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Antigenic and genetic evolution of emerging avian origin influenza A viruses

By

Yifei Xu

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Veterinary Medical Sciences
in the College of Veterinary Medicine

Mississippi State, Mississippi

December 2016

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Antigenic and genetic evolution of emerging avian origin influenza A viruses

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Periodic introductions of influenza A viruses (IAVs) from wild birds contribute to emergence of novel strains that infect domestic poultry, lower mammals, and humans, but the mechanisms of emergence are unclear. The objectives of this dissertation research are to infer the genesis of two emerging IAVs, low pathogenic avian influenza (LPAI) H10N8 and highly pathogenic avian influenza (HPAI) H7N8 viruses, and to characterize the antigenic diversity and genetic evolution of contemporary H7 avian influenza viruses (AIVs) from North America.

First, AIVs that are genetically close to the human H10N8 isolate were recovered at the live poultry market (LPM) visited by the first H10N8 patient. High seroprevalence of H10 virus was observed in ducks and chickens from five LPMs in the region. These findings suggested that LPM was the most probable source of human infection with the H10N8 virus, and this virus appeared to be present throughout the LPM system in the city.

Second, the novel H7N8 virus most likely circulated among diving ducks in the Mississippi flyway during autumn 2015 and was subsequently introduced to Indiana

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turkey, in which it evolved from LPAI into HPAI. H4N8 IAVs from diving ducks possess a gene constellation comprising five H7N8–like gene segments. These findings suggest that viral gene constellations circulating among diving ducks could contribute towards the emergence of IAVs that can affect poultry. Diving ducks may serve as a unique reservoir, contributing to the maintenance, diversification, and transmission of IAVs in wild birds.

Third, antigenic and genetic characterization of 93 H7 AIVs from North America showed limited antigenic diversity. Gradual accumulation of nucleotide and amino acid substitutions in the H7 gene of AIVs from wild and domestic birds caused a wide genetic diversity. These findings suggested that continuous genetic evolution has not led to significant antigenic diversity for contemporary H7 AIVs isolated from wild and domestic birds in North America.

In summary, these findings not only improve our understanding of the ecology and evolution of IAVs but also provide information for formulation of effective disease prevention and control strategies.

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DEDICATION

This dissertation is dedicated to my always encouraging and ever faithful father and mother.

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TABLE OF CONTENTS

DEDICATION ii

ACKNOWLEDGEMENTS iii

LIST OF TABLES ix

LIST OF FIGURES xi

ABBREVIATIONS xiii

CHAPTER

I. INTRODUCTION 1

The Biology of Influenza A Virus 1
Impacts of Influenza Disease Caused by IAVs 2
Ecology of IAVs 4
Wild Birds 4
Domestic Poultry 7
Swine 9
Humans 10
Evolution of IAVs 12
Population and Quasispecies 12
Evolutionary Mechanisms 12
Selection Pressure 14
Immune Pressure 14
Host Switch 16
Vaccination 17
Knowledge Gaps and Objectives of this Dissertation 20
Dissertation Organization 25

II. A WEB-BASED PIPELINE FOR ANALYZING AND ASSEMBLING NGS DATA OF IAVS 26

Introduction 27
Materials and Methods 28
Data Preprocessing 28
Genome Assembly 28
Web-based Application 29

المنارة للاستشارات

www.manaraa.com

Testing Datasets and Performance Evaluation 32
Results 33
Discussion..37

III. THE GENESIS OF A NOVEL LPAI H10N8 VIRUS AND THE SOURCE OF H10N8 HUMAN INFECTIONS IN CHINA.................38

Introduction 39
Materials and Methods 41
Sample Collection 41
RNA Extraction and IAV Screening 42
Viral Isolation 43
Hemagglutination and Hemagglutination Inhibition Assays 43
Reverse Transcription, PCR, and Genomic Sequencing 43
Genomic Assembly 44
Phylogenetic Analysis and Molecular Characterization...............................45
Serological Surveillance 45
Results 45
Discussion..61

IV. THE GENESIS OF A NOVEL HPAI H7N8 VIRUS AND THE EVOLUTIONARY PATHWAY LEADING TO OUTBREAK AMONG DOMESTIC POULTRY IN INDIANA, THE UNITED STATES 64

Introduction 65
Materials and Methods 67
Viruses...67
Phylogenetic Analyses...69
Molecular Clock 70
Discrete Phylogeographic Analyses 70
Phylogenetic Network 71
Genotyping 72
Results 72
H7N8 Virus Originated From IAVs in North American Wild Birds 72
Gene Constellation of H4N8 Virus in Diving Ducks Contributed to Emergence of The Novel H7N8 Virus 80
IAVs From Diving Ducks Are The Probable Genetic Source of The H7N8 Virus in Indiana Turkeys. 85
H7N8 LPAI Virus Evolved Into An HPAI Strain In Turkeys.......................91
Discussion..93

V. THE ANTIGENIC DIVERSITY AND GENETIC EVOLUTION OF CONTEMPORARY H7 AIVS FROM NORTH AMERICA DURING 1971 to 2012 102

المنارة للاستشارات
www.manaraa.com

Introduction 103
Materials and Methods 105
Ethics Statement 105
Viruses 105
Generation of Reference Antisera in Chicken 106
HA and HI Assays 106
Full Genome Sequencing 113
Genomic Assembly 113
Evolutionary Analysis 114
Inference of Amino Acid Sites under Positive Selection 116
Molecular Dating 117
Construction of the Antigenic Cartography and Molecular
Characterization 117
Results 118
H7 HA Genes are Genetically Diverse 118
Limited Antigenic Diversity among Tested H7 AIVs 120
Genetic Evolution Dynamics of the HA Gene 126
Natural Selection for HA Gene of H7 AIVs in North America 131
Frequent Reassortment of Internal Genes of H7 AIVs in NA-WB
Lineage 133
Discussion 139

VI. CONCLUSIONS 147

REFERENCES 149

APPENDIX

A. SUPPLEMENTARY MATERIALS 173

www.manaraa.com

LIST OF TABLES

1 Statistics of influenza surveillance conducted in the LPM visited by the first H10N8 patient...48

2 Summary of genomic sequences recovered from the LPM visited by the first H10N8 patient...49

3 The amino acid diversity in the influenza biomarkers of viruses recovered from the LPM visited by the first H10N8 patient. 57

4 Most closely related genes to eight gene segments of the novel highly pathogenic avian influenza strain A/turkey/Indiana/16-001403-1/2016(H7N8). 78

5 Estimated time to most recent common ancestor and nucleotide substitution rates for eight gene segments of H7N8 viruses detected in Indiana turkeys. 79

6 Sequence identity between A/turkey/Indiana/16-001403-1/2016 and influenza A virus strains isolated from diving ducks..........................84

7 Diffusion pathway of influenza A virus between wild birds and Indian turkeys...89

8 Diffusion pathway of influenza A virus among different host groups in wild birds 90

9 List of H7 AIVs isolated in North America that were subjected to antigenic characterization. 108

10 Sequence identity of HA1 protein of H7 AIVs from distinct genetic clusters from North America. 119

11 Cross-HI data obtained for testing H7 avian influenza viruses against representative chicken sera. The homologous titers were underlined. 121

12 Estimated rates of nucleotide substitution and time to most recent common ancestor for H7 AIVs from distinct genetic clusters from North America...129

المنارة للاستشارات
www.manaraa.com

13 Cross HI data obtained for representative H7 AIVs against chicken serum. 130

14 Selection pressure in HA1 protein of H7 AIVs from North America. 132

15 Amino acid polymorphism at position 189 (H3 numbering) in HA1 protein for H7 AIVs from North America and Eurasia. 132

المنارة للاستشارات

www.manaraa.com

LIST OF FIGURES

1 Pipeline for analyzing and assembling NGS data of influenza virus..............29

2 Reference-based assembly mode submission interface. 31

3 Job status page (job complete). 32

4 Reference-based assembly mode result page. 35

5 Coverage of resulting sequences of *de novo* assembly mode. 36

6 Avian origin influenza A viruses that caused zoonotic infections in Southern China during the past two decades. 41

7 Isolation of H10 influenza A virus from the LPM visited by the first H10N8 patient. 46

8 Maximum likelihood phylogenetic trees of H10 genes recovered from the samples collected at the LPM. 54

9 Maximum likelihood phylogenetic trees of N8 genes recovered from the samples collected at the LPM. 55

10 Distribution of H10 seropositive samples collected from February 25 to March 27, 2014 and across five LPMs in Nanchang city. 61

11 Geographic, temporal, and taxa distribution of 441 influenza A virus strains subjected to genomic sequencing in this study.........................68

12 Maximum likelihood phylogenetic trees for eight gene segments of H7N8 turkey strains and influenza A viruses representing North American and Eurasian genetic lineages. 73

13 Maximum clade credibility phylogenetic trees for HA (a) and NA (b) gene segments. 77

14 Estimated time to most recent common ancestor and nucleotide substitution rates for eight gene segments of H7N8 viruses detected in Indiana turkeys. 79

المنارة للاستشارات
www.manaraa.com

15 Phylogenetic network of H7N8 viruses detected in Indiana turkeys in red font. 81

16 Maximum likelihood phylogenetic tree for N8 gene segment of the H7N8 viruses detected in Indiana turkeys and H4N8 influenza A viruses isolated from wild birds. 83

17 Diffusion of influenza A virus between different host groups of wild birds and Indiana turkey. 86

18 Insertion patterns in the HA cleavage region of the H7 viruses detected in Indiana turkeys and those from four previous outbreaks in the Americas. 92

19 Amino acid substitutions among eight gene segments of the H7N8 strains detected in Indiana turkey. 93

20 Proposed evolutionary pathway leading to the generation of highly pathogenic avian influenza(H7N8) virus that caused outbreak in Indiana turkeys. 96

21 Statistics of influenza A viruses isolated from wild and domestic birds in the Americas. 97

22 Geographic distribution of H7 AIVs selected for antigenic characterization. 112

23 Comparison of genetic and antigenic evolution of H7 AIVs from North America. 125

24 Comparison of temporal genetic and antigenic evolution dynamics for H7 AIVs from North America. 127

25 Maximum likelihood phylogenetic trees for internal gene segments of H7 AIVs from Eurasian and North American lineage. 135

26 Maximum likelihood phylogenetic trees for internal gene segments of H7 AIVs derived from wild birds in North America. 136

27 Congruence in topology among six internal gene segment phylogenetic trees for H7 AIVs derived from wild birds in North America. 137

28 Year-by-year analysis of the evolution dynamics of internal gene constellations for H7 AIVs derived from wild birds in North America during 2001–2013. 138

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ABBREVIATIONS

AIV	Avian influenza virus
APHIS	The United States Department of Agriculture's Animal and Plant Health Inspection Service
BF	Bayes Factor
Gal	Galactose
HA	Hemagglutinin
HI	Hemagglutination inhibition
HPAI	Highly pathogenic avian influenza
HPD	Highest posterior density
IAV	Influenza A virus
LPAI	Low pathogenic avian influenza
LPM	Live poultry market
M	Matrix
NA	Neuraminidase
NGS	Next generation sequencing
NP	Nucleoprotein
NS	Nonstructural
PA	Polymerase acidic
PB1	Polymerase basic 1
PB2	Polymerase basic 2
SA	Sialic acid
SIV	Swine influenza virus
TMRCA	Time to most recent common ancestor
WHO	World Health Organization

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CHAPTER I

INTRODUCTION

The Biology of Influenza A Virus

Emerging infectious diseases can be defined as "infections that have newly appeared in a population or have existed previously but are rapidly increasing in incidence or geographic range" (1). Among many infectious agents, few have as profound effect on humans as influenza viruses (2).

Influenza viruses belong to the *Orthomyxoviridae* family and are enveloped, single-stranded, negative sense RNA viruses containing seven to eight gene segments (3, 4). Influenza viruses are categorized into four distinct serotypes, A, B, C, and D on the basis of antigenic difference in the nucleoprotein (NP) and matrix (M1) protein (5). Among them, serotype A is the most important one due to its wide host range (4). Serotype B, C, and D are reported to infect only mammals (6-8).

The genome of influenza A virus (IAV) comprises eight RNA gene segments (segment one to eight) with varying lengths of 890–2,341 nucleotides. These gene segments encode at least 11 proteins: polymerase basic 2 (PB2), polymerase basic 1 (PB1) and polymerase basic 1 - Frame 2 (PB1-F2), polymerase acidic (PA), hemagglutinin (HA), NP, neuraminidase (NA), matrix (M) 1 and 2, and nonstructural (NS) 1 and 2 proteins are encoded by gene segments one to eight, respectively. IAV contains a host cell-derived lipid membrane, which is embedded with three virus proteins,

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HA, NA, and M2. An inner coat comprised of the M1 protein is found underlying the membrane. Nucleocapsid of the viral genome lies at the center of the virus. Each of eight gene segments is encapsidated by multiple NP proteins to form a nucleocapsid, while three polymerase proteins (PB2, PB1, and PA) sit at the end of the nucleocapsid (4, 9, 10). IAVs could be further divided into distinct subtypes on the basis of the antigenicity of two surface glycoproteins, HA and NA. To date, 18 HA and 11 NA subtypes have been reported (11).

Impacts of Influenza Disease Caused by IAVs

IAVs present a typical one-health challenge, where human health and animal health meet and influence each other (4). IAVs infecting humans could cause both global pandemic and seasonal epidemic. The occasional influenza pandemic could infect 20% to 40% of the world population and cause a significant number of deaths (12). For example, the 1918 influenza pandemic was considered one of the most devastating infectious diseases in human history and it was estimated to have claimed at least 50 million human lives around the world (13, 14). In addition to sporadic pandemic outbreaks, the seasonal epidemic causes a number of morbidity and mortality in humans worldwide annually, including an average of more than 200,000 hospitalizations and 23,000 deaths in the United States alone (15, 16). The direct medical cost associated with seasonal influenza in the United States averages over ten billion US dollars annually (17).

The diseases caused by IAV infection among domestic poultry include asymptomatic infections, mild to severe respiratory disease, and severe disease with high morbidity and mortality. On the basis of virulence in chicken, avian influenza viruses (AIVs) could be divided into two groups: highly pathogenic avian influenza (HPAI) and

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low pathogenic avian influenza (LPAI). HPAI could cause devastating disease among domestic poultry and the mortality may be as high as 100%. HPAI viruses are associated with direct and indirect losses that may be incurred by the poultry industry through mortality of affected birds, culling of flocks to prevent spread, trade restrictions imposed of detection, and increased costs associated with outbreak response. For example, 17 HPAI outbreaks were reported among domestic poultry worldwide during 1955-2000, including six outbreaks among domestic turkey and 11 outbreaks among domestic chicken (18). Among them, seven outbreaks caused more than 100,000 birds affection or depopulation. In 2003, 255 poultry flocks in the Netherlands was affected by HPAI H7N7 virus and it has led to the death or culling of more than 30 million birds (19). The HPAI outbreak among domestic poultry caused by H5 AIVs in the northwestern and mid-western United States in 2015 led to depopulation of more than 48 million birds (https://www.aphis.usda.gov/publications/animal_health/2015/fs-hpai-vaccine-use.pdf).

AIV, such as the HPAI H5N1 virus, could also transmit from birds to humans and cause fatal disease. In spring of 1996, the precursor of currently circulating HPAI H5N1 viruses was identified in farmed geese in Guangdong, China; the mortality caused by the virus among geese was about 40%. Outbreaks among chicken were detected in Hong Kong one year later and led to depopulation of about 1.5 million birds. The first case of human infection with the H5N1 virus was reported in May 1997; H5N1 virus strain was isolated from a boy died of respiratory illness in Hong Kong (20). 17 additional cases of human infection with H5N1 viruses were reported in Hong Kong in November and December of the same year and five cases were fatal. In March 1999, H5N1 viruses were isolated from environmental samples collected from cages that housed geese in Hong

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Kong (21). In 2003, fatal human infections with H5N1 virus were reported for the first time after the outbreak in Hong Kong in 1997 (22). Since December 2003, HPAI H5N1 virus has caused outbreaks among domestic poultry and repeated zoonotic transmissions in Asia (23-26). In May 2005, HPAI H5N1 virus were detected among migratory birds at Qinghai Lake, China (27) and were subsequently found to spread to Europe, the Middle East, and Africa (28, 29). To date, the HPAI H5N1 virus has cause more than 800 laboratory-confirmed human cases worldwide, including over 400 deaths (http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/).

Ecology of IAVs

IAVs exist in a complex ecosystem that involves various hosts, including humans, swine, equine, canine, sea mammals, and numerous wild and domestic birds (4, 9, 30).

Wild Birds

The first isolation of IAV from wild bird was reported in 1961 (31). A HPAI strain, A/tern/South Africa/61(H5N3), was isolated from a common tern in South Africa; and this virus caused infection among 1,300 common terns (18, 31). LPAI strains have been isolated from wild waterfowl since the mid-1970s (30-32). World-wide, LPAI viruses have been recovered from at least 105 wild bird species representing 26 different taxonomic families (33). Most of the 26 families are in the Order *Anseriformes*, followed by the Order *Charadriiformes*; birds in these two orders include ducks, geese, and swans, and shorebirds, gulls, and terns, respectively, which are considered major natural

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reservoirs for IAVs (4, 33, 34). IAVs representing 16 HA and 9 NA subtypes have been identified in wild birds throughout the world (33).

In North America, the southern migration of birds begins as early as August for some species, and in later September, October, and early November for most species. The highest prevalence of IAVs is found in dabbling ducks, particular mallards, which have been the most extensively studied bird species in the ecosystem of IAVs (33). The prevalence of IAVs in mallards fluctuates during the bird migration cycle. Influenza prevalence could reach as high as 60% among the mallards sampled in the breeding areas in Canada prior to the migration in early fall (35). Influenza prevalence falls to less than 2% by the time when mallards reach the wintering ground in lower Mississippi and Louisiana usually around December and January of the following year. Influenza virus were brought back by mallards to the breeding areas in Canada in spring, although the prevalence may fall to as low as 0.25% (4).

The infectious pattern of IAVs in shorebirds and gulls is different from that in waterfowl in the following two major aspects. First, the highest prevalence of IAVs in shorebirds and gulls is usually found in the later spring and early summer (4, 36). Shorebirds and gulls are proposed to bring the virus back to the northern breeding area (36). Thus, shorebirds and gulls may contribute to the overwinter maintenance and long-term persistence of IAVs in wild birds. Second, in addition to H1 to H12, IAVs of the subtype H13 and H16 were primarily isolated from shorebirds and gulls (33, 37, 38). Substantial difference was observed between the genomic sequences of the H13 and H16 gene segments and those of other subtypes, suggest the long time isolation of IAVs of these two HA subtypes in shorebirds and gulls (37).

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The infection caused by most IAV strains in wild birds is asymptomatic, which may due to the long-term adaptation of IAVs in these birds (4). In wild birds, IAVs replicate mainly in the intestinal tract and are excreted in high level in the feces (39, 40). For example, a study in 1978 showed that the load of influenza virus in the feces of mallards could be up to $10^{8.7}$ 50% egg infectious doses per gram (40). In addition, influenza virus could remain infectious for over 30 days in water at 0 degree Celsius (^{o}C) and four days at 22 ^{o}C (40, 41). Wild waterfowl shed virus into the water through feces and fecal-oral route is considered the major way for the transmission of IAVs between waterfowls (4, 9).

The genetic pool of IAVs is divided into two major independently evolving lineages, Eurasian and American, probably due to geographical separation of host species (42-44). The migration of birds between different continents allows the occasional gene flow between these two genetic pools (45). For example, Beringian Crucible is recognized as a common breeding grounds shared by migratory birds from Eurasia and America. Early research showed that around 1.5 to 2.9 million birds migrate from Asia to Alaska annually (46). A 2008 study characterized 38 IAVs isolated from Alaska and found that nearly half (44.7%) possess at least one gene segment more closely related to those recovered from Eurasia instead of those from North America (47). Evidence for intercontinental gene flow was also observed in another study of 25 IAVs isolated from North America, among them, five isolates have two Eurasian origin gene segments and 20 isolates have one Eurasian origin gene segment (48). In addition, there were evidences showing that Eurasian origin H6 IAVs invaded the North American wild bird population and caused outbreaks among domestic poultry in California during 2000-2002 (49, 50).

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Domestic Poultry

Through migration, wild birds can carry IAVs from one area to another and present risks to the host species along the migratory flyway, including domestic poultry, lower mammals, and humans. Frequent introductions of wild bird-origin IAVs to domestic poultry have been well-documented. In Minnesota alone during 1978-2000, there were at least 108 laboratory-confirmed introductions of influenza viruses from wild birds to domestic poultry (51). The direct transmission of IAVs from migratory birds to range-reared turkey was recognized as a major source of IAV outbreaks among domestic turkey in the United States; and this dynamic was also suggested to contribute to the IAV outbreaks among chickens (52). Alternatively, range-reared domestic ducks and geese could serve as intermediate hosts that bridge the ecological gap between wild birds and domestic chickens and turkeys.

Following introduction to domestic poultry, IAVs could cause disease and circulate in domestic poultry for a long time (30, 53); and AIVs of two HA subtypes, H5 and H7, are known to could give rise to HPAI virus. HPAI virus was historically known as fowl plague, which was reported for the first time among chickens in Italy in 1878 (18, 54). The virus was later isolated in 1902, A/Chicken/Brescia/1902(H7N7). Fowl plague was classified as IAV in 1955 and referred to HPAI ever since (18). The major difference in the pathogenicity of HPAI and LPAI viruses is their ability to cause systemic replication in the host. The cleavability of the HA protein is one of the major determinants of the tissue tropism of AIVs (55). The HA protein of HPAI virus can be cleaved by a broad range of enzymes that present in most cells of the body and caused systemic infection. In contrast, the HA protein of LPAI virus can only be cleaved by

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trypsin-like proteases and infection is restricted in the respiratory or intestinal tract (56). The presence of multiple basic amino acids in the cleavage region of the HA protein is the major molecular feature for HPAI strains.

Seventeen HPAI outbreaks were reported among domestic poultry worldwide during 1955-2000 (18). HPAI outbreaks among domestic poultry were also reported in multiple countries after the year 2000. For example, during 2003-2008, HPAI H5N1 virus was reported in at least ten countries in East and Southeast Asia, including China, Japan, South Korea, Cambodia, Indonesia, Laos, Malaysia, Myanmar, Thailand, and Vietnam (57). Outside of the region, HPAI H5N1 virus were reported in multiple countries in Middle East, Europe, and Africa, including Israel, Iraq, Russia, United Kingdom, Germany, France, Egypt, and Nigeria (58, 59). Detection of HPAI H7 viruses were reported in Chile (H7N3, 2002) (60), the Netherlands (H7N7, 2003) (19), Canada (H7N3, 2004 and 2007) (61, 62), and Mexico (H7N3, 2012) (63, 64).

In January 2014, a novel Eurasian lineage clade 2.3.4.4 HPAI H5N8 virus was detected in wild bird and domestic poultry in South Korea (65), and was subsequently detected in China and Japan (66). Since November 2014, the novel HPAI H5N8 virus has been detected in wild bird and domestic poultry in multiple countries in Europe, including Germany, the Netherlands, the United Kingdom, and Italy, (http://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/2014/). In December 2014, HPAI H5N8 virus was first detected in wild bird in the United States (67). Two novel reassortant viruses of the subtype H5N1 and H5N2 have been isolated from wild bird and domestic poultry in the United States and Canada since December 2014 (68-71). The HPAI H5 outbreaks caused depopulation of approximately 7.5 million

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turkeys and 42.1 million chickens in the United States (https://www.aphis.usda.gov/animal_health/emergency_management/downloads/hpai/landfillsandhpairesponse.pdf).

Swine

The infection of influenza virus in swine was first clinically recognized in 1918 in the United States, which overlaps the time period of the 1918 influenza pandemic (72). The first swine influenza virus (SIV) was isolated in 1930 (73, 74); this virus, refers to the classical swine H1N1 virus, has continued to circulate and cause diseases in swine population worldwide. The classical swine H1N1 virus was found to be predominant in the North American swine population until the mid-1990s (75-77); however, this trend changed dramatically with the emergence and establishment of the triple reassortant SIVs of the subtypes H1N2 and H3N2 since 1998 (78-80). The eight gene segments of the triple reassortant SIVs were derived from multiple sources, including the classical swine H1N1, human H3N2, and avian origin IAVs (78, 79). In addition, introduction of AIVs to swine population has been frequently reported. For example, an avian like H1N1 IAVs emerged and led to enzootic disease in the European swine population since 1979 (81-83). In China, serological surveillance results suggested the prevalence of avian origin H3, H4 and H6 IAVs in the swine population (84). Introduction of avian origin H1N1 IAV to swine was reported in Hong Kong in 1993 (85). There were also reported sporadic cases of avian origin IAVs infection in swine, including H4N1 (86), H4N8 (87), H5N1 (88), H6N6 (89, 90), H9N2 (91), and H10N5 viruses (92).

Swine are suggested to play an important role in the ecology of IAVs. The cell of swine respiratory tract possesses receptors recognized by both avian (sialic acid α2,3

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galactose, SA2,3Gal) and human (SA2,6Gal) IAVs (93), and this provides the biological basis for the susceptibility of swine to both avian and human IAVs. Thus, swine is proposed to function as the intermediate hosts, or mixing vessels, for the generation of pandemic strains, including both the 1957 and 1968 pandemic virus (94). The emergence of the 2009 pandemic virus provided further evidence supporting this hypothesis (95); and this virus will be described in the next section.

Humans

The introduction of avian origin influenza gene(s) to influenza viruses that circulating in humans has been associated with the emergence of four documented pandemic strains, including 1918 H1N1, 1957 H2N2, 1968 H3N2, and 2009 H1N1 viruses (95-98).

The 1918 influenza pandemic emerged and swept globally since September, 1918 (13, 99). The origin of the 1918 H1N1 virus remained controversial and probably could never be resolved (12, 100). Although no virus was isolated prior or during the 1918 influenza pandemic, genetic analysis of the reconstructed genome of 1918 H1N1 virus in the 1990s showed that the virus was probably an avian like influenza virus (101). There are still a few key questions not answered, including the origin host of the virus and the potential existence of an intermediate host (12, 13). In contrast to the HPAI viruses, multiple basic amino acids were not observed at the cleavage site of the HA protein of the 1918 H1N1 virus (102, 103).

The 1957 H2N2 and 1968 H3N2 pandemic strains were both originated from southeastern Asia. These two pandemic strains were reassortants generated through reassortment between pre-existing human IAVs and AIVs (96-98). The PB1, HA, and

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NA gene segments of the 1957 H2N2 virus were derived from an AIV, and the remaining five gene segments were the descendant of the 1918 H1N1 strain (97). The 1968 H3N2 virus possess two gene segments (PB1 and HA) originated from an AIV, and the remaining six gene segments were derived from the circulating human H2N2 virus (96).

The first influenza pandemic in the 21th century occurred in 2009. A novel human H1N1 virus was first detected in Mexico in March 2009 (104, 105) and this virus quickly spread to the rest of the world. By July 2009, 162,300 laboratory-confirmed cases and over 1,100 human deaths caused by the 2009 H1N1 pandemic virus were reported in 168 countries (106). The pandemic was estimated to have caused more than 123,000 deaths around the world by the end of 2009 (107). Subsequently, the 2009 H1N1 virus started to co-circulate with seasonal H3N2 and influenza B virus around the world (108-110). Phylogenetic analysis results showed that the 2009 H1N1 virus was a reassortant with the eight gene segments originated from multiple sources (95). The NA and MP gene segments were derived from the European avian like H1N1 SIV, and the remaining six gene segments were originated from the North American triple reassortant SIV; specifically, the HA, NP, and NS gene segments were derived from the classical swine H1N1 virus, PB1 gene segment was originated from seasonal H3N2 virus, and PB2 and PA gene segments were derived from AIVs (111, 112). Each precursor gene segment of the 2009 H1N1 virus had been circulated in the swine population for more than a decade and the direct precursor of the virus may had emerged a few months prior to the detection of the outbreak in humans (112, 113).

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Evolution of IAVs

IAVs are notorious for their capability of rapid evolution in response to the adapting hosts. The evolutionary dynamics of IAVs is largely determined by rapid mutation, frequent reassortment, and multiple selection pressures, including host immune response and host switch (4).

Population and Quasispecies

Infection of IAV could start with a small number of virions and then generated a large number of progeny viruses. An individually-infected cell could release more than 10^4 influenza viruses, although this number is subjected to variation due to difference in host cells (114). This large number of influenza viruses exists as a quasispecies, similar to other RNA viruses (115). Quasispecies is defined as "a cloud of diverse variants that are genetically linked through mutation, interact cooperatively on a functional level, and collectively contribute to the characteristics of the population" (116). Selection pressure acts upon this whole unit rather than individual virus. In addition, the survival of a population is not only determined by its own fitness but also its flexibility to mutate and give rise to progenies that are more fit on average.

Evolutionary Mechanisms

There are three mechanisms that contribute to the evolution and genetic diversity of IAVs: mutation, reassortment, and recombination.

Mutation refers to substitution, insertion, and deletion due to the intrinsic error of the replication. Viral RNA polymerase lacks the proofreading ability and leads to the error-prone replication of RNA viruses. The mutation rate of RNA viruses is

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approximately 10^{-4} mutations per nucleotide copied (117), which is significantly higher than that of DNA viruses (10^{-9} mutations per nucleotide copied). Based on this mutation rate, approximately one mutation is made across the IAV genome during one replication cycle. Given the large population size, mutation contributes to the great genetic diversity of IAVs.

The segmented nature of IAV genome allows exchange of individual gene segment or combination of gene segments between IAVs during mixed infection in the same host cell. **Reassortment** occurs frequently between IAVs and permits rapid generation of progeny viruses with gene segment combination significantly different from that of the parental viruses (48, 118, 119). Reassortment enhances the genetic diversity of IAV, in turn, this increased diversity may facilitate the generation of novel pandemic and epidemic strains. Reassortment has been associated with the emergence of at least three out of four documented pandemic strains as described earlier, including 1957 H2N2, 1968 H3N2, and 2009 H1N1 viruses (95, 96). Moreover, genome-scale evolutionary study showed high degree of reassortment and periodic genome-wide sweeps for the H3N2 seasonal influenza virus (120, 121).

Recombination refers to the exchange of pieces of genetic information between RNA molecules during replication and leads to the production of a RNA molecule with mixed ancestry. Recombination that occurs between regions with high sequence identity is referred as homologous, while recombination between two genetically different regions or non-related RNA molecules is referred as non-homologous. Early study with genomic dataset of human IAV showed that homologous recombination, if occurring at all, is very rare (122). On the other hand, non-homologous recombination has been reported and

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associated with increased pathogenicity of a few AIV strains. The HA gene segment of the HPAI H7N3 viruses isolated during the outbreak in Chile (2002) and Canada (2004) obtained multi-basic amino acids at the cleavage region through non-homologous recombination with NP (60) and MP (123) gene segment, respectively. In addition, HPAI H7N3 virus isolated during the outbreak in Mexico (2012) obtained insert at the HA cleavage region through non-homologous recombination with host genomes (63).

Selection Pressure

Mutation, reassortment, and recombination lead to great genetic diversity of IAVs. This increased genetic diversity provides a platform for selection pressure to select for or against.

Immune Pressure

Although multiple selection pressures were suggested to determine the long-term success of any lineage of IAV, host immune pressure is considered to play a critical role. IAVs could escape the host immune response through its antigenic evolution known as antigenic drift and antigenic shift. Two surface glycoproteins, HA and NA, are the primary targets of the humoral immune response. Accumulation of point mutations within antibody-binding sites in HA and/or NA proteins can lead to a small antigenic change, so called antigenic drift. The switch of HA and/or NA gene segment by reassortment can cause a large antigenic change, so called antigenic shift.

In wild birds, IAVs are proposed to have reached evolutionary stasis and maintained a stable antigenic status (4). For example, a study in 1987 showed that H3 AIVs isolated from mallards within the Pacific flyway during 1977-1985 are highly

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conserved in antigenic properties (124). Similar to that, a limited antigenic diversity was found among 37 H3 AIV strains isolated from migratory birds from Atlantic, Central, Mississippi, and Pacific flyways during 2007-2011 (125). Another study found that antigenic difference among 26 H7 AIVs isolated from shorebirds and gulls at Delaware Bay was within 2-fold change in the hemagglutination inhibition (HI) assays; among the 26 isolates, 24 are of the subtype H7N3, one of subtype H7N4 and one of subtype H7N5 (38). The same study also observed that four mallard-origin H5 AIVs isolated from Sweden and the Netherlands in 1999 and 2002 showed antigenic differences of less than 4-fold change in HI assays.

Probably due to the selection pressure from host immune response and/or host adaptation, the HA protein of IAVs were found to evolve faster in a few species, inducing humans (126), swine (127), and domestic poultry (128, 129). In humans, there are two subtypes of seasonal IAVs, H1N1 and H3N2 viruses. Seasonal H3N2 IAV originated from the 1968 pandemic strain and has continued to circulate ever since. Seasonal H3N2 IAV has undergone considerable antigenic evolution and 11 distinct antigenic clusters were observed during 1968-2003 (130). Antigenic drift between different clusters could be caused by single and multiple amino acid substitutions in the HA protein; and 39 antigenicity associated sites were identified (131). The history of seasonal H1N1 IAV could date back to the 1918 pandemic and it had circulated among humans until replaced by H2N2 virus in 1957 (96, 132, 133). During the post-pandemic period, a major antigenic change was reported in 1947 (134). After 20 years of being undetected, H1N1 virus which is identical to that from the 1950s re-emerged in 1977 (135, 136) and has been co-circulating with H3N2 virus ever since. Compared to the rapid evolution

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demonstrated by seasonal H3N2 IAV, H1N1 virus showed less frequent antigenic drifts (137). In addition to mutations at the antibody binding sites, the change in antigenicity of H1N1 virus was associated with glycosylation in the globular head of the HA protein (138).

Antigenic drift has also been reported for IAVs that infect swine. For example, antigenic variants of classical H1N1 SIVs were reported in the United States during 1992-1994 (139-141). H1N1 SIVs isolated from the North American swine population were divided into multiple antigenic clusters, inducing α, β, γ, δ-1, and δ-2 (142). Similar to that, at least two distinct antigenic clusters, H3N2 SIV-alpha and H3N2 SIV-beta, were identified in the H3N2 SIVs that circulate among the swine population in the United States during 2006-2012 (143). In Europe, antigenic drift was reported for the H3N2 SIVs from multiple countries in the 1990s, including the Netherlands and Belgium (144), and Italy (145).

Host Switch

IAVs present partial restriction in terms of host range with occurrences of interspecies transmission. HA glycoprotein is considered the major determinant for host range due to its role in recognition of and binding to the host cell receptor (9). HAs of human IAVs prefer to bind to SA2,6Gal, whereas those of AIVs preferentially bind to SA2,3Gal. Amino acid mutations in the receptor binding site could confer the change in receptor binding specificity, and specific mutations vary among the HAs of different viruses. For example, amino acid mutations Q226L and G228S (H3 numbering) are linked with increased binding affinity to SA2,6Gal for human H2 and H3 IAVs (146, 147). Amino acid mutations E190D and G225D (H3 numbering) are crucial for increased

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binding affinity to SA2,6Gal for H1 IAVs infecting humans and swine (148). In addition to the HA gene segment, molecular features associated with host switch were identified in other gene segments. For example, approximately 20 amino acids deletion in the stalk region of NA protein is considered one characteristic feature in the host switch of AIVs from waterfowl to domestic poultry (149, 150); and this molecular feature could be a compensatory change to maintain the functional compatibility between HA and NA proteins (151). Molecular changes in the genes that code polymerase proteins, including E627K and D701N substitutions in PB2 gene segment, could be associated with increased replication ability in the cells of new hosts (152-155).

Vaccination

Vaccination is considered one of the primary options to prevent and control influenza outbreaks among humans. The World Health Organization (WHO) Influenza Program was established to address the threat brought to public health by influenza since 1948 (156, 157). Vaccine against influenza A and B viruses were invented in the 1940s (158). The trivalent inactivated vaccine was developed after the 1968 influenza pandemic (159). Currently, the Global Influenza Surveillance and Response System comprises more than 100 national influenza centers and six collaborating centers around the world (160). A large number of influenza samples were collected and analyzed through this network on a yearly basis. Recommendation of the viral seed strain in the vaccine for the upcoming influenza season was made on the basis of antigenic, genetic, and epidemiological data. The vaccine seed strain is determined to be updated when there is significant antigenic difference between the existing vaccine strain and the emerging strain; and the emerging strain may cause human infections in a wide geographic region.

