From transmission to evolution:
low pathogenic avian influenza (LPAI) H9N2 evolving under poultry vaccination

Hongrui Cui

Propositions

1. The most difficult part in the control of avian influenza is that infected wild birds migrate.
   (this thesis)

2. Vaccination in poultry to prevent low pathogenic influenza is economically beneficial but not sustainable.
   (this thesis)

3. It is not necessary for adults to drink animal milk in daily life for the purpose of being healthy.

4. The attacking behavior of coots on their largest and loudest young is to improve the survival rate of offspring.

5. To reach a successful research career, the ability to present is equally important as the research itself.

6. Not following the recipe but thinking outside of the box makes a cook a chef.

Propositions belonging to the thesis, entitled

From transmission to evolution: low pathogenic avian influenza (LPAI) H9N2 evolving under poultry vaccination

Hongrui Cui
Wageningen, 12 November 2021

From transmission to evolution: low pathogenic avian influenza (LPAI) H9N2 evolving under poultry vaccination

Hongrui Cui

Thesis committee

Promotors
Prof. Dr Mart C.M. de Jong
Professor of Quantitative Veterinary Epidemiology (QVE),
Wageningen University & Research

Prof. Dr Monique M. van Oers
Professor of Virology,
Wageningen University & Research

Co-promotors
Dr Nancy Beerens,
Head of NRL for Avian Influenza and Newcastle Disease,
Wageningen Bioveterinary Research

Prof. Dr Zejun Li
Deputy Director, Shanghai Veterinary Research Institute (SHVIR),
Chinese Academy of Agricultural Sciences (CAAS), China

Other members
Prof. Dr J.M. Wells, Wageningen University & Research
Prof. Dr J.A. Stegeman, Utrecht University
Prof. Dr R.A.M. Fouchier, Erasmus MC, Rotterdam
Dr W. Ma, University of Missouri, Columbia, USA

This research was conducted under the auspices of the Graduate School Wageningen Institute of Animal Sciences (WIAS)

From transmission to evolution: low pathogenic avian influenza (LPAI) H9N2 evolving under poultry vaccination

Hongrui Cui

Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 12 November 2021
at 4 p.m. in the Aula.

Hongrui Cui

From transmission to evolution: low pathogenic avian influenza (LPAI) H9N2 evolving under poultry vaccination

PhD thesis, Wageningen University, Wageningen, the Netherlands (2021)
With references, with summary in English

DOI: https://doi.org/10.18174/553900
ISBN: 978-94-6395-995-7

Abstract

The current pandemic reminds us of the shadow of flu caused by avian influenza viruses, which keep circulating and cause outbreaks all over the world. There are two problems with avian influenza viruses (AIVs) that cause flu in poultry: pathogenicity in poultry and possible effects on human health. The high pathogenic avian influenza viruses (HPAIVs) threaten public health directly by high human mortality, and the low pathogenic avian influenza viruses (LPAIVs) play an important role in influenza virus evolution. Due to the mild disease and low death rate, LPAIVs remain circulating in poultry populations, thereby providing a platform and time for the emergence of strains with high transmission, cross host-transmission properties or higher human pathogenicity. More and more human infections, caused by avian influenza virus directly from birds further increase our concerns. The H9N2 subtype LPAIV has attracted attention for its wide range spread in many bird species, as well as for the fact that it has become endemic in commercial poultry in many areas, affecting poultry productivity. As a prevention method, vaccination against the H9N2 subtype virus in poultry was promoted in several countries. However, the persistence of this virus in poultry after vaccination has been reported and it was suggested that the inactivated-virus vaccine might not stop the transmission, but rather trigger the antigenic drift of the virus in poultry. In this thesis, experiments were performed to analyse the effect of the inactivated-virus vaccine on stopping the transmission of H9N2 subtype virus in poultry. In Chapter 2, I showed that the estimated reproduction ratio of H9N2 subtype virus in non-vaccinated and vaccinated chicken was above 1, suggesting that the inactivated-virus vaccine against the H9N2 subtype virus cannot stop the transmission of the homologous virus strain in vaccinated chicken. In Chapter 3, phylogenetic and antigenic analyses of H9N2 subtype virus strains isolated from field markets during 2013-2018 in south China, discovered that a new antigenic group has emerged after 2013. The currently isolated virus strains show a higher number of mutations than the average level observed before, resulting in larger antigenic distances from the vaccine strain. In Chapter 4, I observed evolutionary changes of H9N2 subtype virus strains under continuous selective pressure in different hosts. I also identified changes related to potential antigenic drift events in viruses passaged under antiserum pressure. Then in Chapter 5, I discovered the effects of the PB1 gene from the avian H9N2 strain in the reassortment with a human strain. Corresponding genetic changes were observed in the progeny viruses under selective pressure.

Together, this thesis provided experimental and statistical evidence to the assumption that the inactivated virus vaccine cannot stop the transmission of H9N2 subtype LPAIV in a vaccinated chicken population. The H9N2 subtype virus could evolve as a consequence of continuing transmission under selective pressure from the vaccination, and this was observed in the field and in laboratory simulations. In addition, reassortment events involving the PB1 gene of H9N2 subtype virus may drive the emergence of novel reassortant viruses during this ongoing circulation. Based on these findings, it becomes clear that continuous surveillance for LPAIVs in vaccinated poultry is required during and after outbreaks. Monitoring for reassortment variants between LPAIV and human-adapted virus strains deserves more attention for pandemic preparedness.

Content

Chapter 1	General introduction	1
Chapter 2	Vaccination with inactivated virus against low pathogenic avian influenza subtype H9N2 does not prevent virus transmission in chickens	19
Chapter 3	Molecular characterization of antigenic drift of avian H9N2 viruses in southern China	43
Chapter 4	Identifying evolutionary changes of H9N2 in transmission with/without immune pressure using Next Generation Sequencing	67
Chapter 5	The PB1 gene from H9N2 avian influenza virus showed high compatibility and increased mutation rate after reassorting with a human H1N1 influenza virus	89
Chapter 6	General discussion	111
	Bibliography	125
	Summary	151
	Acknowledgements	155
	Curriculum Vitae	161
	Training and education	164
	Colophon	166

General introduction


Alan

1.1 Influenza — knowledge from history

The flu is now a well-known infectious disease that is causing illness or death in humans and animals all over the world. Its formal name, influenza, was introduced from an Italian word meaning "influence" in English in the mid-eighteenth century during a pan-European epidemic. Even though the record of influenza could be traced back to ancient Egypt and Greece 2500 years ago (Mamelund 2008), the discovery of the causative pathogen and its systemic analysis took place relatively recently, as it first required the development of molecular biology techniques. Due to technical limits, outbreaks of infectious diseases in populations of different host species were separately recorded and studied independently in different host species. However, the pathogen was eventually identified as a virus. Later, it became clear that various strains of influenza viruses exist that can be transmitted from one animal species to another, which may eventually also infect and spread among human beings.

The history of influenza is an interaction between outbreaks and obtaining knowledge from these outbreaks. The well-recorded history of influenza in the human population was said to have started in 1892 when the German bacteriologist Richard Pfeiffer discovered a bacteria that was mistaken to be associated with influenza (Mouritz 1921). Later the real agent was proved to be a virus. After the "first" influenza virus was isolated from swine in a laboratory in 1931 (Shope 1931), the diagnosis and records of influenza in humans became more accessible and convincing. For influenza infections in poultry, which is now known under the common name of avian influenza, the identification history started even earlier. In 1878, a severe disease-causing mass die-offs of chickens and some other poultry in south Italy was reported by Perroncito and co-authors (Perroncito 1878). This is most likely the first recorded avian influenza outbreak but was mentioned at the time as the "fowl plague". Only around the middle 1950s, the filterable virus was identified as type A influenza virus, which was later addressed as highly pathogenic avian influenza (HPAI) at the first International Symposium on Avian Influenza in 1981 (Bankowski 1981).

During the first half of the 20th century, epidemics in humans by this relatively "unknown" virus kept raging worldwide, and the term influenza pandemics was used when referring to such worldwide outbreaks in humans. The most severe outbreak was the 1918 Spanish flu pandemic infecting about one-third of the world's population at that time (Taubenberger et al. 2001). The virus strain that caused the 1918 pandemic and maybe also the earlier (1892) reported flu-like diseases was later identified to be of the H1N1 subtype, which was circulating in pigs and shortly after that also in humans (Shope 1936). This pandemic strain was then replaced by H2N2 causing the Asian flu in 1957 and by H3N2 causing a pandemic in 1968 (Taubenberger et al. 2006). These pandemics of influenza started from specific areas and then spread to almost all the continents (Stafseth 1958, Stubbs 1965). At the time, outbreaks of influenza in poultry/birds were still recognized as "fowl plague", and these were therefore not systemically studied till after the discovery that the upcoming two strains causing human pandemics in 1957 and 1968 were generated from reassortment events between human and avian influenza strains (Kawaoka et al. 1989, Bean et al. 1992, Schäffr et al. 1993). Recently in 2009, we saw again a pandemic of an H1N1 virus reassorted from swine, human and bird strains. It was first detected in the United States and spread globally within a short time (CDC 2019). In addition to the vast death toll during historic influenza pandemics, the seasonal epidemics of influenza that keep circulating in the human population was estimated to introduce three to five million cases of

severe illness globally, including 290,000 to 650,000 respiratory deaths every year, according to a recent report (WHO 2019).

After the 1970s when surveillance of influenza viruses in birds started, the knowledge on influenza in birds started to accumulate. The subsequent definition of avian influenza, causing outbreaks in poultry (instead of the "fowl plague"), contributed to the detailed understanding of the epidemiology of influenza and the mechanism of its evolution within and beyond specific host populations. From then onwards, influenza viruses were classified based on antigenic properties, regardless of the species of origin, as compared to human influenza virus, avian influenza virus (AIV) and swine influenza virus (Memorandum 1980).

Outbreaks caused by avian influenza strains in the wild have been direct reasons for concern for the poultry industry worldwide. The influenza outbreaks in poultry, especially in intensive farming of chickens and turkeys, cause enormous economic losses that ranged from US$32.4 to about 200 million in developing countries (Otte et al. 2008). Moreover, influenza in poultry or other animals is a clear potential threat to public health due to the risk for cross-species transmission and re-assortment events (see explanation below). People became even more concerned when avian influenza strains were recorded to cause disease in humans since the 1990s (Guo et al. 1999, Peiris et al. 1999, Lin et al. 2000, Guo et al. 2001, Lupiani et al. 2009). AIVs of subtypes H5, H6, H7, H9 and H10 had caused human infections and even death in various parts of the world (Short et al. 2015). Remarkably, the avian influenza H5Nx, H7N9 and H9N2 were requested to be reported for risk assessment by WHO due to the frequency of zoonotic infections and/or the severity of disease they caused in humans. From 2003 to 2021, the deaths caused by AIV H5N1 strain have been more than 400 in over 17 countries (WHO 2021).

Due to the identification and better classification of influenza viruses, we can now correlate pandemics in humans with outbreaks in birds, which revealed the hidden threat of avian influenza viruses for human health. The updating reports to WHO about zoonotic infection cases all over the world is timely surveillance on avian influenza virus.

1.2 Molecular details of the causative agent, influenza virus

Influenza viruses belong to the family *Orthomyxoviridae*. This family harbours four types of influenza viruses labelled A, B, C and D (Wolff et al. 2020). Influenza A and B viruses both cause seasonal epidemics of disease (seasonal flu) among humans. Influenza A virus strains are the only type of influenza viruses known to cause flu pandemics, according to the Centers for Disease Control and Prevention (https://www.cdc.gov/flu/about/viruses/types.htm). Therefore this thesis focuses on influenza A virus.

1.2.1 Virus particle and the genetic characteristics of the type A virus

The genome of influenza A viruses consists of single-stranded, negative-sense RNA. Eight gene segments form the genomic viral RNA (vRNA), and these are numbered from one to eight, ordered from large to small (Figure 1.1A). The eight gene segments were found to encode ten essential viral proteins (Palese et al. 1976, Ritchey et al. 1976), including the RNA polymerase subunit 1 and 2 (PB1 and PB2, encoded by segment 2 and 1 respectively), Polymerase Acidic protein (PA, encoded by segment 3), Hemagglutinin (H or HA, encoded by segment 4),

Nucleoprotein (NP, encoded by segment 5), Neuraminidase (N or NA, encoded by segment 6), Matrix protein 1 and 2 (M1 and M2, encoded by segment 7), Non-Structural protein 1 (NSP1) and Non-Structure protein 2 (NS2 or nuclear export protein, NEP) which are both encoded by segment 8. The eight genome segments are also referred to with the names of the corresponding major proteins as the PB2, PB1, PA, HA, NP, NA, M, and NS segments (Muramoto et al. 2013, Dou et al. 2018). During the fundamental research on influenza viruses, several strain-dependent accessory proteins were also discovered and reported as PB1-F2 (Chen et al. 2001), PB1-N40 (Wise et al. 2009), PA-X (Jagger et al. 2012), PA-N155 (Muramoto et al. 2013), PA-N182 (Muramoto et al. 2013), M42 (Wise et al. 2012), NS3 (Selman et al. 2012), amongst others. (Figure 1.1B).

