STUDIES ON INTERSPECIES AND INTRASPECIES TRANSMISSION OF INFLUENZA A VIRUSES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Hadi M. Yassine, M.Sc.

*****

The Ohio State University
2009

Dissertation Committee:
Professor Y.M. Saif, Adviser
Professor D.J. Jackwood
Professor J. Lejeune
Assistant Professor C.W. Lee

Approved by

Adviser
Graduate Program in Veterinary Preventive Medicine
ABSTRACT

Influenza A viruses are enveloped viruses belonging to the family Orthomyxoviridae that encompasses four more genera: Influenza B, Influenza C, Isavirus and Thogotovirus. Type A is the only genus that is highly infectious to variety of animal species, including human, pigs, wild and domestic birds, horses, cats, dogs, ferrets, seals, whales, and others.

Avian viruses are generally thought to preferentially bind the N-acetylneuraminic acid-α2,3-galactose (NeuAcα2,3Gal) form of sialic acid receptors and human viruses preferentially bind to NeuAcα2,6Gal sialic acid receptors. Pigs express substantial amount of both forms of sialic acids on their upper respiratory epithelial cells, and it is believed that both avian and human influenza viruses can attach to the appropriate receptors and infect pigs. Hence, pigs have been postulated to serve as a “mixing vessels” in which two or more influenza viruses can co-infest and undergo reassortment with potential for development of new viruses that can transmit to and infect other species.

An H1N1 influenza A virus, A/swine/Ohio/24366/07, was isolated from pigs in an Ohio County fair. Twenty-six people that came in contact with the infected pigs developed respiratory disease and two of these people were laboratory confirmed as H1N1 by the Centers for Disease Control and Prevention (CDC). Hence, we genetically and antigenically characterized three H1N1 swine influenza viruses isolated from Ohio in
2004, 2006, and 2007. All viruses were triple reassortants, with genes from human, swine, and avian lineage viruses. Although expressed antigenic similarity, viruses showed antigenic changes in their genetic makeup. All three viruses shared multiple amino acids at the receptor binding domain with human viruses, and two of which replicated significantly in human airway epithelial cells.

Triple reassortant H3N2 influenza viruses emerged in swine in 1998 and then in turkeys in 2003. In 2004, we isolated triple reassortant H3N2 viruses from turkey breeder hens in Ohio and Illinois. The Illinois flock was vaccinated twice with an inactivated vaccine containing H1N1 and H3N2 viruses of swine origin before the outbreak. Using the Archetti and Horsfall formula, three turkey viruses were shown to be genetically and antigenically similar to each other but were antigenically distantly similar to swine virus vaccine strain as well as duck and human viruses of the same subtype.

Testing their interspecies transmission between swine and turkeys, we identified viruses (TR H3N2) with different transmission potential between both species. Some viruses were able to transmit both ways between the swine and turkeys, some transmitted only one way from swine to turkeys and some that did not transmit either way. Interestingly, changes were observed on the HA but not the NA protein upon transmission of A/turkey/Ohio/313053/04 virus between swine and turkeys.

Using reverse genetics, we created single gene reassortants between two strains of potentially different transmission behavior, and tested their replication in pigs and
turkeys. It was evident that the hemagglutinin gene plays a critical but not the only role for efficient replication of the above viruses in swine and turkeys.

The transmissible strain between swine the turkeys (A/turkey/Ohio/313053/04) was shown to bind most efficiently to plasma membrane preparations from swine and turkeys tracheal/bronchial epithelial cells as compared to other viruses.
Dedication

To him who taught, inspired and sculpted my personality,
Imam Ali Son of Al-Husain “Zein-el-Abdin”
Peace be upon them
I thank the Almighty GOD for all the blessing in my life.

I would like to express my absolute thanks and gratitude to my advisor throughout my Ph.D. study, Dr. Y.M. Saif. I thank him for his fatherhood attitude, advice, guidance and patience through all the years I spent under his supervision. It was a pleasure to work with such a great person who was a source of knowledge and experience in and outside the science field.

I would like express my grateful appreciation to Dr. Chang Won Lee on his help, insight, and critical evaluation of my work. I thank my committee members Dr. Daral Jackwood and Jeffrey Lejeune for their advice and constructive comments on my work.

Many thanks to current and previous laboratory colleagues, Dr. Yuxin Tang, Dr. Shamaila Ashraf, Abdul Rauf, Maria Murgia, and very special thanks to Robert Dearth for his assistance and help.

I thank Dr. Mahesh Khatri for his contribution to my work.

I thank the members of Dr. Lee laboratory: Leyi Wang, Smitha Pillai and Megan Strother for giving me the help when ever needed.

I would like to express my deep appreciation to all whom helped with my experiments, Dr. Juliette Hanson, Todd Root, Gregory Myers, Kingsly Wanosik, and Janet McCormick.
Special thanks to the front desk cheerful couple, Hannah Gehman and Robin Weimer for being very helpful and patient.

I thank my dearest friends Ali Eid, Ali Al-Toufaily, and Issmat Kassem for having me in their prayers and giving me the advice whenever needed.

I would like to express my deep admiration to my father-in-law Karl Schroeder and mother-in-law Karen Schroeder and the rest of the family for their love, kindness, and warm hearts.

I thank you my dearest, Kristi, for your love, prayers, support, and encouragement. I thank you for your patience, understanding, and for entering the serenity to my life. I thank GOD for having you in my life to share the moments of happiness and success.

I love you my beautiful daughters, Zaynab and Ghadeer (Inshaa Allah), and I ask GOD to bless you with Health, Faith and Wealth in this life and the afterlife.

My special recognition and gratitude are addressed to the two splendid and shining faces that enlighten my whole life, planting it with love, hope and grace, my lovely parents Haj Mohamad and Hajja Fadwa Yassine. I thank you Mahdi, Ammar, Ali, Khadeeja, Diyya, and Kumayle, my lovely brothers and sister, for your prayers, support and all great things you have done to me.
VITA

March 21, 1978………………………..   Born-Kuwait, Kuwait.


2004-present…………………..   Graduate Research Associate, Food Animal Health Research Program, Ohio Agricultural Research and Development Center, Wooster, Ohio.

PUBLICATIONS


FIELDS OF STUDY

Major Field: Veterinary Preventive Medicine

Studies on Molecular Virology and Viral Pathogenesis
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>v</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>vi</td>
</tr>
<tr>
<td>Vita</td>
<td>viii</td>
</tr>
<tr>
<td>List of Content</td>
<td>x</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xiv</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xvi</td>
</tr>
</tbody>
</table>

## CHAPTER 1

Literature review: Interspecies and intraspecies transmission of influenza A viruses: viral, Host and environmental factors

1.1 Introduction..........................................................................................1
1.2 Morphology and genetic constellation................................................2
1.3 Viral proteins and virus replication...................................................2
1.4 Pathotypes and ecology..........................................................................6
  1.4.1 Influenza in wild and domestic birds.............................................6
  1.4.2 Influenza in mammals......................................................................11
1.5 Interspecies and intraspecies transmission of influenza A viruses.........15
  1.5.1 Viral factors..................................................................................15
  1.5.2 Host factors..................................................................................27
  1.5.3 Environmental factors.................................................................33
1.6 Discussion..............................................................................................37
1.7 References                                                             .................................................................39

## CHAPTER 2

Characterization of triple reassortant H1N1 influenza A viruses from swine in Ohio

2.1 Summary..................................................................................................52
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1   Similarity of A/swine/Ohio/24366/2007 (H1N1) genes to other viruses’ based on BLAST search in GenBank Database</td>
<td>68</td>
</tr>
<tr>
<td>2.2   Nucleotide percent similarity between triple reassortant H1N1 swine viruses and human viruses</td>
<td>69</td>
</tr>
<tr>
<td>2.3   Cross-HI and VN results expressed as percentage of antigenic relatedness</td>
<td>70</td>
</tr>
<tr>
<td>3.1   Viruses included in the study</td>
<td>97</td>
</tr>
<tr>
<td>3.2   Cross-HI and VN results expressed as percentage of antigenic relatedness</td>
<td>98</td>
</tr>
<tr>
<td>3.3   Genetic similarity of the Illinois turkey virus to other H3 subtype viruses</td>
<td>99</td>
</tr>
<tr>
<td>4.1   Interspecies Transmission of H3N2 influenza viruses from pigs to turkeys; Virus detection in inoculated pigs</td>
<td>124</td>
</tr>
<tr>
<td>4.2   Interspecies Transmission of H3N2 influenza viruses from pigs to turkeys; Virus detection in turkeys in contact with inoculated pigs</td>
<td>125</td>
</tr>
<tr>
<td>4.3   Interspecies Transmission of H3N2 influenza viruses from turkeys to pigs; Virus detection in inoculated turkeys</td>
<td>126</td>
</tr>
<tr>
<td>4.4   Interspecies Transmission of H3N2 influenza viruses from pigs to turkeys; Virus detection in pigs in contact with inoculated turkeys</td>
<td>127</td>
</tr>
<tr>
<td>4.5   Intraspecies transmission of A/TK/OH/313053/04 (H3N2) influenza virus in chickens, ducks and turkeys</td>
<td>128</td>
</tr>
<tr>
<td>5.1   Reverse genetics generated viruses</td>
<td>150</td>
</tr>
</tbody>
</table>
5.2 Replication and transmission of reassertant strains in turkeys..........................151

5.3 Replication of reassertant strains in pigs.........................................................152
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Phylogenetic relationship of H1N1 influenza viruses based on HA &amp; NA genes</td>
</tr>
<tr>
<td>2.2</td>
<td>Phylogenetic relationship of H1N1 influenza viruses based on PB1, PB2 and M genes</td>
</tr>
<tr>
<td>2.3</td>
<td>Amino acids sequence alignment of the HA1 protein of H1N1 influenza viruses</td>
</tr>
<tr>
<td>2.4</td>
<td>Replication of swine and human H1N1 viruses in HBEpC cells</td>
</tr>
<tr>
<td>2.5</td>
<td>Immunofluorescence for swine viruses in HBEpC cells</td>
</tr>
<tr>
<td>3.1</td>
<td>Phylogenetic relationship based on nucleotide sequences of the H3 gene of different H3 subtype viruses</td>
</tr>
<tr>
<td>3.2</td>
<td>Phylogenetic relationship based on nucleotide sequences of the N2 gene of different H3 subtype viruses</td>
</tr>
<tr>
<td>3.3</td>
<td>Phylogenetic relationship based on nucleotide sequences of the NP gene of different H3 subtype viruses</td>
</tr>
<tr>
<td>3.4</td>
<td>Phylogenetic relationship based on nucleotide sequences of the M gene of different H3 subtype viruses</td>
</tr>
<tr>
<td>3.5</td>
<td>Phylogenetic relationship based on nucleotide sequences of the PB2 gene of different H3 subtype viruses</td>
</tr>
<tr>
<td>3.6</td>
<td>Alignment of the HA1 protein sequences of various H3 subtype influenza A viruses</td>
</tr>
<tr>
<td>3.7</td>
<td>Cartoon representing the amino acid changes at the major antigenic sites of the HA1 Molecule of H3 subtype influenza A viruses</td>
</tr>
<tr>
<td>4.2</td>
<td>Schematic of the room used in the study of interspecies transmission of influenza A viruses between swine and turkeys</td>
</tr>
</tbody>
</table>
4.3 Cartoon representing the amino acid changes at the RBD of H3 molecule of the A/turkey/Ohio/04 virus upon transmission between swine and turkeys........130

5.1 Binding of H3N2 influenza viruses to plasma membrane preparations from MDCK, PEC and TEC cells.................................................................153
Antigenic relatedness (R-values)
Basic Local Alignment Search Tool (BLAST)
Brain heart infusion (BHI)
Cytopathic effect (CPE)
Days post inoculation (DPI)
Embryonated chicken eggs (ECE)
Hemagglutinin (HA)
High pathogenic avian influenza (HPAI)
Hemagglutination inhibition (HI)
Human tracheal/bronchial epithelial cells (HBEpC)
Low pathogenic avian influenza (LPAI)
Maackia amurensis (MAA)
Madin-Darby Kidney Canine (MDCK)
Matrix (NA)
Neuraminidase (NA)
Non-structure (NS)
Nucleoprotein (NP)
Nuclear export protein (NEP)
Open air factors (OAF)
Polymerase basic 1 (PB1)
Phosphate buffered saline (PBS)
Pig tracheal/bronchial epithelial cells (PEC)
Plasma membrane preparations (PMP)
Polymerase basic 2 (PB2)
Polymerase acidic (PA)
Real-time PCR (rRT-PCR)
Receptor binding domain (RBD)
Receptors binding domain (RBD)
Sambucus nigra (SNA)
Sialic acids (S.A.)
Site directed mutagenesis (SDM)
Specific pathogen free (SPF)
Triple reasortants (TR)
Turkey tracheal/bronchial epithelial cells (TEC)
United States (U.S.)
Virus neutralization (VN)
World Health Organization (WHO)
50% tissue culture infectious does (TCID<sub>50</sub>)
50% embryo infectious does (EID<sub>50</sub>)
CHAPTER 1

LITERATURE REVIEW

Interspecies and Intraspecies Transmission of Influenza A Viruses: Viral, Host and Environmental Factors

1.1 INTRODUCTION

Influenza A viruses are enveloped viruses belonging to the family *Orthomyxoviridae* that encompasses four more genera: Influenza B, Influenza C, Isavirus and Thogotovirus (95). Influenza A viruses belongs to the only genus that is highly infectious to a variety of mammalian and avian species. They are divided into subtypes based on two surface glycoproteins, the hemagglutinin (HA) and neuraminidase (NA). So far, 16 HA and 9 NA subtypes have been identified worldwide, making a possible combinations of 144 subtype between both proteins (95). Generally, individual viruses are host specific, however, interspecies transmission of influenza A viruses is not uncommon (69, 129). Most of the HA and NA subtypes have been isolated from wild birds, however, infections to human and other mammalian species are limited to few subtypes. Replication of individual influenza A virus in a specific host is dependent on many factors, including, viral proteins, host system and environmental conditions (69, 95). In this review, key findings that contribute to transmission of influenza A viruses amongst different species are summarized.
1.2 MORPHOLOGY AND GENETIC CONSTELLATION

Influenza A viruses are polymorphic in structure with a diameter of approximately 100-120nm. Like other orthomixoviridae viruses, their genome consists of negative sense, single stranded, and segmented RNA strands. The segmented nature of their genome enables reassortments, and thus exchange of genetic information among different viruses upon co-infection of a host cell (95). The outer layer of the virus structure consists of lipid material (envelop) that is obtained upon the release of the virus from the infected host cell. Beneath it lays the matrix protein (M1) and through both layers projects two spike structures, the hemagglutinin (HA) and neuraminidase (NA) of ratio four to one, respectively (95). The core of the virus encompasses the genetic material in the form of ribonucleoprotein (RNP) that is comprised of eight RNA segments surrounded by the nucleoprotein (NP). The eight RNA segments encode for at least 11 identified proteins recognized as: Polymerase basic 1 (PB1 and PB1-F2), polymerase basic 2 (PB2), polymerase acidic (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M1 and M2) and non-structural (NS1 and NS2) proteins (95).

1.3 VIRAL PROTEINS AND VIRUS REPLICATION

To date, eleven proteins have been identified in the influenza A viruses. The two surface glycoproteins, HA and NA, play roles in virus entry and release from the host cells, respectively. The HA is a trimeric rod-shape molecule that binds to the host cell receptor and has the immunogenic epitops of the virus. It is inserted through its carboxy terminus to the viral membrane, and holds the receptor binding site (RBS) and the antigenic sites (AS) at the globular hydrophobic ends away from the viral surface (95). For its full
activity, the HA protein should be cleaved into two subunits identified as HA1 and HA2 subunit molecules (50, 55).

The HA protein recognizes neuraminic acids (sialic acids) on the host cell surface and it is believed to be a host specific recognition. Upon binding to host cell receptor, the virus enters to the host cell mainly through cathepin-mediated endocytosis (11, 68, 118). A drop in pH occurs due to the inflow of H+ ions from the endosome into the virion particle through the trans-membrane ion channel, M2 tetramer protein. This induces conformational changes in the cleaved HA molecule and enables it to fuse with the endosomal membrane. Viral RNPs are then released into the cytoplasm (116, 126). The acidification process also impairs the M1-RNPs interaction, causing the dissociation of the RNPs that enter the nucleus subsequently (67). RNPs entrance to the nucleus is mediated through the interaction of NP nuclear localization signal (NLS) with its receptor protein in the cytoplasm of the cell, Karyopherin α1 and α2 (155). Viral RNA synthesis in the nucleus occurs through two events, replication and transcription. In the transcription process, the vRNA is transcribed into messenger RNA (mRNA) in a primer dependent process. The mRNAs are incomplete copies of the vRNA template, capped at 5'-ended and polyadenylated at the 3'-end. In the replication process, the negative sense viral RNA (vRNA) serves as a template for the production of full-length complementary RNA (cRNA). This will in turn serve as a template for the production of negative sense vRNA. All of these reactions are catalyzed by the viral RNA polymerase complex made of three protein subunits: PB1, PB2 and PA (84). PB1 forms the central component of the complex and usually binds to both PA and PB1 proteins through its N-and C-terminal domains, respectively (31). PB1 have been shown to be implicated in the sequential
addition of nucleotides during RNA chain elongation. It also possesses endonuclease activity which generates the capped primer required for mRNA synthesis (15, 35, 84). Capped primers are originated from cellular pre-mRNA in an activity defined as “cap-snatching”, involves PB2 cap-binding activity, and PB1 endonuclease activity (22). No specific role has been described for the PA subunit, however, mutations in this gene have been shown to impair replication and transcription (23, 42). The switching mechanism between transcription and replication reactions is not fully understood, and it is believed that the structure of the polymerase complex in the two processes is different. This is evidenced by the fact that different domains of PB1 are needed to bind vRNA versus cRNA. Additionally, the cap-binding and endonuclease activity of PB2 and PB1 are required for one process (transcription) but not the other one (replication) (29, 30, 95).

The two shortest mRNA encoded from segment 7 and 8 are further spliced in the nucleus. For segment 7, the unspliced mRNA encodes for M1 protein, while the spliced form of mRNA encodes for M2 protein. For segment 8, unspliced mRNA produce NS1, while the spliced form produce NS2 protein (95).

The mRNAs are then translated into proteins in the endoplasmic reticulum, folded and modified in the golgi apparatus of the host cell cytoplasm. HA and NA proteins (envelope proteins) have apical sorting signal and get inserted in the lipid raft, while M2 protein gets excluded from lipid rafts (9). The rest of the proteins (non-envelope proteins), move to the nucleus for the formation of RNP complex with the vRNA (11). Export of RNPs from nucleus into the cytoplasm requires two viral proteins, M1 and NS2 (also known as nuclear export protein (NEP)). M1 appears to co-interact with RNPs and NS2 proteins, which from its side binds to the nucleoporins, components of nuclear pore
RNP-M-NS2 complex then mediate the exit of RNP via interaction with Crm1, a nuclear export receptor (21). Imported RNPs from the nucleus are then packaged into budding virion particles. Although the M1 protein has been shown to be the key player in the budding of progeny viruses from infected cell, other proteins like HA and NA were also shown to be essential for this process (13, 28). M1 protein accumulate under the plasma membrane of the infected cell, and initiate binding to the cytoplasmic tails of HA and NA glycoproteins that are inserted into the cell membrane (9). The availability of M1 protein has been shown to affect timing of assembly and maturation. It is believed that both M1 and M2 proteins with the help of the cytoskeleton are needed to capture the RNPs at the site of assembly (5, 74, 96). Full component of eight RNA segments are required for correct assembly and packaging. The accumulation of M1 at the inner leaflet of the lipid raft initiate curvature formation in the plasma membrane. The process is completed by the fusion at the base of the bud, where the enveloped particle is formed and released from the cell (82, 83). To prevent the aggregation of viruses at the release site, the enzymatic activity of the NA (tetramer) protein is needed to remove the sialic acids linked to the glycoproteins at the cell membrane and the newly budded viral particles (97). In addition to its role in cleaving sialic acids, NA can sequester cellular plasminogen, which is then get activated to plasmin, an enzyme that can cleave HA protein without requiring multiple basic amino acids at the cleavage site (32). The NS segment encodes for proteins that are not included into the virion particles. NS1 mediate various activities from modulating cell machinery for virus replication, to inhibiting interferon (IFN) antiviral activity. NS2 protein plays a critical role in transport of viral particles from and into the nucleus. PB1-F2 is a short protein encoded by the PB1
segment in some but not all influenza viruses, and it appears to enhance virus-induced cell death in a cell type-dependent manner (17).

1.4 PATHOTYPES AND ECOLOGY

Influenza A viruses is the only genus of *orthomyxoviridae* that has been shown to infect a great variety of animals including humans, wild and domestic birds, swine, horses, seals, whales, canines, minks and others (162). Infection with influenza A virus results in a wide range of clinical outcomes, depending on virus strain, virus load, host species, host immunity, and environmental factors.

1.4.1 *Influenza in wild and domestic birds*

Based on pathogenicity in chickens, influenza A viruses are classified into two main pathotypes: highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) (1). Infection with LPAI (includes all subtypes) might cause little or no disease, high morbidity and low mortality in some occasions. On the other hand, infection with HPAI viruses results in at least 75% mortality in the infected poultry, and it is solely caused by viruses of H5 and H7 subtype (137). LPAI viruses are characterized by single arginine (basic amino acid) at the cleavage site and another basic amino acid at position three or four upstream from the cleave site (depending on the virus subtype). HA protein of LPAI viruses is limited to cleavage by extracellular proteases (trypsin-like) that are secreted by cells or bacteria at the site of infection (ex. trachea and intestine). On the other hand, HPAI viruses possess multiple basic amino acids at the HA protein cleavage site which make them prone to cleavage by intracellular ubiquitous proteases, like the furin enzyme (110, 128, 150).
Wild birds, especially *Anseriformes* (ducks, geese, swans) and *Charadriiformes* (gulls, terns, surfbirds), are considered the natural reservoir for influenza A viruses. Most recent report showed that these viruses have been isolated from at least 105 wild bird species from 26 families (92). In an older study performed by Stallknecht and Shane (122) a virus isolation recovery rate of 10.9% (2317 of 21318 sample) was observed in samples collected from wild birds. 15.2% recovery rate was noticed for *Anseriformes*, 2.9% for *Passeriformes*, and 2.2% for *Charadriiformes*. LPAI viruses of all subtypes have been isolated from wild birds, where they cause little or no disease in the infected birds. Few HPAI viruses have been isolated from wild birds, represented lately by HPAI H5N1 in Asia, Europe, and Africa (137). Sharp et al. (113) suggested that the waterfowls do not act as a reservoir for all avian influenza viruses, where part of influenza gene pool are maintained in shorebirds and gulls.

Infection of poultry with avian influenza A viruses have been always blamed on the transmission of the viruses from wild bird (1, 137). This could be due a direct contact of wild birds with range-raised commercial poultry, and/or contaminated water, feed, fomites, and human workers with faeces from wild birds (137).

In addition to subclinical infections, LPAI might cause high morbidity (>50%) and low mortality (<5%) in infected poultry (138). However, infection of poultry with HPAI leads to systemic infection with lethality up to 100% in the infected flock (138).

Poultry infection with influenza A virus was first reported in Italy, 1878. The causative agent was first recognized as fowl cholera and it was not identified as influenza A virus (HPAI) until 1955 (137). Thus, HPAI have been recognized for more than 100 years. At least 21 primary outbreaks with HPAI in poultry have been reported, resulting in
death/culling of billions of birds over all continents (2, 16). Milder diseases (primarily respiratory disease) are usually caused by LPAI compared to HPAI viruses, and were first reported in the middle of last century. The oldest existing LPAI was isolated in Germany from chicken in 1960 of H10N7 subtype (137). Since then, LPAI viruses have been frequently isolated from domestic poultry, often from chicken and turkey.