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The WHO consultation is head twice a year, every February for the upcoming Northern hemisphere influenza season and every September for the upcoming Southern hemisphere influenza season, respectively. The trivalent vaccines are recommended to contain two influenza A strains and one influenza B strain; and the quadrivalent vaccines are recommended to include an additional influenza B strain.

Although the existing WHO influenza surveillance program is considered one of the best-developed systems for infectious disease, it still has several shortcomings. First, the selection of vaccine seed strain is based on the prediction of the predominant viral strain for the upcoming influenza season; however, a new antigenic variant could emerge and quickly spread after recommendation of the vaccine seed strain is made. Second, ferret sera were used for antigenic characterization in the existing system, and this may have limitation in accurately predicting vaccination-induced responses in humans (161-163). This was shown by the poor performance of the 2014-2015 Northern Hemisphere influenza vaccines attributed to a mismatch of H3N2 vaccine component with epidemic strains in circulation (161). Third, the existing system mainly consider the two surface glycoproteins, HA and NA; however, genome wide interaction may contribute to the evolutionary dynamics of seasonal influenza virus (120). Fourth, antigenic characterization through current laboratory methods is relatively time-consuming, hence, only a relatively small number of influenza samples can be antigenically characterized.

The conventional strategy for controlling the spread of avian influenza outbreaks among domestic poultry involves enforcement of biosecurity measures, diagnostics and surveillance, and culling of infected birds. Culling of infected birds is one of the conventional approaches to control the HPAI outbreaks. The strategies for control of

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LPAI outbreaks vary from no action to active eradication programs including culling of infected birds. In addition to these conventional methods, vaccination programs have been implemented in multiple countries to control AIV outbreaks among domestic poultry. In North America, vaccine program against AIV was introduced in 1995 to control the HPAI H5N2 virus in Mexico (164); vaccine was used against a 2003 H7N2 AIV outbreak in Connecticut, the United States, and against the on-going H7N3 AIV outbreak in Mexico. In Eurasia, vaccine has been used in Pakistan since 1995 to control H7N3 virus; vaccine was used against H7N1 virus in 2000 and against H7N3 virus in 2002 in Italy. Vaccination program against LPAI H9N2 virus has been implemented in China since 1998. Vaccination program against HPAI H5N1 virus was initially implemented in Hong Kong Special Administrative Region, China in 2012. Afterwards, more than 113 billion doses of vaccine have been used against HPAI H5N1 virus in 14 countries during 2002-2010. Nationwide vaccination programs were carried out in China, Vietnam, Indonesia, and Egypt, all of which account for 99% of the vaccine used against HPAI H5N1 virus (165). The use of vaccine can reduce or prevent clinical disease; reduce or eliminate virus shedding into the environment from infected birds, which would help prevent the spread of virus to uninfected flocks; and increase the resistance of birds to becoming infected. Early experience showed that vaccination program could serve as a valuable component in a successful AIV control strategy.

The implementation of vaccination program has been associated with the antigenic evolution of AIVs circulating among domestic poultry. For instance, vaccination program was suggested to facilitate the genetic evolution of HPAI H5 viruses circulating among domestic poultry; a 2011 study showed that HA gene of HPAI H5N1

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virus isolated from countries where vaccination was implemented genetically evolved faster than those from countries where vaccination was not implemented (166). In addition, vaccination program has been associated with the antigenic evolution of HPAI H5N2 virus in Mexico. Vaccination program was introduced to control the HPAI H5N2 outbreak among domestic chicken in 1995 (167). After that, two novel genetic lineages emerged; viruses in these two lineages underwent antigenic drift and acquired a more than 4-fold antigenic difference from the vaccine strain (168). Moreover, antigenic characterization of 41 H7N3 AIV strains isolated during the 2002-2004 epidemic in Italy showed significant antigenic difference between the viruses isolated prior to the implementation of vaccination program and those after the vaccination program (169).

Knowledge Gaps and Objectives of this Dissertation

Collectively, periodic introductions of IAVs from wild birds contribute to the emergence of novel strains that cause infections in humans, lower mammals, and domestic poultry. Vaccination is considered one of the primary options to prevent and control human influenza outbreaks; and vaccination program could also contribute to a successful AIV control strategy. Antigenic match between vaccine seed strain and circulating viral strains is one of the keys to a successful influenza vaccination program. Thus, it is critical to characterize the genesis of emerging avian origin IAVs and understand the antigenic diversity of contemporary IAVs. Such analyses could provide important information for formulation of effective disease control and prevention strategies. Although our understanding has been greatly improved, there are a few questions not addressed regarding the antigenic and genetic evolution of emerging avian origin IAVs.

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First, a novel LPAI H10N8 virus emerged and caused three human infections in Jiangxi, China since December 2013 (170). Genetic analysis showed that the novel virus was a reassortant with HA and NA gene segments originating from wild bird origin IAVs and the six internal gene segments from the H9N2 virus circulating among domestic poultry in the region. However, the source of human infection with the H10N8 virus was not determined.

Southern China is hypothesized to be an influenza epicenter (171), and the emergence and zoonotic transmission of novel AIVs were favored by a unique set of ecological conditions, including the large domestic duck population and the widespread live poultry market (LPM) system (172-175). The threat brought by the ecosystem in the region was repeatedly demonstrated by the emergence and zoonotic transmission of H5N1, H9N2, and H7N9 viruses (20, 176, 177).

LPMs have been recognized as playing an important role in the epidemiology of IAVs (173, 178, 179). LPMs host various avian species from different sources in a dense environment and serve as an optimal site for maintenance and diversification of influenza virus. For example, LPAI H5N2 viruses had been isolated from LPMs in the United States during 1986–1989 (178, 180). LPAI H7N2 virus was first identified in the LPMs in the Northeast United States in 1994, and it has been continued to circulate until 2006 (129, 181). Evidence for reassortment of H7N2 AIVs with other AIVs circulating in the LPMs was observed. In addition, LPMs provide an environment for contact between humans and infected live animals, which allows the potential zoonotic transmission of AIVs. The association between LPMs and zoonotic transmission was suggested by the earlier human infections of HPAI H5N1 and LPAI H9N2 viruses (182-184). In March

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2013, a novel LPAI H7N9 virus emerged in eastern China (177) and has become enzootic in the region. Since the emergence of H7N9 virus, more than 500 laboratory-confirmed cases in human have been reported, of which more than 100 were fatal (http://www.who.int/influenza/human_animal_interface/influenza_h7n9/en/). LPM was identified as the most probable source of human infections with H7N9 virus (177, 185). This was further supported by the evidence showing temporally closure of LPMs in major Chinese cities after the initial outbreak of H7N9 virus significantly reduced the number of human infections (186, 187). We hypothesized that LPM is the source of H10N8 human infections.

Second, the emergence of HPAI viruses in domestic poultry has been associated with introductions of LPAI viruses from wild birds, but the mechanisms of emergence are unclear. In January 2016, a novel HPAI H7N8 virus emerged and caused high mortality among turkey flock in Indiana, United States. The genesis of this H7N8 virus is not understood.

Following introductions into domestic poultry, LPAI H5 and H7 viruses have the potential to evolve into HPAI viruses through two mechanisms: 1) acquisition of basic amino acids in the cleavage region of the HA protein by insertion or substitution (188), and 2) recombination with another gene segment(s) or host genome (60, 63, 123). HPAI viruses are of concern because of the direct and indirect losses that may be incurred by the poultry industry through mortality of affected birds, culling of flocks to prevent spread, trade restrictions, and increased costs associated with outbreak response.

The consequences of introductions of IAVs from wild birds to domestic poultry has been repeatedly demonstrated by the emergence of novel HPAIs in Eurasia, including

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H5N1 (20), H7N1 (189), and H7N7 viruses (19). In the Americas, HPAI H5 viruses caused outbreaks among domestic poultry in 1983 (190), 1994 (191), and 2014 and 2015 (71). In addition to subtype H5 viruses, HPAI H7 viruses have been frequently reported in the Americas. There were four reported HPAI H7N3 outbreaks between 2002 and present: one outbreak in Chile (2002) (60), two distinct outbreaks in Canada (2004 and 2007) (61, 62), and one outbreak in Mexico (2012). In addition to affect domestic poultry, HPAIs H5N1, H7N3 (Canada in 2004 and Mexico in 2012), and H7N7 viruses also caused human infections (20, 64, 192, 193).

Retrospective studies have often been conducted to determine the putative precursor viruses leading to the emergence of IAV strains that cause outbreak among domestic poultry. Wild birds have been recognized as the most probable source for outbreak strains or at least a few of the gene segments that were incorporated into the genome of the outbreak strains (194, 195). However, in many instances, evidence is lacking regarding the time of emergence, location of reassortment, and bird species associated with the genesis of a particular IAV outbreak strain; limited availability of contemporary wild bird-origin IAV isolates or sequences impedes any attempt to investigate the potential mechanisms underlying the emergence.

We hypothesized that 1) the H7N8 virus identified in turkeys in Indiana was initially introduced from wild birds and developed high pathogenicity within poultry production systems; 2) genetic analysis of a large number of available contemporary wild bird–origin IAV isolates would indicate what wild bird species and IAVs contributed to the emergence of the HPAI H7N8 virus.

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Third, earlier studies showed minor antigenic diversity for H7 AIVs isolated from wild birds in Eurasia (195, 196); however, limited data is available for antigenic diversity of contemporary H7 AIVs from North America. H7 AIVs present a continuous threat to both public and animal health. Vaccination program is considered a useful component of a successful control strategy against AIV and has been implemented in multiple countries. Because IAVs frequently undergo antigenic change, circulating influenza strains should be continually monitored to optimize the antigenic matches between vaccine seed strain and circulating strains. Thus, understating the antigenic diversity of contemporary H7 AIVs is crucial for a successful vaccination program.

The objectives of this dissertation research were to infer the genesis of two emerging avian origin IAVs, LPAI H10N8 and HPAI H7N8 viruses, and to investigate the antigenic diversity and genetic evolution of H7 AIVs from North America. The overall hypotheses of this dissertation were: 1) genetically, a persistent gene constellation of IAVs circulating among wild birds favors the emergence of novel IAVs that cause outbreaks among domestic poultry and human; 2) antigenically, IAVs have reached a point of stasis in wild birds but not in domestic poultry. To validate the hypotheses, four specific objectives were proposed.

First, develop and validate a web-based pipeline for analyzing and assembling next generation sequencing (NGS) data for influenza viruses.

Second, characterize the genesis of a novel LPAI H10N8 virus and identify the source of H10N8 human infection in China.

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Third, characterize the genesis of a novel HPAI H7N8 virus and recover the evolutionary pathway leading to the outbreak of disease among domestic turkey in Indiana, the United States.

Last, characterize the antigenic diversity and genetic evolution of contemporary H7 AIVs isolated from North American wild and domestic birds during 1971-2012.

Dissertation Organization

This dissertation is organized into six chapters. Chapter I introduces the background of IAVs and states the hypotheses, and objectives of this dissertation research. A web-based pipeline for analyzing and assembling NGS data for influenza viruses is presented in Chapter II. Chapter III investigates the genesis of a novel LPAI H10N8 virus that emerged and caused three human infections in China, 2013. Study in chapter IV investigates the potential origins, evolutionary pathways, and introduction routes of a novel HPAI H7N8 virus that caused disease outbreak among turkey flocks in Indiana, the United States, 2016. Chapter V describes the antigenic diversity and genetic evolution of contemporary H7 AIVs from North America. Conclusions of this dissertation study are provided in chapter VI.

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CHAPTER II

A WEB-BASED PIPELINE FOR ANALYZING AND ASSEMBLING NGS DATA OF IAVS

NGS has become a routine method in influenza virus research. To date, there is a lack of publically available pipelines for analyzing and assembling NGS data of influenza virus. There are two major challenges for development of such a pipeline: rapid identification of eight optimal reference sequences from a large influenza database corresponding to eight gene segments in the influenza genome; determination of the origin of short reads and assembly of homologous genomic sequences in mixed infection sample. In this chapter, a web-based pipeline for analyzing and assembling NGS data of influenza viruses is developed and validated. This pipeline could be applied to evaluate the quality of NGS data, to identify the eight suitable reference sequences for influenza genome, to assemble the full genome, and to evaluate whether there is a mixed infection of multiple IAVs in the sample. In addition, the web-based application provides a user-friendly graphic interface and visualization for users. This pipeline is comparable in performance to the commercially available software represented by CLC Genomics Workbench but enabled the genomic analysis and assembly of influenza NGS data in a high throughput manner.

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Introduction

The availability of NGS technology has allowed us to rapidly sequence influenza genomes; and NGS has become a routine method in molecular diagnosis, surveillance, vaccine strain selection, and laboratory research for influenza viruses. The NGS data are characterized by high throughput of relative short reads. For example, the read length is usually less than 300 bp for NGS data generated by Illumina. The challenge in NGS data analyses is to accurately assemble these short reads into the IAV genomic sequences and still represent the polymorphisms of these sequences.

To date, there still lacks of publically available pipelines for analyzing and assembling NGS data of influenza virus. Influenza virus genome is comprised of eight single strand negative-sense RNA gene segments, and thus genomic assembly will require eight closely matched reference sequences. Moreover, influenza virus assembly could be impeded by the mixed infection of multiple influenza viruses in the sample. Considering the relative conservation of influenza gene segments, especially six internal gene segments, it is not a trivial task to differentiate the origin of short reads and then assembly them into homologous genomic sequences.

The objective of this chapter is to develop and validate a web-based pipeline for analyzing and assembling NGS data for influenza viruses. The pipeline is designed to aggregate a series of existing computational tools for NGS data preprocessing, template matching, and genomic assembly, enabling the assembly of influenza genome and the detection of mixed infection in a high throughput manner. The web-based application is expected to provide user-friendly interface and visualization for the users.

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Materials and Methods

Data Preprocessing

The designed web-based pipeline comprises multiple steps, including quality control, trimming, reference search, genome assembly, and mix-infection detection (Figure 1). The pipeline starts with investigation of the overall quality of the raw NGS data with FastQC v0.10.1 (197). It generates composition statistics of the raw data, including read number, read length, and base quality. The reads in the raw data are trimmed with Trimmomatic v0.32 (198). In this step, nucleotide bases are removed from both ends of the read if their quality falls below a pre-defined threshold; and the read is clipped if the average quality within a sliding window falls below a threshold quality. After that, reads above a specified length are retained for further analyses. The quality of the trimmed reads is once again investigated with FastQC v0.10.1.

Genome Assembly

This pipeline enables two modes for sequence assembly: reference-based assembly and *de novo* assembly. The reference-based assembly refers to the case when a reference influenza genome is provided, and then trimmed reads could be directly mapped to the reference with the implementation of Bowtie v2.0 (199). Local alignment and pair-end mode are used for reads mapping. The *de novo* assembly mode is initiated when a reference genome is not available beforehand. In this case, trimmed reads are *de novo* assembled to create contiguous sequence with Velvet v1.2.10 (200). The resulting contigs are searched against the influenza nucleotide database by BLAST (201) and eight reference sequences corresponding to eight gene segments are selected. Subsequently, the influenza genome is assembled as described in the reference-based assembly mode. Each

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nucleotide base in the consensus sequence is determined on the basis of two criteria: (1) it is supported by a specified mapping coverage, and (2) consensus is reached among reads cover this position. The subtype of a sample is determined on the basis of the reference sequences selected for the HA and NA gene segments. When de novo assembly generates multiple HA and/or NA gene segments corresponding to distinct subtypes, the sample is identified as a mixed infection sample.

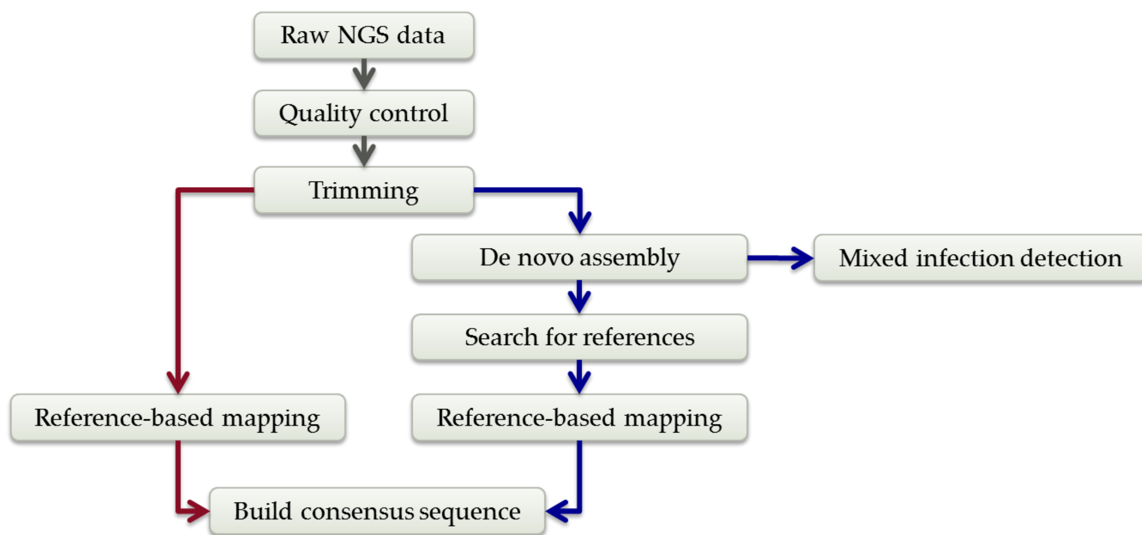

Figure 1 Pipeline for analyzing and assembling NGS data of influenza virus.

Web-based Application

To provide a user-friendly graphic interface and visualization tool, a web-based application was developed for this influenza genome assembly pipeline. Two assembly modes, reference-based assembly and *de novo* assembly, are provided to users as an option. In the reference-based mode (Figure 2), the user will provide NGS data and a table that comprises the GenBank accession numbers of the eight reference sequences for

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each sample. Reference sequences will then be obtained from influenza nucleotide database by the given accession numbers. In addition, users will be asked to provide parameters for each step in the analysis. For example, threshold quality, sliding window size, and read threshold length are needed for quality trimming; threshold coverage and level of consensus are needed for determining the nucleotide base for each position in the consensus sequence. In the de novo assembly mode, users will provide k-mer length for de novo assembly.

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Figure 2 Reference-based assembly mode submission interface.

After the analysis, a number of statistics will be provided to users in form of graphics. These statistics include read number and average base quality in the raw NGS data and trimmed data, percentile of raw reads retained following quality trimming, percentile of read that were mapped to the reference genome, and coverage of the consensus sequences. Users could download these plots and various formats are supported. The resulting genomic sequence files are provided in both gene-specific and

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sample-specific manners. In addition, the mapping profiles of each sample are available for users to assess the mapping quality. On the website, users could monitor the progress of the job and each of its stages in real-time (Figure 3). Our server will notify users when the job starts and completes via emails. The web-based application was written in PHP with a core written in Perl. It is freely accessible on SystemsBio Lab website (http://sysbio.cvm.msstate.edu).

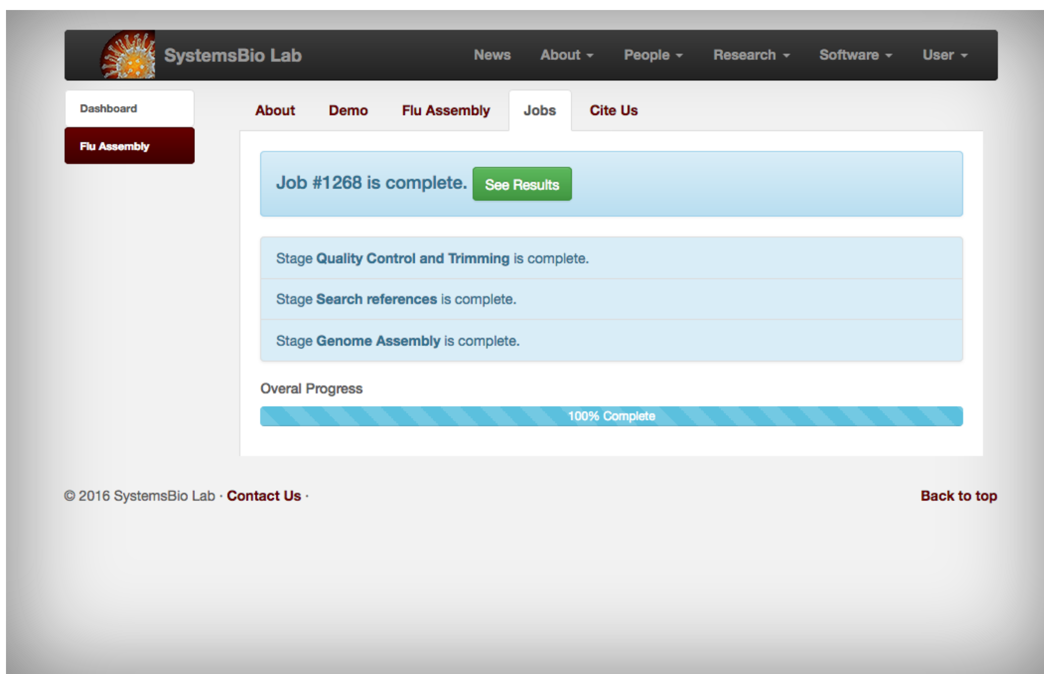

Figure 3 Job status page (job complete).

Testing Datasets and Performance Evaluation

Two datasets were used to evaluate the performance of this web-based influenza genome assembly pipeline. The reference-based mode was investigated with the first dataset that comprises NGS data for five influenza reassortants generated through

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reassortment between a canine H3N2 virus (A/canine/Guangdong/1/2006, H3N2, Guangdong/1) and a 2009 pandemic H1N1 virus (A/California/04/2009, H1N1, California/04). The HA gene segment of these reassortants originated from the canine H3N2 virus and the other seven gene segments were derived from either parental virus. The complete genomes of these two parental strains were downloaded from GenBank under the accession number GU433345 to GU433352 and FJ966079 to FJ966086 and used as references for genome assembly.

The second dataset comprises NGS data for five wild bird origin AIV strains obtained from earlier surveillance. The subtype of these samples and origin of each gene segment are unknown. The genomes of these samples were assembled with the de novo mode imbedded in this pipeline. The resulting genomic sequences were compared with those generated by CLC Genomics Workbench 9.0.1 (CLC bio, Aarhus, Denmark), which is a commercially available software that provides genomic analyses and assembly package. In CLC Genomics Workbench, trimmed reads were de novo assembled to generate continuous sequences using the contig sequences (fast) mode and the contig length threshold was set to 200. Reads were mapped to the references that were found by BLAST the resulting contigs to influenza nucleotide database.

Results

Under the reference-based assembly mode, this pipeline first investigates the quality of the raw NGS data for the five reassortant viruses. Quality control results showed that the number of read pair for each sample ranges from 289,554 to 347,858. The length of reads falls in the range from 32 bp to 301 bp. The average quality score for each position range from 34.45 to 36.64. The quality of the raw NGS data was improved

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through quality trimming. Quality trimming retained around 85% of the reads in the raw data and increased the average base quality to the range between 36.59 and 37.43 (Figure 4).

Examination of the mapping profile showed that more than 98.95% of the trimmed reads were successfully mapped to the eight reference sequences for each sample. A 100% coverage was observed for all eight reference sequences for each sample and the average mapping depth for each reference sequence ranges from 2,855 to 7,642. The consensus sequences generated by this pipeline were compared with those obtained from GenBank. Among the 40 newly generated genomic sequences, 19 sequences corresponding to six gene segments were identical to those from GenBank; and 21 sequences corresponding to the remaining nine gene segments possess one to six nucleotide substitutions. The nucleotide substitutions were consistent for genomic sequences corresponding to gene segment derived from the same parental virus. For example, five genomic sequences of the HA gene segment originated from the canine H3N2 virus all have two nucleotide differences: A695T and T713G. Further investigation of the mapping profile showed adequate mapping depth and consensus for these positions. The nucleotide substitutions were not introduced by this pipeline but may originate from manipulation of these samples such as adaptation during viral culture. These results suggested that the reference-based assembly mode in this pipeline could successfully recover the complete genome from raw NGS data of influenza virus.

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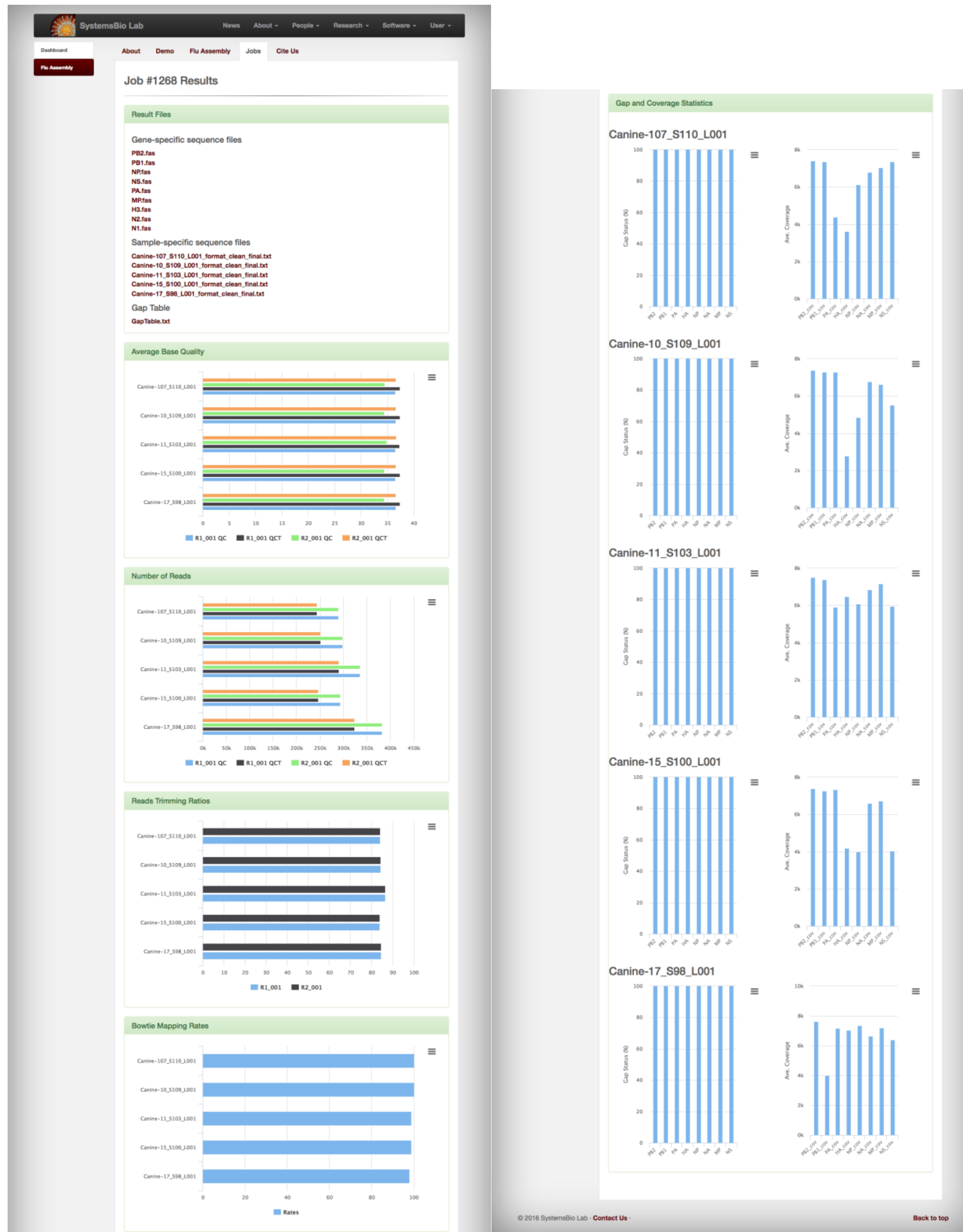

Figure 4 Reference-based assembly mode result page.

Next, the performance of the *de novo* assembly mode was investigated. Among the five tested samples, eight reference sequences corresponding to eight gene segments were found for four samples. Thus, these four samples were designed as single infection.

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More than 99.00% of the trimmed reads were successfully mapped to the references. A 100% coverage was observed for the eight reference sequences of four single infection samples and the average mapping depth for each reference sequence ranges from 862 to 6,817 (Figure 5). Genomic sequences generated by this pipeline were compared with those by Genomics Workbench and results showed a 100% sequence identity. For the last sample, this pipeline found nine reference sequences comprising two references for the NA gene segment corresponding to the subtype N2 and N7, respectively. The resulting sequences from this pipeline comprise two complete NA sequences of the subtype N2 and N7. In addition, one reference sequence of the subtype H7 was identified for the HA gene segment. Thus, this sample was assigned to mixed infection on the basis of these results.

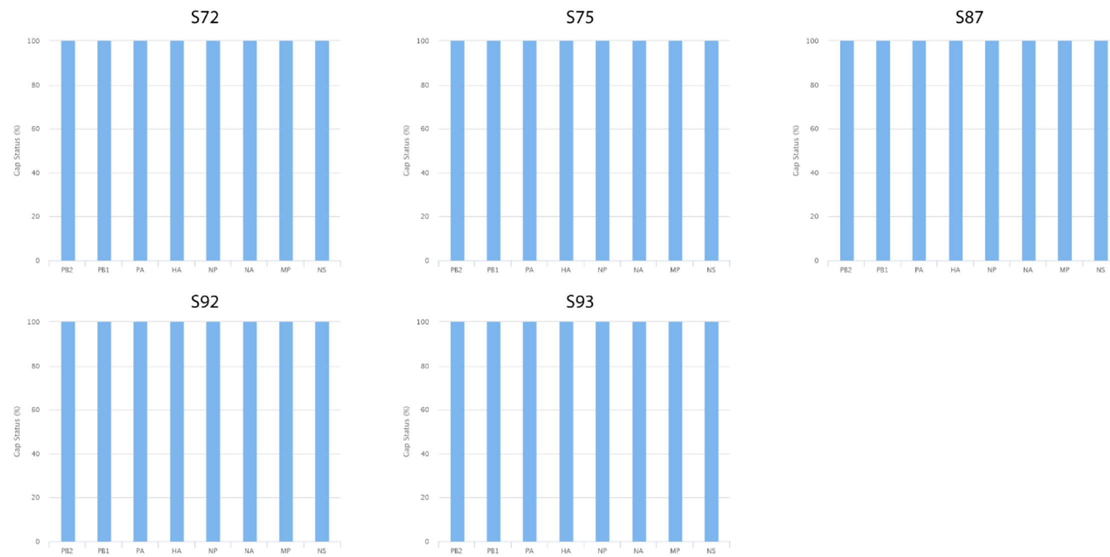

Figure 5 Coverage of resulting sequences of *de novo* assembly mode.

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Discussion

In summary, a publically available web-based pipeline for analyzing and assembling NGS data of influenza virus was developed. This pipeline enables the detection of eight closely matched references, full genomic assembly for influenza genome, and detection of the presence of mixed infection of multiple IAVs in a sample. The web-based application provides a user-friendly graphic interface and visualization for users. The pitfall of this study is that, for a sample with mixed infections, this pipeline still has difficulties in classifying the short reads and recovering homologous genomic sequences.

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CHAPTER III

THE GENESIS OF A NOVEL LPAI H10N8 VIRUS AND THE SOURCE OF H10N8 HUMAN INFECTIONS IN CHINA

In December 2013, a novel LPAI H10N8 virus emerged, causing three human infections in Jiangxi, China (170). Genetic analysis showed that the novel virus was a reassortant comprising HA and NA gene segments from wild bird origin IAVs and the six internal gene segments from the H9N2 viruses circulating among domestic poultry in the region; however, the source of human infections with the novel LPAI H10N8 virus was not determined. LPMs have been identified as a high risk factor for the zoonotic transmission of AIVs in Southern China (182) and we hypothesized that LPMs are the source of H10N8 human infections. We characterized 361 samples collected from April 2013 to January 2014, including 217 samples collected prior to the detection of H10N8 human infection, at the LPM visited by the first index case. Results showed a gradual increase in IAV prevalence and detection of H10 viruses. AIVs that are genetically close to the human H10N8 isolate were recovered from samples collected in this LPM. In addition, we performed HI assays for 800 sera samples collected from chickens and ducks at five LPMs in Nanchang and observed high seroprevalence of H10 virus. These findings suggested that LPM is the most probable source of human infection with this novel H10N8 virus, and this virus appears to present throughout the LPM system in the city. These findings highlight the role of LPMs in the zoonotic transmission of AIVs, and

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suggest influenza ecosystem in Southern China could favor the emergence of novel IAV strains that potentially present risk to both animal and human health. Reduction of the influenza virus burden in LPMs is essential in preventing future emergence of novel IAV strains with zoonotic and pandemic potential.

Introduction

LPMs are considered as an important link in the ecology and epidemiology of IAVs (173). LPMs host a large number of birds among different species in a high-density setting. Live animals are usually brought into the LPMs on a daily basis, and live animals could be carried over in LPMs from one day to the next and even up to weeks for some birds. Thus, LPMs provide an ideal environment for maintenance and transmission of IAVs among these birds and facilitate diversification of IAVs through reassortment. In addition, LPMs provide an environment for direct contact between humans and infected live animals and could serve as the source of potential zoonotic transmission of IAVs.

In the United States, LPAI H7N2 virus was detected in the LPMs in the northeast in 1994, and this virus continued to circulate in the LPMs until 2006 (129, 181). Reassortment was observed between LPAI H7N2 and other IAVs circulating in the LPMs. In 2003, a LPAI H7N2 virus strain was isolated from an immunocompromised man with fever and community-acquired pneumonia in New York (202). Although the source of this human infection case could not be determined, the virus was suspected to originate from the LPMs.

In China, the association between LPMs and zoonotic transmission of AIVs was suggested by reported human infections of HPAI H5N1 and LPAI H9N2 viruses (Figure 6). In May 1997, an avian origin H5N1 IAV strain was isolated from a boy died of

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respiratory illness in Hong Kong (20). A total of 18 cases of human infection with the H5N1 virus were reported in Hong Kong by the end of the year and six cases were fatal. Human infections with LPAI H9N2 virus were reported in China in 1999 (176), 2003 (203), and 2013 (204), respectively. LPMs were indicated to be the source of human infections with H5N1 and H9N2 viruses (182). Human infection with a novel LPAI H7N9 virus was first reported in eastern China in March 2013 (177, 185). LPAI H7N9 virus has caused more than 500 human cases and claimed >100 lives ever since (http://www.who.int/influenza/human_animal_interface/influenza_h7n9/en/). LPMs were also indicated as the most likely source of human infections with the H7N9 virus. This was supported by the significant decrease of mean daily number of infections in major cities in China following measures taken to close LPMs (186).

In December 2013, human infection with a novel avian origin H10N8 virus was reported in Jiangxi, China. The first patient infected with H10N8 virus was hospitalized on November 30, three days after illness onset (170). The patient had visited a local LPM four days prior to the illness onset. Genetic analysis results showed that the novel H10N8 virus was a reassortant possessing HA and NA gene segments from wild bird origin IAVs and six internal gene segments from poultry-origin H9N2 virus. Two more cases of human infections with this novel H10N8 virus were reported in January and February 2014, in Jiangxi. The source of human infections with this virus was not determined. We hypothesize that local LPMs are the source of human infections and we analyzed 361 influenza virus samples collected in the LPM visited by the first patient to infer the possible source of the zoonotic transmission of the novel H10N8 virus.