Each vRNA genome segment is individually packaged by nucleocapsid (NP) protein and accompanied by a trimeric polymerase complex comprising the PB1, PB2 and PA proteins (Figure 1.1C). Each vRNA–NP–polymerase subunit forms a viral ribonucleoprotein (vRNP) complex (Eisfeld et al. 2015). Hereinto, the polymerase complex is the RNA-dependent RNA polymerase (RdRp), which is responsible for the transcription (vRNA → mRNA [messenger RNA]) and replication (vRNA →cRNA [complementary RNA]→vRNA) of all eight segments of the vRNA genome (Lamb et al. 1983) (Figure 1.1C). Hemagglutinin (HA) and neuraminidase (NA) are proteins on the surface of influenza viruses. Because of the exposed surface location of these two proteins, HA and NA are the most prominent “antigens” triggering an immune response in the infected host. In the viral infection cycle, HA is responsible for binding the virus to sialic acid residues on cellular transmembrane glycoproteins. Once bound to this cellular receptor (α-2, 6 and α-2, 3 sialic acids), HA facilitates the viral entry into the target cells by endocytosis and triggers pH-dependent membrane fusion to release the RNPs into the cytoplasm of the host cell, which then travel to the nucleus of these cells. In the nucleus, primary transcription is carried out to generate mRNAs for the functional proteins needed for vRNA replication and progeny particle formation (Fodor et al. 2002). The other surface protein, NA is the enzyme identified to facilitate virus release (Air et al. 1989, Matrosovich et al. 2004), as it removes sialic acid molecules from already infected cells and therefore enables the progeny virus particles to bud out from the cell without immediately being trapped again by receptors of an already infected cell. Hence, NA assists the virus to spread from cell to cell efficiently.

1.2.2 Diversity of influenza A virus

Influenza A viruses are further divided into subtypes based on the two surface proteins, hemagglutinin (H) and neuraminidase (N). Until now, there are 18 H subtypes and 11 N subtypes reported; however, only 131 of the potentially 198 combinations have been isolated naturally. Figure 1.2 shows the subtype diversity and the distribution of different subtypes of influenza A viruses over host categories (Joseph et al. 2017). Almost all known subtypes combinations have been found in birds, except for H17N10 and H18N11 which were found exclusively in bats up to now (Wu et al. 2014).

Therefore, wild aquatic birds are regarded as the natural reservoirs of influenza A viruses and are considered the primary genetic source of all influenza A virus infections in other species (Webster et al. 1992).

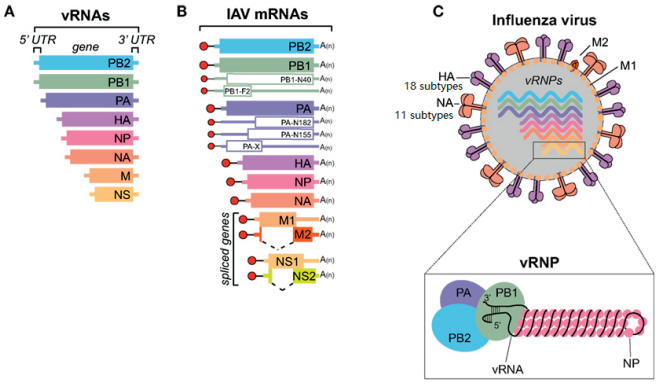


Figure 1.1 Influenza A viruses.
A) Schematic of the eight viral RNA (vRNA) gene segments that comprise the influenza A genome. The 5′ and 3′ untranslated regions (UTRs) are represented with a line, and the box corresponds to the coding region within each vRNA.
B) Diagram of the viral mRNAs that are transcribed from promoters in the 3'UTRs in the IAV (-) vRNA templates. Boxes indicate the viral gene products encoded by each mRNA. Dashed lines show the alternative splicing of the IAV M and NS transcripts. Red circles represent the 5′M7pppG cap, black lines denote the 10–13 nucleotide, host-derived primers that are obtained by a cap-snatching mechanism by the viral polymerase. A(n) corresponds to the 3′ poly-A tail produced by reiterative stuttering of the viral polymerase. The smaller mRNAs (empty boxes) represent transcripts that encode nonessential accessory proteins found in many strains, whereas those that are less prevalent (PB2-S1, M42, and NS3) are not illustrated (Chen et al. 2001, Wise et al. 2009, Jagger et al. 2012, Muramoto et al. 2013, Yamayoshi et al. 2016, Zhang et al. 2017).
C) Diagram of the virion of an influenza A virus. The viral membrane proteins H (HA), N (NA), and M2 are shown, along with the eight viral ribonucleoproteins (vRNPs), and the matrix protein M1 that supports the viral envelope. To highlight the vRNP components, the illustration beneath the virus is not to scale. A single vRNA gene segment is shown wrapped around multiple nucleoprotein (NP) copies with the conserved promoter regions in the 5′ and 3′ UTRs forming a helical hairpin, which is bound by a single heterotrimeric viral RNA-dependent RNA polymerase (PB1, PB2, and PA). The figure was re-used with permission from Robert Daniels (Dou et al. 2018).

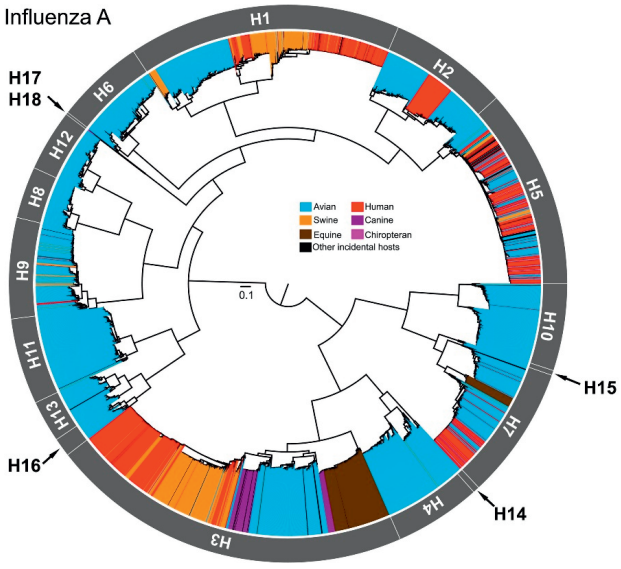


Figure 1.2 Diversity and host distribution of influenza A viruses. Maximum-likelihood (ML) estimation of the haemagglutinin (H) gene sequences of all subtypes of IAVs downloaded from the NCBI GenBank database. Overall data set randomly subsampled to include 200 isolates per subtype per host for the tree reconstruction. Scale for branch length represents number of nucleotide substitution per site (subs/site) in the H alignment. (Joseph et al. 2017) The figure was re-used from open access article based on to Wiley's Open Access Terms and Conditions.

Apart from humans and birds, influenza A virus also brings disease (flu) in other mammals, including swine, dogs, horses, and cats (Fenner et al. 1976, Baigent et al. 2003), and interspecies transmission has been recorded frequently. Figure 1.3 displays the interspecies transmission routes of influenza A viruses and the current host range of particular serotypes (Joseph et al. 2017). After introduction into a new host species, influenza viruses may maintain themselves in that species and further evolve into a host-specific lineage. Therefore, Influenza A viruses are also sub-defined based on the host species to which they have adapted, i.e., human influenza virus, swine influenza virus, equine influenza virus and endemic avian influenza virus (AIV) in poultry.

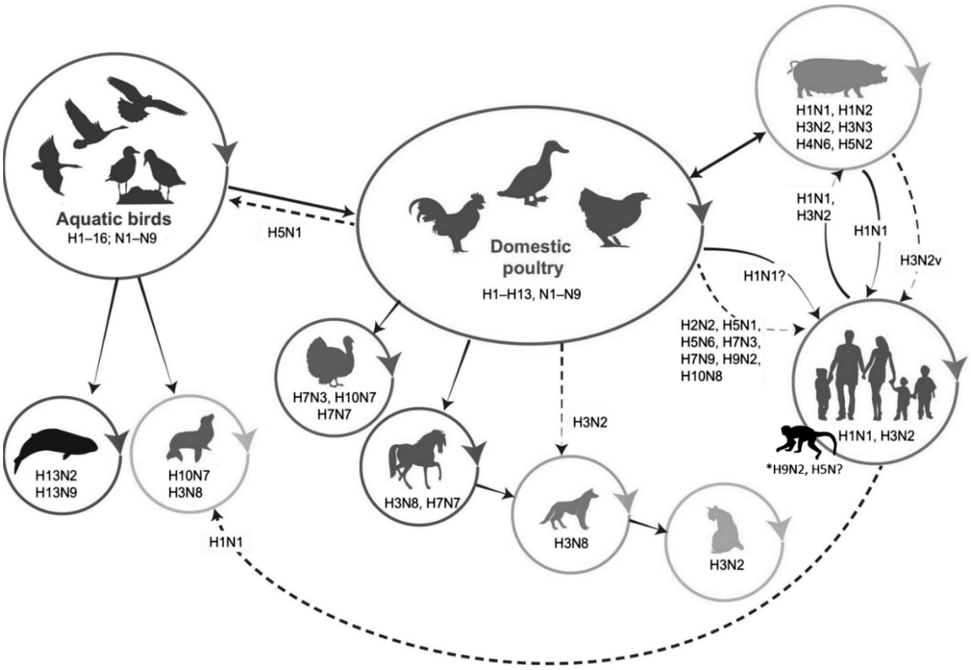


Figure 1.3 Significant interspecies transmission of influenza A viruses.
Representative diagram of interspecies transmission events of IAVs and the subtypes involved in these events. Solid arrows represent direct transmission events that have since been established in the host species. Dashed arrows represent sporadic or limited infection of subtypes where sustained transmission in the new host has not been detected. The figure was re-used from open access article based on Wiley's Open Access Terms and Conditions (Joseph et al. 2017). * The seroprevalence of influenza A virus subtypes in monkeys were detected positive to human H1N1, human H3N2, H9N2, and H5 subtype AIVs (Karlsson et al. 2012). NHPs (Nonhuman Primates) that have contact with humans can be naturally infected with seasonal endemic human influenza viruses (Karlsson et al. 2012), but the circulation and transmission within and without this population were not reported to date.

1.3 Evolution of influenza A virus

Virus evolution is characterized by changes in the "gene pool" of the virus population that can be found in a chain of hosts linked by infection events over time. Evolutionary changes involving random mutation, recombination, reassortment, gene amplification/reduction lead to the occurrence of quasi-species (Domingo et al. 2012). Due to different genetic characteristics and variations in polymerase accuracy, individual viruses display specific ways of genome adaptation and evolution. For influenza A viruses, evolution proceeds via the gradual

accumulation of mutations because of the incorporation of non-complementary nucleotides by the viral RdRP during genome replication, and abruptly, via reassortment of genomic fragments when two influenza viruses infect a single cell (Westgeest et al. 2014). These two phenomena are further explained below.

1.3.1 Mutations cause genetic drift

As for other RNA viruses, numerous mutations might occur during viral RNA replication, mostly due to the nucleotide substitutions (Hanada et al. 2004) introduced by the intrinsic error-proneness of the influenza RdRp and the lack of proofreading capacity (Lauring et al. 2013). For the influenza virus, the mutation rate was estimated to be around 10^{-5} substitutions per nucleotide per infectious cycle (s/n/c) of the whole genome (Parvin et al. 1986, Sanjuán et al. 2010). The frequent generation of mutations in the viral genome during replication is commonly addressed with genetic drift (Masel 2011). This ability to mutate together with subsequent exposure to selective pressure in the host directs the evolution of the virus (Chen et al. 2006).