Chicken and turkeys are not considered natural reservoirs of influenza viruses, however, due to the altered natural ecosystems by human (ways of raising and marketing poultry), new niches were created for occurrence and distribution of influenza infections amongst poultry (137). Until 1998 in the United States (U.S.), when the industry decided to eliminate range rearing of turkeys, wide variety of AI subtypes were isolated from turkeys due direct contact with wild birds (37). Additionally, swine influenza A viruses have been isolated frequently from turkeys since early 1980s in the U.S. The first report of swine influenza (H1N1) in turkeys was in 1981 (77). Since 2003, new form of swine influenza emerged in turkeys and was subtyped as triple reassortant (TR) H3N2 virus (164).

Old and recent findings suggested that turkeys are more susceptible to influenza infections as compared to chicken (2, 102). For example, the H7N2 LPAI isolated from Virginia in 2002 was more infectious in turkeys compared to chicken under laboratory conditions, transmitted more easily amongst turkeys which required lower doses to be infected (137). Influenza was reported in turkeys for the first time in 1963 in the U.S. (2). Since then, various subtypes were isolated from turkeys in many states within the U.S. Isolation of these viruses were mostly reported from states that are heavily populated with turkeys and situated on migratory waterfowl flyways, example California and
Minnesota. In those two states, outbreaks occurred every year since 1966 causing
tremendous economic losses (38). In 1995, two major outbreaks were reported in turkeys
in the U.S. The first in Utah (H7N3) associated with 40% mortality in young birds and
was associated with co-infection with *Escherichia coli* or *Pasteurella Multocida*. The
other outbreak occurred in Minnesota (H9N2) causing tremendous economic losses
estimated in millions of dollars in Minnesota State alone (38). The elimination of outdoor
raising of turkeys in the State of Minnesota in 1998 dramatically reduced the number of
incidences with avian influenza viruses. Recently, infection of turkeys with TR H3N2
rose to be the primary concern in Minnesota as well as other turkey raising states.
Infections with these viruses elicit no clinical signs, but are associated with complete
cessation in egg production in turkey breeder hens. TR H3N2 viruses were first isolated
from swine in 1998 and then isolated from turkeys in 2003. Thus, it was thought that
these viruses had the ability to transmit from swine and infect turkeys (139).
Despite their higher populations compared to domestic turkeys, primary influenza
outbreak incidences in chicken are less compared to turkeys (2). Major outbreaks in
chicken in North America were due to HPAI viruses, like the Pennsylvania outbreak in
1983 and Mexican outbreak in 1995. Both outbreaks were similar in their progress, where
H5N2 was isolated as a low pathogenic virus earlier to the outbreak, and mutated to a
HPAI causing high mortality and severe lesions in the infected flocks (2). On the other
hand, H7 subtype viruses were responsible for multiple outbreaks in poultry in the U.S.
and other countries around the globe. Until the middle of last century, HPAI infection
with H7 in poultry was referred as fowl plague. Starting from 1870 until mid-twentieth
century, fowl plague (H7 HPAI) was isolated from many regions including several parts
of Europe, Switzerland, North Africa, Middle East, Asia, North and South America. Major recent outbreaks were recorded in Pakistan (H7N3; 1995-2003), Netherlands (H7N7, 2003), British Columbia (H7N3, 2004), and North Korea (H7N7, 2005) (2, 16, 98). Chickens were the primary host for the incidence of disease in all of these outbreaks (136).

Between 1965 and 1966, chicken, turkeys and other avian species were diseased with clinical signs indistinguishable from those caused by fowl plague (H7). Interestingly, viruses from these birds were not inhibited by antisera collected from fowl-plague recovered birds. The disease was first referred to as “fowl plague-like” and was then identified as H5 HPAI.

Since 1996, H5N1 has been the cause of the major ongoing pandemic of HPAI worldwide. This virus was first isolated from Guangdong Province from geese in 1996 in China (137). The virus was then spread to infect various poultry species, and broad range of none poultry animals, including human (117). Between 2003 and 2004, outbreaks with H5N1 HPAI were reported almost concurrently at eight counties in South East Asia. This includes China, Cambodia, Indonesia, Japan, Korea, Loa PDR, Thailand and Vietnam (117). The virus was then spread over big parts of Asia, some countries in Europe and Africa. So far, the virus has evolved (genetic drift/ genetic shift) to form at least 20 clades/subclades in the phylogenetic tree and are recognized as (0, 1, 1’, 2, 2.1 (2.1.1-2.1.3), 2.2, 2.3 (2.3.1-2.3.4), 2.4, 2.5, 3-9) (117).

In addition to the above incidences of influenza in poultry, H9 LPAI has been a continuous problem in poultry since mid-1990 worldwide. Outbreaks H9N2 viruses affected chicken, turkeys, and domestic ducks in Germany, turkeys in the U.S., ostriches
in South Africa, Pheasants in Ireland, chickens in Italy, South East Asia, and the Middle East (1).

### 1.4.2 Influenza in mammals

In contrast to birds and most mammalian species, humans are prone to infection with three types of influenza viruses, A, B, and C (95). Type C viruses are less common in human populations and cause milder diseases compared to the other two types. Infections with type A and type B viruses result in mild to acute diseases in human populations. Up to 142,000 hospitalizations are recorded in the U.S. alone every year as a result of influenza infection, with a mortality rate of ~1% (20,000-36,000) of all deaths in the U.S. (134, 147, 148). Human influenza A viruses were first isolated in 1933. Historically, only viruses of H1, H2, H3, N1 and N2 subtypes have circulated widely in human populations (134). Three main pandemics occurred in the last century in humans due to influenza A virus infections: Spanish influenza (1918; H1N1), Asian influenza (H2N2; 1957) and Hong Kong influenza (H3N2; 1968). Spanish influenza was the most severe of all, resulting in deaths of 40-50 million people worldwide. Bacterial infections in lungs were found to be a prominent trait of fatal cases in the 1918 pandemic. Genetic analysis of H1N1 Spanish influenza viruses revealed avian-like genes with mutations for human adaptation (143). Pandemics caused by H2N2 (1957) and H3N2 (1968) were less severe involving four millions and one million deaths, respectively (49). Genetic characterization revealed that both strains arose from reassortments between avian viruses and co-circulating human viruses. H2N2 virus harbored HA, NA, and PB1 of avian influenza and the rest of genes were of circulating human H1N1 virus. On the other
hand, H3N2 virus harbored the HA and PB1 genes from avian influenza with remaining genes from circulating H2N2 viruses. (134). Currently, H5N1 HPAI represents one of the highest threats to human health worldwide. These viruses arose upon circulation of LPAI in poultry, where they mutated into HPAI infecting several animal species over the globe (162). The first human disease caused by H5N1 was reported from Hong Kong in 1997, involving 18 cases, including 6 deaths (99). Until the preparation of this manuscript, at least 417 cases were reported with H5N1 infection from 15 countries, resulting in death of 257 people (161). Seroepidemiological study performed after the 1997 outbreak in Hong Kong showed that some human infections with H5N1 could be asymptomatic or mildly symptomatic (99). Moreover, avian H7 and H9 subtype transmitted and infected humans in Asia, Europe, and North America. After the major outbreak with H7N7 in poultry in Netherlands (2003), 89 human cases were laboratory confirmed with the same subtype virus. Most of the infected humans developed conjunctivitis, few developed influenza-like illnesses, and one veterinarian died from acute respiratory distress syndrome (ARDS) due to the infection. Two people were also infected with H7N3 subtype virus after an outbreak in Canada in 2004, manifesting self-limited conjunctivitis and influenza illnesses (99). Most of the above infections with the three subtypes (H5, H7, and H9) were due direct contact with infected poultry. Human to human transmission was suggested for three individuals in the Netherlands outbreak (99).

Pigs on the other hand are susceptible for infection with both human and avian influenza viruses and have been always hypothesized to serve as an intermediate host for interspecies transmission of these viruses. H1 and H3 subtypes are the most common influenza subtypes in pigs worldwide, but sporadic infections with other subtype viruses
have been recorded (64, 88, 166). Influenza disease was initially recognized in pigs in 1918, in a temporal and special coincidence with Spanish influenza in human (134). First isolation of the virus (H1N1) was in 1930 and it became to be known as classical H1N1 lineage of swine viruses. Recent research has indicated that swine and human viruses of the 1918 were closely related to each other, genetically and antigenically (49, 104). Classical H1N1 (cH1N1) swine viruses in U.S. remained genetically and antigenically stable until the 1990s, where variants of cH1N1 viruses were isolated (162). In Europe, classical H1N1 viruses disappeared in late 1970s with the introduction of avian-like viruses. Avian-like viruses reassorted with human viruses in pigs resulting in new viruses harboring human-like HA and NA genes, and avian-like internal genes. Reasortants between swine and human viruses have been reported in more than one incidence, like Japan (H1N2, 1978) and Europe (H1N2, 1987) (162). H1N2 viruses are still widely circulating with H1N1 and H3N2 subtypes in pigs in Europe. In the U.S., and starting from 1998, a new form of reassortant viruses (H3N2) emerged in swine populations. These viruses harbored genes from human (HA, NA, PB1), avian (PB2, PA) and swine (NP, M, NS) lineage viruses (156). Interestingly, HA and PB1 genes share the same origin (human lineage), is a similar pattern to the 1957 and 1968 human pandemic viruses (47). Human virus genes in the H3N2 viruses were similar to those contained in human vaccine strains used in the mid-1990s (93). Further reassortments occurred between TR H3N2 and cH1N1 viruses resulting in the emergence of H1N2 viruses. Since then, double and/or triple reasortants of H1N1, H1N2, H3N1 and H3N2 have been isolated from swine in the U.S. (57, 162, 164). Interestingly, more than one of these viruses has been isolated from species other than swine, including human and turkeys. H1N1 TR have been
isolated from humans in more than one location in the U.S. including the states of Ohio, Wisconsin, and Texas (33, 86, 93, 94, 108, 109). TR H3N2 was also isolated from one child in Canada in 2007 (105). Additionally, TR H3N2 has been continuously isolated from turkey breeder hens since 2003 in more than one state within the U.S. (164).

Inoculating and feeding domestic pigs with HPAI H5N1 viruses from different origins (human, chicken, duck, and whooper swan) did not result in a severe disease. Virus replication was restricted to the respiratory tract and tonsils in contrast to mouse and ferret animal models, where some of the viruses were highly pathogenic and replicated systemically. Additionally, virus titers were remarkably lower compared with swine influenza viruses.

As with human and swine diseases, equine influenza has been a problem for many years. Two main subtypes have been recognized in horses, equine 1 (H7N7) and equine 2 (H3N8). The disease is usually more associated with equine 2, with symptoms similar to those observed with human and swine influenza, including fever, coughing, loss of appetite, and muscular soreness (162). H7N7 was first isolated in 1956 and the last confirmed outbreak was reported in 1979. However, sporadic detection of anti-H7 antibodies have been detected with unvaccinated horses. On the other hand, H3N8 was first isolated in 1963 and is still wide spread in horse populations. It caused major outbreaks in the U.S. (1963), China (1989), and widespread epidemics in Europe and around the world (162). Moreover, these viruses were isolated from dogs (greyhounds) with respiratory disease in Florida, suggesting interspecies transmission of these viruses (18). Avian H3N2 influenza viruses were also isolated from dogs with severe respiratory disease in Korea in 2007.
Dogs and other canine species were shown to be infected with influenza A viruses. Serologic studies have shown that H3N8 virus has spread to general dog populations (18). Cats (domestic cats, tigers, Owston civets) were shown to be infected with influenza A virus. H5N1 was isolated from all three species and probable virus transmission was reported amongst tigers (145).

Finally, minks have been infected with influenza viruses, naturally and experimentally. Both human and avian viruses can transmit and infect minks. In 1985, a mink died in Sweden due to an infection with avian-like H10N4 subtype. In addition to avian viruses, minks were also experimentally infected with human, swine, and equine viruses, and the viruses were able to transmit to cage mates (162).

1.5 INTERSPECIES AND INTRASPECIES TRANSMISSION OF INFLUENZA A VIRUSES

Transmission of influenza viruses amongst and between species depends on many viral, host and environmental factors. Below is a summary for the most important factors affecting transmission of influenza viruses.

1.5.1 Viral factors

Influenza host-range restriction is a multigenic trait, where most of the genes play a role in influenza virus adaptation to a specific host. The availability of broad number of viruses’ sequences, and utilizing reverse genetics, site directed mutagenesis (SDM), and other molecular techniques, researchers were able to determine at the gene and amino acid levels the contributors in host-range specificity of influenza viruses.
The hemagglutinin (HA) protein is the major surface glycoprotein that mediates two important functions in virus replication: binding to appropriate receptor on host cell and fusion between viral and endosomal membranes. 400-500 trimeric HA spikes are found on each virus which enables simultaneous interaction of many receptors binding domain (RBD) with multiple copies of cell-surface receptors. The RBD that contains the sialic acids (S.A.) binding residues, is a shallow depression on the protein surface located on membrane distal end of the globular head (72). Some residues at the RBD are rather conserved because of their essentiality in virus interaction with the receptor, and others are variable within different subtypes and different lineage viruses. Residues 98, 131-134, 136-138, 153-155, 183-190, 194-195, 218-220, 224, 226-228 have been postulated to be members of RBD. However, the exact location of the RBD depends on virus subtype. Amino acids at 134, 136, and 153 are preserved throughout the evolution of influenza viruses in different hosts. On the other hand, those at positions 190, 225, 226, and 228 are more variable and more prone to mutations. While human viruses usually have Asp190 (H1N1 and H3N2), Asp225 (H1N1), Leu/Ile/Val226, and Ser228 (H3N2 and H2N2), avian viruses harbor Glu190, Gly225, Gln226, and Gly228 at these positions (72). In addition, avian viruses have conserved amino acids Ala138 and Leu194. Any mutations at these positions could affect the binding of the avian viruses to their corresponding receptors. Interestingly, an H7N7 virus was isolated from domestic chickens, with a deletion in residues 221-228 of the HA molecule. Moreover, a laboratory generated mutant with deletion of residues 224-230 was still able to bind receptors and grow in embryonated chicken eggs (ECE) (19, 131). This indicates a less critical role of residues at this site (220-230) for receptor binding, at least for some type A influenza strains.
Generally speaking, avian viruses are thought to preferentially bind the N-acetylneuraminic acid α2,3-galactose (NeuAcα2,3Gal) form of sialic acid receptors and human viruses preferentially bind to NeuAcα2,6Gal sialic acid receptors. Hinshaw et al. (1983) were the first group to show the contribution of HA in determining host range specificity (40). They demonstrated that a reassortant between human (H3N2) and avian (H2N2) viruses, with HA being from human strain and the rest of the genes form the duck strain, failed to replicate in ducks. Introduction of double mutations (Leu226Gln & Ser228Gly) into the HA protein resulted in the replication of the above reassortant strain in duck intestine. Single Leu226Gln mutation was not sufficient to initiate replication in ducks intestine. It was then verified that the Gln226 responsible to the binding to α2,3-linked S.A. and GLy228 enhances the binding affinity (70, 80, 151). Variations in receptor binding specificity were also observed within avian species themselves. Although gull viruses have higher binding affinity to α2,3-linked S.A., few gull viruses were shown to infect and replicate in ducks. Interestingly, H13 subtype, which is the most prevalent subtype in gulls, has been never isolated from ducks. Ser228 and Try229 were shown to be highly conserved in ducks, and to less extent Leu252, and Tyr223 (46, 69).

Various subtypes were isolated from chickens showed variability in receptor binding specificity. An H8N4 isolated from chicken in Ontario (1968) had duck-like receptor binding specificity, while an H9N2 isolated from chicken in Wisconsin (1966) had human-like receptor specificity (89). The latter subtype (H9N2) has spread in many countries in Asia, Europe, Middle East and Africa since early 1990s, deleteriously effecting poultry production in all of these countries. Similar viruses were isolated from human and pigs in South East Asia, raising concerns about interspecies transmission of
these viruses (62, 100, 166). Avian H9N2 viruses isolated from Hong Kong were shown to carry human like amino acid at residue 226 of the RBD (Leu instead of Gln) plus additional mutations at residues 183, 190, and 225 that are supposed to be conserved in avian viruses. These virus showed ability to bind human like receptors ($\alpha$2,6-linked S.A.), that also exists in chicken tracheal epithelial cells (34, 58, 73). Viruses isolated from pigs in China in 2006 were also harboring Leu226, indicating a significant role played by this residue for adaptation of avian H9N2 viruses in mammalian species. In a recent study using ferrets as a transmission model (120), 2 mutations (at residues 226 and 198) occurred in the HA protein upon adaptation of avian H9N2 in ferret, including Leu at residue 226 of the RBD. Both mutations were shown to be required for transmission of the virus amongst ferrets. The same group (153) has previously shown that Leu226 determines cell tropism and replication of H9N2 in human air way epithelial cells. Transmission of avian viruses to humans is not uncommon. Studies have shown that an avian H1N1 virus with human adapted mutations was the cause of the Spanish influenza in 1918. Since 1996, HPAI H5N1 has been isolated from humans in more than one country over the globe. Few mutations at the RBD in both subtypes were shown to alter the receptor binding specificity between $\alpha$2,3 and $\alpha$2,6-liked sialic acid receptors. For the H1N1 (1918) viruses, Asp190 and Asp225 were shown to be important for binding of H1N1 (1918) virus to $\alpha$2,6-linked S.A. receptors, and also for the transmission of these viruses in ferret model. Single mutation (Asp190Glu) decreased the ability of the virus to bind $\alpha$2,6-linked S.A. receptors, while another single mutation (Asp225Gly) increased the ability of the virus to bind $\alpha$2,3-linked S.A. receptors. Double mutations at the above two positions, dramatically decreased the virus ability for binding human-like receptors,
and affected transmission but not replication in ferret model. Interestingly, swine H1N1 isolates from the 1930 showed Asp190, emphasizing the role of Asp at this position for replication in mammals (26).

Since 1996, researchers have been working to understand the mechanisms of transmission of HPAI H5N1 from poultry to human. Although the virus replicate efficiently in the diseased humans, often leading to death, the virus has not yet adapted for human to human transmission. In 2003, H5N1 viruses were isolated from father and son in Hong Kong where the viruses had a replacement at residue 227 of the RBD. Such mutations was shown in the same study to alter the receptor binding specificity, where the human virus was able to bind both α2,3 and α2,6-liked sialic acid receptors (114). Similar mutation (Ser227Asn) was shown to enable an isolate from Vietnam 2004, to bind both α2,3 and α2,6-linked S.A. receptors (127). In a study performed by Yamada et al. (163), different avian and human isolates were analyzed for sequences similarity. Mutations were introduced based on similarities/differences between isolates, and receptor binding specificity to either α2,3 and α2,6-liked sialic acid receptors was evaluated. In vivo analysis revealed that when similar mutations applied to viruses from different clades (I and II), variable results were obtained. Single mutations in Gln192Arg, Gly139Arg, Asn182Lys, and Asn193Lys, enabled a virus from clade I to switch binding specificity to α2,6-linked S.A. receptors. Double and triple mutants in residues 75, 123, 167 (with or without Asn193Lys) were required to enhance binding to 2,6-linked S.A. receptors. On the other hand, only Gln192Arg and Asn193Lys mutations enabled a virus from clade II to support binding to α2,6-linked S.A. receptors. Other mutations, Gly139Arg and Asn182Lys, were not sufficient to enable binding to human receptors,
where the last mutation abolished binding for both $\alpha_{2,3}$ and $\alpha_{2,6}$-linked S.A. receptors. HA specific structure/geometry and the proximity of amino acids residues play a role in which mutations might be suitable for one strain but not the other in determining receptor binding specificity.

In summary, different HA subtypes requires various mutations at the RBD to switch binding specificity between $\alpha_{2,3}$ and $\alpha_{2,6}$-linked S.A. receptors. For H2 and H3, Gln226Leu and Gly228Ser are required to switch from avian to human binding specificity. In H1, Glu190Asp is critical for adaptation to human-like receptor binding. In H5, contradictory findings were recorded depending on the type of strain used in each study, however, mutations close to those happen in H3 might play a role in elevating the binding ability to $\alpha_{2,6}$-linked S.A. without abolishing the binding ability to avian-like receptors.

Receptor affinity can also be modulated by glycosylation and sialylation of the HA head domain. Human H1 viruses show hyperglycosylation compared to viruses from aquatic birds and pigs (43). Hyperglycosylation of HA head was associated with increased virulence of H7 subtype viruses in chicken in Australia and Italy. It has been suggested that hyperglycosylation of HA, combined with compensating NA stalk deletion, modifies the progenitors aquatic bird virus prior the development of virulence during virus evolution in chicken (6-8, 71, 101).

Role played by NA protein in determining host-range specificity is very minor compared to that of HA molecule. However, functional compatibility of receptor binding properties with NA cleavage specificity, along with the stalk length of NA protein, is very critical. As the case with HA, NA can specifically cleave S.A. in the form of $\alpha_{2,3}$ and/or $\alpha_{2,6}$
linkage. The mechanism of this specificity is not well characterized, but in one study it was shown that specificity of NA (N8) is associated with amino acid position 275, as well as glycosilation at residue 144 (107). As another form of compatibility between HA and NA proteins, reduction in NA specific activity is required in human viruses to maintain optimal balance with HA protein that shows less affinity to its receptor compared to avian viruses (6). Changes in the NA protein upon transmission of influenza virus to a new host usually occurs to accommodate compatibility with HA protein in the new host. For example, many of NA stalk deletions have been recorded upon transmission of wild bird viruses (especially duck) into chickens. This has been recorded for three major subtypes that cause diseases in chicken H5, H7 and H9 influenza A viruses (8, 41, 119, 125). Types of gangliosides (with short and long sugar chains) that are distributed in guts of chicken and ducks, and to which influenza viruses bind, may play a role in NA proteins acquiring stalk deletions when the virus transmit from ducks to chickens. NA stalk deletion was also observed in a swine H3N2 isolate from North Carolina in 2003 (165). A study by Luo et al. demonstrated that deletions up to 28 amino acids and insertions up to 41 amino acids of the NA stalk protein did not abolish the formation of progeny viruses in vivo (63). In a separate study (75), a human mutant influenza virus with 24 amino acids NA stalk deletion, was shown to replicate like wild type strain in MDCK cells but not in embryonated chicken eggs (ECE). Upon passaging for several times in ECE, the virus acquired competence in two mechanisms: 1-Some viruses acquired insertions in the NA stalk from PB1, PB2, or NP protein, in a rare RNA/RNA recombination process, while others expressed mutations in the HA protein, affecting its binding to S.A. linked receptors (75). Both mechanisms of adaptation requires interaction between the NA and
HA proteins, emphasizing on balanced HA/NA activities for efficient replication of influenza A viruses. On the other hand, treatment of infected individuals with neuraminidase inhibitors has resulted in multiple mutations in the NA protein (example: Glu119V, Arg292Lys, and His274Tyr). Such mutations were shown to significantly compromise virus infectivity and pathogenicity as compared to wild type. It was also shown that Arg292Lys reduced the transmission of the mutant strain significantly in a ferret model as compared to wild type stain (39).

Contribution of RNPs in determining host range specificity is maintained through three main mechanisms: 1- increasing genetic diversity from which variants with high adaptation and transmission potential are selected in the new host; 2- proper interaction with host proteins, and thus generation of a suitable environment for efficient virus replication; 3- generation of escape mutants to avoid the innate immune system of the new host.

As the case for the surface glycoproteins, adaptation through RNP occurs either directly in the new host, or in an intermediate host between the donor and recipient species (81). Genetic analysis for the NP protein of the 1918 Spanish influenza revealed that its sequence is divergent from that of the wild bird viruses, indicating that the virus (H1N1) evolved for a long time in an intermediate host (distinct from wild birds) before emerging in human population. In contrast, when PB1 gene was introduced (along with HA and NA gene) to human virus genetic background by genetic reassortment in the 1957 (H2N2) and 1968 (H3N2) pandemics, its sequence was more close to that of avian viruses than the concurrent circulating human (H1N1) viruses as evaluated by phylogenetic tree (81). Generally speaking, the PB2, PA, and NP protein seems to share
similar evolutionary pathways, suggesting co-evolution due to strong physical and functional interaction with each other (81).