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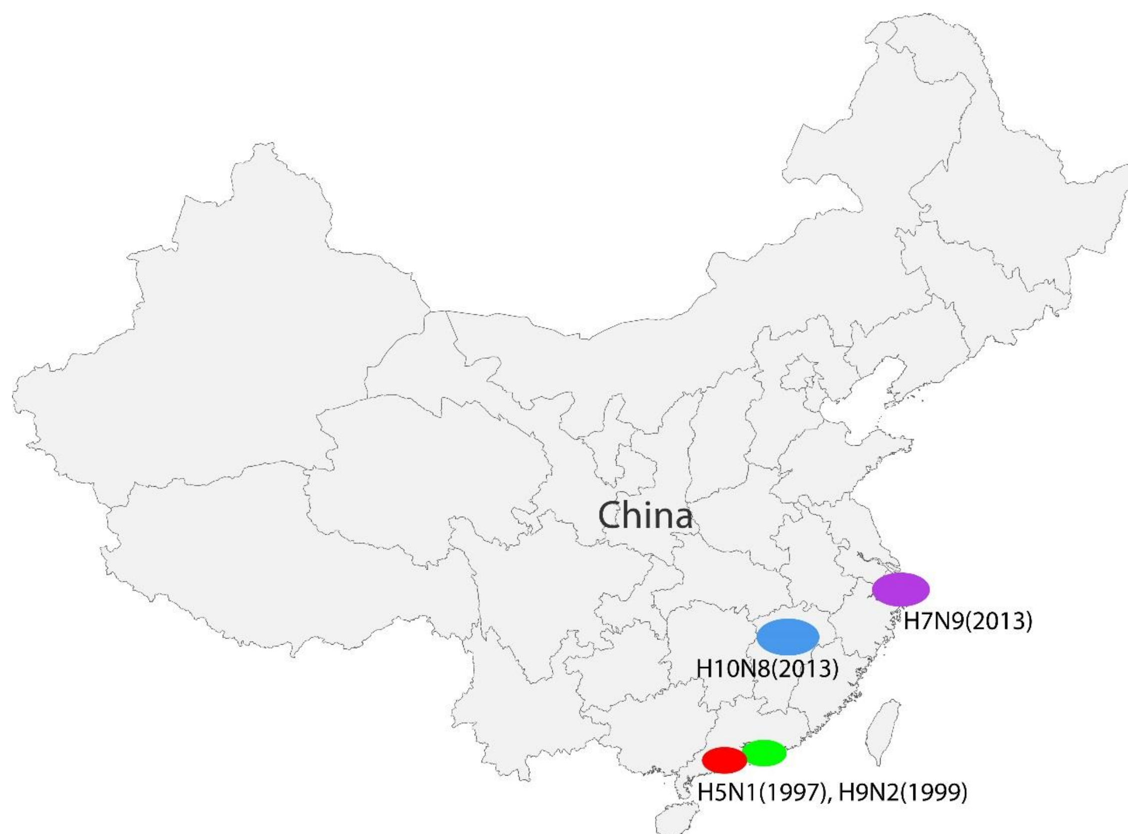

Figure 6 Avian origin influenza A viruses that caused zoonotic infections in Southern China during the past two decades.

Materials and Methods

Sample Collection

Sampling was conducted in the LPM at the Donghu District of Nanchang City, Jiangxi from April 5, 2013 to January 1, 2014. A total of 361 swabs were collected on ten individual time periods, April 5 (number of samples, 47), April 9 (13), June 16 (23), July 25 (64), August 10 (57), August 24 (13), December 12 (46), December 17 (35), December 22, 2013 (36), and January 1, 2014 (27) (Table 1). These samples were collected predominantly from chicken, duck, goose, and four minor species, including pheasant, guinea fowl, pigeon, and turtle doves. In addition, 47 environmental samples

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were collected by swabbing feces in the cages and on the floor of this LPM, especially when samples from live birds were not accessible. The rectal swabs were collected from waterfowl species such as ducks and geese, whereas both oropharyngeal and rectal swabs were taken from the terrestrial birds, including chickens, pigeons, guinea fowl, and turtle doves.

The swabs from each bird or environmental specimen were placed in a tube with M199 transport medium (GIBCO, location) containing 0.5% bovine serum albumin (BSA) and Penicillin G (2 X 106 U/liter), Streptomycin 200 mg/liter. Samples were kept in an ice box before and during shipment to the laboratory and then stored at -80 °C. All manipulations of these samples were conducted under enhanced Biosafety Level 2 (BSL-2) containment facilities at the College of Animal Science and Technology, Jiangxi Agricultural University.

RNA Extraction and IAV Screening

Viral RNA was extracted from the clinical swabs using the QIAamp Viral RNA mini Kit (Qiagen, Valencia, CA) in accordance with manufacturer's instructions. These viral RNA samples were subject to M-gene based IAV screening followed by H7 subtype specific screening using the AgPath-ID™ One-Step RT-PCR Reagent according to the diagnosis manual from WHO)
(http://www.who.int/influenza/resources/documents/molecular_diagnosis_
influenza_virus_humans_update_201108.pdf). The H7 specific primer was adapted from (205).

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Viral Isolation

The non-H7 positive swabs were inoculated into ten day old specific pathogen free chicken embryonated eggs. The eggs were inoculated for 72 hours at 37°C before harvesting.

Hemagglutination and Hemagglutination Inhibition Assays

Both hemagglutination and hemagglutination inhibition assays were conducted according the instruction described in the OIE manual for avian influenza diagnosis using 0.5% chicken red blood cells (http://web.oie.int/fr/normes/mmanual/2008/pdf/2.03.04_AI.pdf). The goat anti-H10 polyclonal antisera were diluted to 1:40 before the HI assays.

Reverse Transcription, PCR, and Genomic Sequencing

The full-length cDNA were amplified from the swabs using SuperScript™ One-Step RT-PCR (Invitrogen, Grand Island, NY). Briefly, total RNA 5 ul, uni-12 2 ul, dNTP 1 ul, and RNase/DNase free water 5 ul, were incubated at 65°C for five minutes and then one minute on ice. Then 1 ul of DTT, 1 ul of RNAse, and 4 ul of buffer, and 1 ul of RT superscript III were added and incubated at 55°C for one hour and then 75°C for 15 minutes. PCR amplification was performed with universal primers U-12 and U-13 Phusion® High-fidelity PCR MMW/HF buffer (New England Biolabs, Ipswich, MA) according to the manufacturer's instruction and followed by eight pairs of gene specific primers (206). PCR product was then purified using QIAquick® PCR Purification Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol.

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All M-gene positive samples were subjective to full genome sequencing using next generation sequencing. Sequencing libraries were prepared from 1 ng cDNA using the Nextera XT kit according to manufacturer's instructions (Illumina, San Diego, CA). Sequencing was performed on an Illumina MiSeq using V2 reagent kits according to the manufacturer's instructions. Sanger sequencing was utilized to fill sequence gaps from the MiSeq run using gene specific primers for the corresponding gene segment. The PCR product was purified by QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA) according to manufacturer's protocol and subjected to sequencing using ABI 3730xl capillary DNA Analyzers.

Genomic Assembly

Genomic assembly was conducted with CLC Genomics Workbench 6 (CLC bio, Aarhus, Denmark). Quality trimming was first conducted to remove reads with two or more ambiguous nucleotides and those below a quality cutoff value of 20. The quality-filtered reads were *de novo* assembled in the fast contig mode and then each assembled contig was BLAST searched against the influenza database to select the best-matched reference sequence. Quality-filtered reads were mapped to the reference sequence and consensus sequences were generated. If two or more genetically distinct references were identified, the quality-filtered reads were matched to each and multiple consensus sequences were generated. If these consensus sequences from a single sample were genetically distinct, this sample was defined as a mixed infection. Each consensus sequence was examined manually to correct potential assembly errors, such as single nucleotide deletions and insertions.

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Phylogenetic Analysis and Molecular Characterization

To recover evolutionary history of H10N8 virus, sequences recovered from this study were combined with those from public database for phylogenetic analyses. Phylogenetic tree was inferred using Maximum Likelihood method implemented in GARLI version 0.951 (207).

Serological Surveillance

To investigate the spread of H10N8 virus in the LPMs system in Nanchang, we collected a total of 800 sera samples from chickens and ducks in five LPMs, from February 25, 2014, to March 27, 2014. These five LPMs cover a geographic area of about 160 square kilometers and the major metropolitan area of Nanchang. Sera positive was determined using HI assay with a H10N8 virus isolated from the surveillance.

Results

The LPM that the first H10N8 patient visited is in the Donghu district of Nanchang City; and we conducted sporadic virologic surveillance at this LPM since April 2013. The number of poultry retailers within this LPM ranges from 20 to 30, and a total of around 1,500 birds are sold per day. The number of poultry retailers and birds being sold vary with season change and other factors. Typically about 90% of birds sold in this market are chickens and ducks with wild birds occasionally being sold as well. Between April 5, 2013, and January 1, 2014, we collected 361 samples from this LPM on ten separate sampling occasions (Table 1). These samples included 192 paired oropharyngeal and cloacal swabs from chickens, pheasant, guinea fowl, pigeon, and turtle doves, as well

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as 122 cloacal swabs from ducks and geese. In addition, 47 environmental samples were also collected by swabbing feces in the cages and from the floor of this LPM.

The proportion of IAV positive samples among these collected samples, as measured by M-gene real time PCR, increased from 4.3% on April 5, to 38.5% on August 24, and to 87.0% on December 12, 2013. The proportion of IAV positive samples, albeit still high, dropped to 48.0% on January 1, 2014 (Table 1). Among these M-gene positive specimens, ten were subtyped as H7 by H7 specific real time PCR (205) and 16 as H10 by H10 specific HI assays (Figure 7, Table 1).

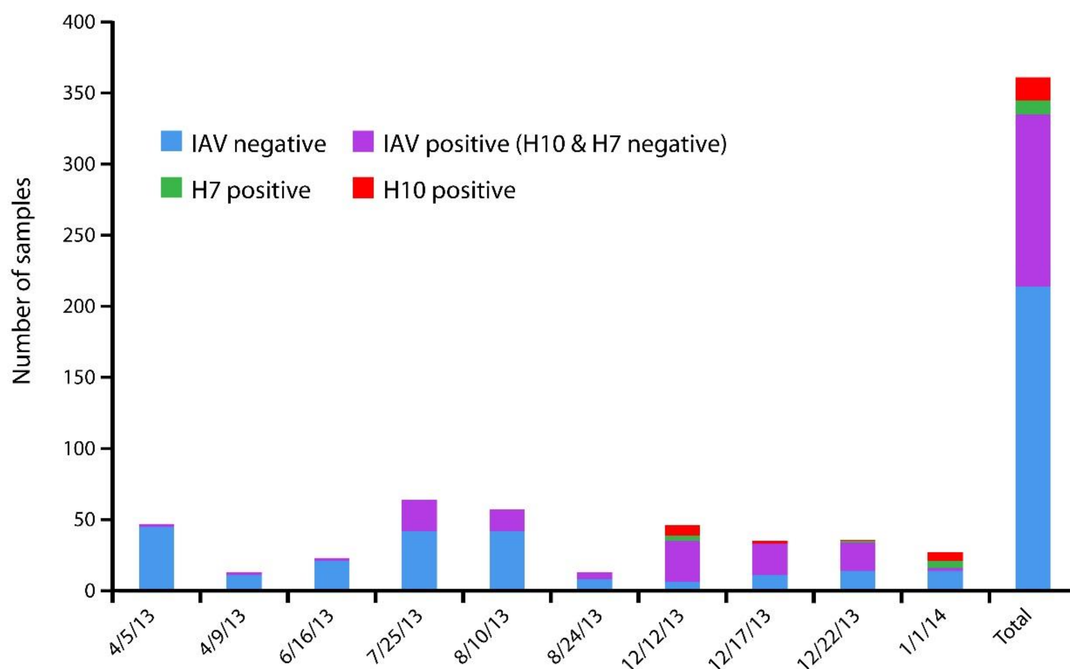

Figure 7 Isolation of H10 influenza A virus from the LPM visited by the first H10N8 patient.

To determine if H10N8 viruses, had been and had continued to circulate in this market, we sequenced positive samples collected on the April, December, and January

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sampling occasions (the first index case was admitted to the hospital in late November). From the April sampling, we sequenced all five positive samples and detected H7 and H9 HA genes in conjunction with N2 and N9 NA genes. Although no H10 or N8 genes were detected, we found PB2, PA, NP, MP, and NS genes were genetically similar to the human H10N8 virus. From the December sampling, we successfully generated sequences from 44 of the 86 positive samples. Twelve samples contained H10 genes, all of which also contained N8 genes, though some did appear to be mixed infections containing gene segments from other viruses such as H9N2. From the January sampling, six samples had both H10 and N8 genes (Table 2).

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Table 1 Statistics of influenza surveillance conducted in the LPM visited by the first H10N8 patient.

Sample type	4/5/13	4/9/13	6/16/13	7/25/13	8/10/13	8/24/13	12/12/13	12/17/13	12/22/13	1/1/14	Total
	Number sampled (%) positive										
Chicken	7 (0%)		9 (0%)	29 (48.3%)	21 (57.1%)		25 (92%)	19 (78.9%)	16 (50%)	15 (40%)	141 (55.3%)
Pheasant	5 (0%)	5 (20%)	1 (100%)								11 (18.2%)
Guinea Fowl	11 (9.1%)										11 (9.1%)
Duck			8 (12.5%)	30 (20%)	18 (0%)		10 (80%)	16 (56.3%)	15 (73.3%)	12 (58.3%)	109 (38.5%)
Pigeon	16 (6.3%)			5 (40%)							21 (14.3%)
Goose		8 (12.5%)	5 (0%)								13 (7.7%)
Turtle Doves	8 (0%)										8 (0%)
Environmental					18 (16.7%)	13 (38.5%)	11 (81.8%)		5 (60%)		47 (42.6%)
Total	47 (4.3%)	13 (15.4%)	23 (8.7%)	64 (34.4%)	57 (26.3%)	13 (38.5%)	46 (87%)	35 (68.6%)	36 (61.1%)	27 (48.0%)	361 (40.7%)
H10							7	2	1	6	16
H7							4		1	5	10

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Table 2 Summary of genomic sequences recovered from the LPM visited by the first H10N8 patient.

Name	Date	GenBank Accession Number									
		HA	NA	PB2	PB1	PA	NP	MP	NS	HA	NA
A/pigeon/Jiangxi/JXA130010/2013(mixed,H7,H9,N2,N9)	4/5/13			KM234652		KM234653	KM234654	KM234655	KM234656; KM234657	H7/H9	N2/N9
A/turtledoves/Jiangxi/JXA130022/2013(H?N?)	4/5/13							KM234660	KM234661		
A/pheasant/Jiangxi/JXA130232/2013(mixed,H9,N2)	4/9/13				KM234662		KM234663	KM234665	KM234666	H9	N2
A/goose/Jiangxi/JXA130250/2013(mixed, H9,N2)	4/9/13						KM234667	KM234669	KM234671	H9	N2
A/goose/Jiangxi/JXA130246/2013(mixed, H?,N1,N2)	4/9/13			KM234672		KM234673	KM234674	KM234677			N1/N2
A/chicken/Jiangxi/JXA131855/2013(mixed,H10,N2,N8)	12/12/13	KM234678	KM234680				KM234679	KM234681	KM234682	H10	N2/N8
A/chicken/Jiangxi/JXA131869/2013(mixed,H10,N2,N8)	12/12/13	KM234686	KM234688	KM234683	KM234684	KM234685	KM234687	KM234689	KM234690	H10	N2/N8
A/chicken/Jiangxi/JXA131890/2013(mixed,H10,N2,N8)	12/12/13	KM234696	KM234698	KM234691; KM234692	KM234693; KM234694	KM234695	KM234697	KM234699	KM234700	H10	N2/N8
A/chicken/Jiangxi/JXA131916/2013(mixed,H10,N2,N8)	12/12/13	KM234701	KM234703				KM234702	KM234704	KM234705	H10	N2/N8
A/chicken/Jiangxi/JXA131919/2013(mixed,H10,N2,N8)	12/12/13	KM234709	KM234711	KM234706	KM234707	KM234708	KM234710	KM234712	KM234713	H10	N2/N8
A/environment/Jiangxi/JXA131877/2013(mixed,H10,N8, N2)	12/12/13	KM234718	KM234720	KM234714; KM234715	KM234716	KM234717	KM234719	KM234721	KM234722	H10	N2/N8
A/environment/Jiangxi/JXA131921/2013(mixed,H10,N2, N8)	12/12/13	KM234726		KM234723	KM234724	KM234725	KM234727	KM234728	KM234729	H10	N2/N8
A/chicken/Jiangxi/JXA131901/2013(mixed,H10,H9,N2,N8)	12/12/13	KM234733	KM234735	KM234730	KM234731	KM234732	KM234734	KM234736	KM234737	H10/H9	N2/N8
A/chicken/Jiangxi/JXA131906/2013(mixed,H10,H9,N2,N8)	12/12/13	KM234742	KM234744	KM234738; KM234739	KM234740	KM234741	KM234743	KM234745	KM234746	H10/H9	N2/N8
A/chicken/Jiangxi/JXA131864/2013(H9N2)	12/12/13			KM234747	KM234748	KM234749	KM234750	KM234751	KM234752	H9	N2
A/chicken/Jiangxi/JXA131923/2013(H9N2)	12/12/13			KM234753	KM234754	KM234755	KM234756; KM234757	KM234758	KM234759; KM234760	H9	N2
A/chicken/Jiangxi/JXA131875/2013(H?N2)	12/12/13						KM234761		KM234762		N2
A/chicken/Jiangxi/JXA131893/2013(H?N2)	12/12/13								KM234763		N2

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Table 2 (continued)

A/chicken/Jiangxi/JXA131964/2013(mixed,H10,N2,N8)	12/17/13	KM234767		KM234764	KM234765	KM234766	KM234768	KM234769	KM234770	H10	N2/N8
A/chicken/Jiangxi/JXA131985/2013(H6N2)	12/17/13			KM234771	KM234772; KM234773	KM234774	KM234775	KM234776	KM234777	H6	N2
A/chicken/Jiangxi/JXA131963/2013(H9N2)	12/17/13			KM234778	KM234779	KM234780	KM234781	KM234782	KM234783	H9	N2
A/chicken/Jiangxi/JXA131973/2013(H9N2)	12/17/13			KM234784	KM234785	KM234786	KM234787	KM234788	KM234789	H9	N2
A/duck/Jiangxi/JXA131977/2013(H9N2)	12/17/13			KM234790	KM234791	KM234792	KM234793	KM234794	KM234795	H9	N2
A/chicken/Jiangxi/JXA131981/2013(mixed,H10,N2,N8)	12/17/13	KM234800	KM234802	KM234796; KM234797	KM234798	KM234799	KM234801	KM234803; KM234804	KM234805; KM234806	H10	N2/N8
A/duck/Jiangxi/JXA131996/2013(H?N2)	12/17/13			KM234807		KM234808	KM234809	KM234810	KM234811		N2
A/duck/Jiangxi/JXA132023/2013(H?N2)	12/17/13			KM234812	KM234813	KM234814	KM234815	KM234816	KM234817		N2
A/duck/Jiangxi/JXA131986/2013(mixed,H6,N2)	12/17/13			KM234818; KM234819	KM234820	KM234821	KM234822; KM234823	KM234824; KM234825	KM234826	H6	N2
A/chicken/Jiangxi/JXA132379/2013(H10,N8,N2)	12/22/13	KM234830	KM234832	KM234827	KM234828	KM234829	KM234831	KM234833	KM234834	H10	N8/N2
A/chicken/Jiangxi/JXA132304/2013(H9N2)	12/22/13			KM234835	KM234836	KM234837	KM234838	KM234839	KM234840	H9	N2
A/chicken/Jiangxi/JXA132363/2013(H9N2)	12/22/13			KM234841	KM234842	KM234843	KM234844	KM234845	KM234846	H9	N2
A/duck/Jiangxi/JXA132308/2013(H9N2)	12/22/13			KM234847	KM234848	KM234849	KM234850	KM234851	KM234852	H9	N2
A/duck/Jiangxi/JXA132362/2013(mixed,H9,N2,N8)	12/22/13		KM234857	KM234853	KM234854	KM234855	KM234856	KM234858	KM234859	H9	N2/N8
A/chicken/Jiangxi/JXA132322/2013(H?N8)	12/22/13		KM234861				KM234860	KM234862	KM234863		N8
A/environment/Jiangxi/JXA132335/2013(H?N2)	12/22/13								KM234864		N2
A/chicken/Jiangxi/JXA132328/2013(H?N2)	12/22/13			KM234865	KM234866	KM234867	KM234868	KM234869	KM234870		N2
A/chicken/Jiangxi/JXA132321/2013(H?N?)	12/22/13			KM234871	KM234872	KM234873	KM234874	KM234875	KM234876		
A/duck/Jiangxi/JXA132323/2013(H?N?)	12/22/13						KM234877				
A/duck/Jiangxi/JXA132348/2013(H?N?)	12/22/13								KM234878		
A/environment/Jiangxi/JXA132330/2013(H?N?)	12/22/13						KM234879		KM234880		

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Table 2 (continued)

A/chicken/Jiangxi/JXA132357/2013(H?N?)	12/22/13										
A/chicken/Jiangxi/JXA132359/2013(H?N2)	12/22/13						KM234881				N2
A/chicken/Jiangxi/JXA132388/2013(H?N2)	12/22/13			KM234882	KM234883	KM234884	KM234885		KM234886		N2
A/duck/Jiangxi/JXA132309/2013(H?N?)	12/22/13								KM234887		
A/duck/Jiangxi/JXA132338/2013(H?N?)	12/22/13										
A/duck/Jiangxi/JXA132339/2013(H?N?)	12/22/13										
A/duck/Jiangxi/JXA132366/2013(H?N?)	12/22/13										
A/environment/Jiangxi/JXA132326/2013(H?N?)	12/22/13								KM234888		
A/environment/Jiangxi/JXA132327/2013(H?N?)	12/22/13										
A/environment/Jiangxi/JXA132345/2013(H?N?)	12/22/13								KM234889		
A/chicken/Jiangxi/JXA132713/2014(mixed,H10,N2,N8)	1/1/14	KM234893	KM234895	KM234890	KM234891	KM234892	KM234894	KM234896	KM234897	H10	N2/N8
A/chicken/Jiangxi/JXA132717/2014(H10N8)	1/1/14	KM234901	KM234903	KM234898	KM234899	KM234900	KM234902	KM234904	KM234905	H10	N8
A/chicken/Jiangxi/JXA132731/2014(mixed,H10,N2,N8)	1/1/14	KM234909	KM234911	KM234906	KM234907	KM234908	KM234910	KM234912	KM234913	H10	N2/N8
A/duck/Jiangxi/JXA132727/2014(H10N8)	1/1/14	KM234917	KM234919	KM234914	KM234915	KM234916	KM234918	KM234920	KM234921	H10	N8
A/duck/Jiangxi/JXA132719/2014(H9N2)	1/1/14			KM234922	KM234923	KM234924	KM234925	KM234926	KM234927	H9	N2
A/duck/Jiangxi/JXA132718/2014(mixed,H10,N2,N8)	1/1/14	KM234931	KM234933	KM234928	KM234929	KM234930	KM234932	KM234934	KM234935	H10	N2/N8
A/duck/Jiangxi/JXA132712/2014(H10N8)	1/1/14	KM234936	KM234937					KM234938	KM234939	H10	N8
A/duck/Jiangxi/JXA132762/2014(mixed, H?,N8,N2)	1/4/14		KM234944	KM234940	KM234941	KM234942	KM234943	KM234945	KM234946		N2/N8
A/duck/Jiangxi/JXA132719/2014(H9N2)	1/1/14			KM234922	KM234923	KM234924	KM234925	KM234926	KM234927	H9	N2
A/duck/Jiangxi/JXA132718/2014(mixed,H10,N2,N8)	1/1/14	KM234931	KM234933	KM234928	KM234929	KM234930	KM234932	KM234934	KM234935	H10	N2/N8
A/duck/Jiangxi/JXA132712/2014(H10N8)	1/1/14	KM234936	KM234937					KM234938	KM234939	H10	N8
A/duck/Jiangxi/JXA132762/2014(mixed, H?,N8,N2)	1/4/14		KM234944	KM234940	KM234941	KM234942	KM234943	KM234945	KM234946		N2/N8

51

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The presence of mixed infections makes it difficult to determine the exact genotypes of the viruses. For example, there were 16 samples that contained H10 genes, 12 that contained H9 genes, two that contained H6 genes, and three that contained two HA genes (two H10/H9 and one H7/H9). Among the 16 samples that contained H10 viruses, only two were of the N8 subtype; the remaining 14 samples were mixed infections with both N2 and N8 genes present. The two mixed H10/H9 samples had both N2 and N8 NA genes as well. Besides the mixed HA and NA samples, 12 had at least two copies of the same gene segments, such as PB2, PB1, NP, MP, and NS, and these gene segments were genetically distinct. Of the 57 samples sequenced, 25 samples were determined to have at least two genetically distinct influenza viruses.

Phylogenetic analyses of the generated sequences showed that 18 H10 and 19 N8 genes forming a monophyletic clade with the corresponding genes of the human H10N8 virus (Figure 8, Figure 9). The H10 and N8 genes were genetically close to those of viruses isolated from wild birds: H10 gene from the Eurasian lineage and N8 gene from the North American lineage. In contrast, the internal genes sequenced were close to those derived from AIVs circulating among domestic poultry and some are similar to those present in the human H10N8 virus (Phylogenetic_trees_of_internal_genes_recovered_from_the_samples_collected_at_the_LPM.pdf). These data confirm that nucleotide sequences of viruses more than 99% identical to those of the human H10N8 virus were circulating in this LPM, supporting the likelihood that the zoonotic transmission had occurred here.

The diversity of internal gene segments revealed that multiple distinct viruses were circulating in this individual LPM. The PB2 genes were clustered into seven distinct

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genetic lineages, PB1 into five, PA into four, NP into six, MP into six, and NS into three (Phylogenetic_trees_of_internal_genes_recovered_from_the_samples_collected_at_the_LPM.pdf). Among these genes, PB2 from the 18 samples alone belong to five distinct genetic lineages. In contrast, NP, MP, and NS gene of those samples with H10 gene were similar. None of the internal genes of these H10N8 samples were unique from those found in H9 and other subtypes of IAV from this LPM. The genetic lineages containing the samples collected on April 5, 2013, were surprisingly associated with the H10 viruses in December 2013.

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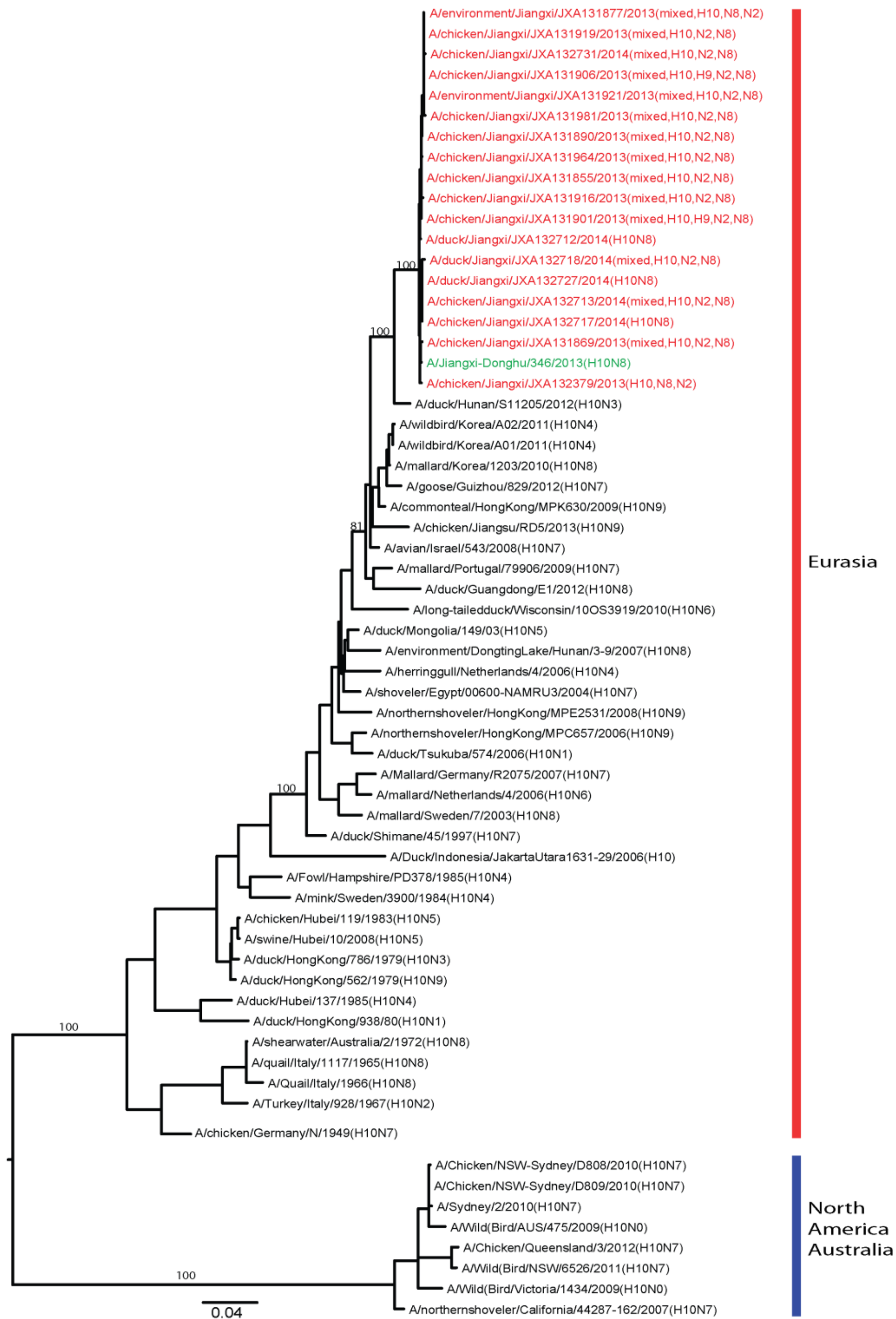

Figure 8 Maximum likelihood phylogenetic trees of H10 genes recovered from the samples collected at the LPM.

The genes of IAV recovered from our surveillance are marked in red and that from the human H10N8 isolate are in green.

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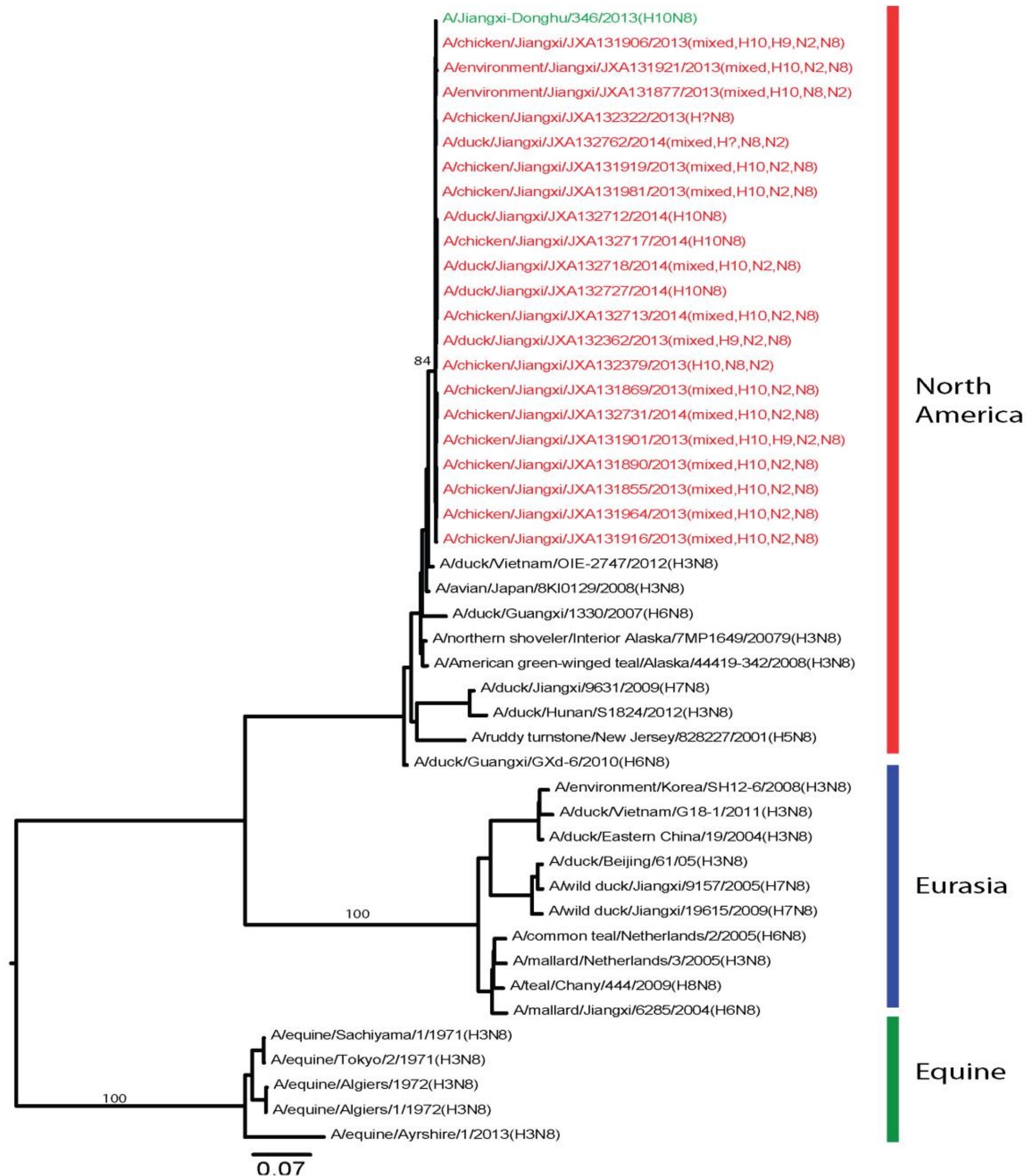

Figure 9 Maximum likelihood phylogenetic trees of N8 genes recovered from the samples collected at the LPM.

The genes of IAV recovered from our surveillance are marked in red and that from the human H10N8 isolate are in green.

The amino acid sequences at the receptor binding sites of the H10 proteins encoded by the genes from this LPM were identical to those in the human H10N8 isolate,

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and the majority of these sites except 128 (137, H3 position) and 181 (193, H3 position) were shown to be divergent from H10 AIVs from public databases: the H10 viruses recovered in this study had R128 (100%) and I181 (61%) while those from public database predominantly had K (67.8%) and T (97.7%), respectively (Table 3). The human H10N8 isolate has R128 and T181. The impact of I181T on host or tissue tropism of the H10N8 virus is unknown; nevertheless, the receptor binding properties are predicted to be avian-like.

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Table 3 The amino acid diversity in the influenza biomarkers of viruses recovered from the LPM visited by the first H10N8 patient.