Mutations in the HA and NA genes might introduce changes in the antigenic surface proteins which are firstly recognized by the host immune system. Consequently, the genetic drift of the HA and NA gene might result in the antigenic drift due to selection pressure. As HA facilitates the recognition of target cells and the entry of the viral genome into those cells, any changes in its sequence could influence the virulence, transmission and even host-specificity. Researchers have explored extensively what kind of crucial amino acid mutations in antigenic epitopes of HA might lead to this antigenic drift (Hampson 2002) so that the virus can escape from existing immune pressure (Sitaras et al. 2014, Peacock et al. 2016) to favour "immune-escape variants". Mutations that affect the receptor binding sites (RBS) of HA might change the tissue tropism (Naeve et al. 1984), which could result in increased pathogenicity when the virus gains the ability to attach to receptors in the lower respiratory tract (e.g. lung) instead of the upper respiratory tract only (Ayora-Talavera et al. 2009). Particular mutations in the RBS may also allow the virus to transmit to other host species (Gao et al. 2009, Liu et al. 2014), leading to cross-species transmission. Mutations in or near the RBS sites might also influence the antigenic properties of the virus (Koel et al. 2013). Apart from antigenic drift, genetic drift may also occur at essential locations of inner gene segments when genes for surface proteins are affected. Such mutations were also identified and shown to influence the replication or host-adaption of the virus, e.g. specific mutations on PB2 (Gastaminza et al. 2003), PB1 (Lin et al. 2019), M (Liu et al. 2002) and NS genes (Ozawa et al. 2011).

1.3.2 Reassortment and genetic shift

Whereas genetic drift results from gradual changes by accumulating point mutations, genetic shifts may occur as a consequence of genome segment reassortment (Webster et al. 1992). Characterized by segmented genomes, influenza viruses are prone to exchange genes when co-infecting one individual host cell (Kilbourne 2006, Sonnberg et al. 2013, Uyeki et al. 2013). Assuming that all the eight gene segments have an equal possibility in reassortment, there will theoretically be 256 (2^8) different offspring viruses possible with only two parental viruses. Compared with genetic drift, the genetic shift has a strong and immediate impact on viral evolution. (Holmes et al. 2005, Dugan et al. 2008) Since reassortment is more efficient in

generating novel viruses (the diversity of the offspring increased suddenly) and some of these new viruses may have competitive advantages under selective pressure. When such a genetic shift involves the gene segments encoding HA and/or NA the term antigenic shift is used as they cause abrupt changes in the ability of the immune system to recognise this virus with new properties. The rapid evolution resulting from reassortment might lead to host-switching (Scholtissek et al. 1978, Garten et al. 2009), immune escaping and resistance to antivirals.

In history, reassortment has always been the major cause of flu pandemics. The pandemic of 1957 was caused by an adapted avian influenza A (H2N2) virus that obtained its virulence and transmitting ability from mixing its PB1 and HA genes with the other six genes from a human-adapted H1N1 virus. Similarly, the Asian H3N2 pandemic, first found in Hong Kong in 1968, also possessed genes from a human H2N2 virus originating from the 1957 pandemic (Kawaoka et al. 1988). This sort of recombination can even happen among more than two different subtypes or strains. For instance, the 2009 H1N1 pandemic was caused by a reassortant virus that had obtained genome fragments from human, avian and swine influenza strains (Cohen 2009). This genomic re-assortment often serves as a "jump" in the evolution of influenza viruses, allowing these to be able to develop into the pre-dominant circulating strain in the years after, and we indeed see the H1N1 strain that emerged in 2009, currently re-appearing seasonably instead of the previous strains (WHO 2018).

1.3.3 Evolution under selective pressure

The individual genomes in the pool of influenza virus genomes (called quasi-species due to the consequence of genetic drift and/or shift) will not be equally successful in replication and host adaptation (Andino et al. 2015), and therefore experience competition from each other. Stabilizing natural selection will occur for the functionality of the encoded proteins and for the correct folding of crucial RNA motifs such as promoter sequences. In addition, immune pressure as will be active in recovered or vaccinated individuals will disfavour those variants that are antigenically too close to previously encountered influenza viruses. As a result, the fitness of the viral population after one round of replication (as the virus unit) might change, due to the fact that viral genomes that mutated into genotypes with certain advantages in that particular environment will be able to produce more progeny. As a result, variants with mutations that allow them to replicate quicker in certain tissues or host, or that allow escaping from neutralizing antibodies, will be able to become dominant in that particular host. Host switching, application of antiviral drugs or immunization of the population by vaccination or a previous infection will change the environment in which the virus is replicating, and therefore modifies the selective pressure acting on the quasi-species, allowing other variants to become dominant. The impact of the variations on functionality and the selective pressure acting on the variants determine together the genome composition of the influenza virus population encountered as the infectious agent.

Another important factor is provided by the "co-evolution with host theory", which states that the adaption of viral genomes to their hosts' cellular environment needs to achieve a balance between viral-replication and remaining cellular functions intact for the host to survive long enough to support high levels of progeny virus production. This is the viral evolution at the individual host level, which seems different to that at "population" level, but it plays a crucial role in transmitting between hosts. Taking influenza virus as a quasi-species, the genome

composition is the result from the survivors in individual host infections (Voskarides et al. 2018) that go on circulating in the host population, to magnify their advantage until achieving equilibrium with their host population. This could be proven by the observation that influenza viruses in their natural host reservoirs show limited evolution at the amino acid level (Webster et al. 1992). Their continued random mutations provide no additional advantages for these viruses in their natural host species, with which they have an ancient relation. However, this balance could be broken when influenza viruses spread to other hosts (different species or a genetically different population of the same species), or when the host obtains a higher level of protective immunity. In such situations, influenza viruses can show rapid diversification with emergence of new antigenic variants that may even form separate clades in phylogenetic trees (Yoon et al. 2014).

1.3.4 Genetic techniques to compare influenza strains

Thanks to the development of phylogenetic methods, it became easier to trace or identify lineage of viruses with specific characteristics that are inherited in their genomes. Combined with data on geography, environment and the host, we can then address the evolutionary pathway of certain genetic changes that are the result of the acting selection pressures. Phylogenetic trees of influenza viruses are generally based on the two surface gene segments (HA and NA) or the six inner segments (PB2, PB1, PA, NP, MP and NS). These treated separately due to the different types of selection mechanisms acting on these groups of genes as a consequence of the variation in the function of their encoded proteins. When comparing the topology of phylogenetic trees of different gene segments, we are also able to reveal the recombination or reassortment of viruses (Chare et al. 2003, Nelson et al. 2007, Boni et al. 2010, Hallinan 2011).

In contrast to Sanger Sequencing that reveals only the most prominent genotypes in a virus population, Next Generation Sequencing (NGS) technologies enable us to analyse the full diversity of the influenza genome segments in the quasi-species in particular environmental or host situations. The large amount of genomic information that is obtained in this way, will contribute to understanding the viral genome population adapts to changing selection pressure (Zhen et al. 2014), such as in the presence of antivirals or vaccination.

1.4 Avian influenza virus -- High versus low pathogenic strains

Avian influenza virus (AIV) is one of the three main sub-categories of influenza A viruses, next to human and swine influenza viruses. AIVs infect wild aquatic birds but also regularly cause infections in poultry and may also infect other animals and humans exposed to infected poultry. The overall pool of AIV strains displays the most H and N subtype combinations, based on 16 subtypes of HA and nine subtypes of NA. All these subtypes of avian influenza viruses have been isolated from wild aquatic birds and/or poultry (Olsen et al. 2006, Yoon et al. 2014, Bergervoet et al. 2019).

To evaluate the pathogenicity of individual strains of AIVs, the "Intravenous Pathogenicity Index" (IVPI) has been introduced as a standard protocol (Avian Influenza Community Reference Laboratory), and displays a mean score per chicken per observation over a 10-day period. Furthermore, this index allows us to divide AIV strains into high pathogenic avian

influenza viruses (HPAIVs) and low pathogenic avian influenza viruses (LPAIVs) (OIE 2019). Highly pathogenic avian influenza is defined by the World Organization for Animal Health [originally addressed as the "Office International des Epizooties" (OIE)] as "an infection of poultry with an IVPI above 1.2. HPAIVs can alternatively be described as influenza strains leading to at least 75% mortality in a defined chicken population during a specified interval of ten days). These HPAIVs are H5 and H7 strains with multiple basic amino acids around the internal cleavage site in the haemagglutinin molecule (OIE 2019). Till now, HPAIV H5 was observed infecting poultry and wild birds, whereas HPAIV H7 was only found in poultry (Hurt et al. 2017). Till now, HPAIV H5 was observed infecting poultry and wild birds, whereas HPAIV H7 was only found in poultry (Hurt et al. 2017, Antigua et al. 2019). Some H5 and H7 subtypes and other non-H5/non-H7 strains, which induce milder flu-like symptoms and are restricted to the respiratory and/or gastrointestinal tracts are defined as LPAIV (meaning an IVPI below 1.2 and showing less than 75% mortality over a 10-days period). As notifiable zoonotic animal diseases, the outbreak of LPAI and HPAI are required to be reported to the OIE.

1.4.1 Economic damage and potential threats of HPAI and LPAI

From the early beginning of the 20th century until now, there have been regular worldwide reports on outbreaks of LPAI and HPAI in wild birds and at poultry farms or live poultry markets (Alexander et al. 2009, Kaleta et al. 2009). Most of the associated influenza viruses were highly pathogenic (HPAIVs) and resulted in high mortality in chickens (up to 90 to 100%, often within 48 hours) (Spackman 2008). Avian influenza virus can rapidly spread in flocks of wild birds and be easily transmitted to poultry. Moreover, the migration of many of these wild birds complicates the prevention and control of avian influenza. Once detected in poultry, the most efficient method to stop the disease is culling of the infected farms (also obligatory by EU law).

Along with the direct death toll from outbreaks of HPAI in poultry, there are therefore also huge economic losses as a result of culling. For instance, the outbreak of HPAIV H7N7 in 2003 resulted in 30 million victims (direct and through culling) in poultry in the Netherlands (Alexander et al. 2008), 2.3 million in Belgium, and 420 thousand in Germany. From 2003 to 2004, many Asian countries suffered from an outbreak of H5N1 HPAIV in poultry. In China, this epidemic caused the death of 20 million poultry, and nine provinces were affected (Smith et al. 2006). In addition to the obvious losses in poultry, these H5N1 HPAIV strains may also cause severe disease in wild birds. A massive die-off caused by HPAIV H5N1 in wild birds in Qinghai Lake (Qinghai, China) shocked the world in 2005. It was identified that this strain showed higher mortality in chicken and mice in laboratory experiments than previous H5 subtype isolates (Liu et al. 2005). Since outbreaks of HPAIVs have led to millions of wild birds and poultry being killed from infection, control measures have been taken throughout Asia, Europe, and Africa (Feare 2007, Salzberg et al. 2007, Brown 2010).

In contrast, the outbreaks of LPAIVs that naturally occur among wild birds and cause no symptoms or only mild disease in poultry seem less severe but deserve our attention. It is still a great threat to the poultry industry due to co-infections with other pathogens (Umar et al. 2017). Moreover, the introduction of H5/H7 LPAIVs from wild birds into domestic poultry might increase the possibility of mutations that would allow an H5/H7 LPAIVs to become HPAIVs during replication (Alexander 2007, Monne et al. 2014) and with the potential to become epidemic (or even pandemic). The Italian epidemic (1999–2000) was a typical example

of such an outbreak, where a mutated avian H7N1 strain converted from a low pathogenic into a high pathogenic strain, resulted in a devastating outbreak in poultry (Capua et al. 2003). For some of the recent outbreaks of HPAIVs in European and Asian countries, phylogenetic evidence has shown that these H5/H7 viruses resulted from reassortment with LPAIVs occurring in wild birds (Suarez et al. 2004, Pasick et al. 2005, Duan et al. 2007, Beerens et al. 2020).

LPAIVs of the other subtypes (not H5/not H7) have not yet been found to be able to mutate directly into HPAIV, and therefore have been given less attention so far. However, due to the migration of many wild birds and their contact with other bird species, LPAIV is able to cross host and geographical boundaries. In addition to the aquatic birds that constitute the major natural reservoir, there are records of LPAIVs detection in over 100 wild bird species from more than 25 families (Webster et al. 1992, Olsen et al. 2006). LPAIVs may also pose a potential threat to public health due to their often-wide host range, longer periods of unnoticed circulation, and unpredictable evolution when transmitted between different hosts. The possibility of bird/poultry to human transmission brings a high risk of gene exchange between poultry and human influenza virus strains. Of all originally avian infecting subtypes, two have established themselves in the human population (H1N1 and H3N2) and now occur as seasonal flu. Five others have been reported to be transmitted from birds to humans occasionally but have not (yet) established themselves in human populations: H5N1, H7N3, H7N7, H7N9, and H9N2 (Capua et al. 2004, Leong et al. 2008).

The devastating historical pandemics of HPAI and the regularly occurring outbreaks of LPAI in birds, which are often associated with human infection cases, have drawn the researchers' attention to surveillance of these viruses in wild birds to study influenza viruses in detail. In the century of fighting against this virus, genetic techniques have become available that ultimately enabled the unravelling of the genomic details of all influenza virus strains isolated in surveillance programs. In-depth studies on the mechanism that drives the evolution of influenza viruses will further contribute to preventing and controlling this centurial plague.