Out of 52 host-associated genetic signatures identified (14), 35 are linked with the RNP (2, 8, 10, and 15 for PB1, PB2, PA and NP, respectively). The first evidence about the contribution of RNPs in determining host range of influenza virus came from Almonds et al. in 1977 (3). Generating reassortant viruses using traditional plaque assay method, it was revealed that extended host range for one strain was conferred on PB2 segment. Utilizing the same technique, reassortants were generated between human and avian viruses to identify genes and their amino acids role in replication of influenza viruses in different host systems. It was shown that, HA and NA genes alone (of human virus lineage) did not confer good replication of the virus in squirrel monkeys if the other six genes were of avian origin (81). Later on, it was shown that the most significant genetic signature for host adaptation is located at residue 627 of PB2. In human viruses, Lys is usually encoded at that residue, while most avian viruses have Glu at this position. Although the PB2 was of avian origin in the 1918 Spanish influenza virus, Lys was recognized at the PB2-627 residue (142). Such substitution was also observed in many of the human H5N1 isolates, where in one study, 23 out of 43 tested isolates had this substitution. Interestingly, Lys was also observed in H5N1 isolates from tigers in Thailand, but not in raccoon dogs isolates from china which maintained avian-like Glu at this position (4, 56, 66, 81, 103). Moreover, during the H7N7 virus outbreak in Netherlands in 2003, same Glu627Lys substitution was found in virus isolated from human with fatal pneumonia, but not with cases of conjunctivitis in the same outbreak (24). On the other hand, swine and equine isolates of avian-like lineages, retained Glu at
this position, suggesting less selective pressure for this residue in both species (115). When PB2 from duck strain (A/Mallard/78) was introduced to a human strain (A/Los Angeles/78), the obtained reassortant had a restricted growth in MDCK cells and squirrel monkey. Revertants from the reassorted strain that were able to replicate in both systems showed single substitution (Glu627Lys) that was responsible for extended host range phenotype (133). Using the ferret model to study influenza transmission, Lys at position 627 of PB2 protein was necessary and sufficient for respiratory droplet transmission, but not required for direct contact transmission, of 1918 H1N1 virus amongst ferrets (149).

Studying the pathogenesis of H5N1 (A/Vietnam/1203/04) and H7N7 (A/Netherlands/219/03) in a mouse model, it was shown that PB2 gene alone, with or without other genes, contributed to virulence of the above two strains in mice. In both cases, substitution of Glu with that of Lys determined the outcome of infection in mice. However, Presence of either Glu or Lys at PB2-627 does not strictly correlate with the severity of human infection with H5N1(27).

In addition to its role in virulence and host range adaptation, PB2-627 plays a role in tissue tropism of the virus. Lys at position 627 of the PB2 proteins enabled H5N1 virus to replicate in nasal turbinates and lungs of infected mice, where as viruses with Glu at this position replicated poorly in nasal turbinates. Human viruses with Lys at PB2-627 replicate well at 33°C, while avian viruses with the same substitution replicate poorly at the above temperature. Taking into account that avian viruses replicate at intestinal tract of the birds with body temperature of 41°C, cold sensitivity of avian viruses polymerase proteins might limit the growth of avian viruses in the upper respiratory tract of human and thus limit human-to-human transmission (81).
Another remarkable residue to support adaption of avian influenza in human is amino acid 375 of PB1. While Asn exist at this position in most avian species, most human viruses have Ser at this position. This substitution was the only conserved mutation observed in PB1 gene (avian origin) in viruses from three pandemics in the last century. Additionally, Gly375Ser modification was observed upon introduction of swine viruses to human (47, 142). Nevertheless, PB1-375 cannot be classified as a genetic signature, where some human H3N2 viruses have Asn at this position, and Ser375 is found in a significant proportion of avian isolates.

In addition to the above, PB2_{701}, PB2_{714}, PA_{615} and NP_{319} were associated with high replication and pathogenicity of at least one H7N7 (A/Seal/Massachussets/1/80) and many H5N1 isolates in mice and humans. Role played by these residues in host-range determinacy is mostly significant in combination with PB2-627 substitution (81). In a recent study performed by Steel et al. (124), it was shown that Asn701 introduction could compensate for the reduced transmission of two human viruses (H3N2 and H5N1) harboring avian-like PB2-627(Glu) in guinea pig model.

The molecular mechanisms involved in the above actions is still not fully understood, however, interaction amongst viral and host proteins, play a critical role in viral adaptation and pathogenesis in different host systems. For example, the crystal structure of the C-terminus domain of PB2 protein complexed with human importin α5 showed how the last 20 residues unfold to permit binding to the importin factor. The domain contains three surface residues (including Asp701) implicated in adaptation from avian to mammalian hosts (140). In a more recent study, adaptive mutations in PB2 (Asp701Asn) and in NP (Asn319Lys) enhances binding of these proteins to importin α1 in mammalian
cells. This was paralleled with increased transport of PB2 and NP to the nucleus of the mammalian cells (25). Tarendeau et al. (141), demonstrated that Lys627Glu mutated domain do not show any structural difference, however, a change in charge disrupt a striking basic patch on the domain. RNPs derived from avian viruses showed low levels of polymerase activity in human cells as compared to their counterparts of human lineage viruses. This was due reduced binding of NP to PB1-PB2-PA complex and to the PB2 alone. Both deficiencies were restored upon introducing Lys to PB2-627 residue, suggesting a pivotal role of PB2 in molecular interactions of viral NP and other cellular proteins (54).

Role of other internal proteins in host-range specificity is limited compared to the above ones. M protein of avian viruses was shown to restrict the replication of avian H2N2 and H3N8 in squirrel monkeys and pigs, respectively. Sequence analysis showed 10 sites with amino acids specific for avian and human influenza strains, but which of these contribute to host-range restriction is not known (12, 48, 79). Determinants of pathogenicity have been identified for NS1 and PB1-F2 proteins, as an interferon antagonist and inductions of apoptosis, respectively. To which extent both proteins contribute to host restriction is not fully understood (17, 85). It is speculated that differences in NS sequences might influenza their activities in different host systems. While most of influenza viruses are sensitive to IFN and TNF-α, most of HPAI H5N1 isolates from human and birds expressed resistance to both cytokines in porcine lung cells. This was associated with Glu at residue 92 of the NS1 protein (111).
1.5.2 Host factors

Influenza replication in a specific host cell starts at the interaction of virus hemagglutinin protein with the host cell receptors. Specific receptors for influenza viruses are still not well characterized; however, sialic acids (S.A.) were identified as receptor determinants long time ago (95). S.A. are family of 9-carbon acid sugars that occupy terminal positions on oligosaccharide chains of glycolipids and glycoproteins, forming exclusively $\alpha$-glycosidic linkage (72). More than 40 elements have been identified in this family which include two main species: N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). The first is the most common form and the biosynthetic precursor of other S.A., and the other one found in all mammals except humans. Generally, S.A. are bound in the form of $\alpha$2-3- of $\alpha$2-6- linkage to the D-galactose (Gal), N-acetyl-D-galactosamine (GalNAc), and N-acetyl-D-glucosamine (GlcNAc) of the penultimate sugar (72). Distribution of these receptors varies between species and on the different organs of the same species. Usage of linkage-specific lectins, like Maackia amurensis (MAA, specific for $\alpha$2-3-linkage) and Sambucus nigra (SNA, specific for $\alpha$2-6-linkage), has enabled to study the distribution of S.A. receptors in different species. Unfortunately, different studies have shown conflicting results due the usage of different lectins manufactured at different companies. Nevertheless, interesting findings have been reported in the last few years regarding the distribution of S.A. in different species. In a recent study (52) pekin ducks showed differential expression of receptors in different organs. $\alpha$2-3-linked S.A. were shown to be exclusively expressed in the intestine, supporting earlier findings. Although tracheal epithelial cells were shown to express the $\alpha$2-6-linkage of S.A., however there ratio was very low compared to that of $\alpha$2-3-linkage.
Interestingly, other organs (Kidney vascular endothelium, kidney tubular cells, endocardium, and alveolar cells) were shown to express substantial amount of both receptors. As the case in ducks, chickens expressed mainly $\alpha_2$-$3$-linked S.A. on their intestinal wall, however, $\alpha_2$-$6$-linked S.A. were dominant in the epithelial cells in a ration of 10:1 to $\alpha_2$-$3$-linked form of S.A. They also expressed substantial amount of both receptors in various amounts in different organs, with dominancy of $\alpha_2$-$6$-linkage in the kidney vascular epithelial cells. In another study preformed on turkeys (102), both types of receptors were expressed on the trachea in almost equal amounts, whereas the $\alpha_2$-$3$-linkage was dominant in the turkey intestine. Examining quail as an intermediate host for influenza zoonotic transmission, Wan et al. (154) reported the abundance of $\alpha_2$-$6$ as well as $\alpha_2$-$3$-linked S.A. receptors in both trachea and intestine.

Human on the other hand, were known to express $\alpha_2$-$6$-linkage on their tracheal epithelial cells. However, recent findings (87) have shown that $\alpha_2$-$3$-linked S.A. are expressed on alveolar pneumocytes, which could explain the susceptibility of humans to H5N1 HPAI viruses. Moreover, human express $\alpha_2$-$3$-linked S.A. receptors on their ocular and lacrimal duct epithelial cells, which could explain the conjunctivitis development in many patients infected with avian influenza viruses, specially H7 and H9 subtypes (91). H5N1 HPAI was shown to replicate ex vivo in human lungs, nasophaynx, adenoid, and tonsil epithelial cells. The mechanism behind this kind of replication is still poorly understood.

Pigs express substantial amounts of both $\alpha_2$-$3$-Gal- and $\alpha_2$-$6$-Gal- linked S.A. receptors on their tracheal epithelial cells. Hence, pigs have been hypothesized to serve as “mixing
vessel” where both avian and human virus can co-infect and from reassortant strains in pigs. Though pigs express both types of receptors, avian-like swine viruses showed a shift in receptors binding specificity over time. Up to 1984, viruses isolated from pigs in Europe recognized both types of receptors, where those isolated after 1985 were strictly binding the Neu5Ac(α2-6)Gal-linked receptors. Ser142Leu mutation was shown to contribute to the loss of binding to Neu5Ac(α2-3)Gal-linked receptors (44). In a further study, Glu190Asp and Gly225Glu were shown to be likely possible for initial changes in receptor binding specificity (70). Although still not proven experimentally, consistent results from two reports suggest that swine H1 and H3 viruses bind Neu5Gc not Neu5Ac receptor types (72).

Equine (H3 and H7 subtype) and seal (H7 subtype) influenza viruses are more like avian viruses than mammalian viruses. H3 equine viruses have stricter receptor specificity compared to their counterparts from duck origin. They only agglutinate erythrocytes with Neu5Ac(α2-3)Gal-terminated receptors, where some duck viruses can agglutinate Neu5Ac(α2-6)Gal-containing erythrocytes. This was supported by the finding that horses express solely α2-3-linked S.A. receptors on their tracheal wall (135). Although equine tracheal tissues are lined with mainly Neu5Gc-containing receptors, equine viruses bind both Neu5Ac and Neu5Gc type of receptors.

Influenza viruses isolated from seals are mostly of avian origin, some of which displayed lower binding affinity to Neu5Ac(α2-3)Gal-containing receptors without increase in binding affinity towards Neu5Ac(α2-6)Gal (72). Dogs also get infected with avian (H5) and equine (H3) influenza viruses. α2-3-linked S.A. were shown to present in dogs
trachea (65), which support the susceptibility of dogs to both avian and equine influenza. No reports have been made about influenza receptor distributions in canine species. The actual virus binding to its receptor has been shown to be greatly affected with a lot of factors that control the chemistry of binding between molecules. Human viruses bind Neu5Ac(α2-6)Gal(β1-4)GlcNAc (6’SLN) essentially stronger than Neu5Ac(α2-6)Gal(β1-4)Glc (6’SL). In contrast, avian viruses bind to both forms, 3’SL and 3’SLN (76). Avian viruses bind strongly to Neu5Ac rather than the Neu5Gc (45). Moreover, duck and shore bird viruses bind with greater affinity to Neu5Ac(α2-3)Gal(β1-3)GlcNAc containing receptors than Neu5Ac(α2-3)Gal(β1-4)GlcNAc. Chicken viruses bind more strongly to gangliosides with longer sugar chains as compared to duck viruses which bind strongly to those with short sugar chains.

To sum up, influenza binding to its receptor on the host cell is a complex interaction including several factors on both sides of the reaction. There are currently more questions than answers to explain the specificity of binding of influenza viruses to host cells receptors. Further investigations are needed on biological, chemical and physical aspects of the binding to understand this process.

Another factor contributing to transmission of influenza virus within and between species is the availability of naïve and susceptible population. Immunity against influenza virus in a specific host is gained by many factors, including the presence of natural barriers that prevent virus replication, vaccination or pre-infection of the host with homologous and/or heterologous virus, genetic makeup of the host, and others. Chimpanzees for example are characterized by respiratory tract secretions containing mucins that can specifically bind viruses before they reach the airway epithelial cells. This could explain the relative
resistance of this species to experimental respiratory exposure to human influenza viruses (53). In a recent study, it was shown that infection of seven laboratory inbred mice with H1N1 (PR8) and H7N7 (SC35M) viruses resulted in different weight loss kinetics and survival rate after in infection. Additionally, the inbred mouse strains showed distinct susceptibility (LD50) to infection, and expressed differential virus replication titers and cytokines production upon infection. Mechanistics for such variations are not identified, and similar studies should be done on other species. It is worth mentioning here that two of the mice strains were deficient for $\text{Mx1}$, a major effector of interferon response to inhibit virus replication, and the other strains carried mutated $\text{Mx1}$. Whether these variations alone, or together with other elements, explain the above findings, requires further investigation (121). $\text{Mx}$ proteins are GTPases that are expressed in the nucleus and cytoplasm of rodent cells, but mostly in the cytoplasm of other vertebrate species. Expression of $\text{Mx}$ protein in mice was associated with high degree of resistance to influenza infections, $\text{in vivo}$ and $\text{in vitro}$ (36). The antiviral activity of $\text{Mx}$ proteins are reported in many species, including mammals, birds, and fishes. They are polymorphic in most species, and thus, they have different modes of action in different species. Their mechanism of action is not fully understood, however, it is suggested that $\text{Mx}$ proteins binds to essential viral components (mostly NP) during virus replication and block their function (36). In an earlier study, the expression of chicken and duck $\text{Mx}$ genes in avian and mammalian cell lines (including mouse 3T3 cells) demonstrated no antiviral activity for several viruses including influenza (132). The $\text{Mx}$ of chicken in that study was cloned from White Leghorn breed in Germany. In a later study, Ko et al. (51) demonstrated the existence of highly polymorphic $\text{Mx}$ gene in different chicken breeds. Some $\text{Mx}$ proteins
expressed resistance to influenza and other viruses, and such activity was associated with residue 631 of the $Mx$ protein. On the other hand, and based on the above findings, duck $Mx$ protein lacks antiviral activity. This is very surprising, since ducks are natural reservoirs for influenza that replicate in their guts without causing evident disease. A comparison study like the one performed in chicken should be done on ducks before drawing any conclusions. Generally speaking, duck immune system is quite different from that of chicken and other poultry species. Ducks have been reported to produce poor antibody response and express low HI antibody activity to natural and experimental infection with avian influenza viruses (132). The inability of ducks to produce hemagglutinating antibody was also associated with other deficiencies in duck antibodies, including precipitation, complement activation, and opsonization. The presence of only two constant regions in the heavy chain of the smaller IgY form in ducks, probably eliminate functions associated with Fc part of antibody, including hemagglutination (132). In a recent study at the University of California, ducks and chickens peripheral blood mononuclear cells expressed distinct levels of cytokines upon infection with avian H11N9 LPAI. How such difference in host response between the two species are related subclinical and persistent infections observed in ducks is something to be further investigated.

Influenza viruses from wild birds (including ducks) show a lower rate of evolution compared to those viruses from poultry and mammals (130). This might be explained by the difference in immune pressure (poorer in duck compared to chickens and human) exerted on the replicating viruses in different hosts. When the virus jumps from one species to another (example from ducks to chickens) it becomes more prone to mutations,
and thus, antigenic drift might result in more transmissible and pathogenic viruses. On the other hand, the pre-existing immunity in the host due to prior immunization or natural infection, might protect against clinical signs from LPAI or HPAI infection but not necessary against shedding of the virus. In such a case, there is a probability of generation of many mutants in the shed material (quasispecies), with potentially different behavioral characteristics than the parental strain.

1.5.3 Environmental Factors

In addition to host and virus specific factors, transmission of influenza viruses depends strongly on many environmental conditions. This includes virus load (in air and on fomites), mode of transmission, stability of the virus in nature, host behavior, social organizations, weather conditions, and many others. So far, three modes of transmission have been subjected for research and discussion: droplet, airborne, and contact transmission. The first requires direct spray of large droplets via sneezing or coughing onto mucosal surfaces of a susceptible host. Airborne transmission occurs through what is known as droplet nuclei (<10μm), where such small droplets can remain airborne for several minutes and can reach the alveolar region of the host. Contact transmission on the other hand, can occur indirectly through secretions/fecal droplets on fomites, or directly through physical contact between the donor and the recipient host. Which of the above modes is responsible for most influenza virus transmissions remains vastly controversial. However, virus inactivation rate in the three modes, which depends on the surrounding environmental conditions, is the key player for virus transmission (157).
Ducks, geese, and shorebirds are the natural habitat of the virus, where they shed large amounts of virus through their fecal material. Several studies have been done to evaluate virus survival rates in water (at laboratory and in nature) of different salinity, pH and temperature. Expectedly, virus infectivity rate was inversely proportional to salinity and pH in water. In one study, virus was isolated after 100 days of inoculation ($10^6$ TCID$_{50}$) into water of $17^\circ$C, 0ppt, and pH8.2 (123). Zhang et al. were able to isolate influenza virus genes from Siberian lake ice, and it was assumed that the virus can be preserved in the ice in between the migration seasons (167). Interestingly, LPAI viruses showed more survival rate than HPAI viruses in water, contradicting the claim that high virulence should be positively correlated with durability outside the host (152, 158). Generally speaking, lakes, ponds and pond sediments could act as a reservoir of influenza viruses, where the virus can survive for several days in such environments. This would enable transmission of influenza viruses amongst wild birds in migration seasons and to commercial poultry in proximity of such water resources. In 2007, an outbreak occurred on multiple age broiler breeder operation in Saskatchewan in Canada. The use of water from a near lake where wild aquatic birds have been observed was linked to the emergence of the H7N3 outbreak.

Sneezing and coughing produce droplet particles up to 2000μm in size. In the most part, expelled droplets are too large (>200μm) and settle down quickly (<60cm), and thus, not available for inhalation. In general, settling time of spherical particle of unit density is: 10sec for 100μm (diameter), 4min for 20μm, 17min for 10μm, 62min for 5μm, and those <3μm essentially do not settle (144). Particles less than 10μm can be aerosol transmitted. They are easily respired and can enter the alveolar region of the host.
Infectivity through droplet (large droplets and droplet nuclei) transmission depends on region of deposition and the minimum number of viruses required to start an infection. In humans, the nasal infectious dose (ID₅₀) is estimated at 100-1000 TCID₅₀ where as airborne ID₅₀ is in the range of 0.6-3 TCID₅₀. Based on various reports, one TCID₅₀ corresponds to 100-650 virion particles, and droplet between 1-10μm can hold between $10^3$-$10^7$ virions. This indicates that a dose of ~ 0.6 TCID₅₀ can fit into one droplet that is enabled to infect alveolar region of the host (158). To an extent, this could explain the ability of HPAI H5N1 influenza viruses to infect human, where avian-like receptors ($\alpha 2,3$-linked S.A.) are expressed in the human lungs. This is taking into consideration that most of the human infections were of those who had close contact with infected poultry, and the virus is usually shed in high titers from nasal secretions, fecal droplets and feather follicles (78). Aerosol transmission of HPAI H5N1 was also demonstrated in laboratory between geese and quails, but not between geese and chickens (160).

Airborne and droplet transmissions however, are highly affected by temperature, relative humidity (RH), sunlight UV radiations, and open air factors (OAF). Most of the studies indicated higher survival rate of influenza virus at lower RH. In an old study, maximum survival time varied between 1 hour at 80% RH to 24 hours at 20% RH (59). Although these findings were supported by recent research (60), however, it contradict observations from more than one area in the globe. In some areas in India, Senegal and Brazil, peak of influenza activity coincides with periods of high rain and humidity in the mentioned regions (157). This implies that outbreaks or seasonal influenza are not exclusively controlled by RH, but by many other factors as indicated above. Using guinea pig as host model, it was shown that cold (5°C) and dry (20-35% RH) conditions favored
transmission of influenza virus in an indoor setting. Additionally, infected guinea pigs at 4°C shed virus for 40 more hours at 5°C than 20°C. The last observation was not linked to impairment of immune system between groups, but was referred to the slowness of mucociliary (physical barrier) clearance of the virus at 4°C and thus its spread in the respiratory tract (60). In another study by the same group, high temperature of 30°C was shown to inhibit aerosol transmission at all humidities (20-80%), however, contact transmission was shown to be equally efficient at 20°C and 30°C (61). In a most recent study, it was shown that absolute humidity (AH) rather than RH modulates influenza survival, transmission, and seasonality. However, the report was based on analysis of previously published observations and it needs validations through laboratory and epidemiological studies (112). Sun UV radiations and OAF are other contributors to virus inactivation in aerosol state. In areas of higher latitudes, winter UV-radiation rates were shown to be low and thus allow aerosolized virions to survive for days (106). As for OAF, outside air has been proven to inactivate microorganisms faster than inside air under the same temperature and RH conditions (20).

Contact transmission depends on the load and survival of the pathogen on inanimate products. Porosity is the major factor influencing survival rate of the virus, where increase of porosity enhances virus of inactivation on hard objects. Influenza virus can survive more than 48 hours on stainless steel and plastic objects, but cannot be detected after 12 hours on papers and cloth tissues at 35-40% humidity (10). Interestingly, human influenza viruses showed low inactivation rate on banknotes; where an H3N2 virus (A/Moscow/10/99) showed no considerable inactivation after 10 days. Nasal mucus is thought to enhance virus survival in this case (146). In case of humans, the highest
inactivation rate was observed on hands, where it appears to be the bottleneck for contact transmission via fomites (159).

In summary, influenza survival rate varies between different modes of transmissions. Aerosolized influenza viruses have a half life of several hours in terms of low RH and low to moderate temperatures. The virus can survive for several days on fomites, depending on their porosities and the surrounding environmental conditions. Persistence could be very high in water especially in cold areas/seasons.