H10: residues in the receptor binding sites				
Residue #	H3 residue #	JXA	Donghu	Animal host H10
88	98	Y 100%	Y	Y 100%
125	134	G 100%	G	G 100%
126	135	T 100%	T	T 97.6%,N 0.2%,D 1.5%,A 0.4%
127	136	T 100%	T	T 100%
128	137	R 100%	R	K 67.8%,R 20.9%,Q 8.5%,I 0.4%,G 0.6%,E 0.6%
129	138	A 100%	A	A 96.7%,S 3.3%
144	153	W 100%	W	W 100%
176	183	H 100%	H	H 100%
181	188	I 61%, T 39%	**T**	T 97.7%,I 0.6%,A 1.7%
182	189	Q 100%	Q	Q 97.5%,K 2.1%,E 0.2%,H 0.2%
183	190	E 100%	E	E 100%
184	191	K 100%	K	K 99.6%,R 0.2%,T 0.2%
185	192	N 100%	N	N 99.6%,S 0.2%,D 0.2%
186	193	D 100%	D	D 97.7%,K 0.2%,N 1.7%,E 0.4%
187	194	L 100%	L	L 100%
188	195	Y 100%	Y	Y 100%
214	221	P 100%	P	P 100%
215	222	Q 100%	Q	Q 99.7%,L 0.2%
216	223	V 100%	V	V 100%
217	224	N 100%	N	N 99.4%,D 0.2%,K 0.4%
218	225	G 100%	G	G 99.7%,S 0.2%
219	226	Q 100%	Q	Q 100%
220	227	S 100%	S	S 99.2%,G 0.2%,R 0.6%
221	228	G 100%	G	G 99.7%,A 0.2%

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Table 3 (continued)

N8: residues potentially being involved in oseltamivir resistance

Residue #	H3 residue #	JXA	Donghu	Animal host H10
115		I 100%	I	I 100%
116		R 100%	R	I 100%
117		E 100%	E	E 100%
134		Q 100%	Q	Q 100%
149		D 100%	D	D 100%
150		R 100%	R	R 100%
221		I 100%	I	I 100%
223		R 100%	R	R 100%
273		H 100%	H	H 100%
275		E 100%	E	E 100%
291		R 100%	R	R 100%
293		N 100%	N	N 100%
312		V 100%	V	V 100%
368		R 100%	R	R 100%

PB2: residues potentially increase viral pathogenesis and enhance viral transmission

Residue #	H3 residue #	JXA	Donghu	Animal host H10
89		V 100%	V	V 99.6%,M 0.4%
357		H 100%	H	H 99.6%,N 0.4%
627		E 100%	K	E 99.3%,0.7%
701		D 100%	D	D 99.3%,N 0.7%

PB1: residues potentially increase replication efficiency

Residue #	H3 residue #	JXA	Donghu	Animal host H10
473		V100%	V	V 99.8%,I 0.2%
598		L 100%	L	L 100%

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Table 3 (continued)

PA: residues potentially increase replication efficiency

Residue #	H3 residue #	JXA	Donghu	Animal host H10
36		A 100%	A	A 99.2%,T 0.4%,V 0.2%,S 0.2%

M1: residues potentially increase influenza pathogenesis

Residue #	H3 residue #	JXA	Donghu	Animal host H10
30		D 100%	D	D 100%
215		A 100%	A	A 100%

M2: residues potentially being involved in amantadine resistance

Residue #	H3 residue #	JXA	Donghu	Animal host H10
26		L 100%	L	L 99.8%,I 0.2%
27		V 100%	V	V 98.2%,I 1.8%
30		A 100%	A	A 100%
31		N 94.1%,S 5.9%	N	S 99.8%,N 0.2%
34		G 100%	G	G 100%

NS1: residues potentially increase viral pathogenesis

Residue #	H3 residue #	JXA	Donghu	Animal host H10
42		S 94.4%,A 5.6%	S	S 90.3%,A 9.7%

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Analysis of NA gene sequences suggests that these viruses are sensitive to oseltamivir and other neuraminidase inhibitors (208). However, 94.1% of the M2 sequences have S31N (209) indicating that they are resistant to amantadine, as was the human H10N8 isolate (210) (Table 3). All PB2 genes had E627 whereas the PB2 gene of the human H10N8 isolate had K627, which is considered a marker of mammalian adaptation (211) (Table 3).

That we were able to detect H10N8 virus in December and early January following human infection raised the question of how widely this virus might be spread within the LPM system in the region. To estimate this, we conducted serologic surveillance at four additional LPMs in Nanchang city. We collected a total of 800 sera from chickens and ducks at these five LPMs, from February 25, 2014, to March 27, 2014 (Figure 10). These five LPMs cover a geographic area of about 160 square kilometers and the major metropolitan area of Nanchang. Using HI assays with a H10N8 virus isolated from the surveillance described above and a cutoff of 1:20, we found that 9.4% of the 800 sera samples were positive. The H10 positive sera were distributed across all five sampled LPMs and from both ducks and chickens. The highest H10 positive percentile on a single market and a single sampling period (34.0%) was detected on March 9, at LPM A, which the index patient visited (Figure 10). Of note, LPM B located across the Gan River from LPM A, had close to 50% H10 positivity by the end of March. These data showed that H10 viruses were widespread in the region's LPM system. Although we are unable to determine the exact nature of the H10 virus from these serologic studies, two human cases with H10N8 infection in Nanchang (in January and February 2014) are consistent with it being the zoonotic virus. Of these two additional

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human cases, the second human case was documented with a visit to a local LPM prior to illness onset whereas the exposure history of the third human case was unclear.

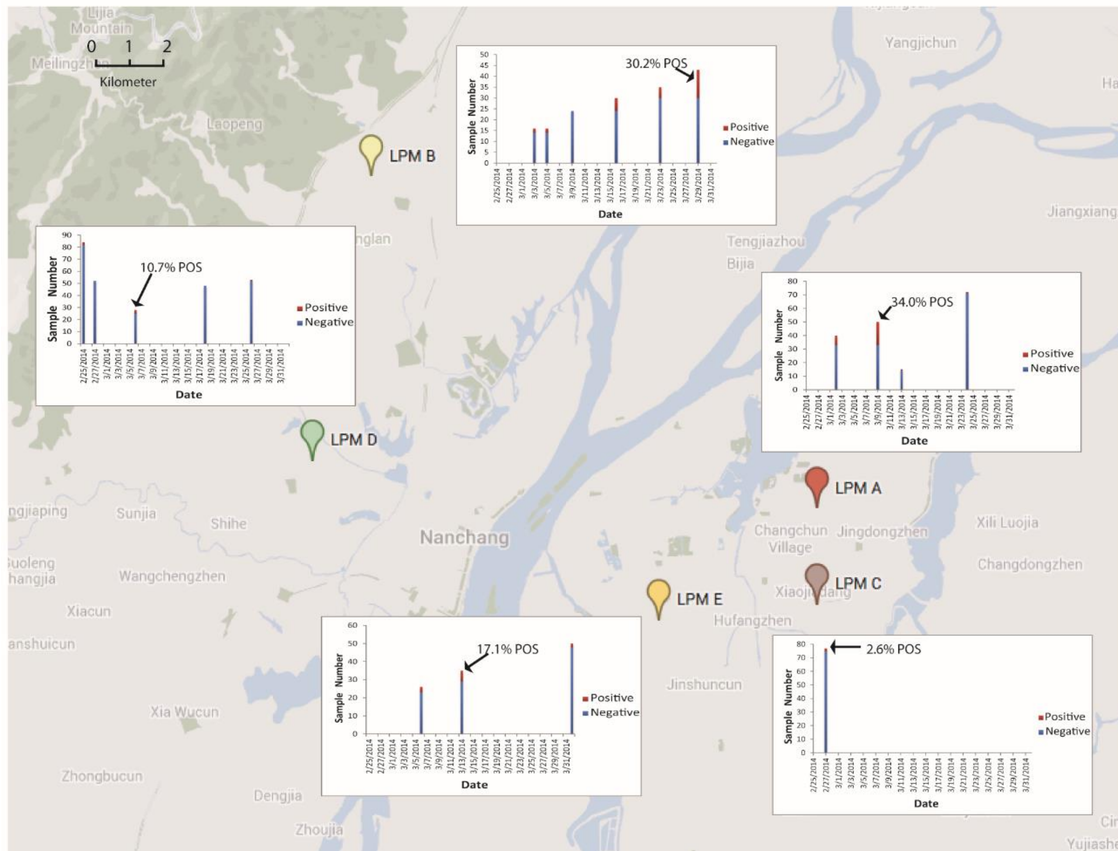

Figure 10 Distribution of H10 seropositive samples collected from February 25 to March 27, 2014 and across five LPMs in Nanchang city.

The seropositive samples were determined by HI assays with a H10N8 virus. LPM A was the one that the first index H10N8 patient visited in November 2013. The highest H10 positive percentile in a single sampling period was marked for each LPM.

Discussion

In summary, we show that the LPM visited by the index H10N8 female patient was very likely the source of her infection and that the virus appears to have been present throughout the LPM system in Nanchang, China. However, the findings in this study

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were limited by the small number of samples we collected and by the fact that the H10N8 virus prior to the emergence of the first human case was lacking. It is unclear whether these H10N8 viruses were introduced into the LPM system or were generated in the LPMs. Continuous influenza surveillance is needed to monitor the epidemiology of the novel H10N8 virus in Nanchang as well as those areas that share poultry movements with Nanchang. In addition to those minor poultry species, there are a variety of chicken and duck species in the LPM; further studies will be needed to identify the reservoir for the H10N8 virus among these bird species, and such information will be useful for developing effective strategies for prevention and control of the H10N8 virus at the LPM.

AIVs were identified in LPMs in China a few decades ago. The first LPAI H9N2 virus was initially isolated from domestic poultry in 1994 (212), and has since been found to be endemic among domestic poultry in China (213). Besides infecting poultry, this H9N2 virus has caused sporadic infections in humans (176, 203). The H9N2 virus has undergone rapid evolution and contemporary H9N2 viruses are both genetically and antigenically diverse (214-216). The internal gene segments of H9N2 viruses contributed to the genomic diversity of HPAI H5N1 viruses in China (217, 218). The novel LPAI H7N9 viruses emerged in Yangze Delta possess six gene segments derived from H9N2 viruses, and mixed infections of H7N9 and H9N2 viruses are very common (185). This study suggested further that novel H10N8 IAVs were frequently co-infected with H9N2 viruses, and their internal gene constellations were similar to each other. It seems that this diverse genetic pool is potentially more dangerous than any single virus. With any chance to interact with other subtypes of HA and NA genes, novel reassortants could emerge, including some strains that are antigenically distinct from the contemporary human IAVs.

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These emerging viruses, including H5N1, H7N9, H9N2, and H10N8, are continuous threats to public health.

A large influenza vaccine campaign against HPAI H5N1 viruses has been conducted in China since 2004. H9N2 vaccines have also been distributed to domestic poultry farms but their implementation has been comparatively less effective. With the substantial subtype and genetic diversity of viruses within LPMs, vaccination is not a realistic strategy to reduce the levels of virus circulation and subsequent zoonotic infections. Other intervention strategies must, therefore, be used to control viral flow into the LPMs. As it seems impossible to close all LPMs simultaneously, more practical policies and approaches such as routine surveillance and regular market disinfection should be urgently implemented.

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CHAPTER IV

THE GENESIS OF A NOVEL HPAI H7N8 VIRUS AND THE EVOLUTIONARY PATHWAY LEADING TO OUTBREAK AMONG DOMESTIC POULTRY IN INDIANA, THE UNITED STATES

Introductions of LPAI viruses of subtypes H5/H7 into poultry from wild birds have the potential to mutate to HPAI, but such viruses' origin is often unclear. In January 2016, a novel H7N8 HPAI virus caused an outbreak in turkeys in Indiana, USA. To determine the virus's origin, we sequenced genomes of 441 wild bird–origin IAVs from North America and subjected them to evolutionary analyses. Results showed that the H7N8 LPAI virus most likely circulated among diving ducks in the Mississippi flyway during autumn 2015 and was subsequently introduced to Indiana turkeys, in which it evolved into a HPAI form. Preceding the outbreak, an isolate with six gene segments (except NP and MP) sharing >99% sequence identity with those of H7N8 turkey viruses was recovered from a diving duck. H4N8 IAVs from diving duck possessed five H7N8–like gene segments (PB2, PB1, NA, MP, and NS). Our findings suggest that viral gene constellations circulating among diving ducks can contribute towards the emergence of IAVs that can affect poultry. Diving ducks may serve as a unique reservoir, contributing to the maintenance, diversification, and transmission of IAVs in wild birds.

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Introduction

IAVs exist in a complex ecosystem that involves various hosts, including humans, swine, horses, dogs, sea mammals, and numerous wild and domestic bird species. Among these hosts, wild aquatic birds are considered the natural reservoir for IAVs and bird migration plays an important role in the dispersal of IAVs. Interactions of migratory bird at congregation sites enable transmission of IAVs and facilitate genetic diversity through reassortment. Periodic introduction of IAVs from wild birds to domestic poultry contributes to emergence of novel IAV strains that occasionally cause outbreaks among domestic poultry. Following such introductions into domestic poultry, LPAI H5 and H7 viruses have the potential to evolve into HPAI viruses through two mechanisms: 1) acquisition of basic amino acids in the cleavage region of the HA protein by insertion or substitution (188), and 2) recombination with another gene segment(s) or host genome (60, 63, 123). HPAI virus is of particular concern because of the devastating consequence it brings to the poultry industry; in some outbreaks, mortality could be as high as 100%.

The threats brought by the introduction of entire IAVs or IAV gene segments from wild birds to domestic poultry has been repeatedly demonstrated by the emergence of novel HPAIs in the Americas. HPAI H5 viruses caused outbreaks of disease among domestic poultry in 1983 (190), 1994 (191), and 2014 and 2015 (71). In addition to subtype H5 viruses, HPAI H7 viruses have been frequently reported in the Americas. There were four reported HPAI H7N3 outbreaks between 2002 and 2016: one outbreak in Chile (2002) (60), two distinct outbreaks in Canada (2004 and 2007) (61, 62), and one outbreak in Mexico (2012). High mortality among domestic poultry was reported for these four HPAI H7N3 outbreaks. Moreover, HPAI H7N3 strains isolated from the

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outbreak in Canada (2004) caused mild respiratory symptoms and conjunctivitis in at least two humans (192). HPAI H7N3 strains from the outbreak in Mexico (2012) caused conjunctivitis in two poultry workers (64).

Studies conducted after outbreaks among domestic poultry have usually been retrospective analyses to determine the identities of the putative precursors of the outbreak strains. However, direct evidence is lacking on the time of emergence, location for reassortment, and wild bird species that contribute to the genesis of a particular HPAI strain. In most cases, the limited surveillance in wild birds prior to the detection of a novel HPAI strain impedes our understanding of the mechanisms underlining the emergence.

On January 15, 2016, The United States Department of Agriculture's Animal and Plant Health Inspection Service (APHIS) announced the detection of a novel HPAI H7N8 virus in a commercial turkey flock experiencing significant mortality in Dubois Country, Indiana. APHIS reported the identification of LPAI H7N8 virus in eight turkey flocks in the control area surrounding the location of the initial HPAI case. The detection of this virus represents the first identification of HPAI H7N8 virus in domestic species. The objective of study in this chapter was to understand the molecular mechanisms leading to the emergence of this novel H7N8 virus.

We hypothesized that 1) the H7N8 virus identified in turkeys in Indiana was initially introduced from wild birds and developed high pathogenicity within poultry production systems; 2) genetic analysis of a large number of available contemporary wild bird-origin IAV isolates would indicate what wild bird species and IAVs contributed to the emergence of the HPAI H7N8 virus. In a collaborative effort, our team sequenced

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441 IAVs obtained through wild bird influenza surveillance during 2007–2016 across North America. We used the sequence data generated in this study together with the IAV genomes recovered in association with the H7N8 outbreak in Indiana turkey and public sequence data to infer the possible origin(s), evolutionary pathway(s), and transmission route(s) of this novel HPAI H7N8 virus.

Materials and Methods

Viruses

To determine the genetic ancestry of the H7N8 IAV strains associated with the outbreak of disease in Indiana turkeys, we sequenced a collection of 441 archived wild bird–origin IAVs obtained throughout North America from influenza surveillance during 2007–2016 (Figure 11). The wild bird–origin isolates represent strains recovered from Alberta, Canada, and from 38 states within the United States of America. IAVs from migratory birds from the Atlantic, Central, Mississippi, and Pacific flyways were included in our dataset. Complete genomes of H7N8 IAVs detected in Indiana turkeys were downloaded from GenBank: (accession nos. KU558903–KU558910 and KU585905–KU585920). One of the strains was a HPAI virus, A/turkey/Indiana/16-001403-1/2016(H7N8) (Indiana/16-001403-1), and the other two strains were LPAI viruses, A/turkey/Indiana/16-001573-2/2016(H7N8) (Indiana/16-001573-2) and A/turkey/Indiana/16-001574-7/2016(H7N8) (16-001574-7).

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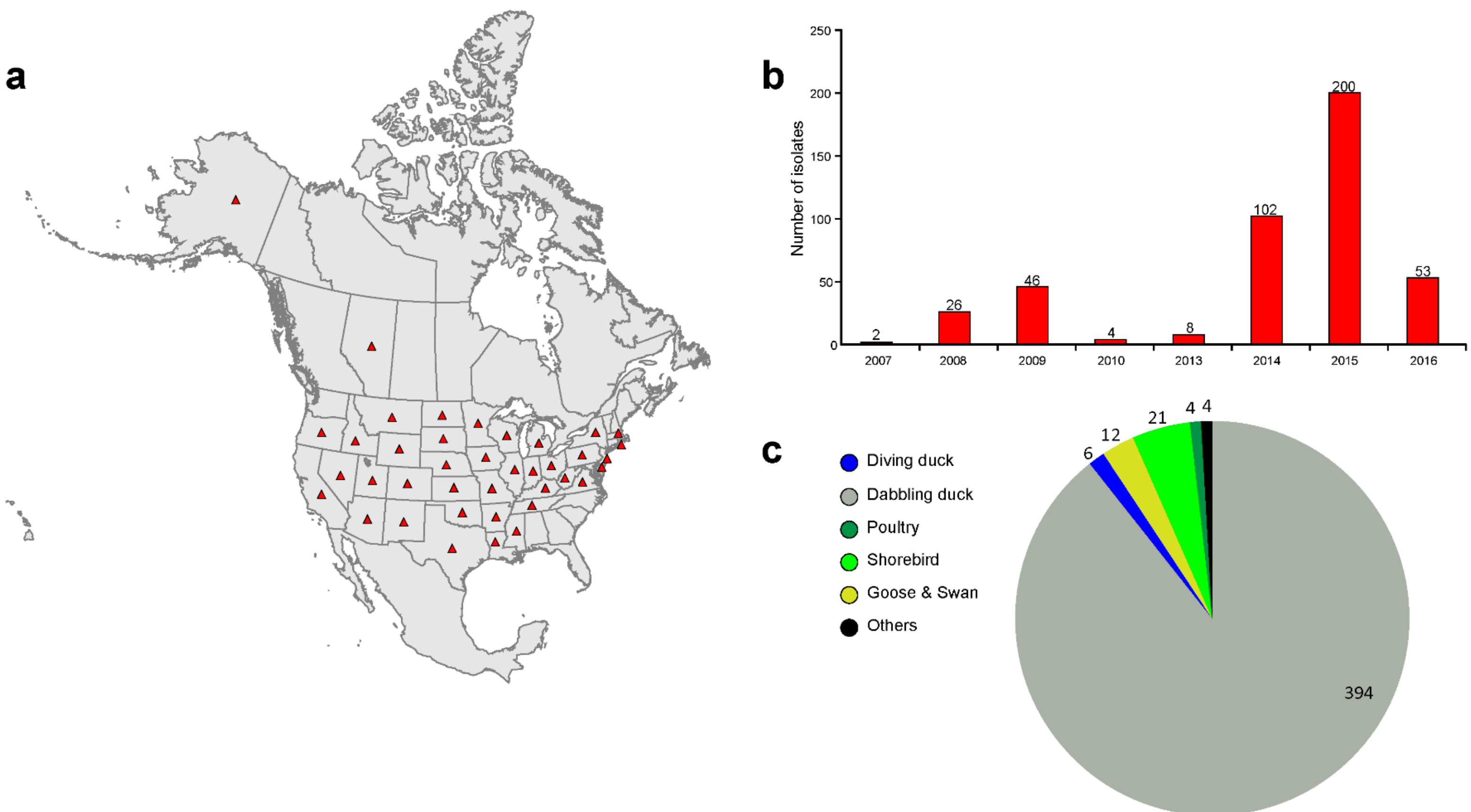

Figure 11 Geographic, temporal, and taxa distribution of 441 influenza A virus strains subjected to genomic sequencing in this study.

(a) Red triangles indicate the state/province where the samples were collected. (b) Numbers above each column indicate the number of influenza A virus isolates from each year. (c) Distribution of strains among distinct host groups. Map of North America with US States, Canadian Provinces, and Mexico by FreeVectorMaps.com, https://freevectormaps.com/world-maps/north-america/WRLD-NA-02-0003.

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Phylogenetic Analyses

Preliminary phylogenetic analyses were performed with the complete genomes of three H7N8 strains originating from turkeys in Indiana and a genomic sequence dataset (219) that represents the two major geographically dependent genetic lineages, North American and Eurasian, for eight gene segments. The next round of phylogenetic analyses were conducted with an integrated dataset that comprised the complete genomes of three H7N8 turkey strains, sequence data for 441 IAVs generated in this study, and sequence data downloaded in May 2016 from the Influenza Research Database (220) for IAVs isolated from wild and domestic birds in the Americas. The number of genomic sequences used in analyses for each gene segment is as follows: PB2 (number=7762), PB1 (n=7813), PA (n=7859), HA (n=1132), NP (n=7327), NA (n=1611), MP (n=7776), and NS (n=7586). In order to perform more detailed analysis on the timing of the emergence of the H7N8 viruses, sequences closely related to the H7N8 turkey strains (referred to henceforth as 'the H7N8–outbreak lineage') were selected for each gene segment from the phylogenetic trees. The number of genomic sequences used in analyses for each gene segment is as follows: PB2 (number=796), PB1 (n=709), PA (n=757), HA (n=721), NP (n=651), NA (n=281), MP (n=742), and NS (n=526).

Gene segment–specific phylogenetic trees were generated using the maximum–likelihood method implemented in RAxML v8.1.17 (221). A general time–reversible model of nucleotide substitution and a gamma–distributed rate variation among sites was applied throughout the analyses. Sequence alignments were performed by using MUSCLE v3.8 (222). We manually examined alignments to ensure accuracy and retained only the coding region for phylogenetic analyses.

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Molecular Clock

We estimated the time–scaled phylogenies, nucleotide substitution rate, and time to most recent common ancestor (TMRCA) using the Bayesian Markov Chain Monte Carlo method implemented in BEAST v1.8.0 (223). Genomic sequences with complete sampling dates (exact month, day, year) were selected for each gene segment from the H7N8–outbreak lineage on the basis of phylogenetic trees, and the number of genomic sequences used in analyses for each gene segment is as follows: PB2 (number=268), PB1 (n=227), PA (n=225), HA (n=206), NP (n=224), NA (n=102), MP (n=220), and NS (n=160). We used the SRD06 partitioned substitution model, uncorrelated lognormal relaxed clock model, and Bayesian skyline coalescent tree prior in the analyses. For each gene segment, we performed two independent runs with a chain length of 100–300 million steps sampled every 10,000 steps (results in >10,000 samples per run). The results were analyzed in Tracer v1.6 (http://tree.bio.ed.ac.uk/software/tracer/). Adequate burn–in was determined from the trace of each run, and 2%–10% of the initial steps, representing poor configuration, were removed from further analysis. Convergence of each run and consistency between two runs was assessed, and results from two runs were combined for analyses to ensure an adequate effective sample size (>200) was reached for relevant parameters. The maximum clade credibility trees were summarized with TreeAnnotator v1.8.0 (http://beast.bio.ed.ac.uk/TreeAnnotator/) and edited in FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

Discrete Phylogeographic Analyses

The hosts of IAVs in our study were categorized into 11 different groups: dabbling duck, diving duck (including sea duck), goose and swan, gull and tern,

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passerine, poultry, raptor, seabird, shorebird, other avian, and other non–avian hosts. In an attempt to keep the number of sequences per host group as balanced as possible, we conducted sub–sampling for sequences within the H7N8–outbreak lineage for each gene segment. For each sampling year, 20 sequences were selected for each host group; if a host group had <20 sequences, all sequences were retained. For the analysis, we used an asymmetric substitution model with the Bayesian Stochastic Search Variable Selection and a strict clock model. Two independent runs of chain length of 150 million steps with sampling every 10,000 steps (results in 15,000 samples per run) were performed for each gene segment. Similar approach for analyzing the resulting log files of molecular clock data was applied to analyze burn–in, convergence of each run, and generate maximum clade credibility phylogenetic trees. Specifically, >10% of initial steps representing poor configuration were removed as burn–in. Mean transition rate and the corresponding indicator were calculated from the resulting log files. Bayes Factor (BF) support was calculated with the indicator to assess statistical support. Significant transition between host groups was determined based on the combination of BF >3 and mean indicator >0.5. Therefore, support levels were defined as support (3≤BF<10); strong support (10≤BF<100); very strong support (100≤BF<1,000); and decisive support (BF≥1,000).

Phylogenetic Network

A phylogenetic network was reconstructed using the quartet–based method implemented in QuartetMethods (224). A sequence dataset was built by concatenating the sequences of six internal genes for 6,693 IAVs isolated from wild and domestic birds in the Americas. The sequences of six internal genes of H7N8 HPAI strain were concatenated and the resulting sequence was BLASTed (201) against the dataset. Fifty

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IAV strains that were most closely related to the H7N8 HPAI stain were selected and included in the analysis.

Genotyping

Genotypes were defined on the basis of the gene segment–specific phylogenetic trees for H7N8 turkey strains and 135 H4N8 IAV strains. A monophyletic clade was identified by two criteria: 1) it was supported by a bootstrap value above 70, and 2) all sequences in the clade had nucleotide sequence identities >98%, as determined by using the hierarchical clustering method implemented in R (https://www.r-project.org/). We implemented a stringent cutoff of 98% to identify IAV strains closely related to the H7N8 turkey strains. The genotype of a genome is the combination of the cluster assignment of eight gene segments.

Results

H7N8 Virus Originated From IAVs in North American Wild Birds

To infer the ancestry of each gene segment of the H7N8 viruses detected in Indiana turkeys, we first performed preliminary phylogenetic analyses with a collection of representative genomic sequences to identify the major genetic lineage to which the H7N8 turkey strains belong. Phylogenetic trees support two geographically dependent lineages of IAV (North American and Eurasian) for eight gene segments (Figure 12). The H7N8 turkey strains were found to share genetic ancestry with IAVs from North America for all eight gene segments.

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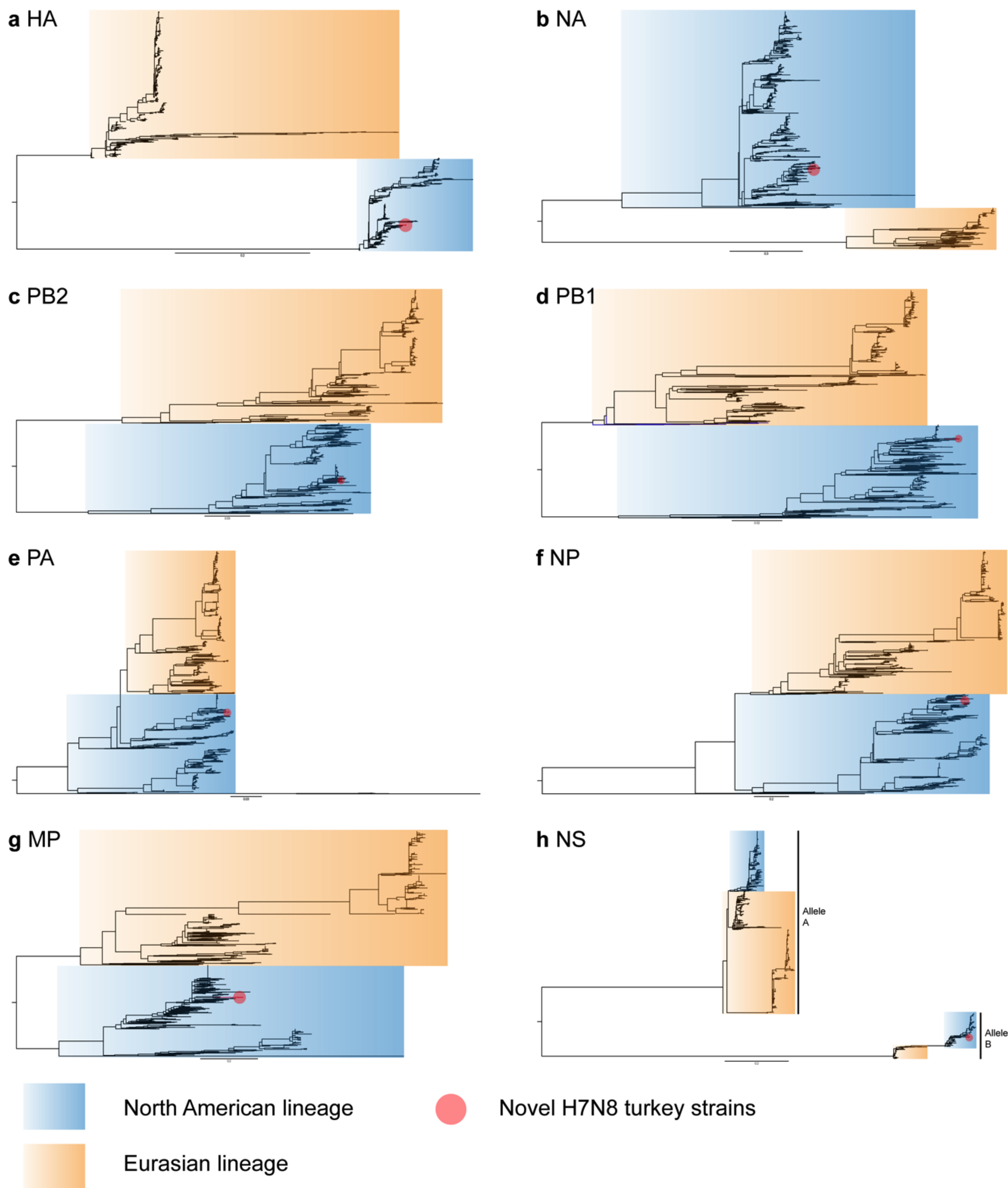

Figure 12 Maximum likelihood phylogenetic trees for eight gene segments of H7N8 turkey strains and influenza A viruses representing North American and Eurasian genetic lineages.

H7N8 strains associated with the outbreak of disease in Indiana turkeys are indicated by red circles. In (h), black bars indicate two alleles for the NS gene segment.

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We then performed phylogenetic analyses, focusing on genomic sequences of IAVs isolated from wild and domestic birds in the Americas. The phylogenetic tree for the H7 gene showed that IAV strains from the Americas could be divided into two genetic sub–lineages: those from North America and those from South America (Phylogenetic_trees_of_IAVs_isolated_from_wild_and_domestic_birds_in_the_Americas.pdf). The South American sub–lineage included H7N3 HPAI viruses isolated during an outbreak in Chile in 2002. Three major genetic clusters were identified in the North American sub–lineage: cluster I was comprised of viruses isolated from wild and domestic birds in North America during the 1970s to the early 1990s; cluster II contained H7N2 IAVs isolated from the live–poultry markets in the northeastern United States during1994–2006; cluster III represents contemporary H7 IAVs circulating in North America, including viruses isolated from wild and domestic birds. The novel H7N8 turkey strains grouped with H7 viruses in cluster III and were most closely related to IAV strains isolated from wild birds (Phylogenetic_trees_of_IAVs_isolated_from_wild_and_domestic_birds_in_the_Americas.pdf, Time_scale_phylogenetic_trees_for_eight_gene_segments.pdf). The H7N3 HPAI viruses isolated during three previous outbreaks in North America also grouped with contemporary H7 IAVs in cluster III; however, these viruses were associated with monophyletic clades divergent from the clade containing H7N8 turkey strains. Phylogenetic analyses of the other seven gene segments also showed that the H7N8 turkey strains clustered with North American wild bird–origin IAV strains and in monophyletic clades different from those containing sequences for H7N3 HPAI viruses (Phylogenetic_trees_of_IAVs_isolated_from_wild_and_domestic_birds_in_the_America

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s.pdf, Time_scale_phylogenetic_trees_for_eight_gene_segments.pdf). Moreover, in the phylogenetic trees for eight gene segments, no poultry–origin IAV gene segments were found to be closely related to the H7N8 turkey strains.

To infer the more detailed evolutionary history of the novel H7N8 viruses, we conducted molecular clock analyses for each gene segment using sequences in the clades containing the H7N8 turkey strains and those most closely related strains. The resulting time–scale maximum clade credibility phylogenetic trees were used to identify viruses most closely related to H7N8 turkey strains for each gene segment (Figure 13, Time_scale_phylogenetic_trees_for_eight_gene_segments.pdf). The most closely related gene segments to H7N8 turkey strains originated from a single isolate, A/Lesser scaup/Kentucky/AH0012935/2015(H7N8) (Kentucky/AH0012935), for six gene segments (PB2, PB1, PA, HA, NA, and NS) (Table 4). This isolate was recovered from a sample collected in Kentucky, USA, on November 28, 2015, approximately seven weeks before the outbreak and approximately 200 kilometers from the Indiana turkey farm where outbreak was detected. The close relatedness between Kentucky/AH0012935 and the novel H7N8 HPAI strain in six gene segments was supported by high nucleotide sequence identities, ranging from 99.00% (HA gene) to 99.95% (PA gene) (Table 4). The NP gene segments of two isolates recovered from samples collected in Ohio, on October 18, 2014, A/Northern pintail/Ohio/14OS2209/2014(H5N9) (Ohio/14OS2209) and A/Northern pintail/Ohio/14OS2210/2014(H5N9) (Ohio/14OS2210), were most closely related (98.40% nucleotide sequence identity) to the NP gene of the novel H7N8 HPAI strain (Table 4). The MP gene segment of A/Bufflehead/Illinois/14OS3567/2014(H4N8)

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was most closely related (99.19% nucleotide sequence identity) to that of the H7N8 HPAI strain (Table 4).

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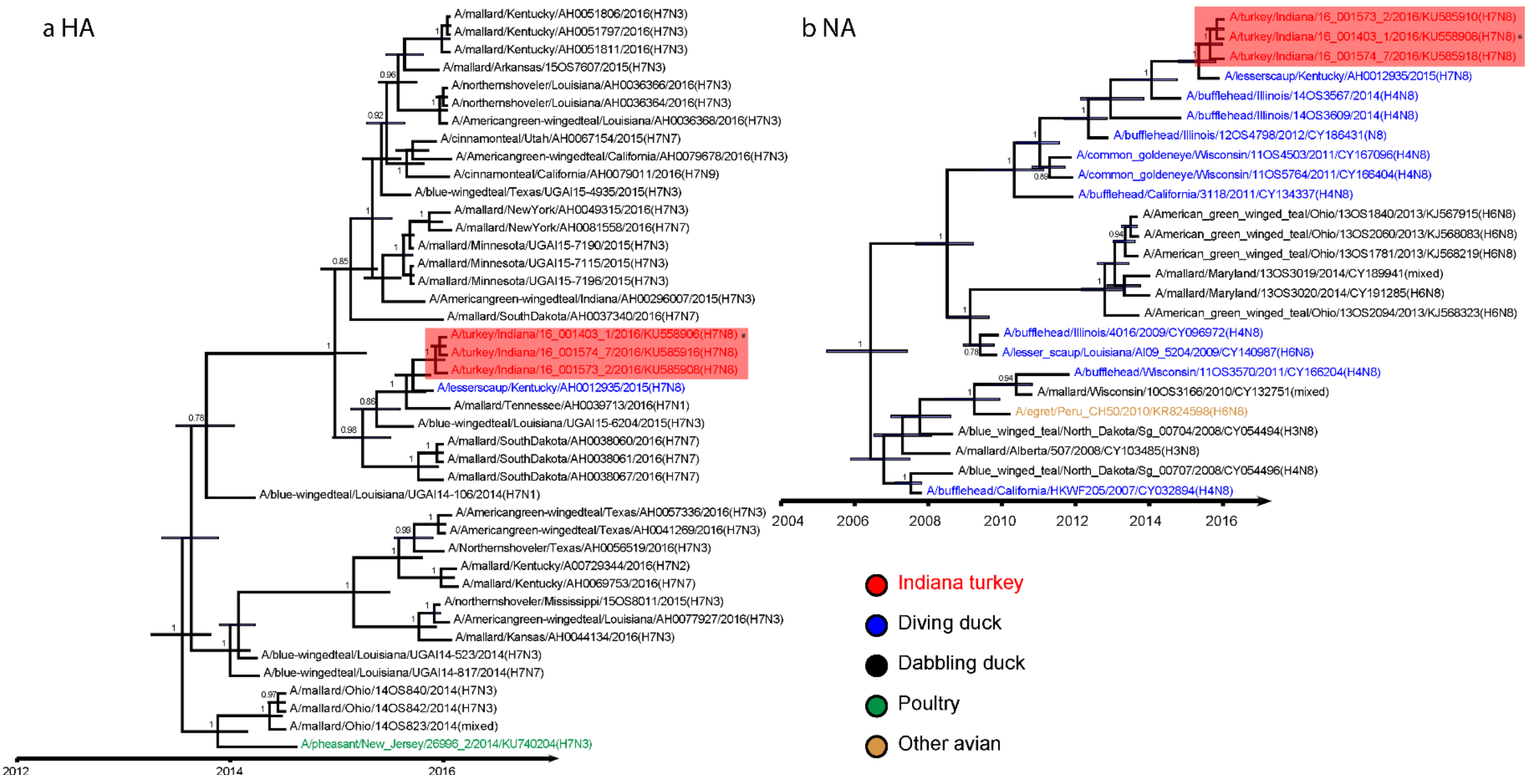

Figure 13 Maximum clade credibility phylogenetic trees for HA (a) and NA (b) gene segments.