1.4.2 Strategies for controlling influenza in poultry

The controlling and prevention of infectious disease largely depends on the host species infected. The measures taken for flu outbreaks in human and mammals are quite different to that for poultry and wild birds. For humans and other mammals, the main strategies to counteract HPAI are hygiene, vaccination, and treatment with anti-flu medicines. To control seasonal flu in humans (H1N1, H3N2, and influenza B virus), vaccines are present and are updated (every 1-5 years) to maintain their effectiveness (Ping et al. 2015). To control HPAIVs in domestic poultry, the current measures are surveillance, biosecurity and culling. In poultry and livestock, the prevention measures are always considered with the economic load, the population density and the mortality caused by the disease. It is more efficient to stop the transmission to a new area than to prevent the spread in an already infected population. Therefore, several papers suggested to reduce the production in poultry industry (Poore et al. 2018), to allow more space in-between farms (Stegeman et al. 2004), to avoid wild bird habitat sharing (Velkers et al. 2020) and to apply vaccination (Tiensin et al. 2007).

The vaccines and vaccination strategies used for poultry are quite different all over the world. In some countries, like China, Indonesia, Vietnam, Egypt, Korea, and Pakistan, poultry is vaccinated routinely (Spackman et al. 2013, Umar et al. 2016, Wei et al. 2016). But the large-scale and long-term use of avian influenza vaccines in those countries mentioned above appears to have already stimulated antigenic drift in AIVs (Sun et al. 2012). Several observations suggest vaccination against the clinical signs of avian influenza may result in virus evolution and escape from vaccination. One is the failure of the commercial vaccine used against H5N2 AIV in Mexico in 1995. After two years of application, the vaccinated animals shed viruses that resulted from a dramatic antigenic drift (Chang-Won et al. 2004). Other reports describe H9N2 circulation in countries that applied H9N2 vaccination in poultry (mostly in the chicken industry) (Hsu et al., Desvaux et al. 2013, Hong et al. 2014). Research in Korea identified that H9N2 AIVs isolated after usage of vaccine were antigenically distinct from early strains and could experimentally escape from the vaccination (Sun et al. 2012). Similar observations were made in China (Li et al. 2005, Xu et al. 2007, Zhang et al. 2008), where vaccination was unable to control the circulation of LPAI H9N2 strains in poultry. In the European Union, vaccination is not allowed, and biosecurity interventions (bio-exclusion/biosecurity) are applied to halt the transmission of LPAIV from wild birds into the poultry industry. The reported LPAIV outbreaks were mostly of the H5 and H7 subtypes in European countries (realize the reporting bias due to the fact that H5 and H7 subtypes are notifiable by OIE and confirmed zoonotic) with the risk of drifting towards high pathogenicity (Gonzales et al. 2014, European Food Safety Authority 2018, European Food Safety Authority 2020, European Food Safety Authority 2021). Reports of H9N2 outbreaks are more frequent in Asian countries, suggesting that this virus is widespread in wild birds and domestic poultry. In summary, H5, H7 and H9 subtypes of avian influenza viruses are under tight surveillance in light of the risk for the poultry industry and possibly for public health. The prevention and control measures taken for avian influenza vary broadly per country.

1.5 H9N2 avian influenza virus -- A new flu threat?

H9N2 avian influenza virus (H9N2 AIV) was largely neglected (comparing to H5 and H7) until more reports appeared indicating its rapid spread over the world, its long-term circulation in both wild and domestic birds and the occurrence of human infections.

Ever since the first isolate in the US state of Wisconsin in 1966 (Homme et al. 1970), H9N2 virus has spread throughout most continents (Shortridge 1992). Currently, H9N2 virus is endemic in domestic poultry across east and west Asia, the Middle East, North and West Africa (Guan et al. 1999). It was also found in wild birds (Kawaoka et al. 1988, Jackwood et al. 2007) across Eurasia and North America (Alexander 2007, Lindh et al. 2014). Figure 1.4 displays the global spread of H9N2 AIV. The continuous circulation of H9N2 AIV between wild birds and poultry populations (Lee et al. 2000, Liu et al. 2004, Perk et al. 2006, Lee et al. 2007) is the second reason for concern for the poultry industry but also for human health. Perez and co-authors experimentally demonstrated that H9N2 AIV could indeed be transmitted from aquatic birds (especially duck) to terrestrial poultry via quail (Perez et al. 2003). Outbreaks of H9N2 AIV in poultry (including chicken (mainly), domestic duck, turkey and ostriches) in Europe, North America, Middle East and Asia occurred ever since the mid-1990s (Werrner 1998, Banks

et al. 2000, Alexander 2003, Alexander 2007). Although avian H9N2 AIV displays a typical avian host range, there is evidence that H9N2 AIV can infect mammalian hosts, including humans. Many clinical cases of human infections with H9N2, led to the understanding that people who have close contact with infected birds have a high risk to become infected with H9N2 AIV (Lin et al. 2000, Butt et al. 2005, Liu et al. 2018, Pan et al. 2018).

The H9N2 AIV thus was shown to have a wide host range, suggesting that, reassortment between H9N2 and other influenza viruses may lead to new viral strains that could adapt to mammalian hosts (e.g., humans). Experimentally generated reassortant viruses containing H9N2 surface genes other viral segments derived from seasonal human H3N2 influenza virus showed efficient transmission in a ferret model after adaptation by serial passage (Sorrell et al. 2009). The massive circulation of H9N2 AIV provides opportunities for the virus to exchange genetic material with other avian, swine (Xu et al. 2004) or human viruses, potentially leading to new pandemic flu viruses. It has already been shown that H9N2 AIV contributed genome fragments to newly emerging reassortant viruses (Chang et al. 2018) such as H7N9 (Pu et al. 2015, Pu et al. 2021) and H10N8 (Chen et al. 2014, Qi et al. 2014), which are zoonotic viruses that threaten public health (Yu et al. 2014, Yu et al. 2014, Zhou et al. 2016). Thus, sufficient surveillance, effective prevention and control of H9N2 avian influenza is crucial. In China, H9N2 subtype influenza viruses are now steadily circulating in poultry as is evident from the annual isolation of this subtype and the H9N2 infections have become endemic in commercial poultry. The first LPAI H9N2 virus detected in China was during the outbreak from 1992 to 1994 (Sun et al. 2015). Since then, the virus has spread in China, and a human infected with H9N2 AIV was reported in 1998 (Guo et al. 1999). In China, the isolation rate of H9N2 AI viruses has been of the same magnitude as that of H5N1 at live poultry markets in 1997; however, the rate of H9N2 started to increase recently.

Genetic studies on HA and NA genes divided H9N2 AIVs into two lineages, North America and Eurasian lineages. The North American lineage was represented by A/Turkey/Wisconsin/1966 (WI/66) (Han et al. 2019). The Eurasian lineage was represented by three differentiated lineages: A/Chicken/Beijing/1/94 (also known variously as the Y280 or G9 lineage), A/Quail/Hong Kong/G1/97 (G1 split into “Western” and “Eastern” sub-lineages) and A/Duck/Hong Kong/Y439/97 (also known as Korean lineage) (Guo et al. 2000, Xu et al. 2007). Due to the current geographical distribution of H9N2, surveillance reports indicate Asian countries (especially China) as the hotspot of Eurasian lineage, and that is where most research is ongoing and where control strategies are applied. In mainland China, the predominant sub-lineages were BJ/94-like and G1-like viruses since the mid-1990s (Choi et al. 2004, Lu et al. 2005, Sun et al. 2010). Li et al. reported that HA genes of some H9N2 isolated from chicken and other hosts were mainly derived from the Y280-like lineage (Li et al. 2012). Recently, the surveillance of H9N2 in China revealed some novel lineages apart from those representative H9 lineages undergoing rapid antigenic drift, which may be driven by selection pressure acting on the virus during endemic circulation in populations of vaccinated birds.

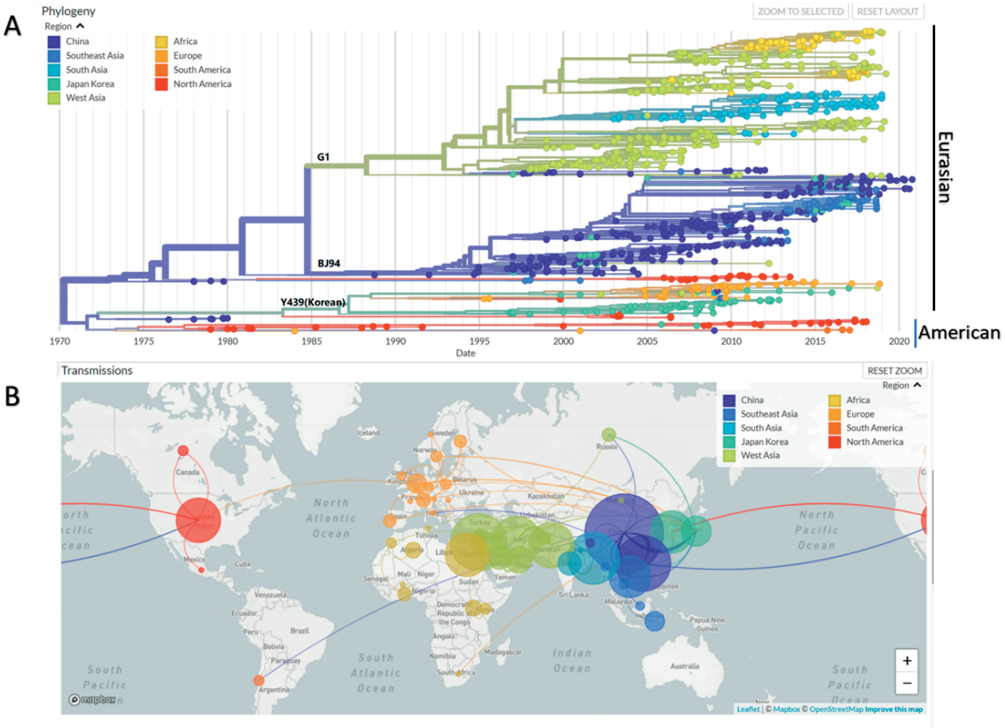


Figure 1.4 The spread and genetic evolution of H9N2 avian influenza virus. A, Phylogenetic tree of H9N2 subtype avian influenza virus. B, Transmission of H9N2 subtype avian influenza virus. There were two main lineages: Eurasian and American lineages. Clades were coloured by regions with the main sub-lineage marked above the main branch. There were 1337 HA gene sequences sampled between 1976 to October 2020. (Time animation of the transmission and clade evolution are available at https://nextstrain.org)

In China, an inactivated H9N2 vaccine has been developed and has been extensively applied in commercial flocks in China since 1998 (Li et al. 2005). Earlier H9N2 isolates such as A/chicken/Shandong/6/96 (SD96) or A/chicken/Shanghai/F/98 (F98) were selected as vaccine strains for commercial vaccines. As acknowledged, the effectiveness of AIV vaccines on individual protection is largely dependent on the antigenic relationship between the vaccine strain and the prevailing ones (Lee et al. 2004). A study of the field isolates of H9N2 AIV from various regions of China led to serious concerns about the efficacy of the H9N2 commercial vaccines. This study compared genetic and antigenic characteristics of the H9N2 field isolates collected between 1998 and 2007 with those of the two commercial vaccine strains, SD96 and F98. The results revealed that antigenic variation had occurred to almost all of the 41 field isolates with only one exception (Zhang et al. 2012).

Genetic and/or antigenic variants of H9N2 AIVs were also frequently isolated from poultry during the period of 2005 to 2009 (Li et al. 2005, Ji et al. 2010, Yu et al. 2014). In addition, it was clearly demonstrated that “heterologous protection among different antigenic groups was suboptimal” (Sharma et al. 2014, Agrawal 2019). Overall, it became clear that the systematic surveillance of the circulating H9N2 AIV is far from sufficient and vaccines are not

updated frequently enough. This situation is in sharp contrast to vaccination control of H5 subtype viruses, under intense surveillance and regular vaccine updates. At this moment, vaccination seems ineffective enough to control the circulation of H9N2 and other virus strains in poultry.

1.5.1 From field problem to the scientific question

A vaccine in the field is often less efficacious than under laboratory conditions. For vaccines against the HPAIVs, the antibody titers achieved in the field were lower. The clinical signs were prevented, but the transmission of homologous viruses can still be estimated. Understanding the dynamics of the environmental conditions, vaccination procedures and other complex factors in the field is helpful to improve the vaccination. The effectiveness of vaccination is often measured by the ability of the antisera raised with the vaccine. The HI titer of the antiserum is a measure for the level of protection against the disease caused by AIV but does not show how much the shedding of the virus is reduced. Previous research has shown that there was a lower amount of virus shedding in vaccinated chickens (Arafat et al. 2018), which could reduce bird-to bird transmission (Swayne et al. 2000). Transmission experiments on HPIAVs (H7N7 (Van der Goot et al. 2005), H5N1 (Sitaras et al. 2016) in chicken) were carried out to study the effect of vaccination on epidemiologically relevant parameters, e.g. transmissibility and the infectious period. Similar research on LPAIVs was needed, especially after the national vaccination against H9N2 in poultry in some countries.