1.6 DISCUSSION

During the preparation of this review, World Health Organization (WHO) reported on a high incidence of human infection with swine influenza (H1N1) in the U.S. and Mexico. Thousands were then affected around the globe, with tens of confirmed deaths. Genetic analysis revealed triple reassortants (TR) with genes from human, swine, and avian lineage viruses. Sporadic cases have been reported on human infection with swine H1N1 TR viruses in the North America in the last few years, but the new “Mexican” virus acquired NA and M genes from Eurasian swine influenza lineage. There are more than one interesting observation to point at in this situation. 1) Although most concerns were directed on the transmission of HPAI H5N1 viruses from birds to human, swine proved itself to be an intermediate host in which influenza viruses are modified to transmit and infect humans. 2) Intercontinental mixing of genes between American and Eurasian lineage viruses, and the role of humans’ behavior (travel and transport of goods) in emergence of new viruses that badly affects them. 3) The rapidness of influenza virus
evolution and spread into human and the rapid plans that should be taken to control such
epidemics and maybe pandemics.
Ecosystems and infrastructures of human life have been dramatically changed in the last
two centuries. Man can travel around the world and transfer of pathogens between the
east and west in a matter of hours. Influenza viruses continue to evolve and emerge in
new hosts, causing disease in dozens of animal species. Expression of influenza receptors
are not unique to every host, but rather a mixture that enables a lot of animals to act as
mixing vessels, including human.
Analysis of influenza and pneumonia mortality data between 1996-2005 in CDC revealed
that a combination between international and national air travel influence the time of
influenza introduction in November into the United States. In another study on 13,000
H3N2 influenza isolates between 2002-2007, it was shown that most of the new strains
originate from east and south East Asia.
Countering any new influenza pandemic requires multiple efforts in more than one
aspect. It will be a matter of a few days before any newly emerged influenza viruses can
spread over the globe. Applying strict security measures is the key point in diminishing
the spread of the virus. Any breach in the above aspect can lead to a disaster for human
and animal populations. Some pandemic influenza plans recommend the use of surgical
but not the N95 masks as a part of personal protective equipment for routine patient care.
Considering the ability of influenza viruses for aerosol transmission, the above measures
are not exactly safe in case of pandemics. Additionally, influenza was shown to cause
conjunctivitis in more than one epidemic. Human ocular and lacrimal epithelial cells
express avian like receptors, and neglecting eye protection would enable avian viruses to
replicate and evolve in human populations causing serious problems. Few mutations in
the HA gene could occur very fast and new antigenic variant strains can be generated due
to the antigenic drift/shift mechanisms. The availability of systems and the power to
develop new vaccines in large amounts and distribute them rapidly, is a crucial aspect in
terms of any pandemic. Continuing the surveillance for new influenza variants and
understanding their mechanism of evolution and their ability to cross species barriers, is
important to establish better plans to encounter influenza virus.

1.7 REFERENCES


161. WHO 2009, posting date. Cumulative Number of Confirmed Human Cases of Avian Influenza A(H5N1) Reported to WHO. WHO. [Online.]
CHAPTER 2

CHARACTERIZATION OF TRIPLE REASSORTANT H1N1 INFLUENZA A VIRUSES FROM SWINE IN OHIO

2.1 SUMMARY
An H1N1 influenza A virus, A/swine/Ohio/24366/07, was isolated from pigs at an Ohio County fair. Twenty-six people that came in contact with the infected pigs developed respiratory disease and two of these people were laboratory confirmed as H1N1 by the Centers for Disease Control and Prevention (CDC). The A/swine/Ohio/24366/07 virus we isolated from swine was shown at the CDC to have 100% identical genome sequence to the human virus associated with the county fair. This prompted us to characterize three swine and two human-origin H1N1 influenza A viruses isolated at different time points in the State of Ohio. The three swine viruses were shown to be triple reassortant viruses harboring genes of human (PB1), swine (HA, NA, NP, M, and NS), and avian (PB2 and PA) lineage viruses. Although viruses evaluated in this study were isolated during a short time interval (3 years), genetic drift was observed within the HA and NA genes, including changes at the receptor binding and antigenic sites of the HA1 protein. Nevertheless, all viruses exhibited antigenic similarity as evaluated with
hemagglutination inhibition and virus neutralizing tests. Internal genes were similar to other reassortant viruses of various subtypes currently circulating in the United States. Interestingly, two of the swine viruses including the 2007 isolate replicated well in human airway epithelial cells, however, another virus isolated in 2006 showed very little replication.

2.2 INTRODUCTION

Influenza A viruses are highly contagious pathogens that have been isolated from a wide variety of animals, including humans, birds, pigs, horses, minks, seals, whales, and most recently from cats and dogs (15, 33, 34, 39, 41). The segmented nature of the influenza A virus genome (8 individual segments of single stranded RNA), enables genetic reassortment which leads to the generation of novel strains of the virus (15). Avian viruses are generally thought to preferentially bind the N-acetylneuraminic acid-\(\alpha 2,3\)-galactose (NeuAc\(\alpha 2,3\)Gal) form of sialic acid receptors and human viruses preferentially bind to NeuAc\(\alpha 2,6\)Gal sialic acid receptors. Pigs express substantial amount of both forms of sialic acids on their respiratory epithelial cells, and it is believed that both avian and human influenza viruses can attach to the appropriate receptor and infect pigs (11, 28, 29). Hence, pigs have been postulated to serve as a “mixing vessel” in which two or more influenza viruses can co-infect and undergo reassortment with potential for development of new viruses that can transmit to and infect humans (2, 15, 39). The classic H1N1 subtype was exclusively prevalent among the swine populations in the United States before 1998 (25), however, since 1998 H3N2 influenza viruses have been continuously isolated from swine populations (14, 44). Genetic analysis of these
H3N2 viruses revealed double and triple reassortants (14, 44). Genes of triple reassortant (TR) H3N2 viruses were derived from human (HA, NA and PB1), swine (M, NS, and NP) and avian (PA and PB2) lineage viruses (14). Subsequently, other reassortant influenza viruses were isolated from swine, including, H1N1, H1N2, H3N1 and most recently H2N3 viruses (13, 16, 40). Although reassortant H1N1 (rH1N1) viruses have been isolated from swine populations in the United States (12, 23), little research has been done to characterize these strains. In this study, isolation and characterization of H1N1 TR viruses from swine in the state of Ohio, with genes from human, swine, and avian lineage viruses are reported. Similar viruses were previously isolated from humans in more than one occasion in the United States (9, 21, 24). Hence, we included two human strains isolated in Ohio for comparison. Swine viruses included in this study were isolated from pigs with influenza-like illnesses, including sneezing, coughing, and nasal and ocular discharge. Two of these viruses originated from 2 different commercial swine operations in the state of Ohio. The third isolate (A/swine/Ohio/24366/07) originated from pigs at a county fair in the state of Ohio (38). Twenty-six people that came in contact with the infected pigs at the fair developed respiratory disease, of which, two were laboratory confirmed with H1N1 by the Centers for Disease Control and Prevention (CDC) (personal communication).

2.3 MATERIALS AND METHODS

Viruses. Three H1N1 viruses of swine origin and two H1N1 viruses of human origin were included in this study. All human and swine viruses (A/swine/Ohio/24366/07, A/swine/Ohio/75004/04, A/swine/Ohio/C62006/06, A/Ohio/K1130/06 and
A/Ohio/K1207/06) were isolated in our laboratory from nasal swab samples using Madin-Darby Kidney Canine (MDCK) cell line maintained in Opti-MEM minimum essential medium (Invitrogen, Grand Island, NY) containing 0.5μg/ml trypsin. Viruses were passaged two or three times in MDCK cells to make working stocks.

**RT-PCR and Sequencing.** RNA extraction was performed from cell culture supernatant using the RNeasy mini kit (Qiagen, Valencia, CA.) based on a modified protocol as previously described (36). All genes were amplified using one-step a RT-PCR kit (Qiagen) with primers specific for influenza A virus. The PB2, PB1, HA, NA and M genes (representing segments originated from different lineage viruses) of the human H1N1 strains were included for comparison. The RT-PCR products were separated by electrophoresis on 1% agarose gel, and the amplicons of the right size were excised from the gel and purified with Qiaquick gel extraction kit (Qiagen). Sequencing was performed at the sequencing facility of the Ohio Agricultural Research and Development Center (OARDC) using the ABI Prism 3100 automated sequencing machine (Applied Biosystems, Foster City, CA 94404).

**Genetic Analysis.** The nucleotide sequences were compared initially by using the MegAlign program (DNASTAR, Madison, Wis.) with the clustal V alignment algorithm. Pairwise sequence alignments were also performed in the MegAlign program to determine nucleotide and amino acid sequence similarity. Phylogenetic comparisons of the aligned sequences for each gene were determined using the maximum parsimony method in a full heuristic search using the PAUP 4.0 b10 program (Sinauer Associates, Inc., Sunderland, Mass.).
**Antigenic relatedness.** Antigenic relatedness amongst swine viruses were calculated based on hemagglutination inhibition (HI) and virus neutralization (VN) tests. Antisera used in both experiments were produced as previously described (43). For HI test, titers were determined by using twofold serial dilutions of antisera (25 μl), 4 HA/25 μl units of homologous and heterologous antigen and a 0.5% suspension of turkey erythrocyte per test well. To obtain more accurate titers, we performed the HI tests with two different starting dilutions of 1:2 and 1:5. The VN test was performed in MDCK cells using serially diluted serum and constant virus procedure. Briefly, constant amount of virus (100 TCID₅₀/25 μl) was mixed with equal volume of twofold serial dilutions of antiserum for 30 min at room temperature and then applied onto MDCK cells grown in 96-well plate. Plates were observed for four days post inoculation to determine the virus neutralization titer. The percent antigenic relatedness between viruses was expressed as R-value based on the Archetti and Horsfall formula (1). The R is equivalent to the square root of r₁ X r₂, where r₁ is the ratio of heterologous titer obtained with virus 2 to homologous titer obtained with virus 1; r₂ is the ratio of the heterologous titer obtained with virus 1 to homologous titer obtained with virus 2.

**Virus replication in human airway epithelial cells.** Human bronchial/tracheal epithelial cells (HBEpC) were purchased from Cell Application (Cell Application, INC) and were maintained in bronchial/tracheal epithelial cells growth medium purchased from the same company. Cells were grown in 24 well plates and were inoculated with swine viruses at a titer of 0.005 TCID₅₀ per cell. Supernatant from inoculated cells was collected at time points 6, 12, 24, and 48 hours post inoculation and was tested with real-time reverse transcriptase PCR (rRT-PCR) as previously described (35, 36). Briefly, rRT-PCR was
performed in 25 μl reaction volume using the Qiagen one-step RT-PCR kit with the following conditions: 10 pmol of each primer, 320 μM each dNTP, 0.12 μM FAM labeled probe, 13 units RNase inhibitor, 1 μl enzyme mix, 8 μl of RNA sample, and water was added to get a total volume of 25 ml. The rRT-PCR conditions were: 50°C for 30 min, 95°C for 15 min, and 45 cycles of 1 sec at 94°C and 20 sec at 60°C. Reactions were run in the Cephid Smartcycler thermocycler (Utech Products, Inc.; Schenectady, NY 12305). To estimate virus titer in the collected samples, we established a standard curve based on one human (A/Ohio/K1207/06) and one swine (A/swine/Ohio/24366/07) isolate, where serially diluted RNA extracted from known virus concentration was used to run the rRT-PCR as described above and the standard curve was made using the machine software.

**Immunofluorescence assay.** Viruses were used to inoculate confluent cells as indicated above. After 24 hour post inoculation, cells were washed with phosphate buffered saline (PBS) and fixed with acetone for 20 minutes at -20°C. After washing with PBS, cells were incubated with 1:100 dilution of fluorescein isothiocynate (FITC)-conjugated mouse/IgG anti-influenza (NP) antibody (Chemicon-Millipore, Billerica, MA Billerica, MA) for 1 hour at room temperature. The cells were then washed and visualized using Olympus 1X70 fluorescent microscope (Olympus 1X70; Olympus America, Inc., Melville, NY)

### 2.4 RESULTS AND DISCUSSION

Considering the importance that pigs can play in the epidemiology and evolution of influenza A viruses, especially by acting as a “mixing vessel”, it is crucial to sustain
surveillance over the types of influenza viruses circulating in pigs. In this paper, we provide characterization of H1N1 TR influenza viruses with genes of human, swine and avian lineage. BLAST (Basic Local Alignment Search Tool) search in GenBank indicated that the HA and NA genes of the most recent isolate, A/swine/Ohio/24366/07, were most similar to the HA and NA genes of A/swine/MN/16419/01 (H1N2) and A/swine/Iowa/17672/88 (H1N1), respectively (Table 2.1). Both genes were highly similar to another recent isolate from our laboratory, A/swine/Ohio/C62006/06, of 99.6% and 98.9% similarity for the HA and NA genes, respectively (Table 2.2). The similarity decreased by 2% and 5% in both genes when comparing the A/swine/Ohio/24366/07 to A/swine/Ohio/75004/04 (Table 2.2). Phylogenetic analysis of the HA and NA genes of the three swine isolates showed that they are of swine lineage, with the HA being more similar to H1N2 reassortant viruses currently circulating in the swine populations in the United States (Figure 1.1a). While all HA genes of the swine viruses grouped together in the phylogenetic tree, the NA of A/swine/Ohio/75004/04 grouped separately from the other two viruses and was mostly related to A/swine/MI/PU243/04 (H3N1) virus (Figure 1.1b). On the other hand, the HA and NA genes of the human H1N1 viruses were highly similar to other human viruses currently circulating in the United States (Figure 1.1a & 1.1b) and were around 76% and 80% similar to HA and NA genes of A/swine/Ohio/24366/07, respectively (Table 2.2). Internal genes of the three swine viruses were similar to other reassortant viruses currently circulating in swine populations in North America (Table 1 and Figures 2.2a, 2.2b, & 2.2c). The PB2 and PA polymerase genes of the most recent isolate, A/swine/Ohio/24366/07, were of avian lineage and were very similar (based on BLAST search) to PB2 and PA genes of
A/turkey/Ohio/313053/04, an H3N2 virus, previously isolated in our laboratory. The PB1 polymerase gene was of human lineage and was also very similar search to A/turkey/Ohio/313053/04 (H3N2) virus. The PB2 and PB1 genes of A/swine/Ohio/24366/07 and A/swine/Ohio/C62006/06 were more similar to each other and separated from A/swine/Ohio/75004/04 virus in the phylogenetic tree (Table 2 and Figures 2a, & b). The rest of the internal genes: NP, M and NS, were of swine lineage viruses (Table 2.1). The M genes of 2006 and 2007 isolates were also more similar to each other as compared to that of 2004 isolate (Figure 2c). On the other hand, PB2, PB1, and M genes of the human isolates were of human lineage and were highly similar to the currently circulating human H1N1 viruses in the United States.

Alignment of the HA1 protein of the swine and human isolates is presented in figure 2.3. Seven amino acids changes were observed at the HA1 protein when comparing the 2007 and 2004 isolates, including two changes at residues 73 and 165 of the antigenic sites Cb and Sa, respectively. Nevertheless, all swine viruses were antigenically similar as evaluated with HI and VN tests. The 2004 isolate was more similar to the 2006 isolate with percentage similarity of ~100% and ≥90% based on HI and VN test respectively. The percentage similarity decreased to 85% and 80% between the 2004 and 2007 isolates with HI and VN tests respectively. The difference in the R-values between the HI and VN test might be due to the fact that the HI test depends on antibodies reaction against HA protein only, however, other proteins might be involved in case of the VN test (5, 10, 22, 30). This includes the NA protein that showed evident amino acid changes between the swine isolates. While the most recent isolates of 2006 and 2007 showed at least four
amino acid changes in the NA protein, 20 or more changes were observed when comparing the isolates from 2004 and 2007 (data not shown). Although the genetic similarity was low between human and swine strains, both lineage viruses shared common amino acids at the receptor binding site (Figure 2.3). Out of 24 amino acids located at the receptor binding domain (7, 32), 15 amino acids were shared between both lineage viruses including critical residues 190, 220, 225, 226 and 228. These amino acids were shown to determine the receptor binding specificity to either NeuAcα2,3Gal or NeuAcα2,6Gal sialic acid receptors. Most importantly, swine viruses harbored Asp (D) at positions 190 and 225 of the receptor binding domain. Presence of D at these positions was shown to critically support the binding to NeuAcα2,6Gal form of sialic acid receptors (the predominant form in human upper respiratory tract) for the 1918 and other H1N1 influenza viruses (8, 17, 26, 27). Tumpey et al. also showed that changes at these two positions, D190E and D225G, would abolish transmission of H1N1 influenza viruses in ferrets, a model for transmission of influenza viruses in human (37). Additionally, Glaser et al. showed that D190E change would increase the affinity of binding to NeuAcα2,3Gal rather than NeuAcα2,6Gal receptors (8). Although residues 226 and 228 play a more critical role in the receptor binding specificity for H2, H3 and H9 than H1 subtype (6, 17, 18), human and swine viruses of H1 subtype shared the same amino acids (Q226 and G228) at these positions (Figure 2.3). Machalova et al. showed that Gln (Q) at position 226 increases the affinity to bind NeuAcα2,6Gal receptors in human H1N1 influenza viruses (20). Last but not least, R220 was also shared between swine and human viruses, and it was previously shown to increase the affinity of binding to NeuAcα2,6Gal receptors in H1N1 influenza viruses (20).
To test the replication potential of these viruses in human airway cells, the three swine viruses were applied onto HBEpC cells. Although all viruses replicated in the epithelial cells as indicated by rRT-PCR (Figure 2.4) and immunocytochemistry (Figure 2.5), the isolates from 2004 and 2007 replicated to higher titers and showed more cytopathic effect (CPE) than the 2006 strain.

At least 20 amino acid changes at all antigenic sites were observed in the HA molecule of human and swine viruses. Both lineage viruses shared four N-glycosylation sites, but the human strains had two additional sites at positions 57 and 163 of the HA1 molecule. The A/swine/Ohio/24366/07 and A/swine/Ohio/75004/04 viruses had non-conserved N-glycosylation sites at position 165 and 279, respectively.

In summary, the three swine viruses included in this study showed distinct genotypic and phenotypic features although they were isolated over a short period of time. The isolates from 2006 and 2007 were more genetically similar to each other than the 2004, but all strains were antigenically similar with percentage relatedness exceeding 85%. Nevertheless, genetic changes might expand and accumulate, eventually leading to phenotypically variant strains that might affect vaccination programs used in swine industry.

The phylogenetic tree of the HA gene grouped the H1N1 TR with the H1N2 TR subtype rather than other recent H1N1 strains (Figure 2.1a), indicating a diversity in HA genes (within the TR strains) and/or reassortments, that are circulating in the swine populations in North America. The same was also observed for the NA gene (Figure 2.1b) where two separate clades are constructed for N1 subtype of swine lineage viruses. This emphasizes
the importance of continuous surveillance and laboratory testing of new strain for their
direct impact on swine as well as human health.

It is worth mentioning that all swine viruses included in this study were selected
randomly and were shown to be triple reassortants. We also isolated other H1N1 TR
strains in the state of Ohio during the preparation of this paper. The frequent isolation of
these viruses raises the question whether they would dominate over the classic swine
influenza (H1N1) in swine populations. These viruses have similar internal genes to other
reassortant viruses of various subtype that are derived from human, swine and avian
lineages. The continuous circulations of various subtypes with similar internal genes as a
backbone elucidate the query about the role played by these genes in reassortment and
transmission of these viruses. Interestingly, BLAST search for the
A/swine/Ohio/24366/07 genes revealed that the three polymerase genes (PB2, PB1 and
PA) were very similar to A/turkey/Ohio/313053/04, an H3N2 strain from our laboratory.
This strain was shown to easily transmit both ways between pigs and turkeys (42), where
other strains of the same subtype transmitted infrequently or not at all. Whether these
polymerase genes play a role in the transmission, reassortment, and pathogenicity of
these viruses is a question to be answered by further investigations.

In a Recent study (38), a strain similar to A/swine/Ohio/24366/07 was shown to
efficiently infect and transmit amongst pigs, inducing more clinical signs and lesions as
compared to many of the previously evaluated swine influenza (H1 subtype) viruses
isolated from United States.

It is important to note that some individuals that had contact with pigs infected with
A/swine/Ohio/24366/07 isolate reported signs consistent with respiratory disease, but
were not hospitalized, indicating the potential for interspecies transmission and pathogenicity for human. CDC confirmed (personal communication) that genomic sequencing in one patient was 100% identical to the virus we isolated from swine, and the second case was nearly identical, with only one nucleotide difference at the non-coding region.

The transmission of H1N1 TR from swine to humans was reported in at least two other states. (9, 19, 21, 31). The shared amino acid at the receptor binding site between the two lineage viruses might explain the ability of these viruses to transmit to human. It is of interest that at least two strains replicated efficiently in human airway epithelial cells in our laboratory.

**2.5 ACKNOWLEDGMENTS**

This study was partially supported by funds from the U.S. Department of Agriculture (USDA), Cooperative State Research, Education, and Extension Service (CSREES), avian influenza–coordinated agricultural project (AI-CAP).

**2.6 REFERENCES**


<table>
<thead>
<tr>
<th>A/swine/Ohio/24366/2007 (H1N1)</th>
<th>Most similar to:</th>
<th>Percent similarity</th>
<th>origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB2</td>
<td>A/turkey/Ohio/313053/04 (H3N2)</td>
<td>98 %</td>
<td>Avian</td>
</tr>
<tr>
<td>PB1</td>
<td>A/turkey/Ohio/313053/04 (H3N2)</td>
<td>99 %</td>
<td>Human</td>
</tr>
<tr>
<td>PA</td>
<td>A/turkey/Ohio/313053/04 (H3N2)</td>
<td>98 %</td>
<td>Avian</td>
</tr>
<tr>
<td>HA</td>
<td>A/swine/MN/16419/01 (H1N2)</td>
<td>97 %</td>
<td>Swine</td>
</tr>
<tr>
<td>NP</td>
<td>A/swine/IN/pu542/04 (H3N1)</td>
<td>97 %</td>
<td>Swine</td>
</tr>
<tr>
<td>NA</td>
<td>A/swine/Iowa/17672/88 (H1N1)</td>
<td>97 %</td>
<td>Swine</td>
</tr>
<tr>
<td>M</td>
<td>A/swine/Ontario/33853/05 (H3N2)</td>
<td>99 %</td>
<td>Swine</td>
</tr>
<tr>
<td>NS</td>
<td>A/swine/IN/14810-S/01 (H1N2)</td>
<td>97%</td>
<td>Swine</td>
</tr>
</tbody>
</table>

**Table 2.1.** Similarity of A/swine/Ohio/24366/2007 (H1N1) genes to other viruses’ based on BLAST search in GenBank Database.
<table>
<thead>
<tr>
<th>Gene</th>
<th>A/swine/Ohio/C62006/06</th>
<th>A/swine/Ohio/75004/04</th>
<th>A/Ohio/K1130/06</th>
<th>A/Ohio/K1207/06</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA*</td>
<td>99.6% (1-1630)**</td>
<td>97.5% (1-1294)</td>
<td>76.2% (1-1648)</td>
<td>76.0% (1-1608)</td>
</tr>
<tr>
<td>NA*</td>
<td>98.9% (1-1413)</td>
<td>94.1% (1-1413)</td>
<td>80.8% (1-1411)</td>
<td>80.7% (1-1432)</td>
</tr>
<tr>
<td>PB2*</td>
<td>98.2% (1-2266)</td>
<td>97.6% (1-2280)</td>
<td>83.1% (1-2280)</td>
<td>83.1% (1-2280)</td>
</tr>
<tr>
<td>PB1*</td>
<td>98.6% (1-2285)</td>
<td>96.5% (1-2294)</td>
<td>80.9% (1-2299)</td>
<td>81.0% (1-2267)</td>
</tr>
<tr>
<td>M*</td>
<td>99.8% (1-920)</td>
<td>99.8% (1-923)</td>
<td>88.4% (1-903)</td>
<td>88.1% (1-898)</td>
</tr>
</tbody>
</table>

* Genes were chosen to represent reassortments of different lineages: HA, NA, and M of swine lineage, PB2 of avian lineage and PB1 of human lineage.
** Numbers indicate the sequence region of the genes included for comparison

**Table 2.2.** Nucleotide percent similarity between triple reassortant H1N1 swine viruses and human viruses.
<table>
<thead>
<tr>
<th>Turkey serum anti-</th>
<th>Turkey serum anti-</th>
<th>Turkey serum anti-</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/swine/Ohio/75004/04</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A/swine/Ohio/C62006/06</td>
<td>~100% *</td>
<td>≥ 85%</td>
</tr>
<tr>
<td>A/swine/Ohio/24366/07</td>
<td>≥ 90%</td>
<td>-</td>
</tr>
<tr>
<td>A/swine/Ohio/75004/04</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A/swine/Ohio/C62006/06</td>
<td>≥ 80%</td>
<td>≥ 85%</td>
</tr>
<tr>
<td>A/swine/Ohio/24366/07</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Archetti and Horsfall formula was used to calculate the R-values. The R is equivalent to the square root of r1 x r2, where r1 is the ratio of heterologous titer obtained with virus 2 to homologous titer obtained with virus 1; r2 is the ratio of the heterologous titer obtained with virus 1 to homologous titer obtained with virus 2. **Bold numbers:** R-**Value based on HI test;** **Italic numbers:** R-**values based on VN test.**

**Table 2.3.** Cross-HI and VN results expressed as percentage of antigenic relatedness (R-Value)
Figure 2.1. Phylogenetic relationship based on nucleotide sequences of: (a) H1 and (b) N1 subtype influenza viruses of human, swine and avian origin. The tree was generated by the maximum parsimony method with 100 bootstrap replicates in a heuristic search with PAUP 4.0b10 software.
Figure 2.1. Continued.