The trees are constructed on the basis of the maximum clade credibility phylogenetic trees shown in Time_scale_phylogenetic_trees_for_eight_gene_segments.pdf. H7N8 strains associated with the outbreak of disease in Indiana turkeys are indicated by a red box and the highly pathogenic strain is marked with an asterisk. Clade posterior probabilities ⩾ 0.7 are shown. Horizontal bars indicate 95% highest posterior density of age estimates of tree nodes.

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Table 4 Most closely related genes to eight gene segments of the novel highly pathogenic avian influenza strain A/turkey/Indiana/16-001403-1/2016(H7N8).

Segment	Virus	Sequence Identity	Subtype	Sampling Date
PB2	A/Lesser scaup/Kentucky/AH0012935/2015	0.9987	H7N8	11-28-2015
PB1	A/Lesser scaup/Kentucky/AH0012935/2015	0.9987	H7N8	11-28-2015
PA	A/Lesser scaup/Kentucky/AH0012935/2015	0.9995	H7N8	11-28-2015
HA	A/Lessers caup/Kentucky/AH0012935/2015	0.9900	H7N8	11-28-2015
NP	A/Northern pintail/Ohio/14OS2209/2014 A/Northern pintail/Ohio/14OS2210/2014	0.9840	H5N9	10-18-2014
NA	A/Lesser scaup/Kentucky/AH0012935/2015	0.9979	H7N8	11-28-2015
MP	A/Bufflehead/Illinois/14OS3567/2014	0.9919	H4N8	11-15-2014
NS	A/Lesser scaup/Kentucky/AH0012935/2015	0.9952	H7N8	11-28-2015

Three H7N8 turkey strains were estimated to share a common ancestor among eight gene segments between March 2015 (MP gene) and December 2015 (HA gene) (Figure 14, Table 5). The mean estimated TMRCAs for seven of eight gene segments (excluding MP gene) was summer and fall (30 June–4 December) of 2015, which overlaps the late breeding and autumn migration period of many wild birds inhabiting North America. The HA gene was estimated to have the latest TMRCA (mean December 4, 2015, 95% highest posterior density [HPD] October 30, 2014–January 4, 2016), which is close to the time when Kentucky/AH0012935 was collected. The mean evolutionary rate for the H7 gene was estimated to be 6.55 x 10−3 substitutions per site per year (95% HPD, 5.74–7.52 x 10−3), which is significantly higher than that for the other seven gene segments (Figure 14, Table 5).

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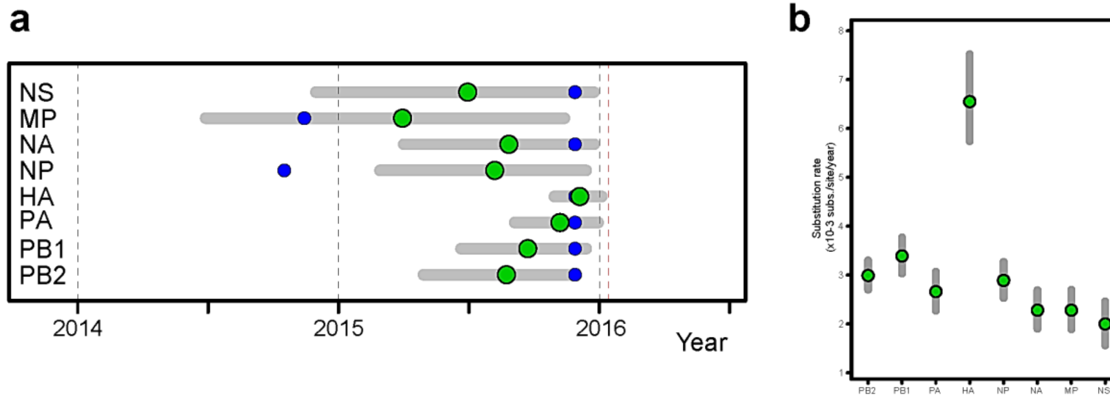

Figure 14 Estimated time to most recent common ancestor and nucleotide substitution rates for eight gene segments of H7N8 viruses detected in Indiana turkeys.

Green circles indicate the estimated mean date (a) or rate (b) and grey bars indicate 95% highest posterior density. Blue circles indicate the sample collection time of influenza A virus isolate containing the most closely related gene segment to the H7N8 turkey strains. Red dash line indicates the sample collection time of the highly pathogenic avian influenza H7N8 virus isolated from Indiana turkeys.

Table 5 Estimated time to most recent common ancestor and nucleotide substitution rates for eight gene segments of H7N8 viruses detected in Indiana turkeys.

	Substitution rate ($x10^{-3}$ subs/site/year)			TMRCA		
Segment	Mean	95% HPD lower	95% HPD upper	Mean	95% HPD lower	95% HPD upper
PB2	2.99	2.69	3.29	2015-08-22	2015-04-30	2015-11-25
PB1	3.39	3.03	3.77	2015-09-22	2015-06-21	2015-12-13
PA	2.66	2.28	3.06	2015-11-06	2015-09-01	2015-12-28
HA	6.55	5.74	7.52	2015-12-04	2015-10-30	2016-01-04
NP	2.89	2.53	3.27	2015-08-07	2015-02-28	2015-12-13
NA	2.28	1.90	2.69	2015-08-27	2015-04-02	2015-12-24
MP	2.28	1.89	2.70	2015-03-31	2014-06-28	2015-11-13
NS	2.00	1.56	2.46	2015-06-30	2014-11-30	2015-12-24

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The sampling time of the most closely related strain for NP gene segment does not overlap with the estimated TMRCA (between February 28, 2015 and August 7, 2015) (Figure 14, Table 5), suggesting a gap in surveillance and that neither Ohio/14OS2209 nor Ohio/14OS2210 are the direct predecessor of H7N8 turkey strains. This observation is consistent with the genetic distance separating H7N8 turkey strains from other viruses in the NP phylogenetic tree (Time_scale_phylogenetic_trees_for_eight_gene_segments.pdf).

Gene Constellation of H4N8 Virus in Diving Ducks Contributed to Emergence of The Novel H7N8 Virus

We investigated possible genetic events that contributed to generation of the H7N8 precursor virus in wild birds. The phylogenetic network showed that, in addition to Kentucky/AH0012935, a group of H4N8 IAVs isolated from diving ducks (referred to as H4N8–DD) during 2011–2014 were closely related, across six gene segments, to the H7N8 turkey strains (Figure 15). Further examination of the eight gene segment–specific phylogenetic trees indicated that the H4N8–DD viruses had five gene segments (PB2, PB1, NA, MP, and NS) closely clustered with the H7N8 turkey strains. The NA, MP, and NS genes of H4N8–DD viruses consistently formed a monophyletic clade with those of the H7N8 turkey strains in phylogenetic trees (Figure 13, Time_scale_phylogenetic_trees_for_eight_gene_segments.pdf). The PB2 gene of H4N8–DD viruses isolated in 2014 and that of the H7N8 turkey strains, and PB1 gene of H4N8–DD viruses isolated in 2012 and 2014 and that of the H7N8 turkey strains were phylogenetically closely related (Time_scale_phylogenetic_trees_for_eight_gene_segments.pdf).

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Figure 15 Phylogenetic network of H7N8 viruses detected in Indiana turkeys in red font.

The network was reconstructed by concatenating sequences of six internal gene segments for each virus strain. Strain names are color-coded with the host group colors of Figure 13. The highly pathogenic H7N8 strain detected in Indiana turkey is marked with an asterisk. H4N8 influenza A viruses isolated from diving ducks in 2011, 2012, and 2014 are indicated by purple, blue, and green shade, respectively. Host species are: AGWT (american green winged teal), BUFF (bufflehead), BWTE (blue winged teal), COGO (common goldeneye), GRSC (greater scaup), LESC (lesser scaup), MALL (mallard), NOSH (northern shoveler), TK (turkey), and WODU (wood duck). Geographic locations are: IL (Illinois), IN (Indiana), KY (Kentucky), LA (Louisiana), MN (Minnesota), OH (Ohio), TX (Texas), and WI (Wisconsin).

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To better understand the contribution of H4N8–DD viruses to the generation of the H7N8 precursor virus, we assigned genotypes to H4N8 IAVs based on the results of phylogenetic analyses. We analyzed the temporal dynamics of gene constellations that possessed at least one gene segment that was assigned to the same cluster as a gene segment in the H7N8 turkey strains. Gene constellation A possessed three gene segments (NA, MP, and NS) that were of the same genotype as those in the H7N8 turkey strains; this constellation was first detected at the end of 2011 and persisted for the next three years (Figure 16). Gene constellation B possessed the same three H7N8–like gene segments as constellation A plus an H7N8–like PB1 gene segment; this constellation was first detected at the end of 2012 and continued to exist in H4N8–DD viruses isolated in 2014. Gene constellation C possessed five H7N8–like gene segments (PB2, PB1, NA, MP, and NS) and was first detected in late 2014. The close genetic relationship between H4N8–DD viruses and the H7N8 HPAI turkey strain at these five gene segments was supported by high nucleotide sequence identities (>99.00%) (Table 6). Taken together, these results provide evidence that gene constellations of H4N8 IAVs isolated primarily from diving ducks contributed to the generation of a H7N8 precursor virus in wild birds. Furthermore, a relatively persistent gene constellation possessing H7N8–like gene segments emerged in or before 2011 and apparently acquired additional gene segments closely related to the H7N8 outbreak strain during 2011–2014.

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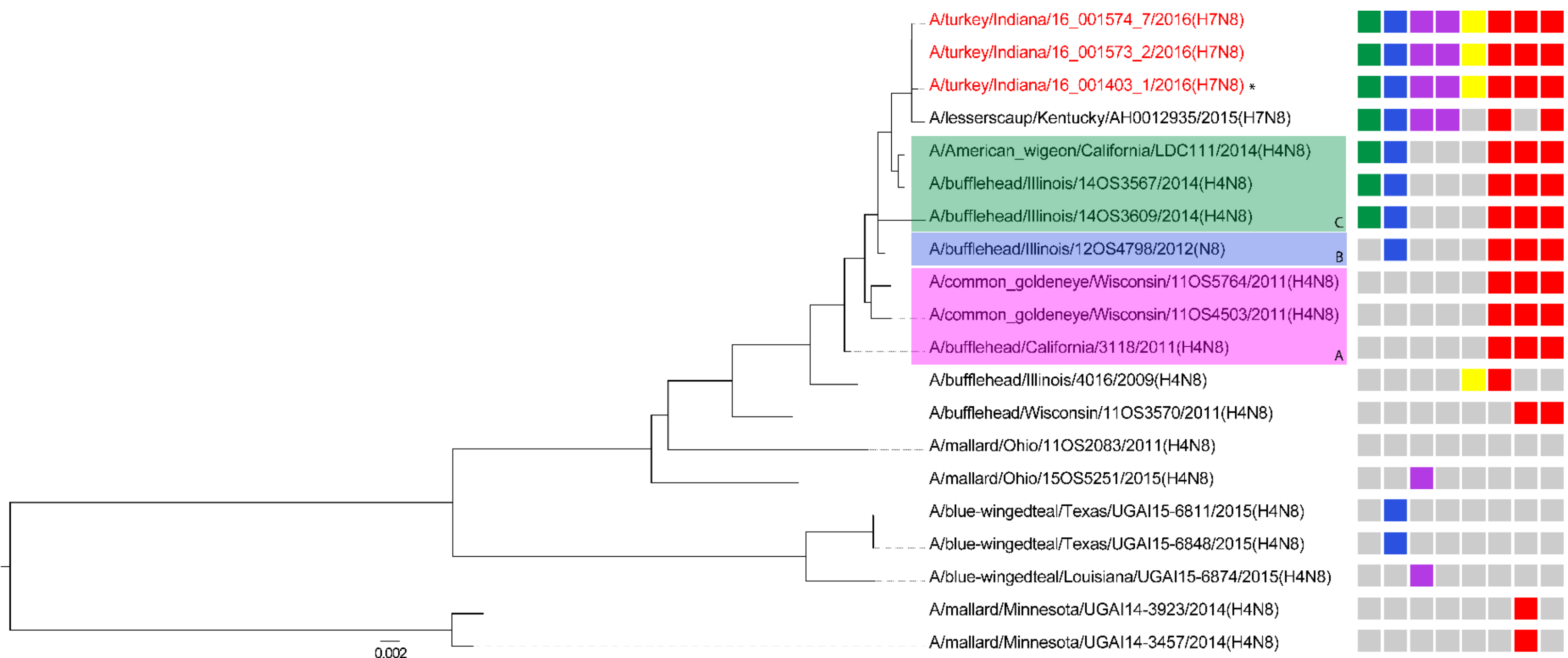

Figure 16 Maximum likelihood phylogenetic tree for N8 gene segment of the H7N8 viruses detected in Indiana turkeys and H4N8 influenza A viruses isolated from wild birds.

H7N8 strains associated with the outbreak of disease in Indiana turkeys are indicated in red and the highly pathogenic strain is marked with an asterisk. Genotype of each strain is shown to the right of the tree, with eight columns of blocks representing eight gene segments (from left to right, PB2, PB1, PA, HA, NP, NA, MP, and NS). Distinct colors denote the cluster assignment and color code among different columns are independent. Grey indicates gene segment that are not same to the H7N8 turkey strains in genotyping analysis. Three gene constellations, A, B, and C are indicated by purple, blue, and green box, respectively.

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Table 6 Sequence identity between A/turkey/Indiana/16-001403-1/2016 and influenza A virus strains isolated from diving ducks.

Virus	Subtype	Sampling date	PB2	PB1	PA	NP	NA	MP	NS
A/turkey/Indiana/16-001403-1/2016	H7N8	1-14-2016							
A/lesser scaup/Kentucky/AH0012935/2015	H7N8	11-28-2015	0.9987	0.9987	0.9995	0.9305	0.9979	0.9745	0.9952
A/bufflehead/ Illinois /14OS3609/2014	H4N8	11-16-2014	0.9947	0.9943	0.9786	0.9352	0.9908	0.9888	0.9917
A/ bufflehead /Illinois/14OS3567/2014	H4N8	11-15-2014	0.9969	0.9965	0.9791	0.9352	0.9958	0.9919	0.9952
A/bufflehead/Illinois/12OS4798/2012	N8	11-23-2012	0.9776	0.9947	0.9809	0.9315	0.9946	0.9929	0.9928
A/bufflehead/California/3118/2011	H4N8	12-7-2011	0.9794	0.9736	0.9066	0.9446	0.9915	0.9919	0.9881
A/common goldeneye/Wisconsin/11OS5764/2011	H4N8	12-4-2011	0.9790	0.9776	0.9823	0.9299	0.9915	0.9919	0.9881
A/common goldeneye/Wisconsin/11OS4503/2011	H4N8	11-20-2011	0.9798	0.9776	0.9828	0.9292	0.9915	0.9929	0.9869
A/bufflehead/Wisconsin/11OS3570/2011	H4N8	10-30-2011	0.9790	0.9780	0.9805	0.9285	0.9767	0.9929	0.9881
A/bufflehead/Illinois/4016/2009	H4N8	11-28-2009	0.9000	0.9701	0.9154	0.9886	0.9844	0.9786	0.9451

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IAVs From Diving Ducks Are The Probable Genetic Source of The H7N8 Virus in Indiana Turkeys.

Our phylogenetic and genotyping analyses provided evidence that IAVs isolated from diving ducks may have contributed to the generation of the H7N8 virus that was detected in Indiana turkeys. Using discrete phylogeographic analyses, we sought to further understand if diving ducks were the most likely source of H7N8 turkey strains. Results showed significant diffusion pathways from diving ducks to domestic turkey flocks in Indiana for seven gene segments: transition with decisive support was observed for the NS gene segment; strongly supported transition was observed for the PB2, PB1, HA, and NA gene segments; and supported transition was observed for the PA and MP gene segments (Figure 17, Table 7, Phylogenetic_trees_for_eight_gene_segments.pdf). No significant transition for NP gene segment was observed between any wild bird species and Indiana turkeys (Figure 17, Table 7, Phylogenetic_trees_for_eight_gene_segments.pdf). In total, the resulting transition patterns suggested IAV gene flow from diving ducks to Indiana turkeys and provide evidence that IAVs from diving ducks were the most likely genetic source of the H7N8 virus that was detected in turkeys in Indiana.

To gain insight into the gene flow pattern of IAVs among various wild bird species, we examined the diffusion pathways among different host groups. Bidirectional transmission was observed between dabbling duck and diving duck, and between gull/tern and shorebird; and gene transmission from diving duck to goose/swan was also observed (Table 8), suggesting extensive mixing of IAVs between these bird species groups in wild birds.

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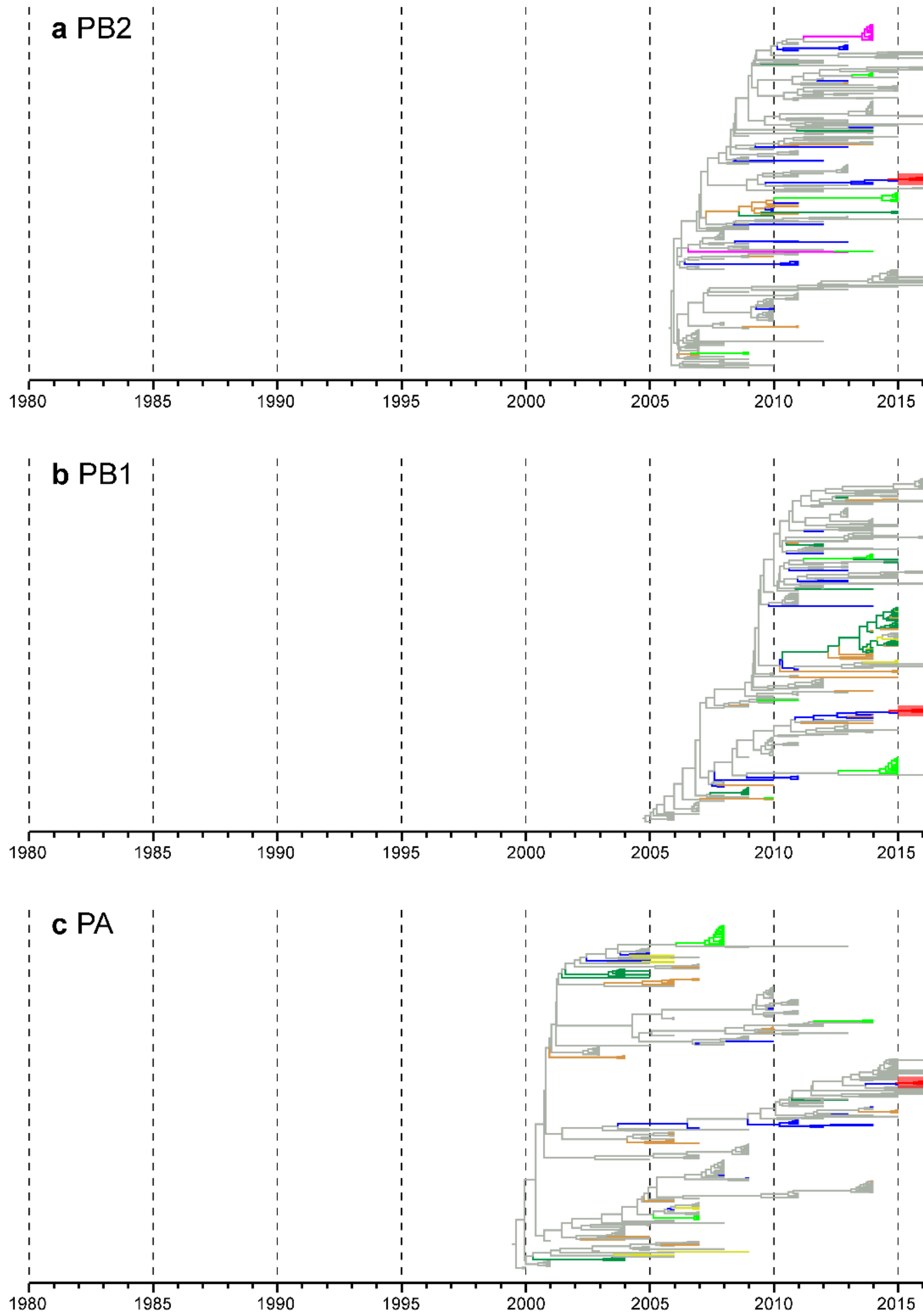

Figure 17 Diffusion of influenza A virus between different host groups of wild birds and Indiana turkey.

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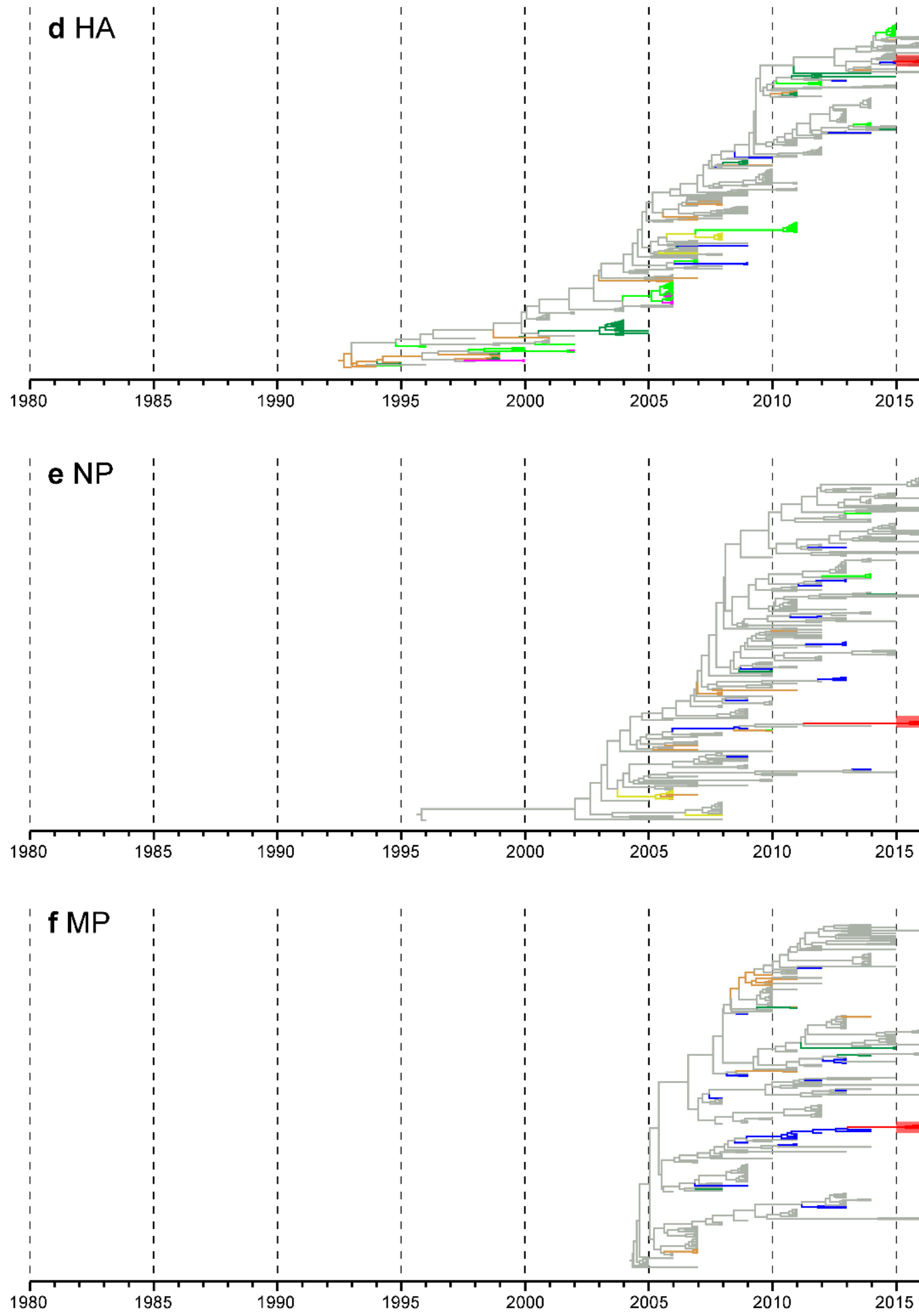

Figure 17 (continued)

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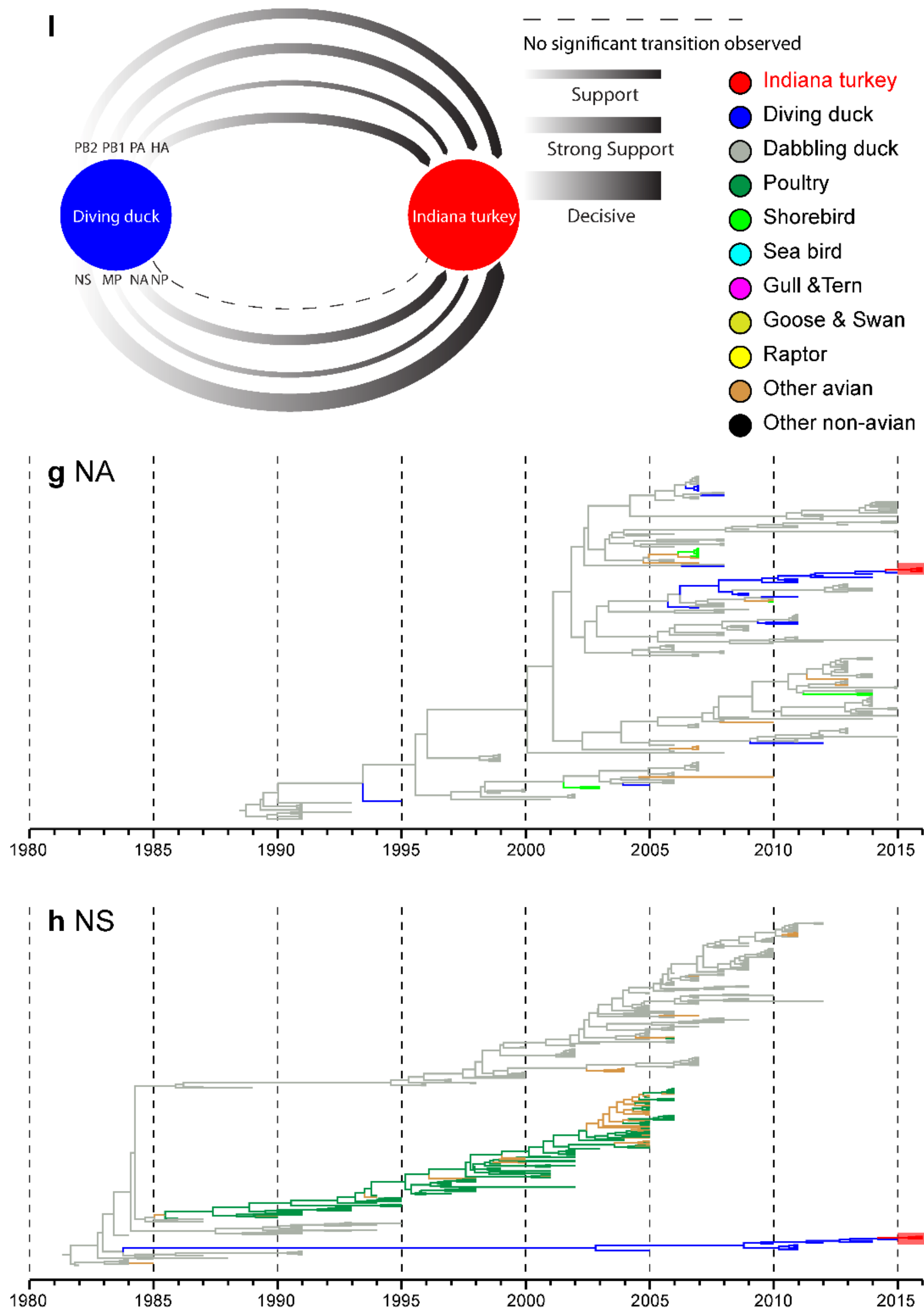

Figure 17 (continued)

(a) – (h) are maximum clade credibility phylogenetic trees for eight gene segments. The trees are constructed on the basis of the maximum clade credibility phylogenetic trees shown in Phylogenetic_trees_for_eight_gene_segments.pdf. (I) summarizes the diffusion pathway of influenza A virus between diving duck and Indiana turkey.

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Table 7 Diffusion pathway of influenza A virus between wild birds and Indian turkeys.

Segment	Host group	Transition rate	Indicator	Bayes factor	Support level
PB2	Diving duck	0.70	0.82	24.12	Strong support
PB1	Diving duck	0.57	0.73	14.39	Strong support
PA	Diving duck	0.73	0.65	9.99	Support
HA	Diving duck	0.93	0.80	24.81	Strong support
NP	No significant transition was observed				
NA	Diving duck	0.44	0.92	37.89	Strong support
MP	Diving duck	0.68	0.55	3.91	Support
NS	Diving duck	0.35	1.00	1045.21	Decisive

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Table 8 Diffusion pathway of influenza A virus among different host groups in wild birds

Segment	Bird-A	Bird-B	Transition rate	Indicator	Bayes factor	Support level
PB2	Dabbling duck	Diving duck	0.54	0.73	14.51	Strong support
	Gull Tern	Shorebird	1.19	0.55	6.50	Support
	Shorebird	Gull Tern	1.05	0.52	5.61	Support
PB1	Dabbling duck	Diving duck	0.34	1.00	1113.82	Decisive
PA	Dabbling duck	Diving duck	0.62	1.00	142441.30	Decisive
	Diving duck	Dabbling duck	1.84	0.66	10.46	Strong support
	Diving duck	Goose Swan	2.13	0.97	192.56	Very strong support
HA	Dabbling duck	Diving duck	0.28	0.94	100.76	Very strong support
	Diving duck	Goose Swan	1.14	0.57	8.49	Support
	Shorebird	Gull Tern	0.93	1.00	50232.67	Decisive
NP	Dabbling duck	Diving duck	0.24	0.92	62.62	Strong support
	Diving duck	Dabbling duck	1.36	0.57	6.85	Support
NA	Dabbling duck	Diving duck	0.39	0.83	15.97	Strong support
	Diving duck	Dabbling duck	1.13	0.74	9.33	Support
MP	Dabbling duck	Diving duck	0.35	0.94	53.01	Strong support
	Diving duck	Dabbling duck	1.11	0.56	4.07	Support

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H7N8 LPAI Virus Evolved Into An HPAI Strain In Turkeys

We also investigated how the H7N8 virus evolved into a highly pathogenic form after its introduction into domestic turkeys. The cleavability of HA protein is considered a major determinant of pathogenicity, although the pathogenicity of IAVs is polygenic. We investigated the sequence in the cleavage region of the HA protein of H7N8 turkey strains. Indiana/16-001403-1 has a three basic amino acids (KRK) insert at the cleavage site that results in a protein sequence motif: PENPKKRKTRGLF (Figure 18). The other two H7N8 turkey strains, Indiana/16-001573-2 and Indiana/16-001574-7, have an identical and typical LPAI cleavage site with no insert. Analyses of H7 HPAI strains from previous outbreaks in the Americas identified multiple insertion patterns and insertions of six to ten amino acids (Figure 18). Comparison showed that the novel H7N8 HPAI strain has a unique cleavage site different from earlier H7 HPAI strains in the Americas.

Comparison of the genome of H7N8 HPAI strain and those of the two H7N8 LPAI turkey strains identified five amino acid substitutions in five distinct proteins (Figure 19). Moreover, five amino acid substitutions in three different proteins were detected between the genome of three H7N8 turkey strains and that of Kentucky/AH0012935 (Figure 19). The profile of three amino acid positions (E105K, F260L, and E278K [H3 numbering]; 95, 251, and 269 [H7 numbering]) in the HA1 protein were analyzed for all H7 IAVs isolated from wild and domestic birds in the influenza database. It is interesting that amino acid K at position 278 [H3 numbering] in the HA1 protein was observed in one poultry–origin isolate, A/chicken/Guanajuato/07437-15/2015(H7N3), which presents multiple basic amino acids

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in the HA protein cleavage region. Amino acid K was also observed in six H7N3 HPAI strains isolated during an outbreak in Pakistan in 2004. These amino acid substitutions were potentially linked to the host adaptation of H7N8 viruses from wild waterfowl to turkey and evolution from low pathogenic to highly pathogenic form.

Cleavage region ↓

Strain																				
A/Citl/Bolivia/4537/01(H7N3)	P	E	K	P	K	-	-	-	-	-	-	-	-	-	-	T	R	G	L	F
A/Ck/Chile/4957/02(H7N3)	P	E	K	P	K	T	C	S	P	L	S	R	C	R	K	T	R	G	L	F
A/Ck/Chile/184240-1/02(H7N3)	P	E	K	P	K	T	C	S	P	L	S	R	C	R	E	T	R	G	L	F
A/Bwt/TX/578585/02(H7N3)	P	E	N	P	K	-	-	-	-	-	-	-	-	-	-	T	R	G	L	F
A/Ck/BC/NS-1319-2/04(H7N3)	P	E	N	P	K	-	-	-	Q	A	Y	H	K	R	M	T	R	G	L	F
A/Ck/BC/NS-2035-12/04(H7N3)	P	E	N	P	K	-	-	-	Q	A	C	Q	K	R	M	T	R	G	L	F
A/Ck/BC/NS-1479-1/04(H7N3)	P	E	N	P	R	-	-	-	Q	A	Y	R	K	R	M	T	R	G	L	F
A/Canada/rv504/04(H7N3)	P	E	N	P	K	-	-	-	Q	A	Y	Q	K	R	M	T	R	G	L	F
A/Ck/BC/CN-7/04(H7N3)	P	E	N	P	K	-	-	-	Q	A	Y	R	K	R	M	T	R	G	L	F
A/Ck/BC/04(H7N3)	P	E	N	P	K	-	-	-	Q	A	Y	Q	K	Q	M	T	R	G	L	F
A/Ck/BC/NS1337-1/04(H7N3)	P	E	N	P	K	-	-	-	Q	A	Y	K	K	R	M	T	R	G	L	F
A/Ck/BC/CN12/04(H7N3)	P	E	N	P	K	-	-	-	Q	A	H	Q	K	R	M	T	R	G	L	F
A/Gadw/AZ/A00663934/09(H7N7)	P	E	N	P	K	-	-	-	-	-	-	-	-	-	-	T	R	G	L	F
A/Ck/SK/HR-00011/07(H7N3)	P	E	N	P	K	-	-	-	-	T	T	K	P	R	P	R	R	G	L	F
A/Redh/OH/13OS0406/13(H7N3)	P	E	N	P	K	-	-	-	-	-	-	-	-	-	-	T	R	G	L	F
A/Ck/Jalisco/12283/12(H7N3)	P	E	N	P	K	-	-	D	R	K	S	R	H	R	R	T	R	G	L	F
A/Mexico/InDRE7218/12(H7N3)	P	E	N	P	K	-	-	D	R	K	S	R	H	R	R	T	R	G	L	F
A/Lesc/KY/AH0012935/15(H7N8)	P	E	N	P	K	-	-	-	-	-	-	-	-	-	-	T	R	G	L	F
A/Tk/IN/16-001403-1/16(H7N8)	P	E	N	P	K	-	-	-	-	-	-	-	K	R	K	T	R	G	L	F
A/Tk/IN/16-001573-2/16(H7N8)	P	E	N	P	K	-	-	-	-	-	-	-	-	-	-	T	R	G	L	F
A/Tk/IN/16-001574-7/16(H7N8)	P	E	N	P	K	-	-	-	-	-	-	-	-	-	-	T	R	G	L	F

Figure 18 Insertion patterns in the HA cleavage region of the H7 viruses detected in Indiana turkeys and those from four previous outbreaks in the Americas.

The H7N8 viruses detected in Indiana turkeys are indicated in red, highly pathogenic avian influenza viruses isolated from previous outbreaks are indicated in green. Wild bird-origin influenza A viruses containing HA gene segment that is most closely related to outbreak strains are indicated in black. Insert of basic amino acids are color-coded accordingly. Host species are: BWTE (blue winged teal), CITE (cinnamon teal), CK (chicken), GADW (gadwall), LESC (lesser scaup), REDH (redhead), and TK (turkey). Geographic locations are: AZ (Arizona), BC (British Columbia), IN (Indiana), KY (Kentucky), OH (Ohio), SK (Saskatchewan), and TX (Texas).