In the field, many poultry farms performed surveillance for HPAI H5 and LPAI H9N2 viruses after vaccination, and the isolation rates of H9N2 were higher than that of H5 viruses, even though an effective HI titer for H9 viruses was measured in the population. Because these companies are being able to produce inactivated-virus vaccines by using the current circulating virus strain to control the transmission of H9N2 AIV in their own farms, the update of the vaccine virus strain is more frequent than for the national commercial vaccines sold in China and the chickens are well protected against disease. Under this situation, where H9N2 associated disease is controlled in the domestic poultry industry, risks arise because the virus may be circulating undiscovered in the field.

Based on this field problem, we can assume that the long-term use of vaccines provides increased selection pressure on the targeted avian influenza viruses. Therefore, the currently circulating LPAI H9N2 strains likely evolved to evade the immune pressure applied by vaccination. To explore how influenza escapes the prevailing immune pressure, previous research focused on the amino acid changes in escape mutants, and many deletions and numerous substitutions in HA were observed *in vitro* (McDonald et al. 2007, Harvey et al. 2016), but for H9N2 this process is much less clear (Peacock et al. 2017). These studies all assumed that rapid evolution would result from the long-term existence of the immune pressure due to vaccination. In real life, the situation may be more complex than in the *in vitro* situation, as there may also be “intrinsic selection” that varies among different host tissues, among genetically different individuals and between different host species.

The living environments and population density and size of farmed poultry are very different from wild birds and vary per country. There are subtle but vital differences between aquatic (e.g. duck) and terrestrial birds (e.g. chicken), even for smaller-scale domestic poultry. Thus, there are differences in the replicating microenvironment where random mistakes occur

to generate diverse genotypes from a molecular view. Under natural conditions, influenza viruses replicate and evolve in the form of quasi-species (Andino et al. 2015) generated due to the rapid speed and error-prone characteristics of viral replication. The theory is that mutations provide the opportunities (resources) for evolution and that selective forces trigger the direction of evolution of AIs. Because of changing selective forces (e.g. immune pressure or host shifts), there is an advantage from having a distribution instead of one genotype.

There have been many studies for other RNA viruses with the aim to determine the influence of host and environmental factors on the appearance of mutations. These studies were focusing on vesicular stomatitis virus (VSV) (Combe et al. 2014), HIV-1 *in vivo* (Cuevas et al. 2015), measles virus (Cattaneo et al. 1988), human parainfluenza virus (Murphy et al. 1991), respiratory syncytial virus, Rift Valley fever virus, and noroviruses (Isidoro et al. 2002, Zahn et al. 2007), rather than influenza A virus. The study on VSV showed that the observed mutation rate varied between cell lines from different host species. Accordingly, this result provides a reference for the hypothesis that there might be differences in the amount of variation and the speed with which these can be found, especially in hosts belonging to different families like chicken and duck.

The genetic diversity of the quasi-species provides a pool for selection. Under intensive immune pressure, the selection pressure acting on the quasi-species may favour certain mutations so that these may become predominant survivors, successful escapers. In this thesis, we like to determine whether the predominant LPAI H9 genotypes differ when H9N2 LPAIV circulates in hosts in which different selection pressures apply. We also want to analyse whether the diversity/composition of the viral quasi-species would change when passaging occurs in different hosts.

1.6 Thesis outline

The prolonged circulation of an influenza virus strain in a partially immunized population might have a direct impact on the evolution of the virus, and particularly on the occurrence and outcome of antigenic drift. However, information on the impact of poultry vaccination on the evolutionary dynamics of AI viruses in the field is limited. In this research project, we will study H9N2 AIV evolution with and without immune pressure.

Since there were no direct research reports that revealed the transmission parameters of H9N2 AIV in vaccinated poultry, we first mimic the natural infection and transmission process of H9N2 AIV in vaccinated and non-vaccinated chickens (**Chapter 2)**. Next, we want to estimate the reproduction ratio (R) in non-vaccinated and vaccinated chicken, for which the statistical (S-R-I) model is used to. Therefore, we analyse surveillance samples collected between 2013 and 2018 from local markets in nine provinces in southern China (**Chapter 3)**. This chapter will also compare these data with the phylogenic and antigenic analysis of H9N2 AIV to determine its current evolution profile in field markets in regions of China where vaccination had been implemented since 1998. To understand whether (and how) selective pressure and mutations (genetic drift) influenced the antigenic drift of H9N2 AIV while circulating in different host species, we performed serial passaging with an H9N2 strain in cells and in chicken (*in vitro* and *in vivo*) **(Chapter 4)**. Finally, we study whether a reassortment (genetic shift) between the avian H9N2 AIV and the human H1N1 virus may give rise to a novel

virus that can readily adapt to novel hosts. We generated reassortant viruses using a reverse genetics system and studied the mutation rate of these viruses during serial passaging experiments in mammalian cells and mice. (**Chapter 5**).

With the newly obtained field information and the evolutionary changes observed in **Chapter 4 & 5**, the discussion will elaborate (i) on how the vaccine potentially influences the evolution (patterns or rate) in the field, (ii) how changes in selection pressure correlate with the evolutionary changes in the HA gene, with and without immune pressure, and (iii) how gene segments from the H9N2 avian strain may lead to evolutionary changes when reassortment occurs with a human strain (H1N1 in this research). In summary, this research will study the transmission of H9N2 in vaccinated chicken to provide information for current surveillance protocols and aims to provide a reference for the evolutionary changes of LPAI H9N2 in different hosts.

Vaccination with inactivated virus against low pathogenic avian influenza subtype H9N2 does not prevent virus transmission in chickens

Abstract

H9N2 subtype avian influenza has spread dramatically in China ever since first reported in the 1990s. A national vaccination program for poultry was initiated in 1998. Field isolation data show that the widely used inactivated H9N2 vaccine does not provide effective control of the transmission of this low pathogenic avian influenza (LPAI) virus in poultry. Current research has focused on two reasons: (i) insufficient immune response triggered by the vaccination with the inactivated virus, (ii) the occurrence of escape mutants selected by vaccine-induced immune pressure. However, few studies reported the effectiveness of the inactivated virus vaccine to reduce the LPAIV transmission in the field. We mimicked the natural infection and transmission process of the H9N2 AIV in vaccinated and non-vaccinated chickens. A statistical model was used to estimate the transmission rate parameters among vaccinated chickens, varying in serum hemagglutinin inhibition titers (HIT) and non-vaccinated chickens. We demonstrate, for the first time, that the transmission is not sufficiently reduced by the homologous H9N2 vaccine, even when vaccinated chickens have an IgG serum titer ($HIT>2^3$), which is considered protective for vaccination against homologous highly pathogenic avian influenza (HPAI) virus. Our study does, on the other hand, cast new light on virus transmission and immune escape of LPAI H9N2 virus in vaccinated chicken populations and shows that new mitigation strategies against LPAI viruses in poultry are needed.

2.1 Introduction

Low pathogenic avian influenza (LPAI) viruses can still cause severe economic losses in the poultry industry (Umar et al. 2018), even though mortality is much lower than that of HPAI. The H9N2 subtype LPAI virus has attracted attention for its wide range spread in many host species, as well as for the fact that it has become endemic in commercial poultry in many areas, affecting poultry productivity. Besides being a threat to the poultry sector, the H9N2 AIV also poses risks for public health as it can transmit from poultry to humans (Peiris et al. 1999, Butt et al. 2005, Pusch et al. 2018).

Theoretically, infectious diseases can be controlled by a stamping-out policy or vaccination (Capua et al. 2003, Capua et al. 2006). Studies show that vaccination can reduce the transmission of HPAI H7N7 (Van der Goot et al. 2005) and H5N1 (Sitaras et al. 2016) in chicken. Hence, a similar reduction in transmission was expected for LPAI viruses after vaccination. To control the spread of H9N2 AIVs in China, inactivated virus vaccines in poultry were licensed and implemented in 1998 (Li et al. 2005). In reality, the poultry industry update privately inactivated vaccines based on their own virus strains noted from timely surveillance, instead of depending on the national commercial H9N2 vaccines in poultry. However, outbreaks of H9N2 AIVs continue to be reported from vaccinated poultry farms (Zhang et al. 2008). The failure of vaccination might be because of inefficient application, low dose, and low vaccination coverage (especially in the household sector) (Peyre et al. 2009, Poland 2018). Moreover, the continuing transmission in combination with the intensive long-term usage of the inactivated virus vaccine may have led to antigenic changes leading to immune escape (Sitaras et al. 2014, Sitaras et al. 2020). Due to these possible vaccination failures in poultry farms, vaccination was suggested as part of an overall integrated control strategy, including continued nationwide surveillance, farm biosecurity, and Differentiating Infected from Vaccinated Animals (DIVA) strategy (Capua et al. 2004, Capua et al. 2006).

The inactivated virus vaccine was shown to be able to induce a strong immune response against the LPAI H9N2 virus in duck and chickens (using $2^{8.5}$ and 2^{6} EID_{50}/0.1 ml of virus for antigen production, respectively), and showed a significant reduction in virus shedding in the lab (Teng et al. 2015, Jing et al. 2017). Studies on virus shedding after vaccination also suggested a reduced shedding after vaccination with the H9N2 vaccine (Tavakkoli et al. 2011, Jing et al. 2017). However, direct information on the effect of vaccination to prevent transmission of H9N2 AIV was yet not available in these reports.

In this study, we quantified the transmission parameters of an H9N2 AIV in chickens vaccinated with a homologous inactivated virus vaccine. Chickens after vaccination were used to study the effect on transmission following an optimized experimental design (Sitaras et al. 2016). The stochastic SIR model was used to estimate the transmission rate parameters among vaccinated chickens using a GLM (Generalized Linear Model) statistical approach (Klinkenberg et al. 2002). No significant reduction of the reproduction ratio (R) was observed in chickens after vaccination, indicating inactivated virus vaccine failed to stop the transmission of LPAI H9N2 virus in this study. The quantified transmission parameters are essential information for vaccine development and vaccination strategies to control LPAI in poultry.

2.2 Method and materials

Ethics statement

All animal experiments were executed according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. Animal welfare in the European Union (Broom 2017) was referred to ameliorate animal suffering. The Animal Care and Use Committee of Shanghai Veterinary Research Institute (SHVRI) reviewed the protocols including operative details of euthanasia via air inhalation (inhalational agent is carbon dioxide). The permit number was SHVRI-C-2019-0628-049. The chickens were regularly fed and maintained by technicians at the Research Team of the Etiologic Ecology of Animal Influenza and Avian Emerging Viral Disease (Research Team for short) at SHVRI. All experiments involving viruses were performed within the Biological Safety Level 2 facility at the Animal Centre of SHVRI.

Virus and animals

The LPAI H9N2 virus, A/chicken/Jiangsu/A2093/2011 (KP866088.1, A2093), used to produce inactivated virus vaccine and viral inoculation in all the transmission experiments was provided by the SHVRI Research Team. This virus was propagated in 10-day-old specific pathogen-free (SPF) embryonated chicken eggs (ECEs) (Beijing Merial Vital Laboratory Animal Technology Co., Ltd). Allantoic fluid was harvested 48h after inoculation and stored at -80°C. The titer of the A2093 virus was determined and the 50% embryo infectious dose (EID_{50}) was calculated using the Reed & Muench method (Reed et al. 1938, Wlliams et al. 2016).

The White Leghorn chickens used in the experiments were hatched from SPF ECEs and raised in high containment facilities with an isolated air-circulation purification system. Enough water, proper food, and room for the chickens' social interaction, foraging, and exercise were provided. Manure was removed at the end of the experiment.

Inactivation and vaccination

The A2093 virus (10^9 EID_{50}/0.1 ml) was inactivated by incubation of the virus stock with 1:2000 β-propiolactone for 12h at 4°C. The residual β-propiolactone was evaporated at 37°C for 2h. Complete inactivation was confirmed by two passages of inoculation in ECEs for at least 72h. Each passage had to be negative in Hemagglutination (HA) assay as a criterion for successful inactivation. The live virus displayed 2^{10} HAU before inactivation and 2^6 HAU after inactivation. The inactivated A2093 strain was then mixed with Montanide VG71 (0.85g/cm^3) adjuvant at a volume ratio of 3:7 to guarantee good antigenicity. A total of 45 three-week-old SPF chickens were vaccinated with this virus emulsion (vaccine) by intramuscular injection in the legs. Twenty chickens were injected with 0.1ml of the 2^6 HAU/ml vaccine with the expectation of a low antibody response, and 25 were injected with 0.8ml of the 2^6 HAU/ml vaccine to achieve a high antibody response. Please note that chickens with a higher vaccine dose can still have a low antibody response.

