted in degenerative and necrotic changes in the surface and glandular epithelia of the oviduct leading to drastic declines in egg production in breeder turkeys (13). Along with confirming these findings, our present study also looked at the duration of viral replication in the oviduct tissues following inoculation. Our results indicated that though virus can be detected in the different oviduct sections as early as 3 DPI, the peak viral titers in different parts of the oviduct were reached at 5 DPI, followed by declines in viral titers especially in the infundibulum and magnum at 7 and 9 DPI. We do not know the reasons for the preferential replication of these viruses in the oviduct as well as their prolonged replication in the distal parts of the oviduct in comparison to the infundibulum and magnum. Previous studies have shown that following infection, influenza viral antigen can be detected in the oviduct and ovaries of layer chickens up to 4 DPI (16). Our observations suggested that that virus could be detected in the oviduct of breeder turkeys as early as 3 DPI and persist at least until 9 DPI, and we believe that differences in viral strains as well as species and the age
of birds used in the experimental infections could affect the duration and extent of viral replication in the oviduct. The high viral titers in the oviduct of breeder turkeys increase the possibility that eggs laid by the infected turkeys can be contaminated before or after oviposition and hence internal or external contamination of the eggs.

Our study demonstrated that viruses could be detected from the albumin of eggs laid by infected turkeys as early as 2 DPI. The detection of virus from albumin suggests that the virus indeed is passed into internal egg contents. Due to difficulties in extracting RNA from yolk of eggs, we did not use egg yolk for viral detection using RRT-PCR. Given the high percentage of albumin samples that were positive for the presence of virus as well as the high viral titers from the oviduct samples and the presence of receptors in the ovaries that could support virus replication (19), it is very likely that egg yolk could also be contaminated with influenza viruses. Influenza viruses have been detected from albumin and egg yolk of chickens and turkeys during highly pathogenic avian influenza outbreaks (2, 8, 10). Considering the high virulence of HPAI virus both for layers and eggs (20), it is unlikely that vertically infected eggs may hatch. However, with low pathogenic strains which normally do not kill embryos in routine propagation of viruses in embryonating eggs, there is a high possibility that the virus may be present in hatched poults.

Influenza viruses have been isolated from day old turkey poults hatched from eggs inoculated with virus at day 18 of incubation (15). The detection of virus from albumin in our studies suggests that vertical transmission of low pathogenic influenza
viruses is a strong possibility. Of the albumin samples collected at different time points, higher positive viral percentages were observed at 3 and 4 DPI, however at 5 DPI, when the peak viral titers were observed in the different oviduct sections, only 1 out of 10 eggs collected were positive for the presence of virus. This might indicate that viral titers in the oviduct do not correspond to egg contamination with viruses. On the other hand, at 5 DPI, we observed that all eggs laid had shell surface contamination. This could be attributed to contamination from the high viral titers in the oviduct while the egg is formed and laid. It could also result from contamination of the egg shell surfaces with feces of infected birds as we observed higher viral titers from cloacal swabs at 5 DPI in comparison to the tracheal swabs.

The presence of influenza virus on the egg shell surfaces as well as albumin indicates possible spread of virus during commercial movement of eggs. Our results indicate a potential for egg associated dissemination of viruses as well as the possibility for vertical transmission of virus and hatchery contamination. Also, potential contamination of food products and biologics where raw eggs are used cannot be ruled out. We believe that our results could serve as a model to initiate similar studies with other low pathogenic influenza viruses that cause egg production problems in different species of poultry. The egg-borne dissemination of influenza viruses and the potential risk associated with human infections indicate the need for additional biosecurity and containment measures while handling infected birds and their products.
5.6 REFERENCES


<table>
<thead>
<tr>
<th>DPI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Viral titers from oviduct sections&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infundibulum</td>
</tr>
<tr>
<td>3</td>
<td>8.69±1.8(1/3)</td>
</tr>
<tr>
<td>5</td>
<td>6.34±1.0(2/3)</td>
</tr>
<tr>
<td>7</td>
<td>3.20(1/3)</td>
</tr>
<tr>
<td>9</td>
<td>3.85±0.2(3/3)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Days post inoculation.

<sup>b</sup>Average viral titers ± standard deviation from positive samples expressed as log<sub>10</sub>50% egg infectious dose (EID<sub>50</sub>) / g of oviduct sample as determined by real time RT-PCR.

<sup>c</sup>Number of real time RT-PCR positive samples / total number of samples tested.

Table 5.1: Virus titers from oviduct samples following inoculation of breeder turkeys with A/turkey/Ohio/313053/04 virus in experiment 1
<table>
<thead>
<tr>
<th>Sample</th>
<th>Viral titer&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oviduct</strong></td>
<td></td>
</tr>
<tr>
<td>Infundibulum</td>
<td>5.64±1.0(3/4)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Magnum</td>
<td>5.48±1.2(4/4)</td>
</tr>
<tr>
<td>Isthmus</td>
<td>6.09±1.0(4/4)</td>
</tr>
<tr>
<td>Uterus</td>
<td>5.51±1.3(4/4)</td>
</tr>
<tr>
<td><strong>Tracheal swab</strong></td>
<td>2.8(2/4)</td>
</tr>
<tr>
<td><strong>Cloacal swab</strong></td>
<td>5.2±0.6(4/4)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Average viral titers ± standard deviation from positive samples expressed as log<sub>10</sub> 50% egg infectious dose (EID<sub>50</sub>/g of oviduct samples or EID<sub>50</sub>/ml of swab supernatant as determined by real time RT-PCR.

<sup>b</sup> Number of real time RT-PCR positive samples/total number of samples tested.

Table 5.2: Virus titers from oviduct samples, tracheal and cloacal swabs at 5 days post inoculation of breeder turkeys with A/turkey/Ohio/313053/04 virus in experiment 2
<table>
<thead>
<tr>
<th>DPI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Virus detection using RRT-PCR</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Albumin</td>
<td>Egg shell swab</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pos/total&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Viral titer&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Pos/total</td>
</tr>
<tr>
<td>2</td>
<td>5/22</td>
<td>4.04±0.7</td>
<td>8/22</td>
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<td>3</td>
<td>6/15</td>
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<td>5</td>
<td>1/10</td>
<td>4.39±1.1</td>
<td>10/10</td>
</tr>
<tr>
<td>6</td>
<td>1/3</td>
<td>3.30</td>
<td>1/3</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Days post inoculation.

<sup>b</sup> Number of real time RT-PCR positive eggs / total number of eggs tested.

<sup>c</sup> Average viral titers ± standard deviation from positive samples expressed as 50% egg infectious dose (EID<sub>50</sub>) / ml of albumin or egg shell surface swab supernatant as determined by real time RT-PCR.

Table 5.3: Virus titers from albumin and shell surfaces of eggs laid by breeder turkeys inoculated with A/turkey/Ohio/313053/04 virus in experiment 2
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