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	PB2		PA	HA			NP	NA	M1	NS1
	54	740	347	**105**	**260**	**278**	377	357	609	**90**
A/Lesc/KY/AH0012935/15(H7N8)	R	D	D	E	F	E	S	V	G	L
A/Tk/IN/16-001403-1/16(H7N8)	K	N	G	K	L	K	N	L	A	I
A/Tk/IN/16-001573-2/16(H7N8)	K	D	D	K	L	K	S	V	G	I
A/Tk/IN/16-001574-7/16(H7N8)	K	D	D	K	L	K	S	V	G	I

Figure 19 Amino acid substitutions among eight gene segments of the H7N8 strains detected in Indiana turkey.

The position of amino acid substitution between Kentucky/AH0012935 and the H7N8 turkey strains are shown in bold, and other positions represent amino acid substitutions between the highly pathogenic avian influenza H7N8 strain and two low pathogenic avian influenza H7N8 strains associated with the outbreak of disease in Indiana turkeys. Amino acids are colored-coded on the basis of Rasmol coloring scheme in CLC sequence Viewer 7, and colors represent the different properties of amino acids. Host species are: LESC (lesser scaup) and TK (turkey). Geographic locations are: IN (Indiana) and KY (Kentucky).

Discussion

Introduction of IAVs from wild birds to domestic poultry presents a continuous threat to livestock health. In this study, we provided evidence that the H7N8 virus associated with the outbreak of disease in Indiana turkeys was generated through a series of genetic events likely occurring in wild birds and subsequently introduced into an Indiana turkey flock, where the virus evolved into a highly pathogenic form.

We propose one possible evolutionary model leading to the generation of H7N8 HPAI virus in Indiana turkeys on the basis of available evidence and emphasize that other models cannot be excluded. The H7N8 precursor virus may have been generated in wild birds through two phase sequential reassortment events (Figure 20). The first phase of reassortment may have occurred between H4N8–DD viruses and other IAVs circulating in North American wild birds during 2011–2014, generating

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A/Bufflehead/Illinois/14OS3609/2014–like virus, possessing five H7N8–like gene segments (PB2, PB1, NA, MP, and NS), in diving ducks. In the second phase, further reassortment between A/Bufflehead/Illinois/14OS3609/2014–like virus and locally circulating IAVs in wild birds that have H7N8–like PA, HA, and NP gene segments lead to the generation of H7N8 precursor virus. Although the direct predecessor virus of the H7N8 turkey viruses could not be identified, we identified one IAV strain that possess six gene segments most closely related to that of the H7N8 HPAI virus, with nucleotide sequence identity ≥99.00%, in a diving duck sampled in Kentucky. IAVs that are most closely related to the H7N8 turkey strains, including Kentucky/AH0012935 and H4N8–DD isolates, were mainly recovered from samples collected in Kentucky, Wisconsin, Illinois, Ohio, and Louisiana, all of which are included within the Mississippi flyway. In addition, sample collection sites for IAVs isolated from diving ducks included locations within all four major flyways of North America (Figure 21). Thus, we predict that the reassortment events leading to the generation of H7N8 precursor viruses likely occurred within the Mississippi flyway. Regarding the time of introduction of H7N8 virus to Indiana turkeys, we observed that the TMRCAs for three H7N8 turkey strains among seven of eight gene segments were summer and fall of 2015. In North America, waterfowl generally begin staging for autumn migration in July and August. Southern migration begins as early as August for blue-winged teal and for most waterfowl in late September, October, and early November. It was likely that waterfowl, including diving ducks, may have carried the H7N8 precursor virus during their southern migration and introduced this virus to Indiana turkeys through an unidentified interface between wild waterfowl and domestic poultry. After introduction to Indiana turkeys, H7N8 LPAI virus

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evolved into highly pathogenic form through acquisition of three basic amino acids (KRK) in the cleavage region of the HA protein.

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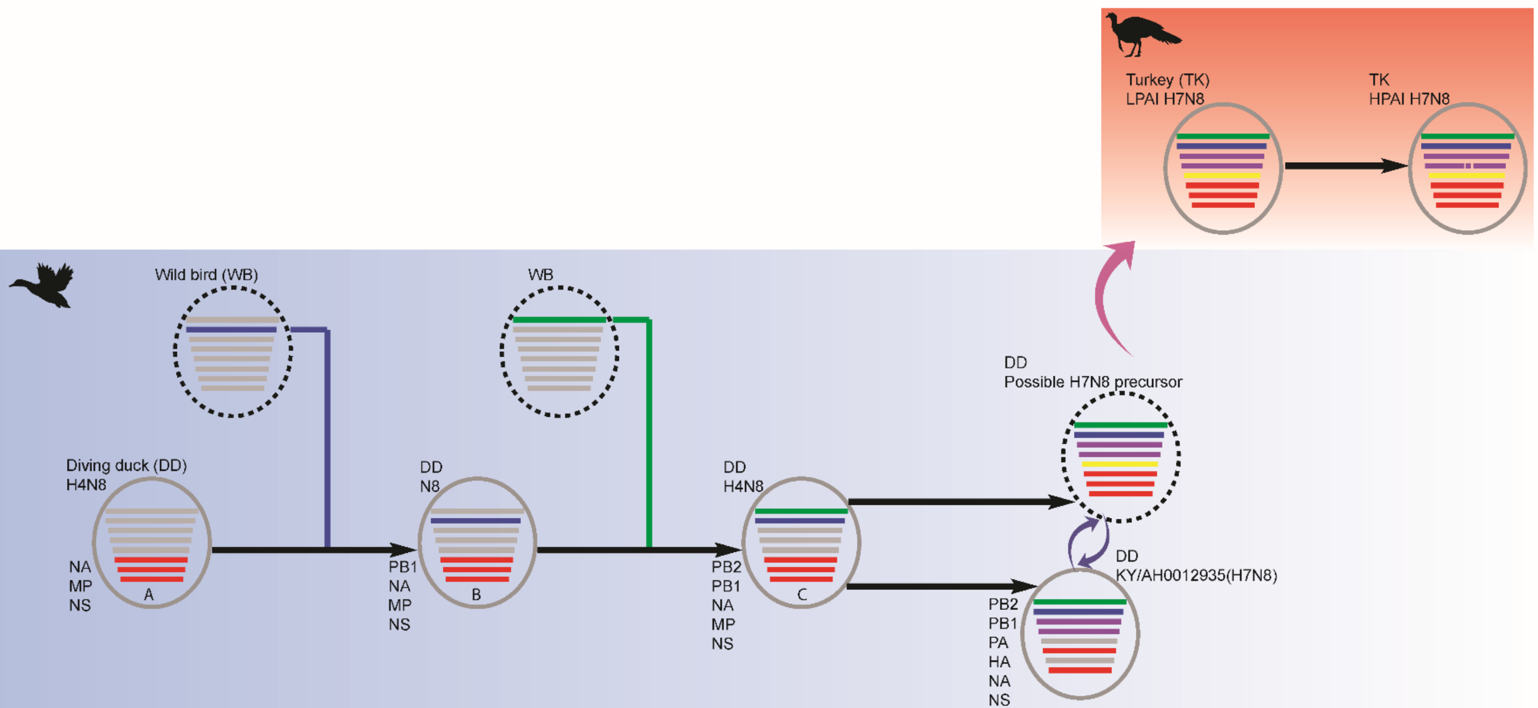

Figure 20 Proposed evolutionary pathway leading to the generation of highly pathogenic avian influenza(H7N8) virus that caused outbreak in Indiana turkeys.

Influenza A viruses are represented by ovals containing horizontal bars that indicate eight gene segments (from top to bottom, PB2, PB1, PA, HA, NP, NA, MP, and NS). Solid ovals represent virus strains isolated from wild and domestic birds, and broken ovals represent hypothetical virus strains. A broken bar in segment four (HA) indicates insert of basic amino acids at the cleavage site. HPAI = highly pathogenic avian influenza, and LPAI = low pathogenic avian influenza. A, B, and C indicate three gene constellations shown in Figure 16. Genetic events occurring in wild and domestic birds are indicated by blue and red shaded boxes, respectively.

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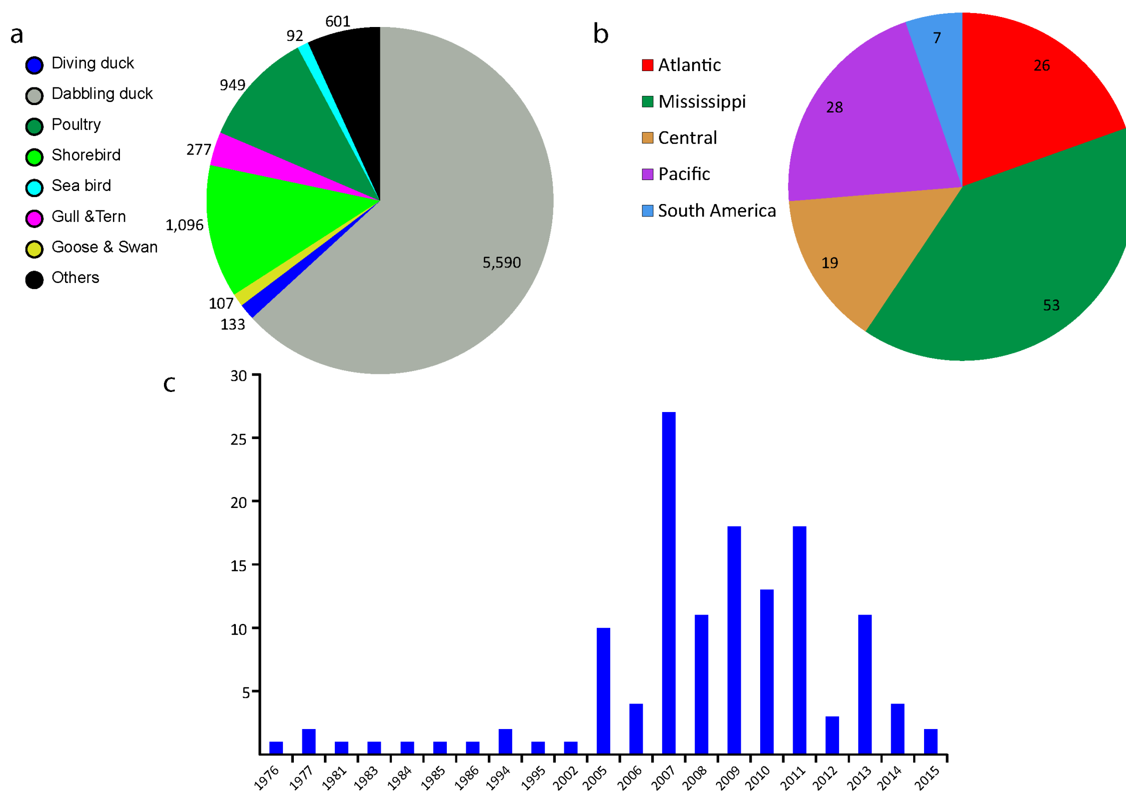

Figure 21 Statistics of influenza A viruses isolated from wild and domestic birds in the Americas.

(a) Distribution of 8,845 influenza A virus strains isolated from wild and domestic birds in the Americas among different bird groups. (b) Distribution of 133 influenza A virus strains isolated from American diving ducks among four bird migratory flyways and South America. (c) Distribution of 133 influenza A virus strains isolated from American diving ducks among different sampling years.

Worldwide, LPAI viruses have been recovered from at least 105 wild bird species representing 26 different taxonomic families (33). Most of the 26 families are in the Order Anseriformes (including ducks, geese, and swans), followed by the Order Charadriiformes (including shorebirds and gulls); both are considered major natural reservoirs for IAVs (4). Among these bird species, shorebirds and gulls were previously suggested to be the source of the precursor of H5 and H7 HPAI viruses in North America

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(225). A more recent study indicated that the precursors of H7N3 HPAI viruses in the Americas most likely originated from wild waterfowl, particularly dabbling ducks (194). In Eurasia, precursors of H7 HPAI viruses were generally found in dabbling ducks (195). Our findings add to the knowledge supporting that introduction of IAVs from wild birds plays an important role in the emergence of IAVs in domestic poultry. This study differs from previous work, though, in providing evidence that diving ducks may also contribute to the emergence of a HPAI strain as compared to dabbling ducks, shorebirds and gulls, and other bird species. Diving ducks appeared to have contributed five gene segments (PB2, PB1, NA, MP, and NS) to the novel H7N8 virus, may have harbored the H7N8 LPAI precursor virus; however, the mechanism of exposure to Indiana turkeys remains unclear. The behavior of diving ducks may contribute to the long-term perpetuation of specific IAV genes or strains. Compared with dabbling ducks and many other birds, diving ducks utilize open water habitats that can include deeper water. It has been shown that IAVs may remain infectious in water for several months (41), but infectivity is adversely affected by repeated freeze-thaw cycles that would be more likely to occur at the shallower water utilized by dabbling ducks (226). In aquatic habitats highly contaminated by waterfowl feces, deeper water habitats that undergo limited or no freeze-thaw cycles could also increase the risk for IAV co-infection, which could lead to virus reassortment and the generation of novel IAV strains as occurred with the H7N8 precursor viruses.

Mutation and reassortment have been two major mechanisms for IAVs to maintain circulation and expand host ranges. Reassortment events have been well documented for IAVs that infect humans, swine, equine, canines, and wild and domestic

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birds. Persistence of a gene constellation has been shown to be associated with emergence of novel IAV strains that infect mammals. The existence of triple-reassortant internal gene constellations has been well established in multiple subtypes of IAVs that infect swine, and such constellations contributed to the genesis of the pandemic influenza A(H1N1) pdm09 virus (95). This internal gene constellation was suggested to possess a selective advantage over other strains (227). In contrast, IAVs that infect wild birds generally form diverse and transient genotypes through reassortment of functionally equivalent gene segments (48). Specific studies devoted to H7 IAVs in wild birds inhabiting North America and Eurasia found similar patterns (196, 219). In addition, extensive gene flow between IAVs in North American wild birds could further facilitate genetic diversity, although the migratory flyway and geographic distance may impose short-term restrictions on gene flow (228, 229). We observed that as many as five H7N8–like gene segments (PB2, PB1, NA, MP, and NS) formed a relatively persistent gene constellation in H4N8 IAVs isolated from diving ducks. This finding suggests that reassortment between IAVs in wild birds may not be random: some gene segments may be more likely to form specific linkages within the context of diverse and transient genotypes. Alternatively, reassortment may be restricted in some groups of birds, like diving ducks, if prevalence and the probability of infection with two or more viruses is unlikely. The significance of the relatively persistent gene constellation, within the context of transient genotypes of IAVs in wild birds, observed in this study is not known.

Influenza surveillance in wild birds is critical for understanding the natural history of IAVs and assessing the ancestry of IAVs infecting domestic poultry, however, the existing surveillance system in wild birds could have led to considerable bias toward bird

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species that are present in large numbers in nature or that are easily caught (33). Diving ducks are underrepresented in both historic and current IAV surveillance. We investigated the host origin of genomic sequences deposited in the Influenza Research Database (220) and found that diving duck-origin strains comprise around 1.50% of the total number of IAV strains isolated from wild and domestic birds in the Americas (Figure 21). Among the 441 IAV strains that we sequenced in this study, only six originated from diving ducks (Figure 11). In addition, data from a large scale surveillance program in wild birds throughout the United States showed that, among the 197,885 samples collected from over 200 wild bird species (2007–2011), diving ducks accounted for only 7.72% of all samples collected and 3.58% of the IAV positive samples determined by M gene based rRT-PCR; dabbling ducks accounted for 62.73% of the samples collected and 86.44% of the IAV positive samples (230). Although data from the same study showed that the IAV positive rate for diving ducks was 5.30% compared to 15.80% for dabbling ducks, our study highlights the contribution of diving ducks to the emergence of the novel H7N8 virus and suggests that diving ducks may serve as a potentially unique IAV reservoir or uniquely contribute to the maintenance, diversification, and transmission of IAVs in wild birds. Thus, we recommend additional surveillance sampling of diving ducks and other bird species of specific ecological significance for IAVs, but acknowledge the difficulty in obtaining these samples from hunter harvested birds since dabblers are usually the preferred bird species of hunters. Moreover, we observed a relatively persistent gene constellation that may be associated with the emergence of the novel H7N8 virus. Genome sequencing and characterization of gene constellation of IAVs in wild birds could serve as a valuable component of a

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successful surveillance strategy. The genomes of 441 wild bird–origin IAVs were sequenced in a collaborative effort in this study. This large number of IAV isolates and sequences enabled us to conduct detailed evolutionary analyses and revealed a few key aspects regarding the evolutionary pathway leading to the novel H7N8 virus. The experience from this study demonstrates the need for a coordinated, systematic, and collaborative approach to active surveillance in wild birds.

In summary, our investigation provides information on the putative viral ancestors, possible evolutionary pathways, and probable host species involved in the emergence of a novel H7N8 virus that caused outbreaks among turkey flocks in Indiana during 2016. Our findings indicate that diving ducks contributed to the emergence of the novel H7N8 virus and may contribute to the maintenance, diversification, and transmission of IAVs in wild birds. The repeated introduction of IAVs into domestic poultry from wild birds highlights important gaps in existing biosecurity systems and provides evidence that surveillance in wild birds can be useful for understanding possible evolutionary pathways of emergence.

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CHAPTER V

THE ANTIGENIC DIVERSITY AND GENETIC EVOLUTION OF CONTEMPORARY H7 AIVS FROM NORTH AMERICA DURING 1971 to 2012

H7 AIVs have caused at least 500 confirmed human infections since 2003 and culling of more than 75 million birds in recent years. Vaccine could serve as a valuable component in a successful AIV control strategy, and antigenic match between vaccine seed strain and circulating viral strains is one of the keys to a successful vaccination program. However, limited data is available for the antigenic diversity of contemporary H7 AIVs from North America. In this chapter, we antigenically and genetically characterized 93 AIV isolates from North America (85 from wild birds [1976–2010], seven from domestic poultry [1971–2012], and one from a seal [1980]). The hemagglutinin genes of these H7 viruses are separated from those from Eurasia. Gradual accumulation of nucleotide and amino acid substitutions was observed in the hemagglutinin of H7 AIVs isolated from wild and domestic birds. Genotype characterization suggested that H7 AIVs circulating in North American wild birds form diverse and transient internal gene constellations. Serologic analyses showed that the 93 isolates cross-reacted with each other to different extents. Antigenic cartography showed that the average antigenic distance among them was 1.14 units (standard deviation [SD], 0.57 unit) and that antigenic diversity among the H7 isolates we tested was limited. Our

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results suggest that the continuous genetic evolution has not led to significant antigenic diversity for H7 AIVs from North America. These findings add to our understanding of the natural history of IAVs and will inform public health decision-making regarding the threat these viruses pose to humans and poultry.

Introduction

H7 AIVs have been frequently reported to cause outbreaks in domestic poultry and humans. The first outbreak of HPAI H7 virus in Pakistan was reported in 1995, and the threat to domestic poultry has persisted in the region since then (231, 232). In 2003, a HPAI H7N7 outbreak in the Netherlands led to the death or culling of more than 30,000,000 birds and 89 infections in humans, one of which was fatal (19, 233). In March 2013, a LPAI H7N9 virus emerged in eastern China (177) and has become enzootic in the region (234). The virus causes asymptomatic infection in domestic poultry, including chickens and waterfowl, but high morbidity and mortality in human infections (235). Since the emergence of H7N9 virus, more than 500 laboratory-confirmed cases in human have been reported, of which more than 100 were fatal (http://www.who.int/influenza/human_animal_interface/influenza_h7n9/en/). Furthermore, epidemics caused by H7N1 and H7N3 AIVs were reported in Italy during 1999–2004 (150, 189). H7 AIVs were also reported to cause outbreaks in domestic poultry in Australia (236), Germany (237), and the United Kingdom (238). In North America, H7N2 AIV was first identified in 1994 in the LPM system in the northeastern United States, and during 1997–2002 it was linked with outbreaks among poultry in Pennsylvania, Virginia, West Virginia, and North Carolina(129, 181). HPAI H7N3

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viruses were responsible for outbreaks in poultry in Canada (in 2004 and 2007) and Mexico (in 2012) (61, 239, 240), and subsequently spilled over to humans (64, 241).

The conventional strategy for controlling the spread of avian influenza outbreaks in domestic poultry involves enforcement of biosecurity measures, diagnostics and surveillance, and culling of infected birds. In addition, vaccination programs have been implemented in multiple countries to control H7 AIV outbreaks among domestic poultry. For example, in Italy, vaccine was used against H7N1 virus in 2000 and against H7N3 virus in 2002; vaccines have been used in Pakistan since 1995 to control H7N3 virus; and in North America, vaccine was used against a 2003 H7N2 virus outbreak in Connecticut, United States, and against the on-going H7N3 virus outbreak in Mexico. Early experience showed that vaccine could serve as a valuable component in a successful AIV control strategy.

IAVs evolve by two major mechanisms: mutation and reassortment. Point mutations within surface glycoproteins HA and NA can lead to a small antigenic change, so called antigenic drift. Reassortment refers to the exchange of individual gene segments or combinations of segments between IAVs during mixed infections in the same cell. The switch of HA and/or NA by reassortment can cause a large antigenic change, so called antigenic shift. Reassortment occurs frequently between IAVs, and it facilitates generation of epidemic and pandemic influenza strains. Both antigenic drift and antigenic shift allow IAVs to evade the herd immunity established from previous influenza infections or vaccination.

Earlier studies have showed minor antigenic diversity for wild bird-origin H7 AIVs from Eurasia (195, 196); however, limited data is available for antigenic diversity

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of contemporary H7 AIVs from North America. Understanding the antigenic diversity of H7 AIVs circulating in North America will facilitate the detection of antigenic variants and development of effective strategies for disease prevention and control. In this chapter, we antigenically characterized 93 H7 AIVs derived from wild waterfowl, domestic poultry, and a seal; the isolates were collected across North America during 1971–2012. The genomic sequences were analyzed to determine the genetic evolution dynamics of H7 AIVs in North America.

Materials and Methods

Ethics Statement

All experiments involved in animals were approved by Institutional Animal Care & Use Committee, Mississippi State University (Project No. 13-090). All experiments were carried out in accordance with the approved guidelines.

Viruses

A total of 93 H7 isolates were included in the study (Table 9): 85 were derived from wild birds during 1976-2010, seven were derived from domestic poultry during 1971-2012, and one was derived from a seal in 1980. Among these isolates, A/cinnamon teal/Mexico/2817/2006(H7N3) was the vaccine strain used during the vaccination campaign against HPAI H7N3 virus in Mexico in 2012, strain A/chicken/British Columbia/314514-2/2004(H7N3) was isolated from the outbreak in domestic poultry in Canada in 2004, and strain A/chicken/Jalisco/CPA-12283/2012(H7N3) was from the outbreak in Mexico in 2012. The wild bird-origin isolates represent those recovered from Canada, Mexico, and 28 states within the United States. They also represent the four

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major bird migratory flyways in North America: the Atlantic, Central, Mississippi, and Pacific flyway (Figure 22).

The isolates were propagated by using nine day old specific pathogen free chicken embryonated eggs; the eggs were inoculated and incubated for 72 hours at 37°C before the virus was harvested. Viruses were then aliquoted and stored at −80°C until use.

Generation of Reference Antisera in Chicken

A total of 15 H7 isolates were selected to generate reference antisera; the isolates were selected to maximize the representative subtype and species diversity and geographic and temporal coverage of the 93 isolates (Table 9). Three-week-old specific pathogen free chickens were used to produce antisera. Chickens were inoculated intranasally with 10^6 50% tissue culture infective doses of an H7 AIV isolate. If the sera titers were ≥ 1:160 at two weeks post inoculation, the sera were collected at three weeks post inoculation; if the viral titers were < 1:160 at two weeks post inoculation, the birds were re-inoculated intranasally with 10^6 50% tissue culture infective doses of the same H7 AIV isolate and the sera were collected at two weeks post re-inoculation. Blood was collected from the chickens' heart four weeks after the first inoculation. Serum was separated from the erythrocytes after centrifugation at 2,000 rpm for ten minutes. All sera were aliquoted and stored at −80°C until use.

HA and HI Assays

Before performing the HI tests, we treated the chicken antisera with 100% packed chicken red blood cells to eliminate non-specific antigen reactions. The HA and HI assays were performed in accordance with World Organisation for Animal Health

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guidelines. HI tests were carried out by using four hemagglutinin units and a 1% chicken red blood cells suspension.

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Table 9 List of H7 AIVs isolated in North America that were subjected to antigenic characterization.

Virus	Date collected	Flyway	Subtype
Isolates from wild birds			
A/American green-winged teal/Colorado/A00551331/2007[a]	10-Nov-07	Central	H7N3
A/northern shoveler/Utah/A00374996/2007	15-Dec-07	Pacific	H7N3
A/domestic duck/West Virginia/A00140912/2008	9-Jul-08	Atlantic	H7N3
A/domestic duck/West Virginia/A00140913/2008	9-Jul-08	Atlantic	H7N3
A/domestic duck/West Virginia/A00140915/2008	9-Jul-08	Atlantic	H7N3
A/mute swan/Rhode Island/A00325105/2008	6-Aug-08	Atlantic	H7N3
A/mute swan/Rhode Island/A00325108/2008	6-Aug-08	Atlantic	H7N3
A/mute swan/Rhode Island/A00325112/2008	6-Aug-08	Atlantic	H7N3
A/mute swan/Rhode Island/A00325114/2008	6-Aug-08	Atlantic	H7N3
A/mute swan/Rhode Island/A00325115/2008	6-Aug-08	Atlantic	H7N3
A/mute swan/Rhode Island/A00325117/2008	6-Aug-08	Atlantic	H7N3
A/mute swan/Rhode Island/A00325125/2008	6-Aug-08	Atlantic	H7N3
A/mute swan/Rhode Island/A00325129/2008	6-Aug-08	Atlantic	H7N3
A/mute swan/Rhode Island/A00325136/2008	6-Aug-08	Atlantic	H7N3
A/mallard/Wisconsin/A00465618/2008	5-Sep-08	Mississippi	H7N3
A/blue-winged teal/Missouri/A00624483/2008	8-Sep-08	Mississippi	H7N3
A/blue-winged teal/Missouri/A00624484/2008	8-Sep-08	Mississippi	H7N3
A/mallard/Iowa/A00558620/2008	18-Oct-08	Mississippi	H7N3
A/American green-winged teal/Colorado/A00660616/2008	9-Nov-08	Central	H7N3
A/mallard/South Dakota/A00649542/2008	19-Nov-08	Central	H7N3

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Table 9 (continued)

Virus	Date collected	Flyway	Subtype
Isolates from wild birds			
A/mallard/New Jersey/A00122457/2008	22-Nov-08	Atlantic	H7N8
A/mallard/Kansas/A00523306/2008	25-Nov-08	Central	H7N3
A/mallard/Indiana/A00142205/2008	26-Nov-08	Mississippi	H7N3
A/northern shoveler/Nevada/A00505416/2008	6-Dec-08	Pacific	H7N6
A/American green-winged teal/Wyoming/A00230796/2008	7-Dec-08	Central	H7N3
A/bufflehead/Virginia/A00120022/2008	9-Dec-08	Atlantic	H7N2
A/northern shoveler/Oregon/A00654616/2008	22-Dec-08	Pacific	H7N3
A/northern shoveler/Mississippi/A00682947/2008	27-Dec-08	Mississippi	H7N7
A/American green-winged teal/Utah/A00833077/2009	2-Jan-09	Pacific	H7N3
A/American green-winged teal/Arizona/A00115994/2009	3-Jan-09	Pacific	H7N3
A/American green-winged teal/Arizona/A00115995/2009	3-Jan-09	Pacific	H7N3
A/American green-winged teal/Utah/A00831743/2009	3-Jan-09	Pacific	H7N3
A/gadwall/Arizona/A00663934/2009	3-Jan-09	Pacific	H7N7
A/northern shoveler/Mississippi/A00602284/2009	3-Jan-09	Mississippi	H7N2,N7
A/northern shoveler/Utah/A00831758/2009	8-Jan-09	Pacific	H7N3
A/American green-winged teal/Utah/A00654391/2009	9-Jan-09	Pacific	H7N3
A/American green-winged teal/Utah/A00461135/2009	15-Jan-09	Pacific	H7N1
A/American green-winged teal/Utah/A00461136/2009	15-Jan-09	Pacific	H7N1
A/American green-winged teal/Utah/A00614935/2009	15-Jan-09	Pacific	H7N3
A/mallard/Oklahoma/A00449368/2009	15-Jan-09	Central	H7N3

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Table 9 (continued)

Virus	Date collected	Flyway	Subtype
Isolates from wild birds			
A/mallard/Oklahoma/A00449455/2009	15-Jan-09	Central	H7N3
A/northern shoveler/Utah/A00461133/2009	15-Jan-09	Pacific	H7N4
A/northern shoveler/Utah/A00468752/2009	15-Jan-09	Pacific	H7N3
A/American green-winged teal/Utah/A00468772/2009	16-Jan-09	Pacific	H7N7
A/northern shoveler/Utah/A00468715/2009	16-Jan-09	Pacific	H7N6
A/northern shoveler/Utah/A00468766/2009	16-Jan-09	Pacific	H7N3
A/northern pintail/Texas/A00466052/2009	18-Jan-09	Central	H7N3
A/ring-necked duck/Texas/A00766403/2009	19-Jan-09	Central	H7N1
A/blue-winged teal/Louisiana/A00637297/2009	22-Jan-09	Mississippi	H7N3
A/American green-winged teal/Texas/A00604024/2009	3-Feb-09	Central	H7N3
A/American green-winged teal/Texas/A00604029/2009	3-Feb-09	Central	H7N3
A/American green-winged teal/Texas/A00604032/2009	3-Feb-09	Central	H7N3
A/American green-winged teal/Texas/A00604814/2009	3-Feb-09	Central	H7N3
A/blue-winged teal/Texas/A00605473/2009	13-Mar-09	Central	H7N3
A/blue-winged teal/Minnesota/A00137660/2009	27-Jul-09	Mississippi	H7N3
A/mallard/Montana/A00750842/2009	16-Sep-09	Central	H7N3
A/blue-winged teal/South Dakota/A00772794/2009	28-Sep-09	Central	H7N7
A/mallard/Michigan/A00869519/2009	14-Oct-09	Mississippi	H7N3
A/mallard/Nebraska/A00709657/2009	28-Nov-09	Central	H7N3
A/mallard/New York/A00723392/2009	12-Dec-09	Atlantic	H7N3
A/mallard/New York/A00723400/2009	12-Dec-09	Atlantic	H7N4

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Table 9 (continued)

Virus	Date collected	Flyway	Subtype
Isolates from wild birds			
A/mallard/Oklahoma/A00749161/2009	12-Dec-09	Central	H7N3
A/mallard/Oklahoma/A00744383/2009	13-Dec-09	Central	H7N3
A/northern shoveler/Oklahoma/A00744384/2009	13-Dec-09	Central	H7N3
A/mallard/Illinois/A00325439/2009	18-Dec-09	Mississippi	H7N3
A/mallard/Illinois/A00755320/2009	18-Dec-09	Mississippi	H7N3
A/mallard/New Jersey/A00926089/2010	17-Feb-10	Atlantic	H7N3
A/American black duck/Delaware/A00870108/2010	18-Feb-10	Atlantic	H7N3
A/blue-winged teal/Texas/A00463679/2010	10-Mar-10	Central	H7N3
A/duck/Alberta/49/1976	1976		H7N3
A/mallard/Ohio/421/1987	1987		H7N8
A/pintail/MN/423/1999	1999		H7N3
A/laughing gull/NJ/2455/2000	2000		H7N3
A/ruddy turnstone/DE/1538/2000	2000		H7N9
A/ruddy turnstone/DE/892/2006	2002		H7N3
A/cinnamon teal/Mexico/2817/2006	2006		H7N3
A/ruddy turnstone/NJ/207/2006	2006		H7N3
Isolates from domestic poultry			
A/turkey/Oregon/1971	1971		H7N3
A/turkey/MN/38429/1988	1988		H7N9
A/turkey/NY/4450-4/1994	1994		H7N2
A/turkey/VA/SEP-67/2002	2002		H7N2
A/chicken/CT/260413-2/2003	2003		H7N2
A/chicken/British Columbia/314514-2/2004[b]	2004		H7N3
A/chicken/Jalisco/CPA-12283/2012[b]	2012		H7N3
Isolate from seal			
A/seal/MA/1/1980	1980		H7N7

[a] Viruses used to generated reference antisera are in bold.
[b] Highly pathogenic avian influenza virus.
Isolates' name was colored-coded according to their genetic clusters. Red indicates virus in cluster I, green indicates virus in cluster II, and blue indicates virus in cluster III.

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Figure 22 Geographic distribution of H7 AIVs selected for antigenic characterization.

The isolates represent strains from Canada, Mexico, and the United States. Triangles indicate viruses isolated from wild birds, dots indicate viruses isolated from domestic poultry, and the square indicates virus isolated from a seal. Map of North America with US States and Canadian Provinces by FreeVectorMaps.com, https://freevectormaps.com/world-maps/north-america/WRLD-NA-02-0003.

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Full Genome Sequencing

Viral RNA was extracted from the allantoic fluid of specific pathogen free embryonated chicken eggs by using the QIAamp Viral RNA Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The full-length cDNA for eight influenza gene segments was amplified by using a SuperScript One-Step RT-PCR kit (Invitrogen, Grand Island, NY) with influenza virus–specific primers (242). PCR products were separated by using agarose gel electrophoresis and purified by using a QIAquick Gel Purification Kit (QIAGEN). Amplified viral DNA products were quantitated by using a High Sensitivity DNA kit on an Agilent 2100 Bioanalyzer system (both from Agilent Technologies, Santa Clara, CA). An equal amount of each sample was used to prepare the sequencing library with the Illumina Nextera DNA Sample Preparation Kit (Illumina, San Diego, CA, USA). Library samples were further quantitated, normalized, and pooled together. Pooled library samples were sequenced by using a MiSeq Reagent Kit v2 (500 cycles) on a MiSeq sequencer (Illumina); the sequencing protocol suggested by the manufacturer was followed. When any gene segment presented more than one copy of sequence, PCR was performed with specifically designed primer to confirm the existence of multiple copies of sequences. These sequences were excluded from further analysis. Sequences obtained in this study are available in GenBank under accession numbers KU289738 to KU290331.

Genomic Assembly

Genomic assembly was conducted with the in-house influenza genome assembly pipeline, which integrates quality trimming by Trimmomatic (198), *de novo* assembly by Velvet (200), reference search by BLAST (201), and mapping by Bowtie v2.0 (199). In

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brief, quality trimming was first conducted by using Trimmomatic, which trims bases from both ends of each read if the quality falls below 30 and clips reads if the average quality drops below 28 (in a sliding window of ten bases). Reads less than 100 nucleotides in length were not included in the downstream analyses. The quality-filtered reads were then *de novo* assembled in Velvet to build long contigs, and resulting contigs were searched against the Influenza Virus Resource (243) by using BLAST to select the reference sequence. Quality-filtered reads were then mapped to the reference sequences by using Bowtie v2.0. Last, consensus sequences were generated with a minimum 10-fold mapping coverage and supported by at least 90% of reads at a given position. The mapping profile was visualized by using the Integrative Genomics Viewer (244) and manually checked to correct potential assembly errors.

Evolutionary Analysis

Phylogenetic trees were inferred by using the maximum likelihood method implemented in RAxML v8.1.17 (245). A general time-reversible model of nucleotide substitution and a γ-distributed rate variation among sites was applied throughout the analysis. Sequence alignments were conducted by using MUSCLE v3.8 (222).

To understand the evolutionary history of the H7 gene of AIVs isolated from North America, two rounds of phylogenetic analyses were conducted. In the first round, all genomic sequences of the H7 gene from AIVs were downloaded from the Influenza Virus Resource in March 2015. A total of 1,315 sequences were available after combining these database sequences with sequences recovered in this study. Two major lineages, North American and Eurasian, were identified from the topology of this preliminary phylogeny. Sequences falling into the North American lineage were kept,

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and 19 sequences were selected to represent the Eurasian lineage. After that, 775 sequences were retained. In the second round, phylogenetic analysis was conducted on the HA1 domain of these 775 nucleotide sequences for a clear comparison of the antigenic and genetic profiles. Topological robustness of the tree was evaluated by 1,000 pseudo-replicates.