Transmission experiments

Sera of vaccinated chickens were checked every day for two weeks post-vaccination and collected until the antiserum level was stable. Hemagglutination Inhibition (HI) assays were carried out with eight HA units of live A2093 virus as antigen to determine the HI titers (HIT) of the sera. All assays were performed in duplicate. Based on their HITs, the vaccinated chickens were separated into the high HIT group (serum HIT higher than 2^3) and low HIT group (HIT lower than or equal to 2^3). This cut-off was based on the titers shown to be effective for HPAI viruses when vaccinated with low doses, which better reflects the field situation. A control group was composed of unvaccinated SPF chickens. In each group, at least ten chickens were selected based on the HIT level; half of the chickens received inoculation, the other half were recipients for transmission.

In Experiment 1, the inoculated chickens were infected with 10^7 EID_{50} of the live A2093 virus at 0.1ml intra-nasally and 0.1ml intratracheally; in Experiment 2, 10^6 EID_{50} viruses were used for inoculation in the same way. A 10-fold lower dose of the A2093 virus was applied in the second experiment to test whether the virus dose used to challenge the birds was too high to be able to see the transmission prevention of the vaccine. On day one post-inoculation (d.p.i.), the contact chickens from the same HI group were added to the containment unit in which the inoculated birds were kept. Chickens in the same group were free to contact each other and to share the food and water supply, and thus were able to have contact with others, for example via excrement.

Oropharyngeal and cloacal swabs of all animals were collected on d.p.i. 1, 3, 5, 7, 9, and 14, and were stocked in 1 ml phosphate-buffered saline (PBS) soon after collection. Each of the samples was mixed thoroughly and centrifuged at 12000 g for 10 mins at 4℃ before the supernatants were collected and stored at -80℃. On d.p.i 14, the chickens were euthanized, and the serum was collected for HI assays.

Virus quantification

In Experiment 1, the viral titers of the oropharyngeal and cloacal swabs were determined using the 10-day-old ECEs as previously described (Klimov et al. 2012) and calculated using the Reed & Muench method (Reed et al. 1938). The detection limit of the method was set at 0.98 log10-EID_{50}/100 ul, when one of the three eggs incubated with the undiluted swab samples was positive in the HA assay.

In Experiment 2, samples were tested by a two-step reverse transcription (RT) quantitative polymerase chain reaction (qPCR) (Wilhelm et al. 2003). Chicken embryo allantoic fluid containing 10^6 EID_{50}/100ul H9N2 virus was diluted into 10^5, 10^4, 10^3, 10^2, and 10 EID_{50}/100 ul for the standard curve. Allantoic fluid from SPF ECEs was used as the negative control. Viral RNA of all samples was extracted using QIAGEN Viral RNA Isolation Kit, and the cDNA was synthesized with Transcriptor High Fidelity cDNA Synthesis Kit (Roche) following the manufacturer's protocol. The qPCR specific to the M gene of the influenza virus was performed by using AceQ Universal U^+ Probe Master Mix V2 (Vazyme) following the manufacturer's protocol. Threshold cycle (Ct) values of all the standard and swab samples and negative control were obtained. Standard curves were generated with the corresponding Ct values as its viral titers. Data were regarded as reliable only when the R-square value was above 0.996 in the data trendline. The Ct values were converted into logarithmic viral titers (log10-EID_{50}/100ul) based

on the standard curves and the corresponding regression line equations. The mean and 95% confidence interval (CI) of the swabs from SPF chicken were referred for a negative background control in the RT-qPCR method. The upper boundary of the 95% CI (1.97 log10-EID_{50}/100ul) was considered positive for virus shedding in this RT-qPCR method.

Rank test on virus shedding

For the total shedding value, we summed up the viral titers (log10-EID_{50}/100ul) of all positive oropharyngeal samples of every chicken in the three Experiment 1 and 2 groups.

Firstly, we compared the total shedding amount of the inoculated and contact individuals in every group to test if the different inoculation doses in two experiments influenced the virus shedding for inoculated chickens compared to the contact ones. If inoculated chickens shed more than the contact infected, the inoculation dose could have been too high compared to the dose the contact chickens were exposed to. The datasets were filtered into high HIT, low HIT, and control groups, setting experiments (Exp1 and Exp 2) as the factor with two levels.

Secondly, virus shedding was also compared between the vaccinated chickens (High HIT group/low HIT group) and non-vaccinated chickens (Control group) to evaluate the effect of this vaccination on reducing virus shedding. Datasets were filtered into inoculated and contact, setting group as the factor with two levels (high HIT vs. control or low HIT vs. control, high HIT combined low HIT vs. control).

A Wilcoxon rank test (Woolson 2007) was performed using RStudio (Dalgaard 2008). The exact p-value was computed for the two-tailed test.

Data collection for SIR model

The in-contact or recipient animals at the start were counted as “susceptible” (S), as is the convention in the SIR model. Furthermore, based on virus quantification, inoculated and any contact animals infected in the course of the experiment were counted as “Infectious” (I) from the first day (Date of Starting, DS) they were found to be positive until the last day a positive sample was found (Date of Ending, DE). After the end of the excretion period, the animals were counted as recovered (R), in a total population with N individuals: N = S+I+R.

A “case” (C) was noted whenever a contact animal was infected, i.e., once an individual changed from the susceptible state (S) to the infected/infectious state (I), the population composition changed into (S-1, I+1). We defined the transmission rate parameter (β) for the transmission rate, βSI/N; and the recovery rate parameter (α) for the recovery rate, αI. We collected the transmission-related data (S, I) to estimate the transmission parameters, especially β in the SIR model. Only the individual chicken with viral titers of the oropharyngeal samples above each cutoff level was counted for statistical analysis. To obtain sufficient statistical power to detect differences between vaccinated and unvaccinated groups, we planned to start the experiment with $I_0 = S_0 = 5$ chickens in each group.

Estimation of transmission parameters

We used the reproduction ratio (R) (Diekmann et al. 1990) to quantify the transmission, i.e. the average number of secondary cases caused by one typical infectious individual during its entire infectious period (T) in a fully susceptible population. The β was estimated with the number of infectious cases (C) in an interval and the number of susceptible (S) and infectious

cases (I) at the beginning of the interval. With the $\alpha = \frac{1}{T}$, we computed the reproduction ratio R = βT. The duration T of the virus excretion was estimated separately for vaccinated and unvaccinated chickens. The β was then estimated using a GLM (generalized linear model) implemented for our analysis in RStudio (Klinkenberg et al. 2002).

The probability that a susceptible individual becomes infected during the observation time interval Δt, is thus given by:

$$p = 1\text{-}e^{-\beta\frac{I\Delta t}{N}} \qquad (1)$$

In GLM analysis, a complementary loglog link function (ln [-ln(1-p)]) was used that transforms (1) into a linear relationship:

$$\text{cloglog}(p) = \ln[-\ln(1\text{-}p)] = \ln(\beta) + \ln(\frac{I\Delta t}{N}) \qquad (2)$$

In this relationship, the dependent variable (p) is the number of cases (C) divided by the binomial total (S), and the offset equals ln ($\frac{I\Delta t}{N}$). Furthermore, the error distribution is binomial. From the GLM analysis, we obtained the estimates of ln (β), its confidence intervals, and standard error.

To achieve a precise analysis of R from estimates of ln (β), ln(R) = ln (β) + ln(T), we counted the T value of every infected chicken in the contact population, of which we took the natural logarithm for the values of ln(T). Then the R and its confidence boundaries were estimated from the ln(R) and its confidence intervals. The variance of the estimator ln(R) was obtained using the following equation, assuming independence of ln(β) and ln(T):

$$\text{Var}[\ln(R)] = \text{Var}[\ln(\beta)] + Var[\ln(T)]$$

The 95% confidence interval can be calculated as:

$$\overline{\ln(R)} \pm 1.96\sqrt{Var[\ln(R)]}.$$

We also used the GLM analysis to find the effect of vaccination by separating the vaccinated chickens into high and low HIT groups with a threshold of 2^3 HI units as previously estimated for HPAI (Sitaras et al. 2016). In this model, we estimated β_{high} for chickens having high HIT, β_{low} for chickens with low HIT, and $\beta_{control}$ for non-vaccinated chickens. The dependent variable is the number of new cases C divided by S (C/S). Two dummy variables indicate either the high HIT group as 1 (0 otherwise) or the low HIT group as 1 (0 otherwise). As groups are homogeneous for the vaccination titer class, the regression coefficient c1 (see equation below) of the dummy variable for high titer gives the extra (or less) transmission in the high HIT group. So, this shows the combined effect of susceptibility and infectivity. The same is implied for c2, but then for the low HIT group. The equation for the model is:

$$\text{cloglog}(p) = c_0 + c_1 Ind_{high} + c_2 Ind_{low} + \ln(\frac{I\Delta t}{N})$$

Herein, $\ln(\beta) = c_0+c_1Ind_{high} +c_2Ind_{low.}$ Three βs can be obtained using the estimated regression coefficients from the GLM analysis:

$\beta_{control} = e^{c0}$

$\beta_{high} = e^{c0+c1}$

$\beta_{low} = e^{c0+c2}$.

Estimation of transmission was performed on both experiments combined after testing for a possible difference between the two experiments.

Comparison of βs in the two experiments

With the two dummy variables set for the high and low HIT and control groups, we compared βs by using the *glm* function in RStudio. The Wald test for regression coefficients was applied to test whether the regression coefficient was different from 0. In the summary table of *glm,* function, z_values were generated with the corresponding probability (Pr (>|z|)) and used to evaluate the differences of the ln(β) values between the control group and the high HIT group (coefficient of Ind_{high}) and or between the control group and the low HIT group (coefficient of Ind_{low}). We used 95% confidence (i.e. 5% error rate) for difference analysis.

To measure the effect of the difference in the virus inoculum in the two experiments as separate factors, we set values 1 (Experiment 1) and 0 (Experiment 2) in the GLM model. We compared the datasets of the high and low HIT and the control groups from the two experiments.

Power calculation of the estimated R value

To draw a conclusion in case no significant difference of the transmission rate parameters between high HIT and control groups was found, we calculated the power of the test for biologically relevant differences. With the parameter βs from the vaccinated and control groups, we calculated the corresponding R_{con} (control group) and R_{vac} (vaccinated group). Practically, we expected a successful vaccination would reduce the R to below 1, meaning that the virus cannot persist in poultry. We took the null hypothesis as H_0: $R_{vac} = R_{con;}$ the alternative hypothesis was H_a: $R_{vac} < R_{con.}$ The power calculation was based on the final size analysis of a corresponding pair-wise transmission experiment. It was shown to be a conservative estimate for the GLM analysis of any transmission experiment with any other group size containing the same number of S and I (Velthuis et al. 2007). The pair-wise final size difference can be analyzed as the difference between two binomial distributions (control vs. vaccinated group) with each having a different infection probability *p* for the contact animal in the pair to become infected. In this case, the p can be calculated using their Rs chosen under the null hypothesis ($R_{vac} = R_{con} = 2.0$) and under the alternative hypothesis ($R_{vac} < 1$ and R_{con} as estimated). By choosing $R_{vac} = R_{con} = 2.0$ under the null hypothesis, we know that we have the worst case for accepting the null hypothesis (Velthuis et al. 2007). This can intuitively be understood as then the probability for contact infection to occur is 0.5, which is the binomial distribution with the highest variance. Under the alternative hypothesis, infection probability is

$p = \frac{R}{R+2}$ (Velthuis et al. 2007).

With probability values of difference for every comparison, we calculated the cutoff value under H_0 and the power under the alternative hypothesis.

2.3 Results

Vaccination with inactivated virus A2093-H9N2

The HIT of the serum from the vaccinated chickens was collected three weeks post-vaccination when the antibody levels were detected to be stable. The HIT of the chickens before inoculation was recorded at 0 d.p.i. (Figure 2.1 and Figure 2.2). The chickens were grouped into high or low HIT groups based on the HI assay (Supplementary Table S2.1) as representative for a good or poor vaccination response. As in the field situation, the HIT varied between individuals after vaccination, but vaccination was considered successful for chickens obtaining HIT > 2^1. In Experiment 1, twelve chickens were included in the low HIT group, ten were included in the high HIT group, and twelve were in the control group. In Experiment 2, only eight chickens obtained HI titers over 2^3 after vaccination, twelve were included in the low HIT group, and ten in the control group. In each group, the number of inoculated and contact chickens was a 50:50 ratio.