B.

Avian

A/duck/NY/13152-13/1994 H1N1
A/duck/NJ/7717-70/1995 H1N1
A/pintail duck/Alberta/210/2002 H1N1
A/Ohio/K1138/96 H1N1
A/Ohio/UR06-0353/2007 H1N1
A/Ohio/K1207/06 H1N1
A/Illinois/UR06-0093/2007 H1N1
A/Ohio/3558/1988 H1N1
A/swine/Wisconsin/238/97 H1N1
A/swine/Iowa/17672/1988 H1N1
A/Wisconsin/10/98 H1N1
A/swine/Ohio/75004/04 H1N1
A/swine/Ml/PU243/04 H3N1
A/swine/North Carolina/36883/2002 H1N1
A/swine/IN/PU542/04 H3N1
A/swine/Minnesota/37866/1999 H1N1
A/swine/Alberta/56626/03 H1N1
A/swine/Ohio/C82006/06 H1N1
A/swine/Ohio/24366/07 H1N1
A/swine/Iowa/CEID23/2005 H1N1
A/swine/Iowa/00239/2004 H1N1
A/swine/Ontario/1111204 H1N1
A/swine/Ontario/23866/04 H1N1
A/swine/Tennessee/48/1977 H1N1
A/swine/Wisconsin/1/1968 H1N1
A/swine/Iowa/1973 H1N1
A/swine/Virginia/671/1987 H1N1

Human

Classic & reasortants/swine

10 changes
Figure 2.2. Phylogenetic relationship based on nucleotide sequences of: (a) PB2, (b) PB1 and (c) M genes of different lineage viruses (human, swine and avian) including reassortant strains. The tree was generated using the maximum parsimony method with 100 bootstrap replicates in a heuristic search in the PAUP 4.0 b10 software.
Figure 2.2. Continued.

B.

Avian

Swine

Human

Reassortants/Human

(Continued)
Figure 2.2. Continued.

C.

Avian

Classic swine

Reassortants/Swine

Human

- 5 changes
Figure 2.3. Amino acids sequence alignment of the HA1 protein from H1N1 swine TR and human influenza viruses. Residues in open boxes: receptor binding domain. Gray shaded area: Antigenic sites of H1 subtype influenza viruses. Bold letters: shared amino acids at the receptor binding domain between swine and human viruses. Underlined letters: N-glycosylation sites. H3 numbering system was used in this figure.
**Figure 2.4.** Replication of swine and human viruses in HBEpC cells. Viruses were applied onto epithelial cells at a titer of 0.005 TCID$_{50}$ per cell for 30 minutes. Inoculums were then removed and cells were washed twice with PBS to remove unattached viruses. HBEpC media was then added over the cells without addition of trypsin. Cells were observed for CPE and supernatant was collected over periods of time and tested with rRT-PCR for virus detection.
Figure 2.5. Immunofluorescence for swine viruses in HBEpC cells. a: negative control, b: A/swine/Ohio/75004/04, c: A/swine/Ohio/C62006/06, d: A/swine/Ohio/24366/07.
CHAPTER 3

GENETIC AND ANTIGENIC RELATEDNESS OF H3 SUBTYPE INFLUENZA A VIRUSES ISOLATED FROM AVIAN AND MAMMALIAN SPECIES

3.1 SUMMARY

In 2004, we isolated triple reassortant H3N2 viruses from turkey breeder hens in Ohio and Illinois. The Illinois flock was vaccinated twice with an inactivated H3N2 vaccine containing a swine origin virus before the outbreak. Additionally, a commercial inactivated vaccine containing an H3N4 virus of duck origin is being used in some turkey farms. This prompted us to initiate a comparative study on the antigenic and genetic relatedness of various H3 subtype influenza viruses isolated from turkeys, ducks, pigs and humans. The antigenic relatedness between the different viruses was evaluated with the Archetti and Horsfall formula, while nucleotide genetic similarities were calculated using pairwise alignments. Results obtained indicated a high degree of antigenic (>90%) and genetic (>99%) similarities among the turkey-origin H3N2 viruses. However, the turkey viruses were antigenically distantly related to the swine-origin vaccine virus (<30%), although they had approximately 95% genetic similarity in the HA1 gene. Additionally, major genetic and antigenic changes were observed between the turkey...
viruses and the H3N4 duck vaccine virus as well as the H3N2 human virus. Such genetic and antigenic differences between the turkey-origin viruses and other H3 subtype viruses including vaccine strains could be the reason for the failure in protection in the Illinois turkey breeders vaccinated with swine origin virus. This also emphasizes the importance of using viruses for vaccines that are antigenically similar to the field strains.

### 3.2 INTRODUCTION

Influenza A viruses belong to the family Orthomyxoviridae that includes four other genera: influenza B, influenza C, Isavirus and Thogotovirus (18). The influenza A viruses are unusual in this group because they can be highly infectious pathogens to a variety of mammalian and avian species (20, 44). They are usually divided into subtypes based on the two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). So far, sixteen HA and nine NA subtypes have been identified (36). Generally, individual viruses are host specific (3, 44). Although the molecular basis of host range restrictions are not completely defined, the compatibility between the HA protein of the virus and its corresponding receptor, sialic acid, on the host cell is known to determine in part the infection of the virus in a specific host (12, 13). Avian influenza A viruses are generally thought to preferentially bind the N-acetylneuraminic acid-\(\alpha\)2,3-galactose (NeuAc\(\alpha\)2,3Gal) form of sialic receptors which is the predominant form of glycosylation of proteins found in birds (27, 29). However, human influenza A viruses preferentially bind to NeuAc\(\alpha\)2,6Gal sialic acid receptors, which is the predominant form of glycosylation expressed on proteins found in the upper respiratory tract in humans (27, 29). Pigs, however, express substantial amount of both forms of sialic acids, and it is
believed that both avian and human influenza viruses can attach to the appropriate receptor and infect pigs (13), potentially allowing them to serve as “mixing vessels” for the generation of reassortant influenza viruses (17, 32). Before 1998, classic swine H1N1 lineage viruses were the dominant cause of influenza in pigs in the United States (U.S.) (26). In 1998, a new lineage of H3N2 influenza viruses was isolated from pigs experiencing severe influenza-like illnesses on a farm in North California (15, 49). Additional outbreaks in pigs were also reported in the same year in Minnesota, Iowa and Texas (43). Genetic analysis of these viruses showed that they are reassortants viruses (49), with gene segments of swine, human and in some cases avian influenza origin (15, 49). Triple reassortant viruses genes were derived from human (HA, NA and PB1), swine (M, NS, and NP) and avian (PA and PB2) viruses (15). The triple reassortant virus has now become the predominant strain found in U.S. swine, although classical H1N1 viruses and other reassortant viruses (H1N1, H1N2, and H3N1) are also isolated (7).

Turkeys are susceptible to a wide range of influenza A viruses and serve as an important host for these viruses, and as a major exception to the host range restriction rule, are routinely infected with swine-like influenza viruses (36). Influenza infections in turkeys range from asymptomatic to severe disease, including respiratory tract disease, depression, drop in eggs production and high mortality (35, 36). Most infections of turkeys with swine influenza are associated with drop in eggs production but with no other clinical signs being observed (38). Natural and experimental infections of turkeys with classic H1N1 swine virus were reported for the first time by our lab in 1978 and 1981 (24, 31). In 2004, we isolated two triple reassortant H3N2 viruses from turkey breeder hens in Ohio (38) and Illinois. Infected turkeys showed no clinical signs but
underwent complete cessation of egg production. Interestingly, the Illinois turkey flock was vaccinated twice with an inactivated H3N2 virus isolated from swine in North Carolina in 2003. The failure of protection in turkeys vaccinated with a swine H3N2 virus against a similar subtype virus, prompted us to initiate a study on antigenic and genetic relatedness of H3N2 viruses isolated from turkeys and swine. Additionally, an H3N4 duck virus (A/mallard duck/Minnesota/79/79) that is being used as a vaccine for turkey breeders, and a recent H3N2 virus isolated from humans in Ohio in 2006 were included in this study since the HA and NA genes in the triple reassortants viruses are of human lineage. Antigenic relatedness assessments were done based on the cross reactivity of the different viruses in the hemagglutinin inhibition (HI) and virus neutralization (VN) tests, while genetic comparisons were done based on gene sequences and phylogenetic analysis.

### 3.3 MATERIALS AND METHODS

**Viruses.** Six viruses were included in this study (Table 3.1): three turkey viruses, one swine, one duck, and one human virus. Two turkey viruses were isolated in our lab, A/turkey/Illinois/04 (H3N2) and A/turkey/Ohio/313053/04 (H3N2) from turkey tracheal swabs on Madin-Darby Canine Kidney (MDCK) cell line maintained in Opti-MEM minimum essential medium (Invitrogen, Grand Island, NY) containing 0.5μg/ml trypsin. The samples were passaged twice in MDCK cells and then used to inoculate 9-10 day old specific pathogen free (SPF) embryonated chicken eggs (ECE) to make a working stock. One turkey virus, A/turkey/North Carolina/03 (H3N2) (passaged twice (P2) in MDCK), and one swine virus (vaccine strain), A/swine/North Carolina/03 (H3N2) (Unknown
passage number), were generously provided by Dr. Eric Gonder (Goldsboro Milling Co.
Goldsboro, NC), and were propagated in 9-10 day old ECE to make a working stock. The
duck vaccine virus, A/mallard duck/Minnesota/79/79 (H3N4) (Unknown passage
number), was obtained from Lohmann Animal Health (Lohmann Animal
Health, Winslow, Maine), and was propagated in 9-10 day old ECE to make a working
stock. The human virus, A/human/Ohio/06 (H3N2) (P2 MDCK), was obtained from Ohio
Department of Health (ODH, Reynoldsburg, Ohio), and was propagated and passaged
twice in MDCK cells.

**Antisera production.** Infectious allantoic fluid was pooled for each virus after
propagation in ECE as described above. Only the human virus was propagated in MDCK
cells. Titers of the viruses were determined as 50% tissue culture infectious dose
(TCID\textsubscript{50}) in MDCK cells as well as HA units prior to inactivation with 0.1%
betapropiolactone (Sigma, St. Louis, Mo.). The inactivated viruses were used to produce
an oil emulsion vaccine as previously described (34). Two week old SPF turkeys were
inoculated subcutaneously with the inactivated vaccine containing either 256 or 512 HA
units depending on the virus used, and were booster vaccinated three weeks later. Two
weeks post the second vaccination, sera was harvested and inactivated at 56\degree C for 30 min
before being used in HI and VN tests. Anti-human virus and anti-duck virus antisera were
produced in chickens using the same protocol as described above. Previous work in our
laboratory showed that antisera produced in turkeys or chickens do not affect non-
specifically HI or VN tests (unpublished data).

**HI and VN tests.** The hemagglutinin inhibition (HI) test was carried out as previously
described (2). Briefly, titers were determined by using twofold serial dilutions of antisera
(25µl), 4 HA/25µl units of homologous and heterologous antigen and a 0.5% suspension of turkey erythrocyte per test well (1% guinea pig erythrocytes was used with the human virus). To obtain more accurate titers, we performed the HI test with two different starting dilutions of 1:2 and 1:5.

The virus neutralization (VN) test was performed in MDCK cells using diluted serum and constant virus procedure (5). Briefly, constant amount of virus (100TCID50/25µl) was mixed with equal volume of twofold serial dilutions of antiserum for 30 min at room temperature and then applied onto MDCK cells grown in 96-well plate. Plates were observed for cytopathic effect (CPE) at days three and four post inoculation to determine the virus neutralization titer. The antigenic relatedness between the different viruses was expressed as R-value based on the Archetti and Horsfall formula (1). The R-value is equivalent to the square root of r1 X r2, where r1 is the ratio of heterologous titer obtained with virus 2 to homologous titer obtained with virus 1; r2 is the ratio of the heterologous titer obtained with virus 1 to homologous titer obtained with virus 2.

**RNA extraction and RT-PCR.** RNA extraction was performed from either allantoic fluid or cell culture supernatant using the RNeasy mini kit (Qiagen, Valencia, CA.) based on a modified protocol as previously described (33). Standard reverse transcription (RT) PCR was carried out with the one-step RT-PCR kit (Qiagen) with primers specific for influenza A virus. Primers sequences are available upon request. The RT-PCR products were separated by electrophoresis on 1% agarose gel, and the amplicons of the right size were excised from the gel and purified with Qiaquick gel extraction kit (Qiagen).

**Sequencing and phylogenetic analysis.** A/turkey/Illinois/04, A/turkey/Ohio/313053/04, and A/swine/North Carolina/03 were sequenced at the Southeast Poultry Research
Laboratory sequencing facility (SEPRL, Athens, GA), while A/mallard
duck/Minnesota/79 and A/human/Ohio/06 were sequenced at the sequencing facility at
the Ohio Agricultural Research and Development Center (OARDC) using the ABI Prism
3100 automated sequencing machine (Applied Biosystems, Foster City, CA 94404). The
entire viral sequence of A/TK/OH/04 virus was published previously (38). The nucleotide
sequences were compared initially by using the MegAlign program (DNASTAR,
Madison, Wis.) with the clustal V alignment algorithm method. Pairwise sequence
alignments were also performed in the MegAlign program to determine nucleotide and
amino acid sequence similarity. Phylogenetic comparisons of the aligned sequences for
each gene were determined using the maximum parsimony method in a full heuristic
search using the PAUP 4.0 b10 program (Sinauer Associates, Inc., Sunderland, Mass.).
Phylogenetic trees were constructed based on HA (human lineage), NP (swine lineage),
NA (human Lineage) and M (swine lineage) genes, because their corresponding proteins
were shown to induce either complete or partial protection when used in vaccination (4,
8, 30, 37, 42). In addition, one phyllogenetic tree was constructed based on the PB2 gene
which is of avian lineage.

**Molecular graphic visualization.** Amino acid changes at the four antigenic sites (A, B,
C, and D (interface)) (40, 45) of the HA1 molecule were determined by amino acid
sequence alignment using the MegAlign program. Changes at the major antigenic site (A,
B, and C) of HA monomer were located using the Rasmol software (v2.6.4)
(Biomolecular Structures Group, Hertfordshire, UK) based on HA structure of H5
subtype influenza virus, A/duck/Singapore/3/97 (10), (1JSM) downloaded from the
Protein Data Bank website (http://www.rcsb.org/pdb/home/home.do). We preferred to
use the H5 structure since it is available as a monomer structure and it would be clearer to visualize the amino acids changes on it than the H3 structure which is only available as a trimer structure.

3.4 RESULTS

Antigenic relatedness study. The R-values (% antigenic relatedness) calculated based on HI and VN tests results indicated that the turkey viruses are highly related to each other but were distantly related to the other viruses of non-turkey origin (Table 3.2). The three turkey viruses were identical as evaluated with HI test, with an R-value equal to 100%. The Ohio virus was also shown to be identical to the Illinois virus as compared with VN test (100%), and both viruses were highly similar to the North Carolina turkey virus (>80%) in VN tests. On the other hand, all the turkey viruses were distantly related to the swine vaccine virus. The R-values between the swine vaccine virus and the three turkey viruses were less than 25% with the HI test and less than 30% with the VN test (Table 3.2). The duck vaccine virus was antigenically totally unrelated to the turkeys and swine viruses with no cross reactivity being observed using the HI test (R<1%) and only low VN cross reactivity (R<20%). As for the human virus, the R-values were ≤10% in the HI tests and ≤20% in the VN tests (Table 2) as compared to the other H3N2 viruses of swine and turkeys origin.

Genetic relatedness. All eight genes of five viruses were amplified, sequenced and compared using the MegAlign program. The turkey Illinois and Ohio viruses had high gene sequence similarity in all 8 genes as indicated in Table 3.3. Nucleotide sequence similarity of the genes of the two viruses ranged between 99.3%-99.7%, except for the
NS gene that was 99.0%. Both viruses were shown to be of the triple reassortant H3N2 subtype that is now circulating in the U.S. (Table 3.3). Phylogenetic analysis of the HA, NP, NA, M and PB2 genes of both viruses (Figure 3.1-3.5), showed that they group together with the newly isolated H3N2 viruses from turkeys and swine in the United States and Canada. Additionally, phylogenetic analysis of the above genes indicated that the TR viruses isolated from swine and turkeys are clustering in clades different from other H3N2 viruses of human, swine and avian origin (Figures 3.1-3.5). This observation is more obvious with the PB2 gene (avian lineage) as well as NP and M genes (swine lineage). The swine vaccine virus was also shown to be a triple reassortant virus, but was grouped separately from the turkey viruses and other recent swine H3N2 viruses. As a result of the BLAST search (Basic Local Alignment Search Tool, National Center for Biotechnology Information, 8600 Rockville Pike, Bethesda, MD 20894; http://www.ncbi.nlm.nih.gov/BLAST), the HA, NA, NP and M genes of the swine vaccine virus were most similar to A/Swine/Illinois/21587/99 (98%), A/Turkey/MO/24093/99 (H1N2) (98%), A/Swine/Iowa/569/99 (98%), and A/Swine/Iowa/569/99 (98%), respectively (Figures 3.1-3.4). Interestingly, a stalk deletion of 39 nucleotide bases (13 amino acids) was detected in NA gene of the swine vaccine virus (bases 115-154). To our knowledge, this is the first observation of its kind in the triple reassortant viruses. The HA gene of the swine vaccine virus was 95.8% similar to that of the Illinois turkey virus, and the NA gene was 94.9% similar between the two viruses. On the other hand, all genes of the duck vaccine virus were of avian lineage (Figures 3.1-3.5). Although this virus was classified as N4 subtype virus, sequence analysis indicated that the NA gene belongs to the N1 avian lineage viruses
The HA gene of the duck vaccine virus was mostly similar to A/mallard duck/New York/174/1982 (98%), and it was 77.9% similar to the Illinois turkey virus HA gene. Phylogenetic analysis based on the HA gene grouped the duck vaccine virus with other duck viruses isolated in the U.S. (Figure 3.1). The Ohio human virus was highly similar to another human virus from New York (A/human/New York/928/2006) with sequence similarity equal to or more than 99.7% for all genes (Figures 3.1-3.4). The HA gene of the Ohio human virus was 91.9% similar to the Illinois turkey virus, while the NA gene was 94.9% similar between the two viruses (Table 3). All the genes of the human virus were of human lineage.

**HA1 amino acids analysis.** HA1 amino acid sequence alignment of five H3 viruses is shown in Figure 3.6. Major amino acid changes were shown at the antigenic sites A, B, C and D (40, 45) between the A/turkey/Illinois/04, A/swine/North Carolina/03, A/mallard duck/Minnesota/79 and A/human/Ohio/06. Eight amino acid changes were observed at the major antigenic sites (A, B, and C) of the HA1 molecule between the Illinois turkey virus and the swine vaccine virus (Figure 3.6 & 3.7). Changes were substantial when comparing the duck vaccine virus to the Illinois turkey virus, with 18 amino acid changes at the four antigenic sites A, B, C and D (Figure 6). Eleven amino acid changes were also observed in the HA1 molecules between the Ohio human virus and both Illinois turkey virus and swine vaccine virus.

The swine vaccine virus and the Illinois turkey virus have six potential N-glycosylation sites on the HA1 molecule, five of which are shared by the two viruses (Figure 3.6). Interestingly, the Ohio human HA1 gene had four additional N-glycosylation sites (total equal to 10) as compared to the TR viruses (Figure 3.6); two of which were at antigenic
site A, and the other two were close to the antigenic site A. The duck vaccine virus has five potential N-glycosylation sites, four of which are at the same positions as the turkey viruses.

3.5 DISCUSSION

This study is the first comparison of antigenic and genetic relatedness of TR H3N2 viruses of swine and turkeys’ origin, as well as other H3 subtype viruses of human and avian origin. It was prompted by the observation of failure of protection in turkeys vaccinated with swine H3N2 virus. Pigs have been hypothesized to play a key role in the interspecies transmission of influenza A viruses by means of antigenic shift mechanism (6, 17). Interspecies transmission of H1N1 and H3N2 viruses between pigs and turkeys has been reported in multiple occasions (9, 23, 38, 48). The geographic proximity of swine and turkey operations in the U.S., especially in the Midwest part of the country, caused a situation that required producers to vaccinate their turkey breeders with swine viruses that are circulating in the field. Vaccination with H1N1 virus in turkey breeders started more than three decades ago (11) to protect turkeys against the cH1N1 swine virus that was shown to transmit to turkeys. Because of outbreaks with triple reassortant H3N2 viruses in 1998, turkey producers started vaccination with autogenous vaccines against these viruses and/or with commercially available H3N4 duck virus vaccine, to prevent infection with the swine viruses. Surprisingly, in 2004 we isolated an H3N2 influenza virus from turkey breeder hens that were vaccinated twice with an inactivated H3N2 virus isolated from pigs in North Carolina in 2003, as well as an H1N1 subtype virus.
Although the turkey viruses and swine vaccine virus were all of TR H3N2iple reassortants (cluster III subtype) circulating in U.S. turkeys and swine (43), antigenically they were shown to be distantly related, although they differed by less than 5% at the amino acid level for the HA1 molecule. A major antigenic difference would be a Archetti-Horsfaul index of less than 30%, and the recent turkey viruses had indexes much lower for the swine and duck vaccines viruses (14) using either HI and VN tests. The antigenic differences provide one likely explanation for the failure of protection by vaccination in the Illinois turkey flocks. Most importantly, we observed 8 amino acid changes at the major antigenic sites (A, B, and C) between the Illinois turkey virus and the swine vaccine virus. Wilson and Cox (46) proposed that a drift variant with $\geq 4$ amino acid changes at $\geq 2$ out of 5 antigenic sites would be of epidemiologic importance. Moreover, several studies have shown that 1-3 amino acid changes in the HA1 molecule of H1N1 and H3N2 viruses, could possibly reduce the cross reactivity and the efficacy of inactivated vaccine in an animal model (16, 19, 25, 47). Although the phylogenetic analysis based on HA gene showed that both turkeys and swine viruses were cluster III H3N2 subtype (43), it seems that the swine vaccine virus is more related to earlier isolates from 1999 (most similar to A/swine/Illinois/21587/99), while the turkey viruses are more similar to recent isolates from 2005 in the U.S. and Canada. Most interestingly, we noticed a unique feature in the swine vaccine virus that has a 13 amino acid stalk deletion in the NA protein. Stalk deletions are commonly found in poultry avian influenza viruses, but to our knowledge, this is the first observation of its kind in a swine influenza virus. Further investigations are needed to elucidate the effect of such deletion on the antigenicity, pathogenicity and transmission of TR H3N2iple reassortants in swine
and turkeys. In comparing the turkey viruses and the duck vaccine virus, 18 amino acid changes were observed at the four antigenic sites A, B, C and D. One of the changes was at residue 156 (KtoA) of antigenic site B, which was described earlier to reduce antigenicity (19, 21). Changes were also observed at the contact region of antigenic site D, including VtoQ change at residue 226 (Figure 3.6) that was shown to affect antigenicity at this region (45). These differences between the turkey and duck viruses could be a result of the HA segments being of human and avian lineage, respectively. Such changes would also explain the lack of any antigenic relatedness between the two viruses as evaluated with the HI test.