Two rounds of phylogenetic analysis were conducted to assess the evolutionary history of the six internal gene segments of H7 AIVs circulating among wild birds in North America. In the first round, 8,545 genomic sequences from 1,030 complete genomes of H7 AIVs were downloaded from the Influenza Virus Resource. When multiple copies of genomic sequences were present for one gene segment, the sequence corresponding to the maximal length was reserved. These database sequences were analyzed with complete genomes recovered in this study. A total of 1,098 complete genomes were retained, and phylogenetic tree was inferred for each gene segment. Topology of the phylogeny was supported by 100 pseudo-replicates. North American and Eurasian lineages were identified from the tree topology. In the second round of analysis, the phylogenetic tree was inferred for 316 complete genomes corresponding to wild bird–origin isolates in the North American NA-WB lineage. Topology of the phylogeny was supported by 1,000 pseudo-replicates. Genotypes were defined on the basis of the phylogenetic tree of each gene segment. A monophyletic clade was identified by two criteria: (1) it was supported by a bootstrap value above 60, and (2) all sequences in the clade had an average genetic distance greater than 95%.

The extent of reassortment among the internal gene segments was determined on the basis of the six gene segment phylogenetic trees for viruses in the NA-WB lineage. A

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maximum likelihood method was implemented to measure the congruence among these trees. Each gene segment tree was fitted to six gene data sets in turn, and the log likelihood value was obtained after optimizing model parameters and branch lengths. The similarity in topology among six gene trees corresponding to the same dataset was determined by the difference in log likelihood values. To put the distribution of log likelihood value in context, 100 random trees were generated for each gene dataset. Log likelihood values were obtained, using the same approach, after fitting them to the reference gene dataset.

Inference of Amino Acid Sites under Positive Selection

Selection pressure for the HA gene segment was investigated by using codon substitution models implemented in the Codeml program of PAML v 4.8 (246). The site models were used, allowing the ratio of non-synonymous/synonymous substitution rates (d_N/d_S) to vary among sites. Four different models were used: M1a, M2a, M7, and M8. Likelihood ratio tests for two pairs of models (M2a versus M1a and M8 versus M7) were conducted according to instructions in the manual. In the test, twice the log likelihood difference between the alternative model and null model was calculated. Small p-values (<0.01) lead to rejection of the null models. If null models were rejected, then Bayes Empirical Bayes analysis was implemented for estimation of specific codons under positive selection. Before analysis, identical sequences and sequences with ambiguous positions were removed. The nucleotide sequences coding the HA1 protein were analyzed.

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Molecular Dating

Molecular dating was conducted by using the Bayesian Markov Chain Monte Carlo method implemented in BEAST v1.8.0 (223). A HKY85 nucleotide substitution model, Bayesian skyline coalescent tree prior, and relaxed uncorrelated lognormal clock model were applied. For each analysis, a chain length of 50 million steps was run and 2,000 samples were generated. The results were analyzed in Tracer v1.6 (http://tree.bio.ed.ac.uk/software/tracer/), and convergence was assessed with a cutoff of 200 for the effective sample size. The mean nucleotide substitution rate and time to the most common ancestor were computed after 10% of the samples were removed as burn-in, and the statistical uncertainty was evaluated by using the values of the 95% HPD.

Construction of the Antigenic Cartography and Molecular Characterization

Antigenic cartography was constructed on the basis of HI data by using AntigenMap (247). Each entry in the HI table was normalized by dividing the maximum HI value for the reference antiserum. Missing HI titers and those below the cutoff value for low reactors were analyzed by low-rank matrix completion. An HI titer of ten was used as the low-reactor cutoff in the HI assay. Antigenic distance between two antigens was defined as the Euclidean distance between the HI values of the two viruses against all the antisera. Each unit of the antigenic distance corresponded to a 2-fold change in HI titer. Multidimensional scaling was used to project viruses to a 2-dimensional map by minimizing the sum-squared error between map distance and antigenic distance. Antigenic distance was subjected to hierarchical clustering analysis implemented in R (https://www.r-project.org/) to determine the potential division of viruses.

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To investigate the difference in antibody binding site within the HA sequences, the protein sequences of these isolates were aligned with the H3 protein sequences and the antibody binding sites were annotated based on those in H3N2 influenza A virus as shown previously (169). 135 amino acid positions corresponding to five antibody binding sites A, B, C, D, and E in the H3 protein were identified.

Results

H7 HA Genes are Genetically Diverse

Phylogenetic analyses showed that HA gene of H7 AIVs was divided into two geographically dependent lineages: Eurasian and North American (Figure 23, Phylogenetic_tree_for_HA1_nucleotide_sequences_of_H7_AIVs.pdf). Sporadic intercontinental gene flow between these two genetic pools was observed. Five H7 viruses isolated in North America fell into the Eurasian lineage, and at least two independent introductions (in 1992 and 1994) were identified. AIVs A/softbill/CA/33445-158/1992(H7N1) and A/softbill/California/33445-136/1992(H7N1) were most closely related to A/non-psittacine/England-Q/1985/89(H7N7), sharing nucleotide sequence identities of 96.3% and 96.4%, respectively. In addition, A/softbill/California/13907-21/1994(H7N1) and A/Pekin robin/California/30412/1994(H7N1) are closely related to a group of AIVs of the same subtype isolated from wild birds in the Netherlands, Singapore, and England in 1994; nucleotide sequence identities ranged from 97.8% to 99.4%. One H7 virus isolated in China, A/duck/Guangdong/1/1996(H7N3), was grouped with bird-origin viruses from North America and was genetically closely related to A/ruddy turnstone/Delaware Bay/135/1996(H7N3), sharing a nucleotide sequence identity of 99.4%.

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Table 10 Sequence identity of HA1 protein of H7 AIVs from distinct genetic clusters from North America.

Genetic cluster	% nucleotide/amino acid identity with viruses in genetic cluster		
	I	II	III
I	94.0/96.8	88.0/90.4	89.4/94.9
II		96.1/95.7	89.5/92.0
III			95.6/98.0

Three major genetic clusters were identified in the North American lineage. Cluster I comprised the viruses isolated from wild and domestic birds during the 1970s into the early 1990s. From the available data, a clear temporal division was observed around 1994: cluster II mainly comprised the domestic poultry-origin H7 viruses isolated during1994–2006, and cluster III comprised the viruses recovered from wild and domestic birds after 1993. The tree topology was supported by the sequence identities: the average shared identities between HA1 nucleotide sequences within these clusters were 94.0% (cluster I), 96.1% (cluster II), and 95.6% (cluster III), and the average identities between viruses from one cluster and another were 88.0% (clusters I and II), 89.5% (clusters II and III), and 89.4% (clusters I and III) (Table 10).

Cluster I was estimated to emerge around 1969 (Table 12), and it circulated in North America for 24 years before its extinction in 1993. This cluster included one isolate recovered from a seal in 1980. Subtype H7N2 was the predominant subtype in cluster II, and the H7N2 viruses were mainly isolated from the LPM system in the northeastern United States and from commercial poultry farms in four US states (Maryland, Pennsylvania, North Carolina, and Virginia). Cluster II was further divided into two clades: II-1 and II-2. Clade II-1 mainly consisted of viruses isolated during 1994-1996, and clade II-2 consisted of viruses isolated during 1996-2006. A clear

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molecular difference separated these two clades: a deletion of eight amino acids at positions 212–219 in the HA1 protein. Cluster II included A/New York/107/2003(H7N2), which infected a human with suspected exposure to poultry (202). A similar 8-amino acid deletion was also observed in the HA1 protein of this virus.

Cluster III represents the contemporary genetic pool of H7 AIVs in North America, including viruses from wild birds and from outbreaks among domestic poultry. H7 viruses isolated during individual outbreaks among domestic poultry formed independent monophyletic clades in the phylogeny, suggesting that they originated from separate introductions from wild birds (194). Three of the H7 viruses evolved into HPAI viruses: A/chicken/Canada/314514-2/2005(H7N3), A/chicken/SK/HR-00011/2007(H7N3), and A/chicken/Jalisco/CPA1/2012(H7N3). Another H7 introduction, A/chicken/Delaware/10851/2014(H7N7), did not evolve into an HPAI virus.

Limited Antigenic Diversity among Tested H7 AIVs

Serologic analyses showed that the antisera generated against 15 selected isolates cross-reacted with the tested H7 isolates to different extents (Table 11, Table 13). Antigenic cartography showed no clear division of these isolates (Figure 23). The average antigenic distance among these isolates was 1.14 units (SD, 0.57 unit), and each unit represented a 2-fold change in HI titer. The hierarchical clustering method grouped these viruses into one cluster at a distance of 1.46 units, except for one outlier, A/laughing gull/NJ/2455/2000(H7N3). The average distance from the outlier to the remaining isolates was 2.01 units. Viruses from three distinct genetic clusters were grouped in this antigenic cluster; no clear correlation between the genetic diversity and antigenic property was observed (Figure 23).

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Table 11 Cross-HI data obtained for testing H7 avian influenza viruses against representative chicken sera. The homologous titers were underlined.

Virus	Titer to chicken antiserum								
	BUFF1 20022	MALL1 22457	MALL4 65618	AGWT 551331	ABDU8 70108	MALL7 50842	MALL7 09657	AGWT 115995	MALL5 58620
A/bufflehead/Virginia/A00120022/2008	<u>80</u>	80	80	160	320	80	80	80	80
A/mallard/New Jersey/A00122457/2008	80	<u>80</u>	80	80	160	80	40	20	80
A/mallard/Wisconsin/A00465618/2008	80	160	<u>160</u>	160	160	160	40	40	80
A/American green-winged teal/Colorado/A00551331/2007	80	160	80	<u>80</u>	160	80	40	20	40
A/American black duck/Delaware/A00870108/2010	80	80	80	160	<u>160</u>	80	80	80	160
A/mallard/Montana/A00750842/2009	160	160	320	320	320	<u>320</u>	160	160	160
A/mallard/Nebraska/A00709657/2009	80	320	80	80	160	80	<u>160</u>	160	80
A/American green-winged teal/Arizona/A00115995/2009	20	80	40	40	80	40	40	<u>40</u>	80
A/mallard/Iowa/A00558620/2008	20	80	40	40	40	40	40	20	<u>40</u>
A/mallard/Indiana/A00142205/2008	160	160	160	160	160	160	160	160	40
A/American green winged teal/Colorado/A00660616/2008	40	160	40	80	160	40	40	40	40
A/American green-winged teal/Utah/A00461136/2009	20	160	160	160	160	40	40	40	40
A/blue winged teal/Missouri/A00624484/2008	160	160	160	160	160	160	160	160	80
A/ring-necked duck/Texas/A00766403/2009	40	160	80	80	160	80	40	40	40
A/blue-winged teal/South Dakota/A00772794/2009	160	640	320	320	320	320	320	320	640
A/seal/MA/1/1980	40	160	80	160	80	40	40	40	80
A/chicken/CT/260413-2/2003	40	40	40	80	40	40	20	20	40
A/chicken/British Columbia/314514-2/2004	80	160	160	160	160	160	80	160	160
A/turkey/Oregon/1971	160	320	320	640	640	160	160	320	320
A/turkey/MN/38429/1988	40	40	80	80	80	40	40	40	40
A/chicken/Jalisco/CPA-12283/2012	40	40	80	80	80	40	40	40	40
A/turkey/VA/SEP-67/2002	40	40	80	80	80	80	80	80	80
A/turkey/NY/4450-4/1994	80	80	80	160	80	80	80	40	80
A/duck/Alberta/49/1976	80	320	160	160	160	160	160	80	320
A/pintail/MN/423/1999	80	160	320	640	320	160	80	80	320
A/laughing gull/NJ/2455/2000	10	10	320	640	320	160	320	160	320
A/ruddy turnstone/DE/1538/2000	40	80	80	80	80	40	40	80	160
A/mallard/Ohio/421/1987	40	160	80	80	80	40	40	40	80
A/cinnamon teal/Mexico/2817/2006	80	160	320	320	320	160	160	160	320
A/ruddy turnstone/DE/892/2006	20	40	40	40	80	20	20	20	40
A/ruddy turnstone/NJ/207/06	20	40	40	40	40	40	20	20	40
A/American green-winged teal/Arizona/A00115994/2009	40	80	80	160	80	80	80	40	80
A/blue-winged teal/Minnesota/A00137660/2009	80	160	80	80	80	80	80	40	320

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Table 11 (continued)

Virus									
A/domestic duck/West Virginia/A00140912/2008	40	80	80	80	80	40	160	20	40
A/domestic duck/West Virginia/A00140913/2008	80	80	80	80	80	80	40	40	40
A/domestic duck/West Virginia/A00140915/2008	40	80	80	80	80	80	40	20	40
A/American green-winged teal/Wyoming/A00230796/2008	40	80	80	80	80	80	40	40	80
A/mute swan/Rhode Island/A00325105/2008	160	160	160	80	160	40	40	40	40
A/mute swan/Rhode Island/A00325108/2008	40	80	40	40	40	20	20	20	20
A/mute swan/Rhode Island/A00325112/2008	20	40	40	40	40	20	20	20	80
A/mute swan/Rhode Island/A00325114/2008	20	40	40	40	40	20	40	40	80
A/mute swan/Rhode Island/A00325115/2008	160	160	40	80	160	40	40	40	40
A/mute swan/Rhode Island/A00325117/2008	40	80	40	40	40	20	20	20	20
A/mute swan/Rhode Island/A00325125/2008	40	80	40	40	80	40	40	20	40
A/mute swan/Rhode Island/A00325129/2008	20	40	40	40	40	20	20	20	40
A/mute swan/Rhode Island/A00325136/2008	40	80	80	80	80	40	40	20	40
A/mallard/Illinois/A00325439/2009	40	80	80	80	80	40	40	40	40
A/northern shoveler/Utah/A00374996/2007	80	160	160	160	160	160	80	40	80
A/mallard/Oklahoma/A00449368/2009	80	160	80	80	80	40	40	40	80
A/mallard/Oklahoma/A00449455/2009	16	20	20	20	20	20	20	20	20
A/mallard/Delaware/A00456271/2009	80	320	320	320	320	160	80	80	160
A/northern shoveler/Utah/A00461133/2009	80	80	80	80	80	80	80	80	80
A/American green-winged teal/Utah/A00461135/2009	80	80	80	80	80	80	80	80	80
A/blue-winged teal/Texas/A00463679/2010	80	320	160	160	160	80	80	40	160
A/northern pintail/Texas/A00466052/2009	40	80	40	80	160	40	40	20	40
A/American green-winged teal/Mississippi/A00468514/2009	160	160	320	320	320	160	320	160	160
A/northern shoveler/Utah/A00468715/2009	20	20	20	20	40	20	20	20	40
A/northern shoveler/Utah/A00468752/2009	80	80	80	80	80	80	80	80	80
A/northern shoveler/Utah/A00468766/2009	80	160	80	80	160	80	40	40	20
A/American green-winged teal/Utah/A00468772/2009	40	80	80	80	80	40	40	40	160
A/northern shoveler/Nevada/A00505416/2008	40	80	80	80	80	40	40	80	40
A/mallard/Kansas/A00523306/2008	80	160	80	160	160	80	40	40	40
A/blue-winged teal/Louisiana/A00557206/2009	160	160	160	160	160	160	40	80	160
A/northern shoveler/Louisiana/A00557321/2009	40	80	40	40	40	40	40	20	320
A/American green-winged teal/Texas/A00586649/2009	40	80	80	80	80	40	40	40	20
A/northern shoveler/Mississippi/A00602284/2009	160	640	320	640	640	160	320	160	160
A/American green-winged teal/Texas/A00604024/2009	80	160	160	160	160	80	80	40	40
A/American green-winged teal/Texas/A00604029/2009	40	80	80	80	80	40	40	40	40
A/American green-winged teal/Texas/A00604032/2009	320	160	320	320	320	160	160	80	320
A/American green-winged teal/Texas/A00604814/2009	160	320	160	320	160	160	80	80	320
A/blue-winged teal/Texas/A00605473/2009	20	80	40	40	40	20	20	20	20
A/American green-winged teal/Utah/A00614935/2009	160	320	160	160	320	160	160	160	320

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Table 11 (continued)

A/blue-winged teal/Missouri/A00624483/2008	160	80	40	40	160	40	20	20	20
A/American green-winged teal/Mississippi/A00630203/2009	20	160	160	160	160	160	40	20	160
A/blue-winged teal/Louisiana/A00637297/2009	40	80	40	40	40	20	20	20	40
A/mallard/South Dakota/A00649542/2008	80	160	80	80	160	80	40	20	40
A/American green-winged teal/Utah/A00654391/2009	80	80	80	80	80	80	40	40	40
A/northern shoveler/Oregon/A00654616/2008	80	320	80	80	160	80	80	160	160
A/gadwall/Arizona/A00663934/2009	80	80	80	160	160	80	40	20	40
A/blue-winged teal/Texas/A00676566/2009	40	320	80	80	80	80	80	80	80
A/northern shoveler/Mississippi/A00682947/2008	160	80	320	320	160	320	160	160	640
A/mallard/New York/A00723392/2009	20	80	40	40	40	20	20	20	40
A/mallard/New York/A00723400/2009	40	80	80	160	160	80	40	40	64
A/mallard/Oklahoma/A00744383/2009	80	160	80	160	160	80	160	160	320
A/northern shoveler/Oklahoma/A00744384/2009	80	320	80	80	160	80	80	80	320
A/mallard/Oklahoma/A00749161/2009	40	80	40	40	80	40	40	40	40
A/mallard/Illinois/A00755320/2009	80	320	160	160	320	160	80	80	160
A/American green-winged teal/Utah/A00831743/2009	160	160	160	160	160	160	40	40	40
A/northern shoveler/Utah/A00831758/2009	80	160	320	320	160	160	160	160	160
A/American green winged teal/Utah/A00833077/2009	40	160	160	40	160	160	40	40	20
A/mallard/Michigan/A00869519/2009	80	320	80	80	80	80	80	80	320
A/mallard/New Jersey/A00926089/2010	80	160	40	80	80	40	40	20	40
A/northern shoveler/Mississippi/A00630207/2009	160	320	320	320	320	160	80	80	80

Red indicates virus in cluster I, green indicates virus in cluster II, and blue indicates virus in cluster III.

Abbreviations: BUFF120022, A/bufflehead/VA/A00120022/2008(H7N2); MALL122457, A/mallard/NJ/A00122457/2008(H7N8);
MALL465618, A/mallard/WI/A00465618/2008(H7N3); AGWT551331, A/American green winged teal/CO/A00551331/2007(H7N3);
ABDU870108, A/black duck/DE/A00870108/2010(H7N3); MALL750842, A/mallard/MT/A00750842/2009(H7N3);
MALL709657, A/mallard/NE/A00709657/2009(H7N3); AGWT115995, A/American green winged teal/AZ/A00115995/2009(H7N7);
MALL558620, A/mallard/IA/A00558620/2008(H7N3); MALL142205, A/mallard/IN/A00142205/2008(H7N3);
AGWT660616, A/American green-winged teal/Colorado/A00660616/2008(H7N3);
AGWT461136, A/American green-winged teal/Utah/A00461136/2009(H7N1);
BWTE624484, A/blue-winged teal/Missouri/A00624484/2008(H7N3);
RNDU766403, A/ring-necked duck/Texas/A00766403/2009(H7N1);
BWTE772794, A/blue-winged teal/South Dakota/A00772794/2009(H7N7).

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Viruses isolated from wild and domestic birds lacked antigenic diversity. The average antigenic distance among wild bird-origin isolates was 1.14 units (SD, 0.57 unit). With the exception of one outlier, A/laughing gull/NJ/2455/2000(H7N3), hierarchical clustering showed that these isolates merged into one cluster at a distance of 1.47 units. The average antigenic distance among seven poultry-origin isolates was 1.13 units (SD, 0.71 unit). These viruses were grouped into one cluster, at a distance of 1.63 units, by the hierarchical clustering method. Poultry-origin isolates were antigenically similar to those from wild birds; the distance between poultry-origin isolates and the most antigenically similar wild bird-origin isolates was 0.18–0.86 units.

Limited antigenic diversity was supported by the comparison of amino acid sequence of the 135 residues corresponding to those in the reported antibody binding sites in influenza HA protein. Results showed that the average shared identity among tested isolates was 96.6%, and lack of divergence of these amino acid positions (Amino_acid_variations_in_antibody_binding_sites_of_H7_AIVs.pdf).

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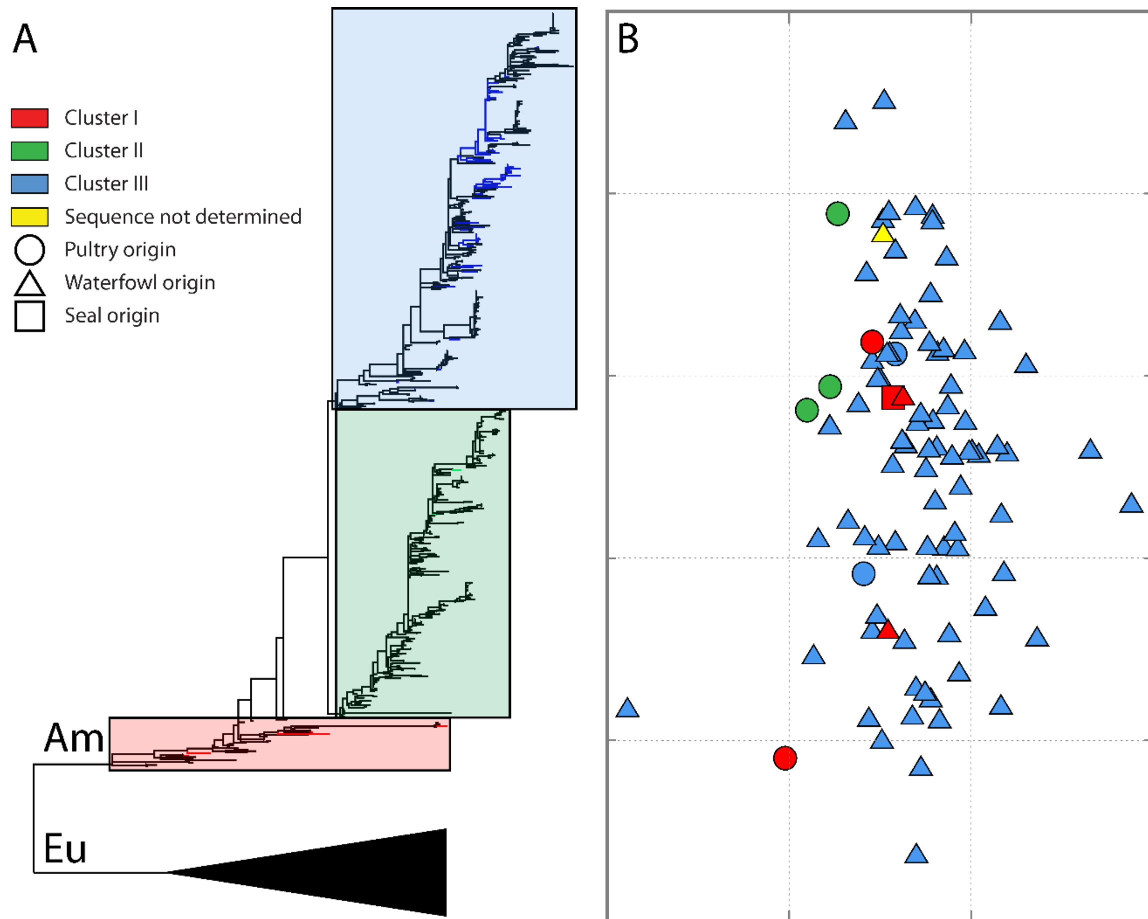

Figure 23 Comparison of genetic and antigenic evolution of H7 AIVs from North America.

(A) Schematic phylogenetic tree of the HA1 nucleotide sequences of H7 AIVs. Boxes represent the three major genetic clusters; branches corresponding to viruses subjected to antigenic characterization are colored-coded as described in the key; the same color code is used in (B). The Eurasian lineage (EU) is represented by the large black triangle, and Am represents North American lineage. (B) Antigenic cartography constructed on the basis of HI data. Each gridline (horizontal and vertical) represents a 2-fold difference in HI titer.

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Genetic Evolution Dynamics of the HA Gene

To evaluate the genetic evolution dynamics for the HA gene of H7 AIVs isolated from North America, we analyzed (in temporal order) the genetic distance from each virus to A/turkey/Oregon/1971(H7N3), the oldest isolate in the phylogeny (Figure 24). The average evolutionary rate on the nucleotide level was determined by the slope of the linear regression line that fits the data. Viruses in cluster I demonstrated a gradual and stable increase of genetic distance to A/turkey/Oregon/1971(H7N3). The regression line for cluster I had a slope of 0.0061 (adjusted $R^2 = 0.88$; $p < 2.20E-16$). The lack of continuations from 1971 to 1976 was due to the limited sampling during that time period. Viruses in cluster II evolved at a faster rate than those in cluster III (0.0052 [adjusted $R^2 = 0.82$; $p < 2.20E-16$] vs. 0.00218 [adjusted $R^2 = 0.42$; $p < 2.20E-16$], respectively). Cluster II was separated into two clades at the 1996 time point, and an elevated increase in genetic distance was identified at that time. To determine the fluctuation of evolutionary dynamics in cluster II, we independently analyzed viruses in two clades. Regression analysis showed that viruses in clade II-1 evolved faster than those in clade II-2 (0.008 [adjusted $R^2 = 0.59$; $p < 4.07E-9$] vs. 0.0046 [adjusted $R^2 = 0.67$; $p <$2 .20E-16], respectively). Cluster III consisted of viruses isolated from wild birds and individual outbreaks among domestic poultry. To evaluate the evolutionary status for H7 AIVs in wild birds more precisely, we conducted additional analyses for wild bird-origin viruses in this cluster. The slope of the regression line was 0.0019 (adjusted $R^2 = 0.31$; $p < 2.20E-16$), which is lower than that for the entire population.

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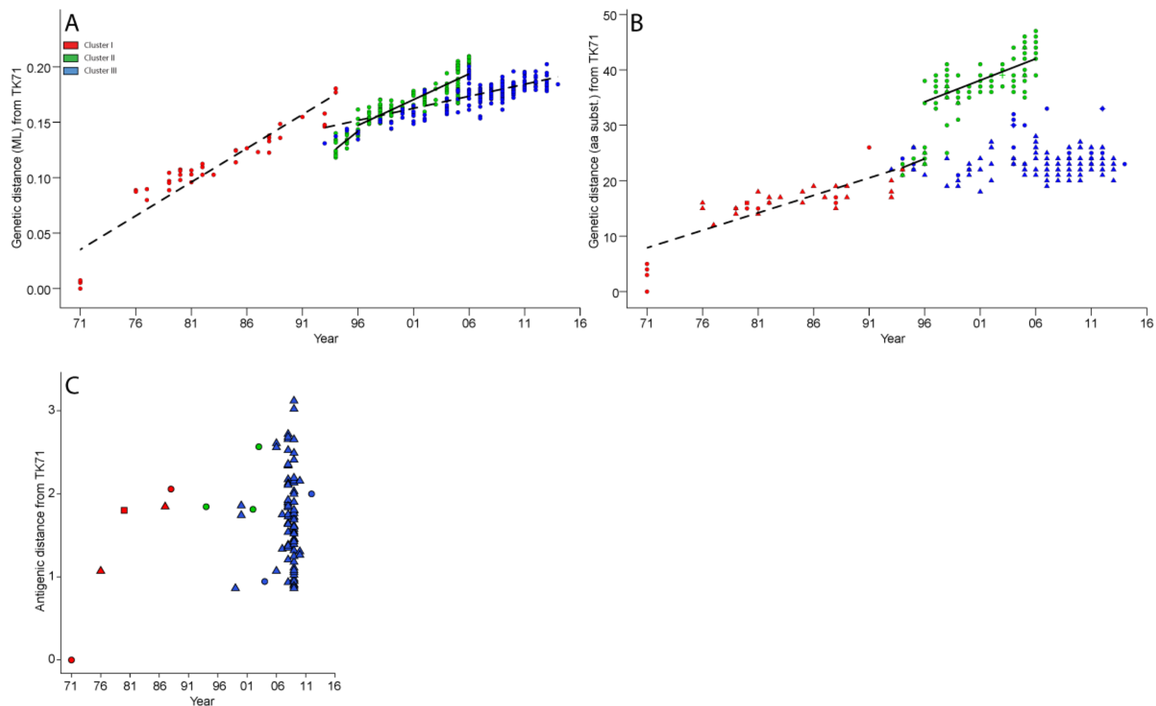

Figure 24 Comparison of temporal genetic and antigenic evolution dynamics for H7 AIVs from North America.

(A) Year-by-year analysis of genetic evolution dynamics. Genetic distances between each virus and A/turkey/Oregon/1971 (TK/71) were determined from phylogenetic analysis. Dashed line indicates linear regression fit for viruses in genetic clusters I and III; solid lines indicate linear regression fit for two clades in genetic cluster II. (B) Same as panel A, but genetic distances are characterized by the number of amino acid substitutions (aa subst.) in the HA1 protein. (C) Year-by-year analysis of antigenic evolution dynamics. Antigenic distances were calculated from antigenic cartography. 71 (1971), and 16 (2016). In panel B and C, dots indicate viruses isolated from domestic poultry, triangles indicate viruses isolated from wild birds, the square indicates virus isolated from a seal, and crosses indicate virus isolated from human.

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Similar analyses were conducted at the amino acid level by characterizing the genetic distance by the number of amino acid substitutions (Figure 24). A consistent evolution trend was observed for cluster I, which showed an average of 0.63 amino acid substitutions per year (adjusted $R^2 = 0.70$; $p < 3.27E\text{-}14$). Cluster II had an average change of 1.47 amino acids per year (adjusted $R^2 = 0.71$; $p < 2.20E\text{-}16$). A dramatic increase in the number of amino acid substitutions was observed in 1996; this finding is consistent with the observed deletion of eight amino acids in the HA1 protein at the same time. However, viruses in clades II-1 and II-2 had a similar average rate of evolution. The slopes of regression lines for these two clades were 0.80 (adjusted $R^2 = 0.26$; $p < 0.00044$) and 0.78 (adjusted $R^2 = 0.46$; $p < 2.20E\text{-}16$), respectively. The temporal fluctuation of amino acid substitutions for viruses in cluster III was not well fitted by the linear regression model. However, it was observed that the number of amino acid substitutions in viruses isolated from poultry and humans is larger than that in wild bird-origin viruses.

Rates of mean nucleotide substitution and the time to the most recent common ancestor were also estimated by using the Bayesian Markov Chain Monte Carlo method (Table 12). The mean evolutionary rate for the cluster I was estimated as 5.11×10^{-3} substitutions per site per year (sub/site/year) (95% HPD, $3.94–6.25 \times 10^{-3}$). Cluster II evolved at a higher mean evolutionary rate (5.34×10^{-3} sub/site/year; 95% HPD, $4.68–6.11 \times 10^{-3}$) than cluster III (4.84×10^{-3} sub/site/year; 95% HPD, $4.34–5.31 \times 10^{-3}$). For cluster II, the estimated evolutionary rate for a collection of viruses in clade II-2 decreased to 4.90×10^{-3} sub/site/year (95% HPD, $4.19–5.67 \times 10^{-3}$). This result suggested that cluster II experienced a higher evolutionary rate during 1994–1996 and a

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lower evolutionary rate afterwards. For cluster III, the estimated evolutionary rate for a collection of wild bird-origin viruses was 4.60×10^{-3} sub/site/year (95% HPD, 4.10–5.11 $\times 10^{-3}$), which is lower than that for the entire cluster III population.

The corresponding analysis of antigenic evolution dynamics showed that antigenicity was relatively stable during 1971–2012 (Figure 24). No clear correspondence was observed between the genetic and antigenic evolution dynamics. Of the 93 characterized viruses, 72 were isolated in 2008 and 2009; the antigenic distances between these viruses and A/turkey/Oregon/1971(H7N3) ranged from 0.86 to 3.12 units. The antigenic distances between the other 21 isolates and A/turkey/Oregon/1971(H7N3) also fall into this range.

Table 12 Estimated rates of nucleotide substitution and time to most recent common ancestor for H7 AIVs from distinct genetic clusters from North America.

Genetic cluster	Model	Substitution rate ($\times 10^{-3}$ substitutions/site/year)			TMRCA (calendar year)		
		Mean	95% HPD[a]	ESS[b]	Mean	95% HPD	ESS
I	SRD06-UCL–Skyline[c]	5.11	3.94–6.25	347	1969	1963–1970	289
II	SRD06-UCL–Skyline	5.34	4.68–6.11	693	1993	1990–1993	406
II[d]	SRD06-UCL–Skyline	4.90	4.19–5.67	856	1996	1995–1996	1070
III	SRD06-UCL–Skyline	4.84	4.34–5.31	389	1992	1991–1992	780
III[e]	SRD06-UCL–Skyline	4.60	4.10–5.11	299	1992	1990– 1992	693

[a] HPD: highest probability density.
[b] Effective sample size.
[c] SRD06: HKY substitution model; UCL: uncorrelated lognormal molecular clock, Skyline Coalescent Bayesian Skyline tree model.
[d] Includes only viruses isolated after 1996.
[e] Includes only viruses isolated from wild birds.

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Table 13 Cross HI data obtained for representative H7 AIVs against chicken serum.

Virus	Titer to chicken antiserum generated against 15 selected isolates														
	BUFF120022	MALL122457	MALL465618	AGWT551331	ABDU870108	MALL750842	MALL709657	AGWT115995	MALL558620	MALL142205	AGWT660616	AGWT461136	BWTE624484	RNDU766403	BWTE772794
A/turkey/Oregon/1971	160	320	320	640	640	160	160	320	320	320	40	40	160	320	640
A/duck/Alberta/49/1976	80	320	160	160	160	160	160	80	320	320	160	160	80	160	320
A/seal/MA/1/1980	40	160	80	160	80	40	40	40	80	80	80	80	20	80	160
A/mallard/Ohio/421/1987	40	160	80	80	80	40	40	40	80	160	80	80	20	80	160
A/turkey/MN/38429/1988	40	40	80	80	80	40	40	40	40	80	40	80	40	80	80
A/turkey/NY/4450-4/1994	80	80	80	160	80	80	80	40	80	80	160	80	40	80	40
A/pintail/MN/423/1999	80	160	320	640	320	160	80	80	320	320	160	80	80	160	320
A/laughing gull/NJ/2455/2000	10	10	320	640	320	160	320	160	320	320	320	640	160	320	640
A/turkey/VA/SEP-67/2002	40	40	80	80	80	80	80	80	80	80	40	80	40	80	80
A/chicken/CT/260413-2/2003	40	40	40	80	40	40	20	20	40	40	40	20	20	40	40
A/chicken/British Columbia/314514-2/2004	80	160	160	160	160	160	80	160	160	160	80	80	80	160	320
A/cinnamon teal/Mexico/2817/2006	80	160	320	320	320	160	160	160	320	320	320	320	160	320	320
A/northern shoveler/Utah/A00374996/2007	80	160	160	160	160	160	80	40	80	160	80	320	40	320	320
A/bufflehead/Virginia/A00120022/2008	80	80	80	160	320	80	80	80	80	640	160	320	80	160	320
A/American green-winged teal/Arizona/A00115994/2009	40	80	80	160	80	80	80	40	80	160	40	160	40	160	160
A/blue-winged teal/Texas/A00463679/2010	80	320	160	160	160	80	80	40	160	320	80	320	80	160	320
A/chicken/Jalisco/CPA-12283/2012	40	40	80	80	80	40	40	40	40	80	40	80	40	80	160

The 17 selected H7 AIVs represent the maximal temporal coverage of 93 viruses isolated during 1971-2012.
Abbreviations:
BUFF120022, A/bufflehead/VA/A00120022/2008(H7N2); MALL122457, A/mallard/NJ/A00122457/2008(H7N8); MALL465618, A/mallard/WI/A00465618/2008(H7N3); AGWT551331, A/American green winged teal/CO/A00551331/2007(H7N3); ABDU870108, A/black duck/DE/A00870108/2010(H7N3); MALL750842, A/mallard/MT/A00750842/2009(H7N3);
MALL709657, A/mallard/NE/A00709657/2009(H7N3); AGWT115995, A/American green winged teal/AZ/A00115995/2009(H7N7);MALL558620, A/mallard/IA/A00558620/2008(H7N3);
MALL142205, A/mallard/IN/A00142205/2008(H7N3); AGWT660616, A/American green-winged teal/Colorado/A00660616/2008(H7N3);
AGWT461136, A/American green-winged teal/Utah/A00461136/2009(H7N1); BWTE624484, A/blue-winged teal/Missouri/A00624484/2008(H7N3);
RNDU766403, A/ring-necked duck/Texas/A00766403/2009(H7N1);BWTE772794, A/blue-winged teal/South Dakota/A00772794/2009(H7N7).