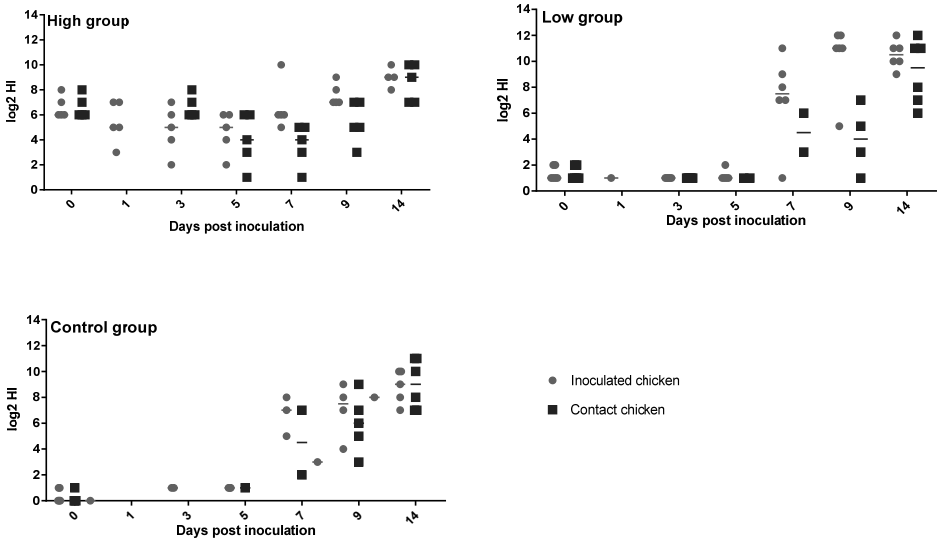


Figure 2.1 HI titers in the sera of the chickens before and after inoculation in Experiment 1. The high group and low group represent the high HIT group (serum HIT higher than 2^3) and low HIT group (HIT lower than or equal to 2^3), respectively. The Control group displayed the chickens without vaccination. All the HI titers of serum samples from individual chickens are displayed with median (bar) and range. Both the inoculated and contact chickens are shown.

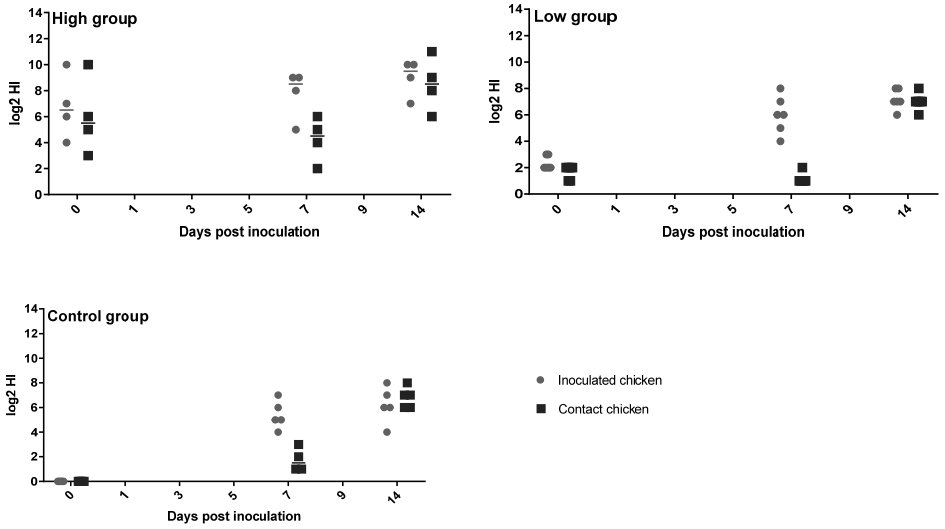


Figure 2.2 HI titers in the sera of the chickens before and after inoculation in Experiment 2. The high group and low group represent the high HIT group (serum HIT higher than 2^3) and low HIT group (HIT lower than or equal to 2^3), respectively. The Control group displayed the chickens without vaccination. All the HI titers of serum samples from individual chickens are displayed with median (bar) and range. Both the inoculated and contact chickens are shown.

Virus shedding

Antibody titers in the serum of both inoculated and contact chickens were checked in two experiments. All the contact chickens were infected according to a significantly increased HIT in serum on 14 d.p.i, (Figure 2.1 and Figure 2.2), indicating a successful infection with the virus. We observed no clinical signs in all the groups during the experiment. Only low levels of virus shedding were observed in cloacal swabs for this H9N2 strain in both experiments. Therefore, statistical analysis was performed on the data obtained from oropharyngeal swabs.

In Experiment 1, all the inoculated chickens from three groups tested virus positive in the oropharyngeal swabs from 1 d.p.i. Contact chickens in the high HIT group were all tested positive at 7 d.p.i. (Figure 2.3b); in the low HIT group (Figure 2.3c), all contact chickens were positive at 5 d.p.i., similar to the control group, which had 5 of 6 individuals infected (Figure 2.3a). All the details of the viral titers in the positive swabs are presented in Supplementary Table S2.2.

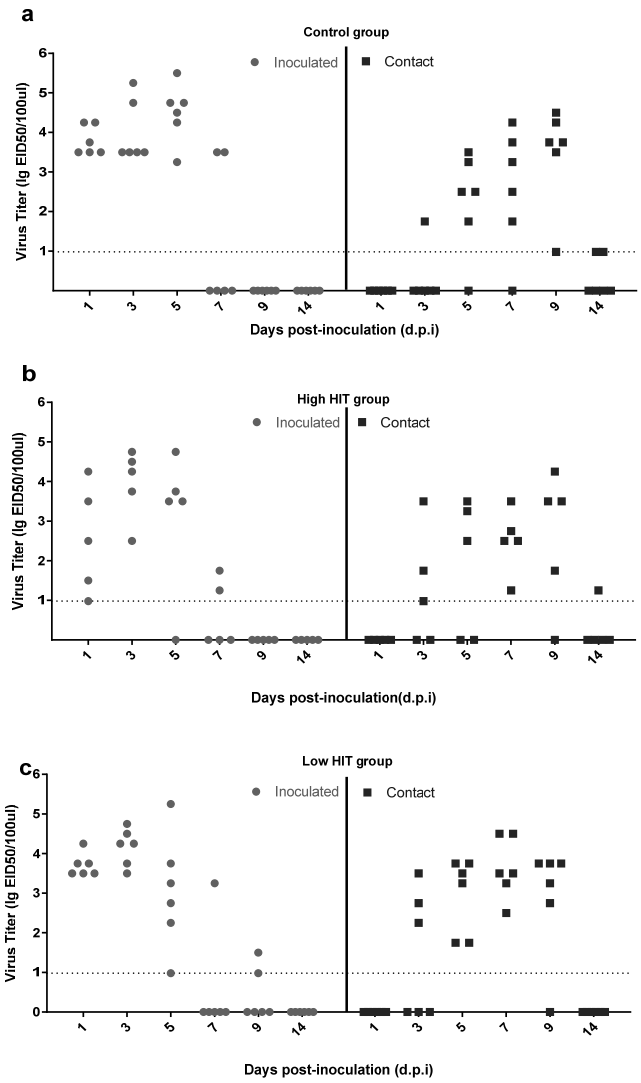


Figure 2.3 Viral titers of the oropharyngeal swabs after inoculation in Experiment 1. a Viral titers of the oropharyngeal swabs in the control group; b, Viral titers of the oropharyngeal swabs in the high HIT group; c, Viral titers of the oropharyngeal swabs in the low HIT group. Viral titers were determined in ECEs and calculated using the Reed-Muench mathematical method. The background cut-off line is 0.98 $lgEID_{50}$/100ul. All individuals which survived in this experiment and those with HI titers below the threshold are displayed on the x-axis.

Based on the detection method, the cut-off value of 1.97 log10-EID_{50}/100ul was set to count infectious cases and estimate the duration of virus shedding. The individuals with virus shedding below the cut-off were set as 0 (Figure 2.4). Not all the inoculated chickens were infected after 24 hours in each group, and they also had variable individual viral titers. In the control group, virus shedding was detectable in all of the contact chickens after 6 d.p.i. (Figure 2.4a). In the high HIT group, only one had a titer above the cutoff line after 24 hours (Figure 2.4b). On 3 d.p.i., three of the inoculated chickens showed significant virus shedding and two contact chickens became infected on 5 d.p.i. In the low HIT group (Figure 2.4c), two inoculated chickens shed viruses on four and 5 d.p.i and four of six contact chickens became infected on 6 d.p.i. Details in Supplementary Table S2.3.

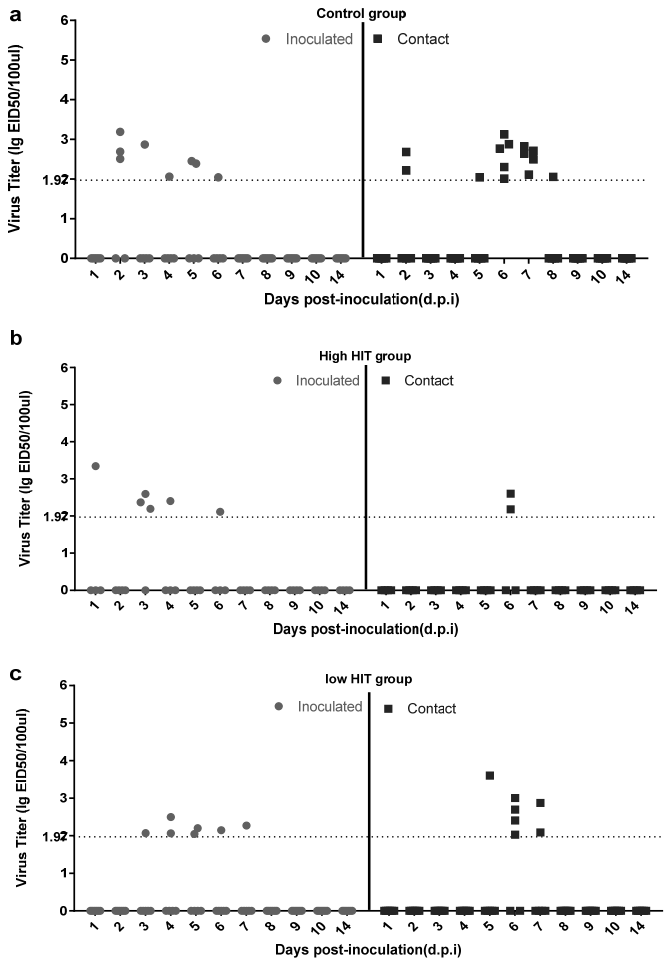


Figure 2.4 Viral titers of the oropharyngeal swabs after inoculation in Experiment 2. a Viral titers of the oropharyngeal swabs in the control group; b, Viral titers of the oropharyngeal swabs in the high HIT group; c, Viral titers of the oropharyngeal swabs in the low HIT group. Viral titers were determined using qPCR and calculated into EID_{50} values based on the standard curve. The background cutoff line is 1.97 lgEID_{50}/100ul. All the individuals were alive, but only those with viral titers above the cut-off are shown in the figure.

Comparisons of virus shedding in rank test

For the analysis of the transmission, we assumed that contact infected and inoculated chickens in the different groups had the same infectivity. However, the infectivity of inoculation may depend on the inoculation dose. An indication for too high inoculation dose (e.g. 10^7 EID_{50} in Experiment 1) would be when inoculated individuals shed more than the recipient contact individuals. Therefore, we compared the virus-shedding levels between the inoculated and contact individuals in the different groups in two experiments. The null hypothesis was that inoculated and contact chickens have the same virus-shedding level. In the Wilcoxon rank test (Table 2.1), p-values for the comparison between inoculated and contact chickens in Experiment 1 indicated the same distribution of the inoculated and contact population in the vaccinated population (p = 0.35 in high HIT group, p = 0.75 in low HIT group), but a biased shedding level in the control group (p = 0.0065). Comparing Experiment 1 with Experiment 2 showed that in experiment 1, virus shedding was significantly higher than in Experiment 2.

Table 2.1 Wilcoxon rank test of virus excretion comparing inoculated versus contact and Experiment 1 (Exp1) versus Experiment 2 (Exp2).

Comparisons	Datasets	Exp 1	Exp 2
Inoculated vs. Contact	High HIT Group	W = 8, p-value = 0.35	W = 0, p-value = 0.050
	Low HIT Group	W = 16, p-value = 0.75	W = 10, p-value = 0.56
	Control Group	W = 1, p-value = 0.0065#	W = 19, p-value = 0.17
Exp1 vs. Exp2*	High HIT Group	W = 58, p-value = 0.0024#	
	Low HIT Group	W = 95, p-value = 0.00029#	
	Control Group	W = 96, p-value = 0.018#	

* Sum of virus shedding of inoculated and contact chickens. # P-value < 0.05 indicated significant difference.