Although the HA genes of swine and turkey viruses were originally from human influenza virus lineage, the swine H3N2 viruses are now evolving separately from the human viruses and considerable antigenic drift has occurred in the last decade. Eleven amino acid changes were observed between the human and the triple reassortant viruses that were clustered in separate clades in the phylogenetic tree based on the HA gene. Interestingly, triple reassortants of swine and turkey origin retained the receptor binding specificity to NeuAcα2,6Gal receptors like human viruses. Residues 226 and 228 on the receptor binding domain of the HA1 molecule were shown to play a critical role in determining receptor specificity (28, 39, 41). Val226 and Ser228 were expressed in the HA1 molecules of both turkey and swine triple reassortants, while Leu/Ile226 and Ser228 are usually expressed in the human viruses (22). Leu, Ile, and Val are neutral non-polar amino acids, and substitutions between them most likely maintain the hydrophobic interactions and the proper conformation at the binding domain. The Gln226 and Gly228 are usually found in the HA1 molecules of avian viruses (41).
Vaccination of breeder turkeys is the only routine vaccination done for influenza in poultry in the U.S. Currently there are no effective commercial vaccines (the H3N4 duck strain is the only one licensed vaccine for use in the U.S.) available for turkeys as indicated in our study. The use of a swine-origin virus for vaccination, although closer in sequence, also did not appear to be effective in protecting turkey breeders, because of the major antigenic changes between the two viruses of swine and turkey origin. Considering our study, it is emphasized that using viruses for vaccines that are antigenically similar to the field strains is of utmost importance.

3.6 ACKNOWLEDGMENTS

The authors are grateful to Dr. Eric Gonder (Goldsboro Milling Company, Goldsboro, North Carolina, USA), Dr. Kathleen A. Smith (The Ohio Department of Health, Reynoldsburg, Ohio, USA) and Lohmann Animal Health (Winslow, Main, USA) for providing the strains used in this study. We would also like to thank Mr. Robert Dearth for his help in the animal work for antisera production. This study was partially supported by funds from USDA, CSREES, AI-CAP project.

3.7 REFERENCES


nucleoprotein (NP) and herpes simplex virus genes (VP22) induces highly protective immunity against different subtypes of influenza virus. Virology 354:48-57.
<table>
<thead>
<tr>
<th>Virus</th>
<th>Origin</th>
<th>Year of isolation</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/turkey/Illinois/2004 (H3N2)</td>
<td>Turkey</td>
<td>2004</td>
<td>FAHRP/OSU</td>
</tr>
<tr>
<td>A/turkey/Ohio/313053/2004 (H3N2)</td>
<td>Turkey</td>
<td>2004</td>
<td>FAHRP/OSU</td>
</tr>
<tr>
<td>A/turkey/North Carolina/2003 (H3N2)</td>
<td>Turkey</td>
<td>2003</td>
<td>Dr. Eric Gonder</td>
</tr>
<tr>
<td>A/swine/North Carolina/2003 (H3N2)</td>
<td>Swine</td>
<td>2003</td>
<td>Dr. Eric Gonder</td>
</tr>
<tr>
<td>A/mallard duck/Minnesota/79 (H3N4)</td>
<td>Duck</td>
<td>1979</td>
<td>Lohmann Animal Health</td>
</tr>
<tr>
<td>A/human/Ohio/2006 (H3N2)</td>
<td>Human</td>
<td>2006</td>
<td>Ohio Department of Health</td>
</tr>
</tbody>
</table>

Table 3.1. Viruses included in the study.
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A/turkey/Illinois/04</td>
<td>-</td>
<td>100*</td>
<td>100</td>
<td>18</td>
<td>&lt;1</td>
<td>&lt;10</td>
</tr>
<tr>
<td>A/turkey/Ohio/313053/04</td>
<td>100*</td>
<td>-</td>
<td>100</td>
<td>13</td>
<td>&lt;1</td>
<td>&lt;10</td>
</tr>
<tr>
<td>A/turkey/North Carolina/03</td>
<td>86</td>
<td>80</td>
<td>-</td>
<td>25</td>
<td>&lt;1</td>
<td>&lt;10</td>
</tr>
<tr>
<td>A/swine/North Carolina/03 (H3N2/Vaccine)</td>
<td>30</td>
<td>24</td>
<td>25</td>
<td>-</td>
<td>&lt;1</td>
<td>&lt;10</td>
</tr>
<tr>
<td>A/mallard duck/Minnesota/79 (H3N4/Vaccine)</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>-</td>
<td>&lt;10</td>
</tr>
<tr>
<td>A/human/Ohio/06</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.2. Cross-HI and VN results expressed as percentage of antigenic relatedness.* The Archetti and Horsfall formula was used to calculate R-values. **Bold numbers:** R-Values based on HI tests; **Italic numbers:** R-values based on VN tests.
The percentage similarities were determined using the MegAlign-DNASTAR program. The words between brackets represent the virus lineage to which each gene belongs.

<table>
<thead>
<tr>
<th>Gene</th>
<th>A/turkey/Ohio/04 (H3N2)</th>
<th>A/swine/North Carolina/03 (H3N2/Vaccine)</th>
<th>A/mallard duck/Minnesota/79 (H3N4/Vaccine)</th>
<th>A/human/Ohio/06 (H3N2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB2</td>
<td>99.5 (Avian)*</td>
<td>96.6 (Avian)</td>
<td>91.1 (Avian)</td>
<td>84 (Human)</td>
</tr>
<tr>
<td>PB1</td>
<td>99.6 (Human)</td>
<td>96.8 (Human)</td>
<td>88.8 (Avian)</td>
<td>94.4 (Human)</td>
</tr>
<tr>
<td>PA</td>
<td>99.4 (Avian)</td>
<td>96.5 (Avian)</td>
<td>93.1 (Avian)</td>
<td>83.2 (Human)</td>
</tr>
<tr>
<td>HA1</td>
<td>99.3 (Human)</td>
<td>95.8 (Human)</td>
<td>77.9 (Avian)</td>
<td>91.9 (Human)</td>
</tr>
<tr>
<td>NP</td>
<td>99.7 (Swine)</td>
<td>96.6 (Swine)</td>
<td>83.6 (Avian)</td>
<td>82.1 (Human)</td>
</tr>
<tr>
<td>NA</td>
<td>99.5 (Human)</td>
<td>94.9 (Human)</td>
<td>53.6 (Avian)</td>
<td>94.9 (Human)</td>
</tr>
<tr>
<td>M</td>
<td>99.7 (Swine)</td>
<td>96.4 (Swine)</td>
<td>90.5 (Avian)</td>
<td>87.4 (Human)</td>
</tr>
<tr>
<td>NS</td>
<td>99.0 (Swine)</td>
<td>98.9 (Swine)</td>
<td>70.0 (Avian)</td>
<td>81.9 (Human)</td>
</tr>
</tbody>
</table>

* The percentage similarities were determined using the MegAlign-DNASTAR program. The words between brackets represent the virus lineage to which each gene belongs.

**Table 3.3.** Genetic similarity of the Illinois turkey virus to other H3 subtype viruses
Figure 3.1. Phylogenetic relationship based on nucleotide sequences of the H3 gene of different TR H3N2 viruses as compared to other H3N2 viruses of human, swine and avian origin. The tree was generated by the maximum parsimony method with 100 bootstrap replicates in a heuristic search with PAUP 4.0b10 software.
Figure 3.2. Phylogenetic relationship based on nucleotide sequences of the N2 gene of different TR H3N2 viruses as compared to other H3N2 viruses of human, swine and avian origin. N1 and N2 of avian lineage were included to define the lineage to which the NA gene of the duck strain belongs. The tree was generated by the maximum parsimony method with 100 bootstrap replicates in a heuristic search with PAUP 4.0b10 software.
Figure 3.3. Phylogenetic relationship based on nucleotide sequences of the NP gene of different TR H3N2 viruses as compared to other H3N2 viruses of human, swine and avian origin. The tree was generated by the maximum parsimony method with 100 bootstrap replicates in a heuristic search with PAUP 4.0b10 software.
Figure 3.4. Phylogenetic relationship based on nucleotide sequences of M gene of different TR H3N2 viruses as compared to other H3N2 viruses of human, swine and avian origin. The tree was generated using the maximum parsimony method with 100 bootstrap replicates in a heuristic search in the PAUP 4.0 b10 software.
Figure 3.5. Phylogenetic relationship based on nucleotide sequences of PB2 gene of different TR H3N2 viruses as compared to other H3N2 viruses of human, swine and avian origin. The tree was generated using the maximum parsimony method with 100 bootstrap replicates in a heuristic search in the PAUP 4.0 b10 software.
Figure 3.6. Alignment of HA1 amino acid sequences. Residues in the open boxes represent previously identified antigenic sites with H3 molecule. **Bold letters:** substitutions occurred at the four antigenic sites A, B, C and D. **Underlined residues:** Possible N-glycosylation sites.
Figure 3.7. Cartoon diagram representing the amino acid changes at the major antigenic sites of the HA1 molecules of H3 subtype influenza A viruses. **RBD**: receptor binding domain. a, b, and c: major antigenic sites at the HA molecules. **Yellow color**: conserved amino acids and **red color**: changed amino acids. I) TK/IL/04 VS. SW/NC/03, II) TK/IL/04 VS. DK/MN/79, III) TK/IL/04 VS. HUM/OH/06, IV) SW/NC/03 VS. HUM/OH/06.
CHAPTER 4

INTERSPECIES AND INTRASPECIES TRANSMISSION OF TRIPLE REASSORTANT H3N2 INFLUENZA VIRUSES

4.1 SUMMARY

The triple reassortant H3N2 viruses were isolated for the first time from pigs in 1998 and are known to be endemic in swine and turkeys populations in the United States. In 2004, we isolated two TR H3N2iple reassortant viruses from two turkey breeder flocks in Ohio and Illinois. Infected hens showed no clinical signs, but experienced a complete cessation of egg production. In this study, we evaluated three triple reassortant H3N2 isolates of turkey origin and one isolate of swine origin for their transmission between swine and turkeys. Although all 4 viruses tested share high genetic similarity in all 8 genes, only the Ohio strain (A/turkey/Ohio/313053/04) was shown to transmit efficiently both ways between swine and turkeys. One isolate, A/turkey/North Carolina/03, was able to transmit from pigs to turkeys but not vice versa. Neither of the other two viruses transmitted either way. Sequence analysis of the HA1 gene of the Ohio strain showed one amino acid change (D to A) at residue 190 of the receptor binding domain upon transmission from turkeys to pigs. The Ohio virus was then tested for intraspecies transmission in three different avian species. The virus was shown to replicate and transmit among turkeys,
replicate but does not transmit among chickens, and did not replicate in ducks.

Identifying viruses with varying inter- and intra-species transmission potential should be useful for further studies on the molecular basis of interspecies transmission.

4.2 INTRODUCTION

Influenza A viruses are highly contagious pathogens that have been isolated from a wide variety of animals, including man, birds, swine, horses, minks, seals, whales, and most recently from cats and dogs (26, 27, 36). Influenza A viruses are rarely known to cross species barriers (4, 21), however, their interspecies transmission has always been a major concern. Although determinants of interspecies transmission are still not fully identified, many studies showed that the compatibility between the hemagglutinin (HA) protein of the virus and its corresponding receptor on the host cell is essential for establishing an infection in a specific host (10-12). Pigs are known to be a major reservoir for H1N1 and H3N2 influenza viruses and have been hypothesized to act as intermediate host for interspecies transmission of influenza A viruses (5, 11, 25). Turkeys on the other hand, are susceptible to a wide range of influenza A viruses and serve as an important host for these viruses (31, 32). Influenza infections in turkeys range from asymptomatic to severe disease, including respiratory tract disorder, depression, drop in eggs production and high mortality (32). Between 1978 and 1981, our laboratory was the first to report on experimental and natural infections of turkeys with H1N1 swine influenza viruses (20, 37). In 1998, a new lineage of swine influenza viruses, triple reassortants (TR) H3N2, were isolated for the first time from pigs in the United States (U.S.) (38). These viruses had genes derived from human (HA, NA, and PB1), Swine (NP, M, and NS) and avian...
viruses (PA and PB2) (13, 35). The TR H3N2 viruses are now endemic in swine populations in North America (23, 35). In 2003 and 2004, similar viruses (TR H3N2) were isolated from turkeys in two different locations in the U.S. (7, 33). Later in the same year, we isolated another TR H3N2 virus from turkey breeder hens in Illinois that were vaccinated twice with a swine TR H3N2 virus. Infected turkeys experienced complete cessation of egg production, but had no other clinical signs. In a previous study we observed major antigenic differences between turkey and swine TR H3N2 viruses. The antigenic relatedness (R-value) between the turkey viruses and the swine virus (vaccine strain) was less than 30% as expressed by the Archetti and Horsfall formula (2) based on hemagglutinin inhibition (HI) and virus neutralization (VN) tests. At least eight amino acid changes were observed at the antigenic sites of the HA1 molecule between the turkey viruses and the swine vaccine virus. Although the transmission of TR H3N2 viruses from pigs to turkeys was suggested in previous reports (7, 33), no experimental work has been done to support this premise. Hence, we initiated this study to evaluate the interspecies transmission of these viruses between swine and turkeys, and to determine at the molecular level the basis for such transmission. Additionally, we tested one strain, A/turkey/Ohio/313053/04, that was shown to transmit between swine and turkeys for its intraspecies transmission in turkeys, chickens and ducks. Identifying viruses with different transmission potential between swine and turkeys will help in identifying the molecular determinants that control such transmission using the reverse genetics techniques.
4.3 MATERIALS AND METHODS

Viruses. Four TR H3N2 viruses of turkey or swine origin were included in this study. Additionally, two H1N1 viruses (one turkey origin and one swine origin) were included for comparison. Two H3N2 turkey viruses, A/turkey/Illinois/04 and A/turkey/Ohio/313053/04, were isolated in MDCK cells in our laboratory in 2004, and were propagated in 9-10 days old embryonated chicken eggs (ECE) to make working stocks. One turkey virus, A/turkey/North Carolina/03 (H3N2) (passaged twice (P2) in MDCK cells), and one swine virus, A/swine/North Carolina/03 (H3N2) (unknown passage number), were kindly provided by Dr. Eric Gonder (Goldsboro Milling Co. Goldsboro, NC), and were propagated in 9-10 days old ECE to make working stocks. The turkey H1N1 (A/turkey/Ohio/1988) and swine H1N1 (A/swine/Ohio/06) viruses were isolated in ECE in our lab in 1988 and 2006, respectively. Both viruses were propagated once in ECE to make working stocks. The two H1N1 influenza viruses were included as controls for the transmission from pig to turkey, but not in the turkey to pig transmission study.

Virus isolation. Turkey tracheal and pig nasal swabs were used for inoculation of MDCK cell line maintained in Opti-MEM minimum essential medium (Invitrogen, Grand Island, NY) containing 0.5 μg/ml trypsin. The samples were passaged twice in MDCK cells and then used to inoculate 9-10 days old specific pathogen free (SPF) ECE to make working stocks.

Transmission studies. A schematic layout of the room used for the transmission studies is presented in Figure 4.2. The rooms were mechanically ventilated and the air was HEPA filtered at the intake and the exhaust. Briefly, the infected and contact animals
were placed close to each other in two different cages (with rubber coated floors) to study the indirect transmission of TR H3N2 viruses between swine (large white breed) and specific pathogen free (SPF) turkeys. The direction of the air current was always from the infected animals’ side to the contact animals’ side. The animals received a virus titer of $10^7$ TCID50 contained in 0.5 ml, and the contact animals were placed in the same room close to the infected animals at one day post inoculation (1 DPI). Nasal swabs from pigs and tracheal swabs from turkeys were collected on daily basis and were maintained in Brain Heart Infusion (BHI) media and were directly used for RNA extractions.

Intraspecies transmission experiments with the Ohio virus (A/turkey/Ohio/313053/04) were performed in SPF turkeys, SPF chickens and commercial pekin ducks. The individual bird (n=15 for turkeys and ducks, and n=20 for chickens) received a virus titer of $10^7$ TCID50 contained in 0.5ml, and the contact animals (10 turkeys, 10 chickens and 15 ducks) animals were placed in the same cage at 1 DPI. Tracheal swabs were collected on daily basis and were maintained in BHI media and were directly used to do RNA extractions. Non-inoculated negative control animals were placed in a separate room and were treated like infected animals.

**Antisera collection and HI test.** Blood was collected from all animals at zero and fourteen days post inoculation/exposure (DPI/DPE) to test for antibodies to H3N2 and H1N1 influenza viruses. Sera were harvested and inactivated at 56°C for 30 min before being used in hemagglutinin inhibition (HI) test. The HI test was carried out as previously described (3). Titers were determined by using twofold serial dilutions of antisera (25μl), 4 HA/25μl units of homologous antigen and a 0.5% suspension of turkey erythrocyte per test well.
RNA extraction and real-time RT-PCR. RNA extraction and rRT-PCR reactions were performed as previously described (15, 28, 30). Briefly, swab samples in 1.5 ml BHI media were vortexed for 5 seconds then left standing for 15 min to precipitate the debris. Of the 1.5 ml swab sample, 300 μl were used for RNA extraction using the RNeasy kit (Qiagen, Valencia, CA). rRT-PCR was performed in 25 μl reaction volume using the Qiagen one-step RT-PCR kit with the following conditions: 10 pmol of each primer, 320 μM each dNTP, 0.12 μM FAM labeled probe, 13 units RNase inhibitor, 1 μl enzyme mix, 8 μl of RNA sample, and water was added to get a total volume of 25 μl. The rRT-PCR conditions were: 50°C for 30 min, 95°C for 15 min, and 45 cycles of 1 sec at 94°C and 20 sec at 60°C. Reactions were run in the Cephid Smartcycler thermocycler (Utech Products, Inc.; Schenectady, NY 12305).

Standard curve for virus titer estimation. To estimate the virus titer in the infected animals, we established a standard curve based on one turkey and one swine H3N2 viruses of known TCID₅₀ titer. Briefly, RNA was extracted from A/turkey/Illinois/04 and A/swine/North Carolina/03 and serial dilutions were prepared. The serially diluted RNA was used to run the rRT-PCR as described above and a standard curve was established.

Sequence analysis and molecular graphic visualization. The HA1 and NA genes of the A/turkey/Ohio/313053/04 virus were amplified from viruses obtained from directly inoculated pigs, pigs in contact with infected turkeys, directly inoculated turkeys and turkeys in contact with infected pigs. Both genes were amplified with standard reverse transcription (RT) PCR using influenza specific primers and one-step RT-PCR kit (Qiagen) following the manufacturer instructions. The RT-PCR products were separated by electrophoresis on 1% agarose gel, and amplicons of the right size were excised from
the gel and purified with Qiaquick gel extraction kit (Qiagen). Sequencing was done at the Ohio Agricultural Research and Development Center (OARDC) sequencing facility using the ABI Prism 3100 automated sequencing machine (Applied Biosystems, Foster City, CA 94404). Pairwise sequence alignments were performed in the MegAlign program (DNASTAR, Madison, Wis.) to determine nucleotides and amino acids sequences similarity. Amino acid changes in the HA protein of different isolates were located using the Rasmol software (v2.6.4) (Biomolecular Structures Group, Hertfordshire, UK) on the HA structure of H3 subtype influenza virus, A/Aichi/2/68, (1HGG) downloaded from the Protein Data Bank website (9, 24).

4.4 RESULTS

Interspecies transmission of H3N2 influenza viruses from pigs to turkeys. Three H3N2 influenza isolates of turkey origin and one H3N2 influenza isolate of swine origin were evaluated for their transmission from pigs to turkeys. Additionally, two H1N1 isolates of swine and turkey origins were included for comparison. All viruses were shown to replicate in pigs but with different efficiencies (Table 4.1). The A/turkey/Ohio/313053/04 and A/turkey/North Carolina/03 viruses replicated more efficiently than the other H3N2 viruses, with nasal swab titer of 2\times10^6 and 2\times10^{6.6} 50% tissue culture infectious dose (TCID_{50}) per ml, respectively (Table 4.1). The H1N1 turkey strain, A/turkey/Ohio/88, showed the highest replication titer (2\times10^{8.1} TCID_{50}/ml) among all viruses tested. The Ohio strain, A/turkey/Ohio/313053/04, elicited the highest antibody titer (1:360 HI) among all the H3N2 viruses tested (Table 4.1). Different patterns of transmission from pigs to turkeys were observed among the TR H3N2 viruses
The H3N2 Ohio strain was transmitted from pigs to turkeys and virus was detected for more than two days in turkeys using the real-time reverse-transcription PCR (rRT-PCR). Four out of the eight contact turkeys got infected and three of them seroconverted to an average HI titer of 1:80. The A/turkey/North Carolina/03 virus was detected in three out of eight contact turkeys using the rRT-PCR, with one turkey detected positive for two days; however, none of the infected turkeys seroconverted. Viruses were successfully re-isolated using Madin-Darby Canine Kidney (MDCK) cells from the contact turkeys infected with A/turkey/Ohio/313035/04 and A/turkey/North Carolina/03 H3N2 viruses. On the other hand, although three out of eight and four out of eight swab samples were AIV-positive with rRT-PCR at two days post exposure (DPE) from turkeys in contact with pigs infected with A/turkey/Illinois/04 and A/swine/North Carolina/03, respectively, no viruses were isolated from any of the rRT-PCR positive samples and none of the turkeys seroconverted (Table 4.2). Both H1N1 viruses replicated in pigs, but none of them were detected in the contact turkeys as evaluated with rRT-PCR and HI tests.

**Interspecies transmission of H3N2 influenza viruses from turkeys to pigs.** We also evaluated the transmission of the H3N2 viruses from turkeys to pigs (Tables 4.3 and 4.4). As expected, all H3N2 viruses replicated in turkeys regardless of their isolation origin and were detected in the inoculated turkeys for at least six days, except for the A/swine/North Carolina/03 virus that was detected for only four days. In general, the swab viral titers were lower than that from pigs, ranging from 2X10^{2.8} to 2X10^{3.3} TCID_{50}/ml. Again, the Ohio and North Carolina turkey isolates replicated at the highest titers of 2X10^{3.3} TCID_{50}/ml. All viruses were shown to elicit antibody response in turkeys
with the highest titer observed against the Ohio strain at 1:420 HI. Only the Ohio strain transmitted from the infected turkeys to the contact pigs as determined by rRT-PCR, HI test and virus isolation (Table 4.4). The first positive pig was detected at the 3 DPE, and the rest became positive at 5 DPE. All pigs infected with the Ohio strain seroconverted with an average HI titer of 1:320.

**Sequence analysis.** The two surface glycoproteins encoding genes, HA and NA, were amplified and sequenced from A/turkey/Ohio/313053/04 H3N2 virus isolated from directly inoculated pigs, pigs in contact with infected turkeys, directly inoculated turkeys and turkeys in contact with infected pigs. Pairwise sequence alignment showed two changes in the HA gene sequence upon replication and transmission of the virus from pigs and turkeys. The first change was observed at residue 190 (D to A) of the receptor binding domain (RBD) in viruses isolated from pigs in contact with infected turkeys (Figure 4.1). The other change was observed at residue 246 (S to N) in two of the inoculated pigs and one of the turkeys in contact with inoculated pigs (Figure 4.1). No changes were observed in the NA gene upon replication and transmission of the virus from pigs to turkeys and vise versa.

**Intraspecies transmission of A/turkey/Ohio/313053/04 H3N2 virus in turkeys, chickens and ducks.** To evaluate the transmission potential of H3N2 viruses in different avian species, we tested the intraspecies transmission of A/turkey/Ohio/313053/04 virus (strain that showed efficient transmission between pigs and turkeys) in turkeys, chickens and ducks (Table 4.5). The virus behaved differently in different avian species, where it was capable of replication in turkeys and chickens, but not in ducks. Although the replication titers in chickens were higher than those in turkeys, $2 \times 10^6$ and $2 \times 10^{3.4}$
TCID$_{50}$/ml, respectively, no transmission was detected among chickens. The virus was detected for more than one day in 90% of the inoculated chickens, of which 62% seroconverted to an average titer of 1:216 HI. On the other hand, 80% of the inoculated turkeys were positive with rRT-PCR for influenza virus for more than two days, and all of them seroconverted to an average HI titer of 1:990. The very high HI average titer of the turkey serum was due to two turkeys that showed an HI titer of 5120 and 2560 respectively. Nine of the ten contact turkeys in the same cage became positive, two of which were positive at 3 DPE, while the rest were positive between 7 DPE and 9 DPE. Only two of the contact turkeys seroconverted to an HI titer of 1:120 HI units. The delay in infection in most of the contact turkeys would explain the negative HI tests (only two of the contact turkeys were positive) that were performed on serum samples collected at 14 day post exposure (DPE).