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Natural Selection for HA Gene of H7 AIVs in North America

The selection pressure for the HA gene was investigated independently for each of the three genetic clusters (Table 14). The mean d_N/d_S for cluster II (d_N/d_S = 0.1875−0.2154) was higher than that for cluster III (d_N/d_S = 0.1232−0.1407). The HA gene of viruses in clade II-2 had a lower mean d_N/d_S (0.1821−0.2121) than the entire population in cluster II. This finding suggests that the purifying selection for cluster II was lower during 1994–1996 than afterwards. Wild bird-origin isolates in cluster III were considered separately, and the results showed that the mean d_N/d_S was lower than that for the entire population in cluster III. This finding suggests that the purifying selection pressure was greater for wild bird-origin viruses than for poultry-origin viruses. Overall, the HA gene of H7 AIVs circulating in North America is under strong purifying selection, although there is variation in selection pressure for distinct genetic clusters.

Amino acid position 189 (H3 numbering; H7 numbering, 180) in the HA1 protein was found to be under positive selection for the wild bird-origin viruses in cluster III. The amino acid profile of this position for H7 AIVs from Eurasia and North America was analyzed. Viruses isolated from land-based poultry and waterfowl were considered separately (Table 15). For viruses in the North American lineage, position 189 was found to be highly polymorphic in genetic clusters I and III: three and five distinct amino acids were observed, respectively, and T was the major amino acid in both clusters. HA gene in cluster II showed a distinct and conserved profile for position 189; with only one exception, S was the predominant amino acid. Amino acid profiles were also analyzed for three H7 viruses isolated from humans in North America: A/NewYork/107/2003(H7N2),

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which is from cluster II, and A/Mexico/InDRE7218/2012(H7N3) and A/Canada/rv504/2004(H7N3), which are from cluster III. Amino acid S was observed at position 189 in A/NewYork/107/2003(H7N2), and amino acid T was observed in the other two viruses. Amino acid S was not observed at position 189 of Eurasian lineage H7 AIVs.

Table 14 Selection pressure in HA1 protein of H7 AIVs from North America.

Genetic cluster, model	d_N/d_S	Positively selected sites (probability)
I		
M1a	0.1048	
M7	0.0999	
II		
M1a	0.2154	
M7	0.1875	
II[a]		
M1a	0.2121	
M7	0.1821	
III		
M1a	0.1407	
M7	0.1232	
III[b]		
M1a	0.1242	
M8	0.1086	189[c] (99.7%)

[a] Include only viruses isolated after 1996.
[b] Include only viruses isolated from waterfowl.
[c] H3 numbering.

Table 15 Amino acid polymorphism at position 189 (H3 numbering) in HA1 protein for H7 AIVs from North America and Eurasia.

Genetic group	Species	No. of sequences	Relevant amino acids	No. of corresponding amino acids
NA I[a]	L[b]	16	T	16
	W	32	A/N/T	5/4/23
NA II	L	221	I/S	1/220
	W	15	S	15
NA III	L	39	A/N/T	1/6/32
	W	351	A/D/I/N/T	11/48/1/20/271
NA human isolates	H[e]	3	S/T	1/2
EA[c]	L/W	1,118	A/G/N/T	670/1/1/446

[a] Genetic cluster I in North American lineage.
[b] L, land-based poultry; W, waterfowl; H, human.
[c] Eurasian lineage.

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Frequent Reassortment of Internal Genes of H7 AIVs in NA-WB Lineage

Panoramic phylogenetic analyses of all H7 complete genomes showed a clear division of the two major lineages (North American and Eurasian) for each gene segment (Figure 25). It is noteworthy that the topology of the NS gene segment phylogeny showed a deep divergence between the A and B alleles: within each allele, virus isolated from North America and Eurasia was separated. Phylogenetic analysis of internal genes in the NA-WB lineage demonstrated a high level of heterogeneity (Figure 26). Multiple distinct clades could be identified for each gene segment. The largest number of clades was observed in the PB2 phylogeny, which had 14 clades. Phylogeny of PB1, PA, and NP gene segments could be separated into 12 clades. Less genetic diversity was observed for the matrix protein and NS gene segments, which had three and two distinct clades, respectively.

The frequencies and patterns of reassortment of the six internal gene segments were assessed by determining the congruence among each gene's phylogenetic tree. Results showed that the topologies of the internal gene trees were more similar to each other than to random phylogenetic trees (Figure 27), suggesting that the internal gene segments are not completely independent from each other. However, the dissimilarities in tree topology were extensive. The most significant incongruence was observed between NS and other gene segments; the NS gene tree was found to be closer in topology to random trees rather than to the other five internal gene trees. These results indicate frequent reassortment of the internal genes for H7 AIVs in lineage NA-WB; no clear link among specific gene segments was observed.

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We assigned genotypes to viruses in the NA-WB lineage and analyzed the dynamics of these genotypes. Because 91% of the viruses were isolated during 2001–2013, our analysis was constrained to viruses from this time period. H7 AIVs in the NA-WB lineage demonstrated diverse genotypes: we identified 104 distinct genotypes for the internal gene constellation (Figure 28). Multiple genotypes were observed in the same year, and the largest number of genotype (29) was observed in 2009. Internal gene constellations were transient rather than stable, and no individual genotype existed throughout 2001–2013. A total of 92 genotypes existed for only one year, and two genotypes existed for the maximal time span of five years.

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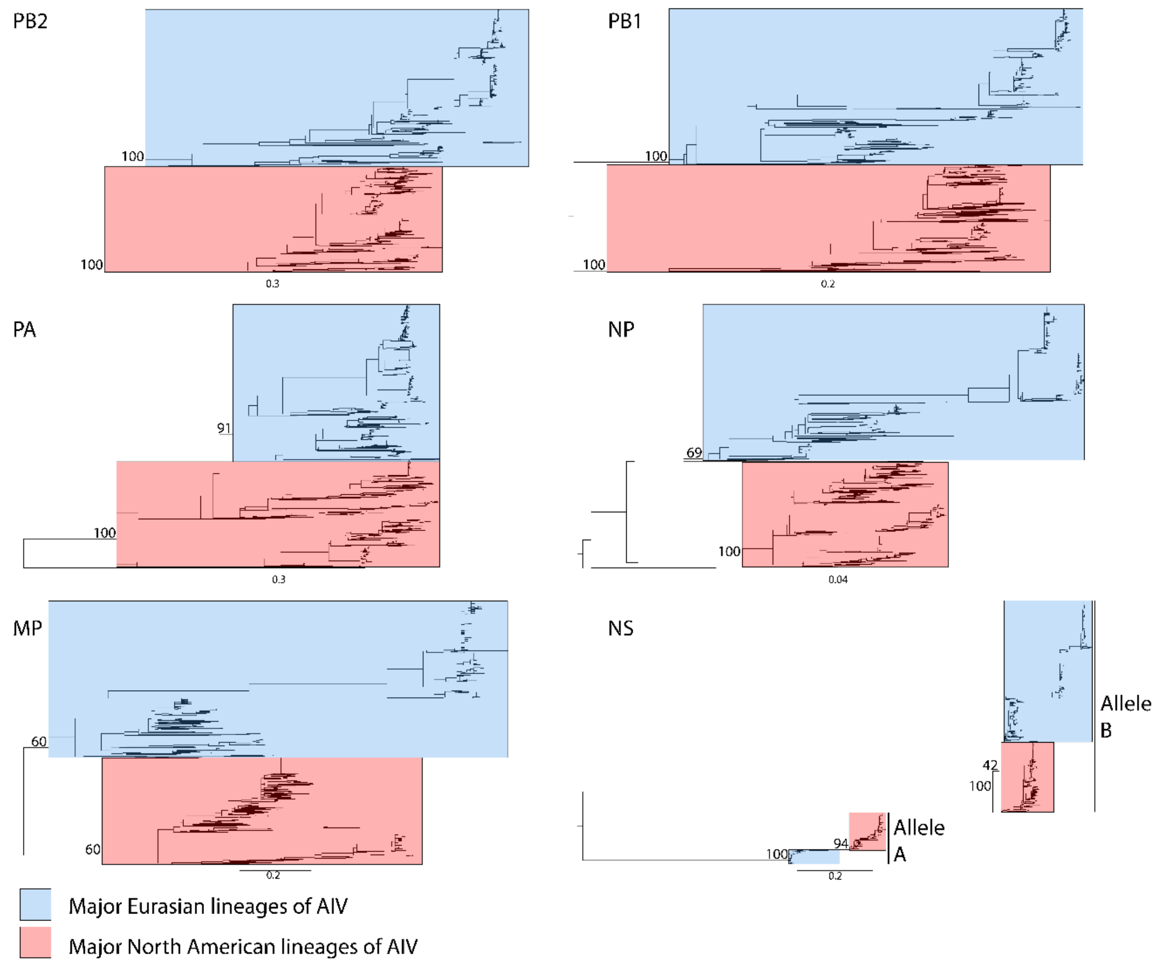

Figure 25 Maximum likelihood phylogenetic trees for internal gene segments of H7 AIVs from Eurasian and North American lineage.

Black bars on the lower right indicate two alleles in the NS gene segment phylogenetic tree. Bootstrap values estimated from 100 resamplings of the sequence data are shown adjacent to selected nodes.

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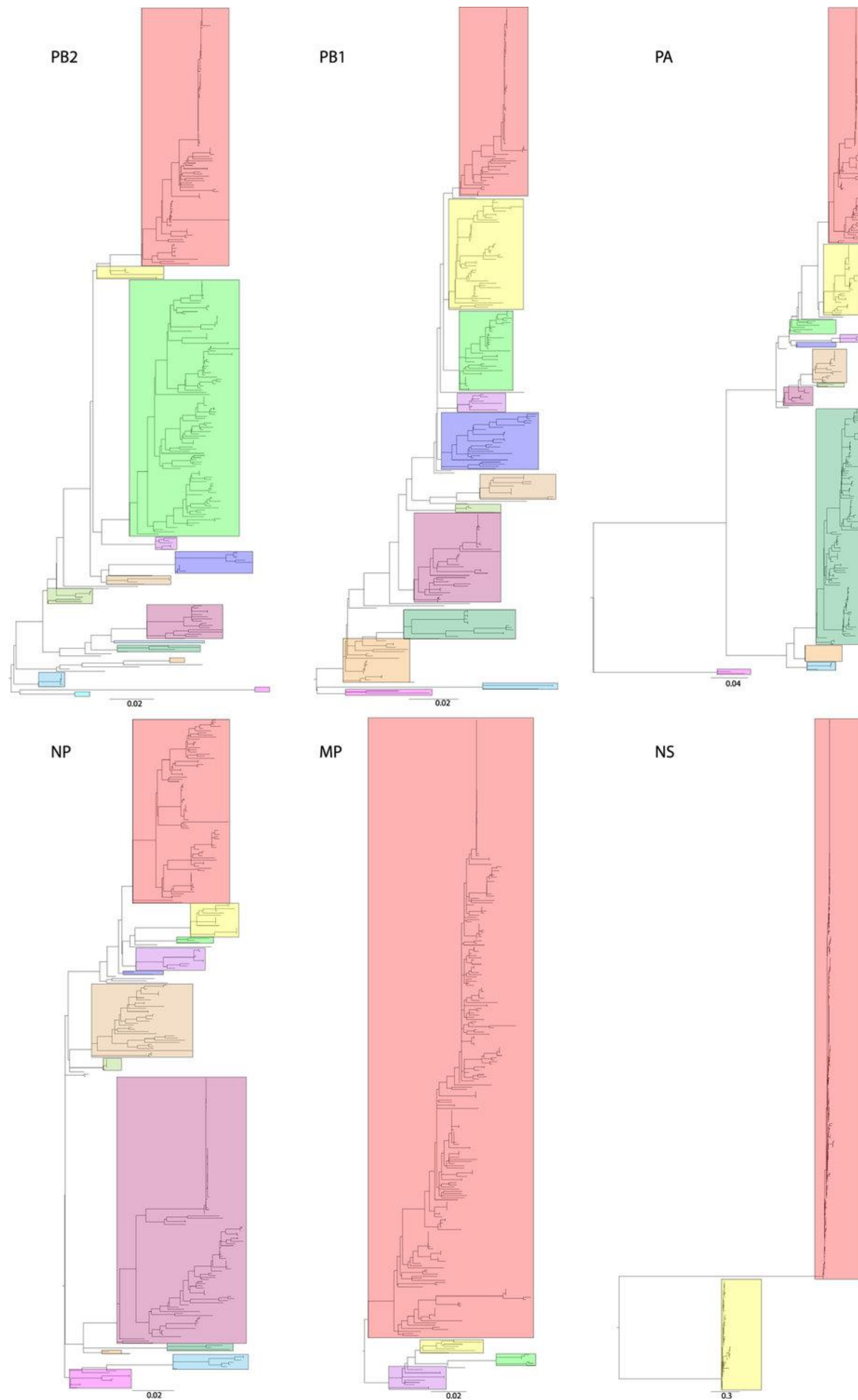

Figure 26 Maximum likelihood phylogenetic trees for internal gene segments of H7 AIVs derived from wild birds in North America.

Boxes represent the genetic clades. Scale bars indicate substitutions per site.

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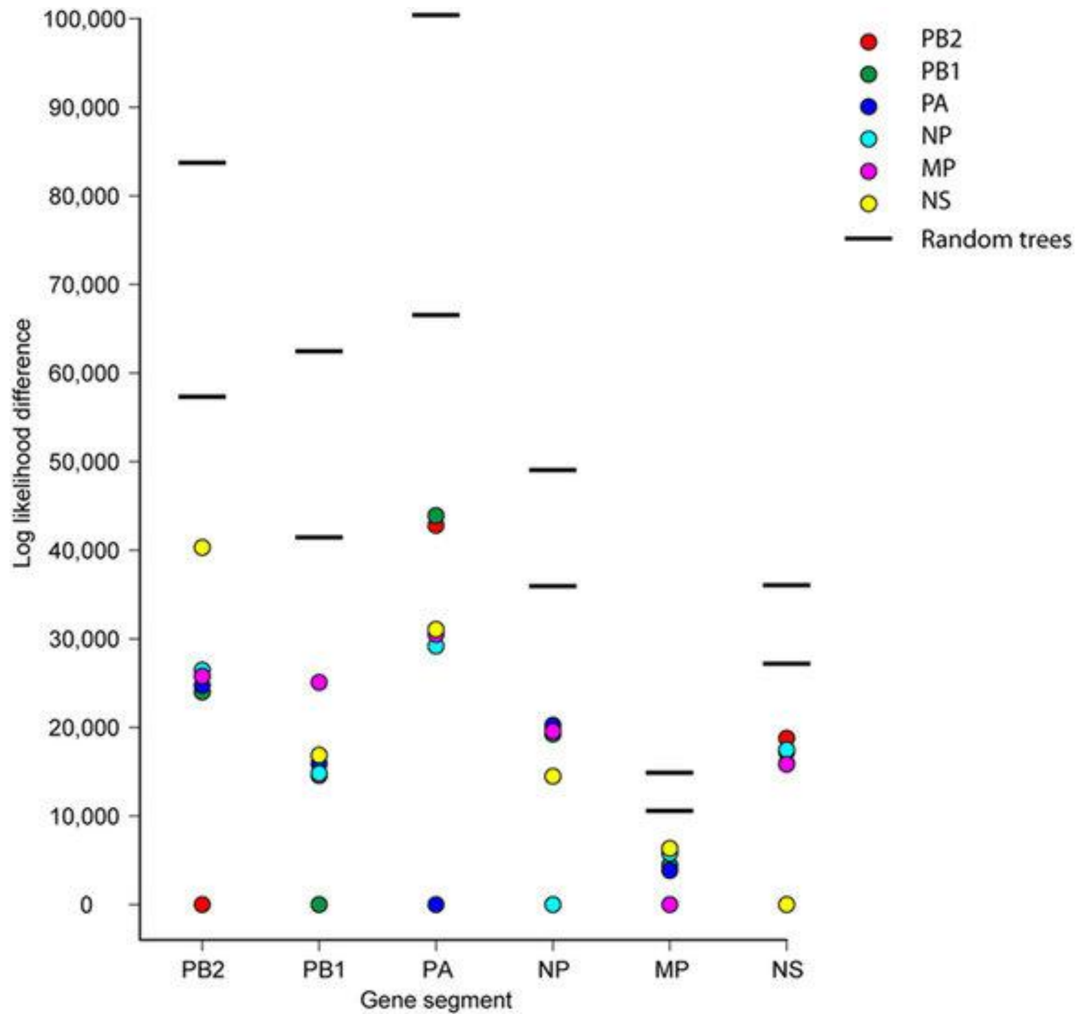

Figure 27 Congruence in topology among six internal gene segment phylogenetic trees for H7 AIVs derived from wild birds in North America.

Each column represents the difference in log likelihood value when six internal gene phylogenies and 100 random phylogenies were fitted to the same dataset. The difference in log likelihood value for each gene phylogeny is indicated by a colored dot. Range for random phylogenies is represented by two solid lines.

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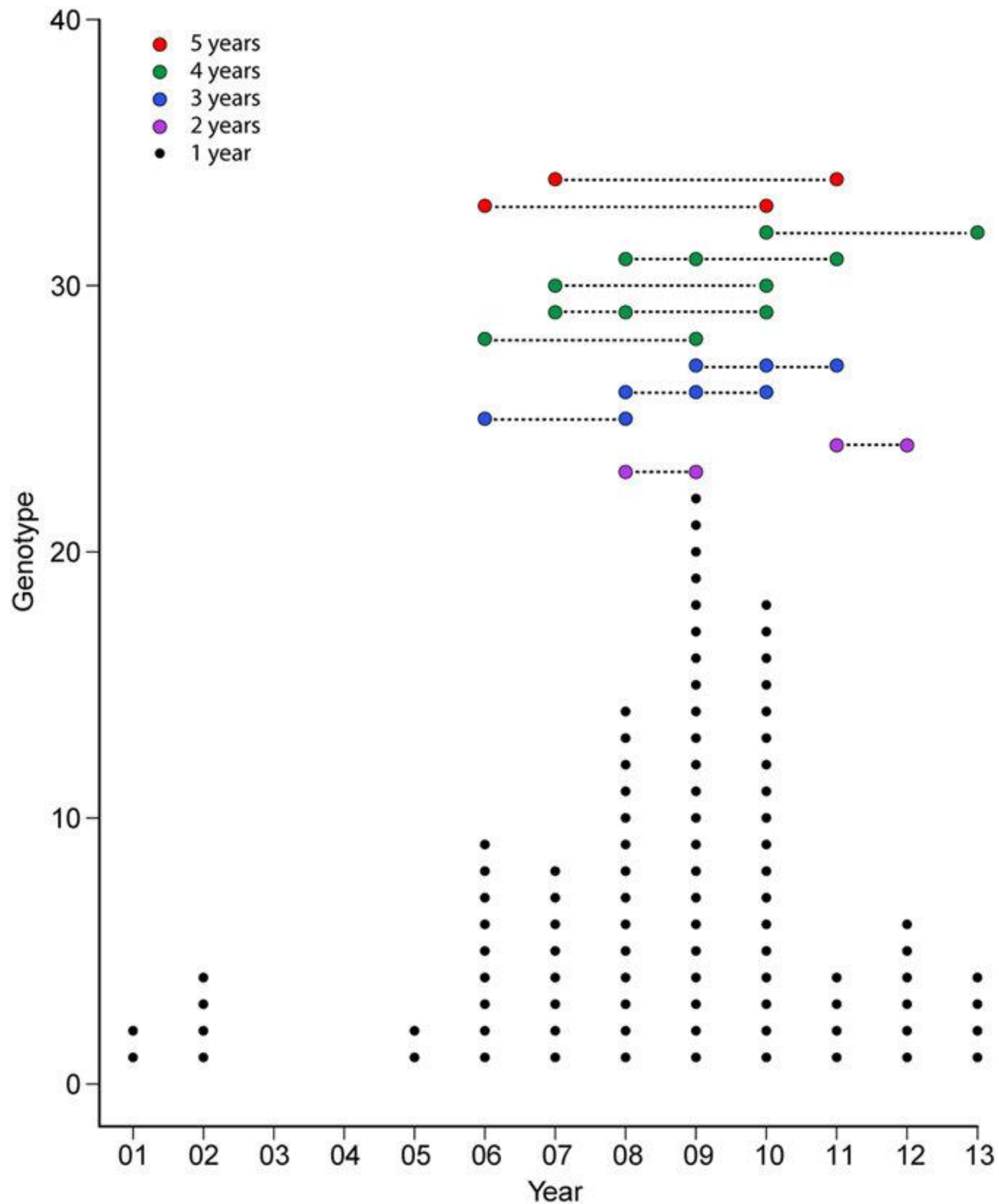

Figure 28 Year-by-year analysis of the evolution dynamics of internal gene constellations for H7 AIVs derived from wild birds in North America during 2001–2013.

Small black dots indicate gene constellations that existed for only one year. Larger colored dots indicate gene constellations that were observed in multiple years; the span of years is indicated by a dashed line between the dots, and colors indicate the number of years constellations existed.

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Discussion

We genetically and antigenically characterized 93 H7 AIVs isolated from North America. Our results show that H7 AIVs in North America have wide genetic diversity. Gradual accumulation of nucleotide and amino acid substitutions is observed for the HA gene of H7 AIVs isolated from wild and domestic birds. Our results also show a limited antigenic diversity among the H7 viruses we tested.

In wild birds, the limited antigenic diversity for H7 AIVs is consistent with the concept of evolutionary stasis (248). These results are consistent with those from other studies of wild bird-origin H7 viruses in other regions. For example, a 2005 study showed that four H7 AIVs isolated from mallards in Sweden and the Netherlands in 2000 and 2002 had relatively conserved antigenic properties (249). In addition, antigenic differences between these mallard-derived H7 isolates and an HPAI H7N7 strain isolated in the Netherlands in 2003 were within a 4-fold change. No significant antigenic differences (i.e., within a 4-fold change) were observed for nine H7 AIVs isolated in Italy and China during 1999–2005 (196); of the nine isolates, four were H7N7 viruses derived from ducks in China in 2003, three were H7 isolates derived from mallards in Italy during 2001–2005, and two were H7 viruses derived from turkeys in Italy in 1999 and 2002.

Our molecular characterization results suggest that the genetic evolution pattern in the isolates we tested is gradual and stable. The estimated mean rate of evolution for the H7 gene of AIVs circulating among wild birds in North America is 4.60×10^{-3} sub/site/year, which is similar to the rate for Eurasian lineage H7 viruses (5.75×10^{-3} sub/site/year) (234). Selection analysis showed that the H7 gene is under strong purifying selection in wild birds; however, no amino acids at known antibody binding sites were

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under positive selection. The limited antigenic diversity among the H7 isolates might be associated with the absence of certain pattern recognition receptors in the immune response system of wild birds. These receptors are triggered by influenza virus and could initiate the activation of innate immune responses. A 2010 study (250) showed that the presence of the retinoic acid–inducible gene 1 (RIG-I) in ducks induces the production of IFN-β and expression of downstream IFN-stimulated antiviral genes; however, the absence of RIG-I in chickens contributes to their increased susceptibility (compared with that of ducks) to influenza virus. In addition, Toll-like receptors (TLR) induce the expression of type I IFN and proinfammatory cytokines (251). TLRs 3,7,8, and 9 upregulate in naturally occurring influenza, and they are associated with innate virus inhibitory and proinflammatory responses (252). TLRs in birds differ from those in mammals. Furthermore, TLR9 is absent in avian species, and many TLRs in wild birds have yet to be identified (253).

Vaccination is a key component of the control strategy for HPAI viruses. However, antigenic variations in AIVs from domestic poultry have been reported, and these variations seem to be caused by the use of vaccine in poultry flocks. In 2002, poultry flocks across Italy were vaccinated to prevent an outbreak of H7N3 virus; the vaccine contained an inactivated H7N1 AIV (i.e., a vaccine with the same HA subtype as the outbreak virus but with an antigenically and genetically different NA subtype). Antigenic characterization of a longitudinal collection of 41 isolates showed large antigenic difference between viruses isolated before and after implementation of the vaccination program (169). Antigenic change was mirrored by the simultaneous appearance of four amino acid substitutions within the antibody binding sites, and one

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amino acid was positively selected after the use of vaccine. In addition, the poultry vaccination program in Mexico has facilitated the antigenic evolution of HPAI H5N2 virus in that country. An outbreak of HPAI H5N2 virus was detected in 1994 in Mexico, and a vaccination program, using inactivated vaccine, was implemented in 1995. Phylogenetic analysis of 52 H5N2 viruses isolated during 1993–2002 showed that two genetically different sub-lineages had emerged after introduction of the vaccine program and replaced the early sub-lineages (168). Viruses in these two new sub-lineages had undergone antigenic drift and acquired a more than 4-fold antigenic change from the vaccine strain. Six amino acid substitutions located within antibody sites A, B, and C were detected for these two novel sub-lineages. Similar changes were observed for the emergence of an H5N1 influenza variant in China. Since September 2005, routine vaccination programs against H5N1 virus in domestic poultry have been conducted nationwide in China. However, results of serologic testing of serum samples from 1,113 chickens in Guangdong and Guizhou, China, suggested that among the vaccinated poultry, protection against an FJ-like H5N1 variant was poor compared with that against other co-circulating H5N1 viruses (254). The presence of vaccine pressure probably selected the FJ-like H5N1 viruses, and these viruses became predominant in the region.

The change of antigenicity in these outbreaks was mirrored by the observed fast rate of evolution. The H7N3 AIVs from the Italian epidemic were estimated to evolve at a mean rate of $8.04 \times 10 \times 10^{-3}$ sub/site/year, whereas the evolutionary rates for two emerging H5N2 sub-lineages in Mexico were estimated to be 12×10^{-3} and 10×10^{-3} sub/site/year, respectively. These evolutionary rates are significantly higher than those reported for H7 and H5 viruses from domestic poultry in the absence of vaccine program,

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as exemplified by the range of evolutionary rates for H5N1 viruses isolated from Thailand, Turkey, and Nigeria: 2.5×10^{-3} to 5.2×10^{-3} sub/site/year (166). The findings from these studies (166, 168, 169, 254) suggest that the presence of vaccine pressure may drive antigenic drift of AIVs in domestic poultry. In our study, antigenic cartography showed that the average antigenic distance among seven poultry-origin H7 isolates was 1.13 units (SD, 0.71 unit). The limited antigenic diversity may be attributable to the absence of large-scale vaccination programs against H7 AIVs in North America, with the exception of Mexico. The estimated mean evolution rate for the H7 gene of AIVs from domestic poultry in the United States was 5.34×10^{-3} sub/site/year. This rate is significantly lower than that found in the presence of a vaccination program but similar to that found in the absence of vaccine program.

Adaptations are required when IAVs are transmitted across species, including from waterfowl to land-based birds (e.g., chickens and turkeys) (9). HA protein plays an important role in host-cell recognition, and mutation in HA protein has been identified as a major determinant of host shift to domestic poultry. Previous research identified two amino acid substitutions in the HA1 protein for H7 AIVs after their introduction from wild birds to domestic poultry (150). Furthermore, a 1997 study showed that evolution of the HA gene could increase significantly after introduction into domestic poultry; the evolution rate for the HA gene of H5 AIVs increased significantly after introduction into domestic poultry in Mexico (255). Our results showed the same trend for the H7 gene of AIVs from North America: the HA gene of poultry-origin H7 AIVs evolves faster than that of waterfowl-origin viruses. Moreover, results showed that the evolution rate for the H7 gene was faster during 1994–1996 than in subsequent years. The rapid evolution

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during 1994–1996 was mirrored by an 8–amino acid deletion in 1996 at positions 212–219 in the HA1 protein. This deletion removes five of six consecutive amino acids in part of the receptor binding site. These findings suggest that the H7 gene underwent rapid adaptation in the receptor binding domain after introduction into domestic poultry.

Of interest, position 189 (H3 numbering; H7 numbering, 180) in the HA1 protein was identified as being under positive selection pressure, and this site is located in the receptor binding site for H7 IAVs (256). Extensive polymorphisms, representing six distinct amino acids (A, D, I, N, S, and T), are present at position 189. A previous study showed that propagation of human H1N1 influenza virus in embryonated chicken eggs could cause the substitution E189K at the HA1 protein (257). R189K was found to have contributed to the antigenic drift of H3N2 IAV (258), and position 189 plays an important role for the antigenicity of H3 equine influenza virus (259). Another study showed that single amino acid changes at position A186D (H3 numbering, 189) could increase yield of A/California/7/09(H1N1) virus in eggs (260). Most influenza viruses recovered from avian samples have been propagated by using chicken embryonated eggs, and a previous study demonstrated frequent adaptation of H1N1 waterfowl-origin AIVs during propagation in embryonated eggs (261). The receptors in chicken embryonated eggs and those in the mallard gastrointestinal track are not exactly the same. In addition, expression of sialic acids showed substantial host-specific distinctions among avian species. Expression of α2,3-linked sialic acids and α2,6-linked sialic acids were observed in chicken trachea, whereas α2,3-linked sialic acids were predominant in ducks (262, 263). Thus, it is possible that the polymorphisms and positive selection detected in position 189 in the HA1 protein were due to viral propagation in embryonated eggs.

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Our phylogenetic analysis identified the 2-way intercontinental flow of the AIV H7 gene through the migration of wild birds. At least two independent introductions (in 1992 and 1994) from the Eurasian genetic pool to the North American genetic pool were identified. These introductions were not observed in earlier studies with smaller datasets (264, 265). Early research detected a subtype H6 IAV in the United States with an HA gene derived from the Eurasian gene pool; the virus subsequently caused an outbreak among poultry in California during 2000–2002 (50). The introduced Eurasian H6 virus has led to the replacement of the endemic H6 AIVs in North America. Recently, outbreaks of novel HPAI H5 viruses have been detected in the United States and Canada. These viruses originated from a wholly Eurasia-origin H5N8 virus introduced to North America by migratory birds through Beringia in 2014 (266). Two novel reassortants (H5N2 and H5N1) were generated by reassortments with viruses circulating in North America. The Beringian Crucible, including Alaska and the Russian Far East, serves as a common breeding area for diverse bird species from Asia and North America. This area provides an ideal environment for reassortment between IAVs carried by migratory birds from distinct gene pools, and it allows for the intercontinental transfer of gene segments or whole virus. Gene flow increases the diversity in individual genetic pools, and diversity could enhance the risk of generating novel strains that can spread more efficiently among birds and even cross the species barrier and cause transmission to mammals.

Our evolutionary analysis revealed frequent reassortment of six internal gene segments of H7 AIVs in the NA-WB lineage; no clear link was identified for any specific gene segment. The internal gene constellation was diverse and transient. This finding was

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concordant with those from a 2008 study conducted with a smaller dataset of AIVs and without differentiating the HA subtype (48). However, our finding of a diverse and transient gene constellation differed from the finding of a limited number of stable internal gene cassettes for IAVs adapted to mammals. The eight gene segments of IAVs could evolve differently due to different selection pressures. The evolution of the internal gene segments may be determined by functional constraints rather than immune pressure for the genes coded for the two surface proteins. In wild birds, internal protein genes are highly conserved on the amino acid level, and they could form a large pool of functionally equivalent gene segments. Such a pool would allow frequent reassortment because the exchange of functionally equivalent gene segments is not likely to attenuate the relative fitness of the reassorted viruses. For mammalian adapted IAVs, certain internal gene constellations were suggested to confer a selective advantage to the virus (227). Mutations may be acquired for internal genes after adaptation to a new host, and those mutations would separate the viruses from those in the natural reservoir. The protein–protein interaction could force the co-evolution of these gene segments and maintain the existence of a specific internal gene constellation.

In summary, our findings demonstrate a limited antigenic diversity among contemporary H7 avian-origin IAVs from North America. H7 IAVs from wild birds, domestic poultry, and a seal, which together represent a diverse geographic and temporal coverage, were included in this study. The limitation of this study was the small number of H7N3 isolates from the ongoing AIV outbreak in Mexico. Additional studies on a longitudinal collection of H7N3 viruses from the outbreak in Mexico would add to our understanding of the influence vaccination programs have on genetic and antigenic

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evolutionary dynamics. LPAI H7N9 viruses are enzootic in China (234), but the antigenic properties of these viruses have not been characterized. An antigenic comparison of H7 AIVs from the United State and those from outbreaks in other regions, including China and Europe, is also lacking. Due to the possible transmission of H7 AIVs across regions and continents, constant monitoring of emerging H7 AIVs in North America must continue.

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CHAPTER VI

CONCLUSIONS

The emergence of novel avian origin IAVs presents a continuous threat to animal and public health. The objectives of this dissertation research are to infer the genesis of two emerging avian origin IAVs, LPAI H10N8 and HPAI H7N8 viruses, and to investigate the antigenic diversity and genetic evolution of H7 AIVs isolated from North America over the past 40 years. The findings and contributions are concluded as following.

First, a gradual increase in IAV prevalence and detection of H10 viruses was observed in the LPM visited by the first patient infected with the novel H10N8 virus. AIVs that are genetically close to the human H10N8 isolate were recovered from this LPM. High seroprevalence of H10 virus was observed in chickens and ducks from five LPMs in the city. These findings suggested that LPM is the most probable source of human infection with this novel H10N8 virus, and this virus appears to present throughout the LPM system in the city. These findings also highlight the role of LPMs in the zoonotic transmission of AIVs and suggest that the existing influenza ecosystem in Southern China could favor the emergence of novel IAVs that present potential risk to animal and human health.

Second, the novel H7N8 virus most likely circulated among diving ducks in the Mississippi flyway during autumn 2015 and was subsequently introduced to Indiana

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turkey, in which it evolved from LPAI into HPAI. H4N8 IAVs circulating among diving duck possess a gene constellation comprising five H7N8–like gene segments (except PA, HA, and NP, >98% sequence identity). Preceding the outbreak, an isolate with six gene segments (except NP and MP) sharing >99% sequence identity with those of H7N8 turkey viruses was recovered from a diving duck. These findings suggest that viral gene constellations circulating among diving ducks could contribute towards the emergence of IAVs that can affect domestic poultry, and diving ducks may serve as the potentially unique IAV reservoir or uniquely contribute to the maintenance, diversification, and transmission of IAVs in wild birds. These findings also highlight the importance of genomic sequencing and gene constellation characterization in wild bird IAV surveillance.

Third, a gradual accumulation of nucleotide and amino acid substitutions in the HA gene of H7 AIVs isolated from wild and domestic birds in North America has led to wide genetic diversity. Limited antigenic diversity was observed among the 93 North American contemporary H7 AIVs we tested. These findings suggested that continuous genetic evolution has not led to significant antigenic diversity for contemporary H7 AIVs from North America.

In summary, this dissertation study addresses the immediate public and animal health problem presented by the emergence of two novel avian origin IAVs, LPAI H10N8 and HPAI H7N8 viruses. In addition, it investigates the long-term antigenic and genetic evolution of H7 AIVs from North America over the past 40 years. The findings in this dissertation add to our knowledge of the natural history of IAVs and are critical for formulation of effective disease prevention and control strategies.

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APPENDIX A

SUPPLEMENTARY MATERIALS

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All supplemental materials are shown in the following PDF files. Reading these files requires Adobe Reader, v6.10.

- Phylogenetic_trees_of_internal_genes_recovered_from_the_samples_collected_at_the_LPM.pdf
- Phylogenetic_trees_of_IAVs_isolated_from_wild_and_domestic_birds_in_the_Americas.pdf
- Time_scale_phylogenetic_trees_for_eight_gene_segments.pdf
- Phylogenetic_trees_for_eight_gene_segments.pdf
- Phylogenetic_tree_for_HA1_nucleotide_sequences_of_H7_AIVs.pdf
- Amino_acid_variations_in_antibody_binding_sites_of_H7_AIVs.pdf

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