4.5 DISCUSSION

Generally, influenza A viruses are considered host specific, nevertheless, some can overcome the species barrier and infect a new host. The mechanisms by which the influenza A viruses cross the species barriers and the molecular determinants that control such transmission are not well identified. Pigs have been hypothesized to play a role in interspecies transmission by acting as “mixing vessels” for the generation of reassortant viruses that might have the potential to jump from one species to another (6, 14). In 1998, a new lineage of swine viruses, TR H3N2, emerged and caused influenza like illnesses in pig populations in the U.S. (13, 38). Similar viruses were later isolated from turkey
breeder hens experiencing drop in eggs production and it was hypothesized that these
viruses were transmitted from pigs to turkeys (7, 33).

Our findings indicated the ability of certain TR H3N2 viruses to transmit between pigs
and turkeys. Despite the high degree of molecular similarity between some of these
viruses, like A/turkey/Illinois/04 and A/turkey/Ohio/313053/04 (>99% similarity in all
genes), they behaved differently in the transmission experiments, with the
A/turkey/Ohio/313053/04 transmitting both ways between the two species and the
A/turkey/Illinois/04 virus not transmitting either way.

Regardless of the differences in transmission, all viruses were capable of replication in
turkeys and pigs but to different titers. Furthermore, the A/turkey/Ohio/313053/04, the
strain transmissible between pigs and turkeys, was shown to infect and transmit among
turkeys, infect but did not transmit among chickens, and did not infect ducks.

We speculate that the TR H3N2 viruses, which have the HA gene from human lineage
viruses, retain the receptor binding specificity to NeuAcα2,6Gal receptors similar to
human influenza viruses. Val226 and Ser228 were expressed in the HA1 molecules of
both turkey and swine triple reassortants, while Leu/Ile226 and Ser228 are usually
expressed in the human viruses (17). Leu, Ile, and Val are neutral non-polar amino acids,
and substitutions between them most likely maintain the hydrophobic interactions and the
proper conformation at the binding domain (34). Gln226 and Gly228 are usually found in
the HA1 molecules of avian viruses amino acids at these positions and are known to play
a critical role in determining the receptor binding specificity (34). Our studies (data not
shown) demonstrated the presence of substantial amount of NeuAcα2,6Gal receptors in
turkey tracheas, which would explain the ability of these viruses to replicate in turkeys as
well as in pigs that are known to express these receptors (11). Although ducks were shown to express few NeuAcα2,6Gal receptors in their tracheas, the A/turkey/Ohio/313053/04 H3N2 virus was not able to replicate in ducks. The absence of a large number of NeuAcα2,6Gal receptors in ducks’ tracheas may explain the inability of the A/turkey/Ohio/313053/04 H3N2 virus to replicate in ducks. However, there may be factors other than receptors distribution that contribute to host tropism of influenza viruses and more work is needed in this area.

While all viruses had the Asp (D) amino acid at residue 190 of the receptor binding domain (RBD), a D to A (Ala) change occurred upon the transmission of the A/turkey/Ohio/313053/04 virus from turkeys to pigs. The presence of either D (specific for SAα2,6gal) or E (specific for SAα2,3gal) at amino acid position 190 of the HA molecule in the H3 subtypes was reported in previous studies (19, 22), however, our observation of (A) residue at this position is the first of its kind to our knowledge (sequencing was performed on the HA gene of the Ohio virus isolated from three different pigs in contact with infected turkeys). The role of (A) residue at position 190 in determining receptor binding specificity should be further investigated. In addition, the role of Asn (N) residue at position 246 of the HA molecule is not known and will be further studied in our laboratory.

Although all viruses were shed by pigs for more than 6 days, the A/turkey/Ohio/313053/04 and A/turkey/North Carolina/03 viruses replicated to higher titers than A/turkey/Illinois/04 and A/swine/North Carolina/03 viruses. This might be one of the possible reasons that allowed A/turkey/Ohio/313053/04 and A/turkey/North Carolina/03 viruses to transmit from pigs to turkeys (all animals were inoculated with the
same virus titer). The A/turkey/Illinois/04 and A/swine/North Carolina/03 viruses were detected only on one day in contact turkeys by rRT-PCR, however, no viruses were obtained upon isolation attempts. The high sensitivity of the rRT-PCR might explain the ability to detect these viruses in contact turkeys, whereas the viruses were inefficient in replicating to a high titer in turkeys. In contrast, the A/turkey/Ohio/1988 H1N1 virus was shown to replicate to a very high titer in pigs ($10^{8.1} \text{TCID}_{50}$), but it did not transmit to turkeys. The above observations indicate the specificity of individual influenza A viruses, even within the same subtype (TR H3N2 in this case), in their ability to transmit between species.

Previous analysis of the A/swine/North Carolina/03 virus in our laboratory showed that it has a 13 amino acids stalk deletion in the NA protein. Shortened NA stalks might result in less efficient virus release, and hence lower virus titers (8, 18). This might explain our results from pigs and turkeys. However, the exact effect of NA stalk deletion is not clear because many chicken adapted H5, H7, and H9 viruses show different length stalk deletions and replicate to very high titer in poultry (1, 16, 29).

The identification of viruses with varying potential for interspecies transmission should be useful for reverse genetic studies to identify the gene(s) and the amino acid(s) residues that contribute to the transmission of these viruses between swine and turkeys. The use of the reverse genetics and site directed mutagenesis could also be helpful in deciphering the role of residues 190 and 246 of the HA molecule in receptor binding specificity and transmission of these viruses between swine and turkeys.

Interspecies transmission studies between swine (mammalian) and turkeys (avian) will enhance our understanding of the genetic factors that control transmission of influenza
viruses and would help in improvement of surveillance strategies for early detection of influenza A viruses.

4.6 ACKNOWLEDGEMENTS

The authors are grateful to Dr. Eric Gonder for providing two of the strains used in this study. We would also like to thank Mr. Robert Dearth and Mr. Abul Rauf for their help in animal work. This study was partially supported by funds from USDA, CSREES, AI-CAP project.

4.7 REFERENCES


<table>
<thead>
<tr>
<th>Virus</th>
<th>1 to 3 DPI*</th>
<th>4 to 6 DPI</th>
<th>No. positives for 2 or more days</th>
<th>Peak day of virus detection</th>
<th>Estimated average virus titer on peak day/ml</th>
<th>No. of animals seroconverted/total inoculated</th>
<th>HI**** average titer at 14 DPI</th>
<th>Virus isolation from swab samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/TK/IL/04 (H3N2)</td>
<td>5/5**</td>
<td>3/3***</td>
<td>5/5</td>
<td>4DPI</td>
<td>2X10^4.5</td>
<td>3/3</td>
<td>1:160</td>
<td>Positive</td>
</tr>
<tr>
<td>A/TK/OH/04 (H3N2)</td>
<td>5/5</td>
<td>4/4***</td>
<td>5/5</td>
<td>5DPI</td>
<td>2X10^6.0</td>
<td>4/4</td>
<td>1:360</td>
<td>Positive</td>
</tr>
<tr>
<td>A/TK/NC/03 (H3N2)</td>
<td>5/5</td>
<td>4/4***</td>
<td>5/5</td>
<td>4DPI</td>
<td>2X10^4.6</td>
<td>4/4</td>
<td>1:220</td>
<td>Positive</td>
</tr>
<tr>
<td>A/SW/NC/03 (H3N2)</td>
<td>5/5</td>
<td>3/3***</td>
<td>5/5</td>
<td>4DPI</td>
<td>2X10^4.7</td>
<td>3/3</td>
<td>1:340</td>
<td>Positive</td>
</tr>
<tr>
<td>A/TK/OH/88 (H1N1)</td>
<td>5/5</td>
<td>4/4***</td>
<td>5/5</td>
<td>4DPI</td>
<td>2X10^5.1</td>
<td>4/4</td>
<td>1:320</td>
<td>NT</td>
</tr>
<tr>
<td>A/SW/OH/06 (H1N1)</td>
<td>5/5</td>
<td>4/4***</td>
<td>5/5</td>
<td>4DPI</td>
<td>2X10^5.6</td>
<td>4/4</td>
<td>1:160</td>
<td>NT</td>
</tr>
</tbody>
</table>

* Days post inoculation. Swabs were collected on daily bases and results are displayed in three days intervals.

** No. of pigs positive with rRT-PCR/No. of pigs inoculated.

*** Some pigs were euthanized at 3 DPI to collect organs and tissues for other studies.

**** Hemagglutinin inhibition.

NT Not Tested.

**Table 4.1.** Interspecies transmission of H3N2 and H1N1 influenza viruses from pigs to turkeys; virus detection in inoculated pigs.
<table>
<thead>
<tr>
<th>Virus</th>
<th>1 to 3 DPE</th>
<th>4 to 6 DPE</th>
<th>7 to 9 DPE</th>
<th>No. positives for 2 or more days</th>
<th>Peak day of virus detection</th>
<th>Estimated average virus titer on peak day/ml</th>
<th>No. of animals seroconverted / total exposed</th>
<th>HI*** average titer at 14 DPE</th>
<th>Virus isolation from swab samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/TK/IL/04 (H3N2)</td>
<td>3/8**</td>
<td>0/8</td>
<td>0/8</td>
<td>2DPE</td>
<td>2X10³</td>
<td>0/8</td>
<td>-</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>A/TK/OH/04 (H3N2)</td>
<td>0/8</td>
<td>4/8</td>
<td>2/8</td>
<td>6DPE</td>
<td>2X10³.12</td>
<td>3/8</td>
<td>1:80</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>A/TK/NC/03 (H3N2)</td>
<td>0/8</td>
<td>2/8</td>
<td>1/8</td>
<td>5DPE</td>
<td>2X10³.8</td>
<td>0/8</td>
<td>-</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>A/SW/NC/03 (H3N2)</td>
<td>4/8</td>
<td>0/8</td>
<td>0/8</td>
<td>2DPE</td>
<td>2X10³</td>
<td>0/8</td>
<td>-</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>A/TK/OH/88 (H1N1)</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>A/SW/OH/06 (H1N1)</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NT</td>
</tr>
</tbody>
</table>

* Days post exposure. Swabs were collected on daily bases and results are displayed in three days intervals.

** No. of turkeys positive with rRT-PCR / total No. of contact turkeys

*** Hemagglutinin inhibition

NT Not Tested

**Table 4.2.** Interspecies transmission of H3N2 and H1N1 influenza viruses from pigs to turkeys; virus detection in turkeys in contact with inoculated pigs.
<table>
<thead>
<tr>
<th>Virus</th>
<th>1 to 3 DPI*</th>
<th>4 to 6 DPI</th>
<th>7 to 9 DPI</th>
<th>No. positives for 2 or more days</th>
<th>Peak day of virus detection</th>
<th>Estimated average virus titer on peak day/ml</th>
<th>No. of animals seroconverted/total inoculated</th>
<th>HI*** average titer at 14DPI</th>
<th>Virus isolation from swab samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/TK/IL/04 (H3N2)</td>
<td>6/10**</td>
<td>7/10</td>
<td>NT</td>
<td>8/10</td>
<td>5DPI</td>
<td>2X10^2</td>
<td>9/10</td>
<td>1:300</td>
<td>Positive</td>
</tr>
<tr>
<td>A/TK/OH/04 (H3N2)</td>
<td>7/10</td>
<td>5/10</td>
<td>2/10</td>
<td>8/10</td>
<td>4DPI</td>
<td>2X10^1.3</td>
<td>4/6</td>
<td>1:420</td>
<td>Positive</td>
</tr>
<tr>
<td>A/TK/NC/03 (H3N2)</td>
<td>6/10</td>
<td>6/10</td>
<td>NT</td>
<td>6/10</td>
<td>4DPI</td>
<td>2X10^1.3</td>
<td>3/10</td>
<td>1:80</td>
<td>Positive</td>
</tr>
<tr>
<td>A/SW/NC/03 (H3N2)</td>
<td>4/10</td>
<td>1/10</td>
<td>0/10</td>
<td>3/10</td>
<td>3DPI</td>
<td>2X10^0.8</td>
<td>2/10</td>
<td>1:80</td>
<td>Positive</td>
</tr>
</tbody>
</table>

* Days post inoculation. Swabs were collected on daily bases and results are displayed in three days intervals.
** No. of turkeys positive with rRT-PCR / No. of inoculated turkeys
*** Hemagglutinin inhibition
NT Not Tested

Table 4.3. Interspecies transmission of H3N2 influenza viruses from turkeys to pigs; virus detection in inoculated turkeys.
### Table 4.4. Interspecies transmission of H3N2 influenza viruses from turkeys to pigs; virus detection in pigs in contact with inoculated turkeys.

<table>
<thead>
<tr>
<th>Virus</th>
<th>1 to 3 DPE*</th>
<th>4 to 6 DPE</th>
<th>7 to 9 DPE</th>
<th>No. positives for 2 or more days</th>
<th>Peak day of virus detection</th>
<th>Estimated average virus titer on peak day/ml</th>
<th>No. of animals seroconverted/total exposed</th>
<th>HI*** average titer at 14DPI</th>
<th>Virus isolation from swab samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/TK/IL/04 (H3N2)</td>
<td>0/5**</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>-</td>
<td>-</td>
<td>0/5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A/TK/OH/04 (H3N2)</td>
<td>1/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5DPE</td>
<td>2x10^4</td>
<td>5/5</td>
<td>1:320</td>
<td>Positive</td>
</tr>
<tr>
<td>A/TK/NC/03 (H3N2)</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>-</td>
<td>-</td>
<td>0/5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A/SW/NC/03 (H3N2)</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>-</td>
<td>-</td>
<td>0/5</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Days post exposure. Swabs were collected on daily bases and results are displayed in three days intervals.

** No. of pigs positive with rRT-PCR /total No. of contact pigs

*** Hemagglutinin inhibition
<table>
<thead>
<tr>
<th>Virus TK/OH/04 (H3N2)</th>
<th>No. positive 1 to 3 DPI/DPE</th>
<th>No. positive 4 to 6 DPI/DPE</th>
<th>No. positive 7 to 9 DPI/DPE</th>
<th>No. positive 10 to 12 DPI/DPE</th>
<th>Peak day of virus detection</th>
<th>Estimated average virus titer on peak day/mL</th>
<th>No. of animals seroconverted/total exposed</th>
<th>HI average titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected chickens</td>
<td>19/20</td>
<td>10/16*</td>
<td>NT</td>
<td>NT</td>
<td>2DPI</td>
<td>2X10^6</td>
<td>10/16</td>
<td>1:216</td>
</tr>
<tr>
<td>Contact chickens</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>NT</td>
<td>-</td>
<td>-</td>
<td>0/10</td>
<td>-</td>
</tr>
<tr>
<td>Infected ducks</td>
<td>0/15</td>
<td>0/15</td>
<td>0/15</td>
<td>NT</td>
<td>-</td>
<td>-</td>
<td>0/15</td>
<td>-</td>
</tr>
<tr>
<td>Contact ducks</td>
<td>0/15</td>
<td>0/15</td>
<td>0/15</td>
<td>NT</td>
<td>-</td>
<td>-</td>
<td>0/15</td>
<td>-</td>
</tr>
<tr>
<td>Infected turkeys</td>
<td>13/15</td>
<td>8/10*</td>
<td>NT</td>
<td>NT</td>
<td>3DPI</td>
<td>2X10^3.4</td>
<td>10/10</td>
<td>1:990</td>
</tr>
<tr>
<td>Contact turkeys</td>
<td>2/10</td>
<td>2/10</td>
<td>6/10</td>
<td>7/10</td>
<td>8DPE</td>
<td>2X10^1.5</td>
<td>2/10</td>
<td>1:120</td>
</tr>
</tbody>
</table>

* Some of the inoculated turkeys and chickens were euthanized at 3DPI to collect tracheas for other studies

Table 4.5. Intraspecies transmission of A/TK/OH/313053/04 (H3N2) Influenza virus in chickens, ducks and turkeys.
Figure 4.1. Schematic of the room used in the study of interspecies transmission of influenza viruses (swine to turkey transmission setting in this case). The air flowed from the experimentally infected animals to the contact uninfected animals. Scale is not proportional.
Figure 4.2. Cartoon representing the amino acid changes at the HA molecule of the A/turkey/Ohio/313053/04 H3N2 virus, that occurred upon replication and transmission of the virus between turkeys and pigs. Red: receptor binding domain (RBD). Yellow: the change at residue 190 that occurred upon transmission of the virus from turkeys to pigs. Violet: the change at residue 246 that occurred in two of the inoculated pigs and one of the contact turkeys with inoculated pigs.
CHAPTER 5

DETERMINANTS FOR INTERSPECIES TRANSMISSION OF TRIPLE REASSORTANT H3N2 INFLUENZA A VIRUSES BETWEEN SWINE AND TURKEYS

5.1 SUMMARY

Triple reassortant H3N2 influenza viruses emerged in swine in 1998 and then in turkeys in 2003. It was hypothesized that the virus had the ability to cross the species barrier to transmit from pigs and infect turkeys. In previous work we identified viruses with different transmission potential between both species. A/turkey/Ohio/313053/04 was shown to be transmissible both ways between swine and turkeys while A/swine/North Carolina/03 did not transmit either way between the two species. Using the 12-plasmid reverse genetics system, we rescued the two viruses and switched the hemagglutinin or neuraminidase genes between the rescued strains. In vivo experiments indicated that the hemagglutinin gene of A/turkey/Ohio/313053/04 plays a significant role in its replication and transmission amongst turkeys. Additionally, it was shown that the hemagglutinin gene was a key but not the only player that determines the efficient replication of A/turkey/Ohio/313053/04 virus in pigs. A/turkey/Ohio/313053/04 showed greater binding affinity to plasma membrane preparations from pig and turkey tracheal/bronchial epithelial cells, as compared to A/swine/North Carolina/03 virus.
5.2 INTRODUCTION

Influenza A viruses are enveloped viruses belonging to the family Orthomyxoviridae that encompasses four more genera: Influenza B, Influenza C, Isavirus and Thogotovirus. Type A is the only genus that is highly infectious to variety of animal species, including human, pigs, wild and domestic birds, horses, cats, dogs, ferrets, seals, whales, and may be others (24, 39). They are divided into subtypes based on two surface glycoprotein, the hemagglutinin (HA) and the neuraminidase (NA). So far, 16 HA and 9 NA subtypes have been identified.

Wild aquatic birds are considered natural reservoirs for influenza A viruses, from which all subtypes have been isolated. Infection with influenza A viruses in other animal species is limited to few subtypes, example: human (H1, H2, H3, N1 and N2), swine (H1, H3, N1, and N2), and equine (H3, H7, N7, N8) (24, 39). In some occasions, the virus can cross the species barrier and infect a new host, like infection of human with avian viruses (H5, H7, and H9) (38), infection of dogs with equine viruses (H3) (6), and infection of pigs with both human and avian viruses (H3 human, H4 and H5 avian) (11, 16, 32, 42).

Avian viruses are generally thought to preferentially bind the N-acetylneuraminic acid-α2,3-galactose (NeuAcα2,3Gal) form of sialic acid receptors and human viruses preferentially bind to NeuAcα2,6Gal sialic acid receptors. Pigs express substantial amount of both forms of sialic acids on their respiratory epithelial cells, and it is believed that both avian and human influenza viruses can attach to the appropriate receptor and infect pigs. Hence, pigs have been postulated to serve as “mixing vessels” in which two or more influenza viruses can co-infect and reassort in epithelial cells in the upper respiratory tract, with a potential for development of new strains that can transmit to and
infect other species (10). First isolation of the virus from pigs (H1N1) was in 1930 and they become to be known as classical H1N1 lineage of swine viruses (cH1N1). In the United States (U.S.), cH1N1 viruses remained genetically and antigenically stable until the 1990s, when variants of cH1N1 viruses were isolated (13, 35, 39). In 1998, a new lineage of influenza viruses (H3N2) was isolated for the first time from pigs with influenza like illnesses in the U.S. These viruses were triple reassortants (TR) with genes from human (HA, NA, and PB1), swine (NP, M, and NS), and avian (PB2 and PA) lineage viruses (12, 44). Starting from 2003, similar viruses were isolated from turkey breeder hens experiencing drastic drop in egg production, with no other clinical signs observed (5, 33). Based on previous observations on transmission of cH1N1 viruses from swine to turkeys (20), it was hypothesized that TR H3N2 viruses were able to cross species barrier to transmit from pigs and infect turkeys (33). In previous work, we identified viruses (TR H3N2) with different transmission potential between swine and turkeys. Some viruses were able to transmit both ways between the two species, some transmitted only one way from swine to turkeys and some that did not transmit either way. Interestingly, changes were observed in the HA but not in the NA protein upon transmission of A/turkey/Ohio/313053/04 virus between swine and turkeys. One of these changes was observed at the receptor binding domain of the HA molecule (D190A), and the other was at the residue 246 of the HA molecule (40). Based on the previous observations, we generated two viruses with potentially different transmission behavior using the 12-plasmid reverse genetic system. Focusing on HA and NA genes, we generated single gene reassortants (SGR) between the two strains and tested their replication and transmission amongst turkeys as well as replication in pigs.
Additionally, we evaluated the two original viruses for their binding affinity to plasma membrane preparations from pig and turkey tracheal/bronchial epithelial cells. Identifying molecular markers that contribute to interspecies transmission of influenza viruses would help in improving the surveillance strategies for early detection of variant strains that can cross species barriers. It would also help in improving pandemic preparedness plans as well as vaccine development strategies to encounter infections with influenza A viruses.

5.3 MATERIALS AND METHODS

**Viruses.** Three viruses were included in this study: A/turkey/Ohio/313053/04, A/swine/North Carolina/03, and A/turkey/Ohio/313053/04(D190A). All viruses are part of our laboratory repository and are propagated in 10-days old embryonated chicken eggs (ECE). The first two viruses (A/turkey/Ohio/313053/04 and A/swine/North Carolina/03) were used to generate reassortant strains with reverse genetics, and were used in the animal experiments as described below. The third virus (A/turkey/Ohio/313053/04(D190A)) was originated upon the transmission of A/turkey/Ohio/313053/04 from turkeys to pigs in a previous study (40) and it harbor D190A mutation at the receptor binding domain (RBD). It was included in the plasma membrane binding assay for comparison.

**Virus rescue with reverse genetics.** Two viruses (A/turkey/Ohio/313053/04 and A/swine/North Carolina/03) with potentially different transmission behavior between swine and turkeys were rescued using 12-plasmid reverse genetics (RG) system based on previously established protocol (15, 21). Briefly, RNA was extracted from viruses in
allantoic fluid using RNeasy mini kit (Qiagen, Valencia, CA). HA, NP, NA, M, and NS genes were amplified using one-step RT-PCR kit (Qiagen, Valencia, CA), while PB1, PB2, and PA were amplified with two-steps RT-PCR using SuperscriptIII First Strand Synthesis System and Elongase Enzyme Mix (Invitrogen, San Diego, CA). RT-PCR products were separated on 1% agarose gel, and amplicons of the right size were excised from the gel and purified using Qiaquick gel extraction kit (Qiagen, Valencia, CA). Products were then digested with BsmBI restriction enzyme (BsmBI restriction site was designed within primers sequences) and cloned into pH21 vector between promoter and terminator sequences of RNA polymerase I. Eight plasmids harboring eight segments were transfected along with four expression plasmids (pCAGGS-WSN-NP, pcDNA774-PB1, pcDNA762-PB2, and pcDNA787-PA) into 293T cells with the help of Lipofectamine 2000 reagent (Invitrogen, San Diego, CA). Supernatant from transfected cells was collected at 48 hour post transfection and was subsequently inoculated into 10-days old ECE. After 72 hour of incubation, the allantoic fluid was harvested from the eggs, tested for hemagglutination activity, and stored at -70°C for further experiments.

For generation of single gene reassortants (SGR), HA or NA gene (cloned on pH21 vector) from one strain was replaced by its correspondent gene from the other strain during the transfection process. The rest of procedure is the same. Reassortants were confirmed by sequencing.

**Animal experiments.** Reassortants with changes in the HA or NA genes were tested for replication and transmission in turkeys. Six 3-week old specific pathogen free (SPF) turkeys were inoculated intranasally with a virus titer of 5×10^6 EID_{50}/ml. Three turkeys were then placed in contact with the inoculated group in the same cage at one day post
inoculation (1 DPI). Tracheal swabs were collected from inoculated and contact turkeys at 2, 4, and 7 DPI and were tested with real-time PCR (rRT-PCR) for influenza A virus detection. Turkeys were euthanized at 15 DPI, serum was collected and tested for antibodies against influenza A virus using HI test.

Reassortants with changes in HA gene were evaluated for replication in pigs deprived from sow’s colostrums. We tested pigs from more than four sources and they were all seropositive for influenza A virus (high hemagglutination inhibition (HI) titers for H1 subtype and low to moderate titers for H3 subtype). Hence, we naturally delivered pigs from two sows at our facility and fed them with cow’s colostrum-COLOSTRX (AgriLabs, St. Joseph, MO) for three days and then with SPF-LAC (PET-AG, Hampshire, IL) until 2-weeks old. All pigs had HI titer <4 before the start of the experiment. Pigs were separated into four groups, each with three pigs receiving a virus titer of 5X10^6 EID_{50}/ml per pig, using intranasal rout. Nasal swabs were collected from the pigs at 2, 4, 7, and 9 DPI and were tested with rRT-PCR for influenza virus detection. All animals were euthanized at 15 DPI, serum was collected and tested for antibodies against influenza A virus using HI test.

**RNA extraction RRT- PCR for influenza detection.** RNA extraction and rRT-PCR reactions were performed as previously described (29, 30). Briefly, swab samples in 2ml brain heart infusion (BHI) media were vortexed for 5 seconds then left standing for 15 min to precipitate the debris. Of the 2ml swab sample, 200 μl were used for RNA extraction using the RNeasy kit (Qiagen, Valencia, CA). rRT-PCR was performed in 25 μl reaction volume using the Qiagen one-step RT-PCR kit with the following conditions: 10 pmol of each primer (matrix gene specific primers), 320 μM each dNTP, 0.12 μM
FAM labeled probe, 13 units RNase inhibitor, 1 μl enzyme mix, 8 μl of RNA sample, and water was added to get a total volume of 25 μl. The rRT-PCR conditions were: 50°C for 30 min, 95°C for 15 min, and 45 cycles of 1 sec at 94°C and 20 sec at 60°C. Reactions were run in the Cephid Smartcycler thermocycler (Utech Products, Inc.; Schenectady, NY 12305). To estimate the virus titer in the infected animals, we established a standard curve based on a virus of known EID₅₀ titer. Briefly, RNA was extracted and ten-fold serial dilutions were prepared. The serially diluted RNA was used to run the rRT-PCR as described above and a standard curve was automatically generated by the machine software.

**Generation of swine and turkeys tracheal/bronchial epithelial cells.**

Tracheal/bronchial epithelial cells were generated based on recently published protocols (2, 14, 43). Briefly, distal tracheal/proximal primary bronchial air way tissues were collected in phosphate buffered saline (PBS) from 5-week old healthy pigs and one-week old SPF turkey poults. After washing with PBS, tissues were cut into small fragments (~1 cm long) and treated with 0.25% trypsin-1mM EDTA (Invitrogen, San Diego, CA) in PBS supplemented with Penicillin-Streptomycin antibiotic mixture (Invitrogen, San Diego, CA) for 24-48 hours at 4°C. Trypsin activity was stopped by adding 10% FBS, cells were washed with PBS and then suspended in serum free mammary epithelial growth media supplemented with bovine pituitary extract, human epidermal growth factor, insulin and hydrocortisone (MEGM, Lonza, Walkersville, MD). Cells were incubated for 2-6 hours at 37°C and 5% CO₂, non-adherent cells (epithelial cells) were collected and seeded into new culture flask while the adherent cells (fibroblasts) were discarded. Cells were propagated up to five times prior to use in the further experiments.
**Plasma membrane binding assay.** Plasma membranes were prepared from Madin-Darby canine kidney (MDCK), pig tracheal/bronchial epithelial cells (PEC), and turkey tracheal/bronchial epithelial cells (TEC) as previously described (7-9). Based on Wan et al. protocol (37), binding assay was performed as follows. 96-wells microplates (Costar, USA) were coated with plasma membrane protein (25μg/ml) overnight at 4°C. Plates were rinsed once with PBS and blocked with 0.2% BSA in PBS for 2 hours at 37°C. 50μl of two-folds serially diluted virus (64-4 HA) in reaction buffer (0.02% BSA in PBS) were added to wells and incubated at 4°C for 1 hour. After washing four times with ice-cold washing buffer (0.2XPBS containing 0.05% tween-80), 50μl of peroxidase-labeled fetuin in PBS containing 0.02% BSA and 0.05% Tween-80 were added and incubated 1 hour at 4°C [fetuin (Sigma, St. Louis, MO) was labeled using peroxidase labeling kit (Roche Applied Science, Indianapolis, IN) based on manufacturer’s protocol]. Plates were then washed as indicated above and color was developed by adding 100μl SureBlue TM TMB substrate (KPL, Gaithersburg, MD) for 10 min at 37°C. The reaction was terminated with 100μl 2N H2SO4 and OD450 measurement was obtained. Dose-response curves were generated by plotting OD450 values on y-axis and virus concentration (in HA units) on x-axis.

**5.4 RESULTS**

**Replication and transmission of reassortant viruses in turkeys.** Six viruses (Table 5.1) including A/turkey/Ohio/313053/04 (TK04), A/swine/North Carolina/03 (SW03), and single gene reassortants (SGR) with HA or NA gene changes between the two viruses, were evaluated for replication and transmission amongst turkeys (Table 5.2).
TK04 (was shown previously to transmit both ways between swine and turkeys) replicated most efficiently than other viruses and persisted in all inoculated turkeys for at least seven days. It was also transmissible to contact turkeys as indicated with rRT-PCR and HI tests. SW03 showed less replication than TK04 and it was detected only in one inoculated turkey at 7 DPI. Three of the contact turkeys in the SW03 group were positive with rRT-PCR for one day and none of them seroconverted as tested with HI test. Replacing the HA or NA genes of TK04 with those of SW03 (TK04/SW.HA and TK04/SW.NA, respectively) reduced replication efficiency and abolished transmission to contact turkeys. Substituting SW03-HA with that of TK04 (SW03/OH.HA) enabled the reassortant strain to transmit to contact turkeys, although the replication titers of the virus were lower than TK04 strain. Interestingly, SW03/OH.HA showed delay in initiation of replication, where none of the inoculated turkeys were positive at 2DPI, but all were positive at 7DPI. Having NA of the TK04 virus in SW03 backbone (SW03/OH.NA) dramatically affected virus replication in trachea as indicated with rRT-PCR, although inoculated turkeys with this virus showed higher HI titer than those inoculated with SW03 strain. In summary, strains with HA genes from A/turkey/Ohio/313053/04 (TK04 and SW03/OH.HA) showed higher replication titers in turkeys as indicated with both rRT-PCR and HI tests, and were the only transmissible strains amongst turkeys.

**Replication of reassortant viruses in pigs.** Based on the above observations, it was shown that the HA is a key player in the replication and transmission of TR H3N2 in turkeys. Hence, SGR with changes in the HA gene between TK04 and SW03 were evaluated for replication in pigs (table 5.3). Again, TK04 persisted longer in inoculated pigs than other viruses, where all of three were positive at 7 DPI and one stayed positive
until 9 DPI. The highest titer was observed on 2 DPI, recording $10^{4.5}$ EID$_{50}$/ml as estimated with rRT-PCR.

Two out of three pigs inoculated with SW03 were positive with rRT-PCR on 4DPI and only one pig was positive at 7 DPI. The virus replicated to a lower titer compared to TK04, reaching it maximum titer at 4 DPI, $10^{3.2}$ EID$_{50}$/ml. Infection with TK04/SW.HA persisted for a shorter time compared to TK04 but longer than SW03 strain. Maximum replication titer of the virus was recorded at 4 DPI at $10^{5.0}$ EID$_{50}$/ml. Only one pig was positive at 7 DPI and none were positive at 9 DPI. As was the case in turkeys, one pig inoculated with SW03/OH.HA showed delayed infection with the virus (negative at 2DPI) but the virus was then detected until 9 DPI. Although this strain had the HA from TK04, its replication was less than TK04 but better than SW03 virus. Only one pig was positive at 7DPI, and the maximum titer of the virus was recorded at 2 DPI at $10^{5.0}$ EID$_{50}$/ml.

**Binding of viruses to plasma membrane preparations from MDCK, TEC, and PEC.**

In previous work, a D190A change at the RBD was observed upon transmission of TK04 from turkeys to pigs. This stain (TK04(D190A)), as well as the TK04, and SW03 viruses were tested for their binding efficiency to plasma membrane preparations (PMP) from MDCK, TEC, and PEC (Figure 5.1a, 5.1b and 5.1c). TK04 showed more efficient binding to the three PMP compared to the other two viruses. Additionally, TK04 bound with the same efficiency to PMP form MDCK, TEC, and PEC. Although TK04(D190A) bound as efficiently as TK04 to all three PMP at 64HA, its binding was less by at least 1 unit and 2.5 units at 32 and 16 HA, respectively, as compared to the original strain.
SW03 showed the lowest binding efficiency between all viruses, with at least 2.5 units difference at 64 HA as compared to the TK04 strain.

5.5 DISCUSSION

Pigs have always been hypothesized to act as “mixing vessels” where two or more influenza viruses can co-infect and reassort to generate new viruses with potentially wider host-range specificity (1, 10). cH1N1 influenza viruses were the dominant cause of influenza in pigs until the 1990s, when new subtypes emerged and spread widely among pig populations. The first of these was the TR H3N2 virus that emerged in 1998 and it was characterized by having genes from human, swine and avian lineage viruses. Subsequently, TR H3N2 reassorted with H1N1 viruses and some other viruses, generating a wide range of reassortants that are continuously isolated from pigs in the U.S. This included double and triple reassortants H1N1, H1N2, H2N3, H3N1, and H3N2 viruses (4, 17, 18, 23, 36, 40). Some of these viruses managed to cross the species barrier and infect other hosts, like human infections with H1N1 and H3N2 viruses (22, 27) and turkey infections with H1N2 and H3N2 viruses (31, 33). Infections of turkeys with swine viruses have been reported since the early 1980s, starting with H1N1 and currently with H3N2 viruses. Infection of turkeys with these viruses results in no clinical signs, but turkey breeder hens experience a drastic drop in egg production (40). As the case in pigs, turkey trachea express avian and human like α2,3- and α2,6-linked sialic acids receptors, respectively (25, 26). This in part explains the susceptibility of turkeys to swine viruses. On the other hand, molecular determinants that control the transmission of TR H3N2 viruses between the two species are not yet identified. In a previous study, we identified
viruses with different transmission potential between swine and turkeys, including A/turkey/Ohio/313053/04 that showed transmissibility both ways between the two species, and A/swine/North Carolina/03, that was not transmissible either way between swine and turkeys (40). In this study, we evaluated the role of the surface glycoproteins (HA and NA) in the replication of TR H3N2 viruses in swine and turkeys. The two above viruses (TK04 and SW03) were rescued with the 12-plasmid reverse genetics system, and HA or NA segments were switched between the two strains. Results obtained from \textit{in vivo} experiments indicated that the HA segment play a critical but not the only role for efficient replication of the above viruses in pigs and turkeys. Although TK04-HA segment in SW03 backbone enabled the transmission of the virus (SW03/TK.HA) to the contact turkeys, virus titers in inoculated and contact turkeys were lower than those infected with TK04 strain. Additionally, HA from SW03 in the TK04 backbone abolished its transmission and affected virus replication in the inoculated turkeys as compared to the original TK04 virus. However, TK04/SW.HA replicated better than SW03 in terms of virus titer and numbers of positive turkeys throughout the experiment. Switching the NA gene between the two strains dramatically diminished virus replication and transmission amongst turkeys. Interestingly, having the HA from TK04 and the NA from SW03 in different backbone virus (TK04/SW.NA & SW03/TK.HA) revealed different outcomes in terms of virus replication and transmission amongst turkeys. Previously we recorded a 13 amino acids stalk deletion in the NA protein of the SW03 virus (41). That was the only observation in TR H3N2 viruses. Compatibility between the HA and NA proteins is pivotal for replication of influenza A virus in a specific host. We assume that the compatibility between the HA and NA proteins might have affected the replication of the
SGR viruses in turkeys. We also propose that the compatibility between the two proteins might be controlled in part by other player(s) (example M1 protein), where a combination of the same HA and NA proteins in different backbone viruses (TK04/SW.NA & SW03/TK.HA) resulted in different outcomes of virus replication and transmission amongst turkeys. The disruption of the protein-protein interaction in the SGR strains could be a possible explanation for the above observations, where a single amino acid mutation might alter protein function and its interaction network. In more than one study, it was shown that single gene change in a virus alters its phenotype in more than one aspect (3, 19, 28, 34). In a recent study, Chen et al. generated 63 possible reassortments between avian H5N1 and human H3N2 viruses, with surface glycoproteins from the avian virus, and tested their replication *in vivo* and *in vitro* (3). Nearly half of the reassortants showed defective phenotype compared to the wildtype virus *in vitro*, and many showed differential phenotypes in a mouse model. For example, replacing only the M protein in the avian virus with that from human origin, not only diminished virus replication in mice, but also altered the tropism of the virus in the infected animals. While wild type virus replicated to high titers in lung and was isolated from spleen, lung and nasal turbinate, the SGR with M gene exchange showed at least two log_{10} lower titer in lung and was not isolated neither from spleen nor brain. The effect of M protein on the functional compatibility between HA and NA protein in TR H3N2 viruses requires further investigations.

SGR with HA gene changes were further assessed for replication in pigs. It is worth mentioning that it was difficult to obtain seronegative pigs from our area to start the experiment. Sera was collected from pigs at different places in Ohio and all were
seropositive for influenza A antibodies, with high antibody titers against H1N1 viruses, and low to moderate titers against H3N2 viruses. This indicates the wide spread nature of these viruses among pig populations although they might not cause any significant disease. Hence, piglets were delivered and deprived from sow’s colostrum. Patterns of viruses’ replication in pigs were quite similar to those observed in turkeys. TK04 replicated most efficiently amongst all reassortant viruses, with one pig remaining positive until 9 DPI as tested with rRT-PCR. HA gene from TK04 enhanced the replication when introduced to SW03, but was still less than TK04 strain. Interestingly, one pig inoculated with SW03/TK.HA showed a delay in virus replication (negative at 2 DPI), similar to what happened in turkeys inoculated with the same virus (all negative at 2 DPI). The pig then tested positive until 9 DPI. The molecular mechanism for such a delay in the replication is not understood and needs to be identified. TK04/SW.HA strain which has the HA from SW03 with the rest of the genes from TK04 replicated to higher titers and persisted longer in pigs compared to SW03, but its persistence was shorter compared to TK04 virus. This indicates that the HA gene is not the only player that determines the efficient replication of TR H3N2 in pigs as well as turkeys. Looking at genetic signatures in genes other than HA which were previously shown to determine host range specificity, we noticed no difference in these signatures between TK04 and SW03 viruses. This included PB2627, PB2701, PB2714, PB1375, PA615, and NP319. Interestingly, all of the residues except for PB1375, showed avian like residues in the corresponding positions. Performing reassortants with two or more gene changes and testing their replication \textit{in vivo} is the only way to determine what other player(s) besides HA might contribute to the efficient replication of the above viruses in both species.
Performing binding assay, at least 2.5 fold difference was noticed in the binding affinity of TK04 to PMP from MDCK, TEC and PEC compared to SW03. This in part explains the ability of TK04 to replicate and persist longer in turkeys and pigs. Comparing the RBD between the two viruses we noticed two changes at residues 186 and 189 of the RBD and 8 more changes close to the RBD. Whether these changes affected the binding affinity of the SW03 strain is not clear and requires further investigation. Interestingly, TK04(D190A) bound as efficient as TK04 to the three PM preparations at 64 HA, however, its binding affinity decreased dramatically at lower HA compared to the wild type strain (TK04). Hence, D190A mutation seems to affect binding ability of the virus to epithelial cells of different origins. Combining the above two observations (from SW03 and TK04(D190A)) , it seems that residues 190 and those around it at the RBD are essential in terms of binding affinity of the H3N2 viruses to airway epithelial cells in pigs and turkeys.

In conclusion, hemagglutinin protein was shown to be a key but not the only player for efficient replication of TR H3N2 viruses in swine and turkeys. In a recent study (34) it was shown that PB2 of the same origin as HA and NA enhances replication and is necessary for aerosol transmission of the virus (1918 Spanish influenza) in a ferret model. It could be the PB2 or other gene(s) are required for efficient replication and may be for transmission of H3N2 viruses between swine and turkeys. Performing more in vivo experiments with various reassortments between the above two strains might answer more questions.
5.6 ACKNOWLEDGMENTS

We would like to thank Maria Murgia for her help in the animal work. This study was partially supported by funds from USDA, CSREES, AI-CAP project.

5.7 REFERENCES


<table>
<thead>
<tr>
<th>Description</th>
<th>Abbreviations</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/turkey/Ohio/313053/04 virus generated by reverse genetics</td>
<td>TK04</td>
</tr>
<tr>
<td>SGR* strain with turkey.OH.04 backbone and SW.NC.03-HA gene</td>
<td>TK04/SW.HA</td>
</tr>
<tr>
<td>SGR strain with TK.OH.04 backbone and SW.NC.03-NA gene</td>
<td>TK04/SW.NA</td>
</tr>
<tr>
<td>A/swine/North Carolina/03 virus generated by reverse genetics</td>
<td>SW03</td>
</tr>
<tr>
<td>SGR strain with SW.NC.03 backbone and TK.OH.04-HA gene</td>
<td>SW03/OH.HA</td>
</tr>
<tr>
<td>SGR strain with SW.NC.03 backbone and TK.OH.04-NA gene</td>
<td>SW03/OH.NA</td>
</tr>
</tbody>
</table>

* Single gene reassortant

**Table 5.1.** Reverse genetics generated viruses
<table>
<thead>
<tr>
<th>Virus</th>
<th>Animal group (Turkeys)</th>
<th>Positives at 2DPI*</th>
<th>Positives at 4DPI</th>
<th>Positives at 7DPI</th>
<th>No. of animals seroconverted (Average HI**)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TK04</strong></td>
<td>Inoculated (n=6)</td>
<td>6 (2.4 (^a))</td>
<td>6 (3.0)</td>
<td>6 (2.70)</td>
<td>6 (67)</td>
</tr>
<tr>
<td></td>
<td>Contact (n=3)</td>
<td>3 (2.30)</td>
<td>3 (2.6)</td>
<td>3 (2.70)</td>
<td>3 (16)</td>
</tr>
<tr>
<td></td>
<td>TK04/SW.HA</td>
<td>Inoculated (n=6)</td>
<td>6 (2.5)</td>
<td>4 (2.3)</td>
<td>1 (2.6)</td>
</tr>
<tr>
<td></td>
<td>Contact (n=3)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>TK04/SW.NA</td>
<td>Inoculated (n=6)</td>
<td>4 (2.6)</td>
<td>1 (1.9)</td>
<td>1 (2.6)</td>
</tr>
<tr>
<td></td>
<td>Contact (n=3)</td>
<td>0 (0.0)</td>
<td>1 (2.6)</td>
<td>1 (1.90)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>SW03</strong></td>
<td>Inoculated (n=6)</td>
<td>3 (2.1)</td>
<td>5 (1.9)</td>
<td>1 (1.8)</td>
<td>5 (28)</td>
</tr>
<tr>
<td></td>
<td>Contact (n=3)</td>
<td>1 (1.9)</td>
<td>2 (1.7)</td>
<td>0 (0.0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>SW03/OH.HA</td>
<td>Inoculated (n=6)</td>
<td>0 (0.0)</td>
<td>4 (2.2)</td>
<td>6 (2.0)</td>
</tr>
<tr>
<td></td>
<td>Contact (n=3)</td>
<td>1 (1.6)</td>
<td>3 (2.0)</td>
<td>2 (1.7)</td>
<td>3 (16)</td>
</tr>
<tr>
<td></td>
<td>SW03/OH.NA</td>
<td>Inoculated (n=6)</td>
<td>1 (1.4)</td>
<td>2 (1.6)</td>
<td>5 (1.9)</td>
</tr>
<tr>
<td></td>
<td>Contact (n=3)</td>
<td>0 (0.0)</td>
<td>1 (1.6)</td>
<td>3 (2.0)</td>
<td>0 (3)</td>
</tr>
</tbody>
</table>

* Days post inoculation  
** Hemagglutination inhibition results with sera collected at 15 DPI.  
\(^a\) Log\(_{10}\) virus titers/ml as estimated with rRT-PCR

**Table 5.2.** Replication and transmission of reassortant strains in turkeys
<table>
<thead>
<tr>
<th>Virus</th>
<th>Animal group (pigs)</th>
<th>Positive at 2DPI*</th>
<th>Positive at 4DPI</th>
<th>Positive at 7DPI</th>
<th>Positive at 9DPI</th>
<th>No. of animals seroconverted (Average HI**)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TK.OH.04</td>
<td>Inoculated (n=3)</td>
<td>3 (4.5&lt;sup&gt;a&lt;/sup&gt;)</td>
<td>3 (3.9)</td>
<td>3 (2.5)</td>
<td>1 (2.0)</td>
<td>3 (64)</td>
</tr>
<tr>
<td>TK.OH.04/ SW.NC-HA</td>
<td>Inoculated (n=3)</td>
<td>3 (4.5)</td>
<td>3 (5.0)</td>
<td>1 (2.3)</td>
<td>0 (0.0)</td>
<td>3 (64)</td>
</tr>
<tr>
<td>SW.NC.03</td>
<td>Inoculated (n=3)</td>
<td>3 (3.0)</td>
<td>2 (3.2)</td>
<td>1 (2.6)</td>
<td>0 (0.0)</td>
<td>3 (53)</td>
</tr>
<tr>
<td>SW.NC.03/ TK.OH-HA</td>
<td>Inoculated (n=3)</td>
<td>2 (5.0)</td>
<td>3 (4.3)</td>
<td>1 (2.9)</td>
<td>1 (2.2)</td>
<td>3 (64)</td>
</tr>
</tbody>
</table>

* Days post inoculation  
** Hemagglutination inhibition results with sera collected at 15 DPI.  
<sup>a</sup> Log<sub>10</sub> virus titers/ml as estimated with rRT-PCR

Table 5.3. Replication of reassertant strains in pigs
Figure 5.1. Binding of H3N2 viruses to plasma membrane (PM) preparations from: (A) Madin-Darby Kidney Canine (MDCK) cells, (B) Turkey Tracheal Epithelial Cells, and (C) Pig Tracheal Epithelial Cells.
BIBLIOGRAPHY


neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy. Arch Virol 146:963-73.


Brooksby, J.B. 1967. Presented at the Int. Symp. on foot and mouth disease, variants and immunity, Lyon, France.


Sylte, M. J., B. Hubby, and D. L. Suarez. 2007. Influenza neuraminidase antibodies provide partial protection for chickens against high pathogenic avian influenza infection. Vaccine.


WHO 2009, posting date. Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) Reported to WHO. WHO. [Online.]