Considering the influence of inoculation doses, a comparison of virus shedding from vaccinated versus non-vaccinated chickens was carried out separately for the two experiments. Table 2.2 shows that the virus shedding of the chickens in the vaccination group with relatively high HIT ($>2^3$) was reduced compared to the non-vaccinated group in some comparisons: i.e. for experiment 1, this was in the inoculated group and experiment 2 in the contact infected group. In detail, in Experiment 1, six of the ten vaccinated chickens (high HIT group) obtained antisera titer at 2^6 HIT, and four were above 2^6 HIT, but the virus shedding level was not significantly different from the un-vaccinated group for both inoculated (p = 0.10) and contact chickens (p = 0.78). With less virus dose for inoculation in Experiment 2, virus shedding of chickens in the vaccinated group (high HIT group with five of eight chickens achieving more than 2^6 HIT) was significantly less in contact chickens, at only (p = 0.025). Moreover, combining the data of high HIT and low HIT group as a vaccinated population (mixed individuals with good and bad antibody responses), the virus shedding was also reduced with a significant difference in inoculated chickens but not in contact chickens (W = 53, p-value = 0.044) from Experiment 1. However, in Experiment 2 vaccination significantly reduced the virus shedding in contact chickens (p-value = 0.042) compared to inoculated ones. All these biases could be due to the influences from different inoculation doses. After combining data from the two experiments ("Experiments combined" in Table 2.2), no significant difference was found with the Wilcoxon rank test for both contact and inoculated chickens.

Table 2.2 Wilcoxon rank test of virus excretion comparing the vaccinated (High HIT and low HIT group) versus non-vaccinated (Control group) chickens.

Comparisons	Datasets	Exp 1	Exp 2	Combined Experiments
High HIT vs. Non-vaccinated	Inoculated	W = 24, p-value = 0.10	W = 6, p-value = 0.65	W = 51, p-value = 0.56
	Contact	W = 16.5, p-value = 0.78	W = 15, p-value = 0.025#	W = 59.5, p-value = 0.20
Low HIT vs. Non-vaccinated	Inoculated	W = 29, p-value = 0.078	W = 14, p-value = 0.33	W = 67, p-value = 0.40
	Contact	W = 10, p-value = 0.20	W = 15, p-value = 0.22	W = 58, p-value = 0.83
Vaccinated vs. Non-vaccinated*	Inoculated	W = 53, p-value = 0.044#	W = 20, p-value = 0.68	W = 118, p-value = 0.39
	Contact	W = 26.5, p-value = 0.51	W = 30, p-value = 0.042#	W = 117.5, p-value = 0.41

* The vaccinated data was the sum of virus shedding from the High HIT and low HIT group; Non-vaccinated data was from the control group. # P-value < 0.05 indicated significant difference.

Estimation of transmission rate parameters

The data of the transmission experiments used to estimate transmission parameters were observed as S, I, C, and N as described in the M&M above. The results are given in Table 2.3 and the result of the analysis is described below.

Table 2.3 Data abstracted from the transmission experiment for parameter estimation for the stochastic transmission model.

Experiment	Group	DS[a]	DE[b]	S	I	C	N
Exp 1	Control	1	3	6	6	1	12
	Control	3	5	5	7	4	12
	Control	5	7	1	11	1	12
	High	1	3	5	5	3	10
	High	3	5	2	8	1	10
	High	5	7	1	8	1	10
	Low	1	3	6	6	3	12
	Low	3	5	3	9	3	12
Exp 2	Control	2	3	3	5	0	10
	Control	3	4	3	3	0	10
	Control	4	5	3	4	1	10
	Control	5	6	2	5	2	10
	High	1	2	4	1	0	8
	High	2	3	4	1	0	8
	High	3	4	4	3	0	8
	High	4	5	4	2	0	8
	High	5	6	4	1	2	8
	High	6	7	2	3	0	8
	Low	3	4	6	1	0	12
	Low	4	5	6	3	1	12
	Low	5	6	5	3	3	12
	Low	6	7	2	5	0	12
	Low	7	8	2	3	0	12

* [a], Date of Start, the date when infection case(s) is observed; [b], Date of Ending, the date when the infection case ends.

Supplementary Table S2.4 displays the basic datasets of the two experiments for estimation and pairwise comparison between groups in RStudio. The average duration of the observed shedding (T), the estimated βs, and the corresponding R values of the different groups in both experiments are listed in Table 2.4. In experiment 1, the average infectious periods were 7.6, 7.8, 6.5 in the high, low HIT, and control groups, respectively. Whereas, in Experiment 2, the average T of both high and low HIT groups dropped (2 for the high HIT group and 2.8 for the low HIT group) compared to that of the control group (T = 5.0 days).

Table 2.4 Three transmission rate parameters of three groups in different datasets from two experiments.

Experiments	Group	Coefficient	β[a] (day^{-1})	T (days)	R[b]	0.95 CI of R	
						down	up
Exp 1	High HIT Group	-0.210	0.811	7.6	6.090	2.276	16.295
	Low HIT Group	0.007	1.007	7.8	7.727	3.007	19.860
	Control Group	-0.450	0.638	6.5	4.857	2.079	11.349
Exp 2	High HIT Group	-0.840	0.433	2.0	0.865	0.212	3.536
	Low HIT Group	-0.016	0.985	2.8	2.591	0.790	8.496
	Control Group	-0.251	0.778	5.0	3.562	0.833	15.227
Exp 1&2	High HIT Group	-0.442	0.643	6.0	3.305	0.740	14.766
	Low HIT Group	-0.003	0.997	5.8	4.988	1.290	19.284
	Control Group	-0.385	0.680	6.5	4.110	1.458	11.586
	Control & Low Group	-0.203	0.816	6.1	4.466	1.490	13.386

[a] β is calculated from exp (Coefficient).
[b] R is calculated from exp(lnR), lnR = lnβ+lnT

In the pairwise comparison of the β between groups in the two separate experiments, no significant differences between the high HIT and control groups were found, nor between the low HIT and control groups (Table 2.5). Combining the data for the control and low HIT group (C&L), gave a combined estimated $\beta_{C\&L}$ for unvaccinated/poor-vaccinated chickens in each experiment. Even then, no significant differences were found comparing the combined group to that of well-vaccinated (high HIT group) chickens in either experiment (Table 2.5, p= 0.931 in Experiment 1, p= 0.379 in Experiment 2).

We then conducted difference analysis between the two experiments separately for paired high, low HIT, and control groups, respectively. No significant differences were found in transmission parameters between paired HI level groups from the two experiments (Table 2.5). Practically, we combined the paired data of both experiments to estimate the transmission parameters without considering the inoculation dose. With the combined basic dataset (Table S4), we obtained more precise estimations for the transmission parameters of high, low HIT, and control groups, $\beta_{con} = 0.680\ day^{-1}$, $\beta_{high} = 0.643\ day^{-1}$, and $\beta_{low} = 0.997\ day^{-1}$. As in Table 2.4, the estimated T for the high HIT group was $T_{high} = 6.0$ days, $R_{high} = 3.31$ (95% CI, 0.740 -- 14.766), for the low HIT group was $T_{low} = 5.8$ days, $R_{low} = 4.99$ (95% CI, 1.290 -- 19.284); and for the control group it was $T_{con} = 6.46$ days, $R_{con} = 4.11$ (95% CI, 1.458 -- 11.586). Pairwise comparisons were analyzed between the high HIT and control group using the same method, but again no significant differences were observed (p-value = 0.911); we also found no significant difference between the low HIT and control group (p-value = 0.415) (Table 2.5). We then combined the statistics of the control and low HIT groups (C&L), obtaining the combined estimated $\beta_{C\&L} = 0.82\ day^{-1}$, and R = 4.47 for control combined with the low HIT group (95% CI, 1.490 -- 13.386). Still, we found no significant differences between vaccinated and unvaccinated chickens (p-value = 0.594).

Table 2.5 Comparison analysis of lnβ between groups.

Experiments	Comparisons of lnβ	p value	Effect (lnβ)
Exp1	Control vs. High Group	0.702	0.240
	Control vs. Low Group	0.451	0.457
	Low Group vs. High Group	0.735	0.216
	High Group vs. (C&L)	0.931	0.042
Exp 2	Control vs. High Group	0.524	-0.587
	Control vs. Low Group	0.758	0.236
	Low Group vs. High Group	0.348	-0.823
	High Group vs. (C&L)	0.379	-0.715
Exp 1&2	Control vs. High Group	0.911	-0.057
	Control vs. Low Group	0.415	0.383
	Low Group vs. High Group	0.594	-0.239
	High Group vs. (C&L)	0.594	-0.239
High HIT Group	Exp 1 vs. Exp 2	0.464	0.629
Low HIT Group	Exp 1 vs. Exp 2	0.972	0.023
Control Group	Exp 1 vs. Exp 2	0.780	-0.198
High Group vs. (C&L)	Trial I vs. Trial II	0.747	0.177

Power calculation

For the comparison of the R values between the vaccinated group with high titer and the unvaccinated control, we compared (S, I) = (6, 6) for control to (S, I) = (5, 5) for the high HIT group in Experiment 1; and (S, I) = (5, 5) for control and (S, I) = (4, 4) for the high vaccinated group in Experiment 2. In terms of power, this is the same as comparing between 9 pairs of (S, I) = (1, 1) for the vaccinated high titer group and 11 pairs of (S, I) = (1, 1) for the control group (Velthuis et al. 2007). The experimental data of the control group gave an estimated $R_C = 4.1$ with the number of sample pairs, $N_C = 11$. With the sample size of 9 pairs for the high HIT population and 11 pairs for the control, we obtained a p_value < 0.05 (0.04965) rejecting the null hypothesis ($R_{vac} = R_{con} = 2$). Then, under the alternative hypothesis with $R_{con} = 4.1$ and expected $R_{vac} = 0.6$, we obtained the power = 0.651.

After having found no significant differences between the low HIT and control groups in the transmission parameter, we combined the data from the low HIT and control groups for precise estimation of the R parameter and a larger sample size for higher power value. The experimental data of the unvaccinated population had a combined estimation $\beta_{C\&L} = 0.82$ day^{-1}, and an average T of 6.1 days, with $R_{C\&L} = 4.47$. The number of the sample pairs in this poorly or non-vaccinated population was $N_{C\&L} = 23$, and an estimated power of 0.781 was obtained with p_value as 0.049.

Therefore, we had more than 0.78 of power i.e. if vaccination after achieving HIT>2^3 in the high HIT experimental groups would have stopped the transmission of LPAI H9N2 virus (R<1), we would have detected it.

2.4 Discussion

In this study, we investigated the effect of the inactive-virus vaccine against LPAI H9N2 on the transmission of the homologous virus in chickens by combining the transmission experiments and SIR model for reproduction ratio (R). The starting assumption was that the transmission could successfully be stopped (R < 1) in a vaccinated chicken population if there is no antigenic difference between virus and vaccine. The stochastic SIR model was used to estimate transmission parameters from experimental data (De Jong et al. 1994). In this study, we estimated the transmission rate parameter based on the H9N2 virus from oropharyngeal shedding, given that it is difficult to evaluate clinical protection for LPAI viruses via observing clinical signs from infected chickens (Foster 2018, Germeraad et al. 2019).

In the transmission experiments, virus shedding was detected and used to determine the infectious status of the chickens for both inoculation and contact (susceptible individuals). The SIR model does not include an exact value for virus shedding; the infected case is defined based on virus detection of either 0 (negative) or > 0 (positive). Therefore, the viral titers determined using the 10-day-old ECEs with Reed & Muench method (Experiment 1) and by RT-qPCR (Experiment 2) were referred based on the methodology thresholds, respectively. When challenged with 10^7 EID_{50} of virus (Experiment 1), the transmission of LPAI H9N2 did not result in an R value below 1 in well-vaccinated chickens. With this high viral dose, all the inoculated chickens from the vaccinated groups were shedding virus via the oropharyngeal pathway, and total shedding levels between inoculated and contacts were not significantly different according to the Wilcoxon rank test. However, the high virus dose contributed to the high shedding of inoculated chickens in the control group (Table 2.1). We performed another independent transmission experiment with 10^6 EID_{50} viruses to avoid the bias of data from a single high inoculation dose. In this study, where we simulated the natural infection route via contact, virus shedding was still observed for both inoculated and contact chickens. No significant difference in the total shedding amount was found between inoculated and contact chickens in the Wilcoxon rank test, based on the combined data in two experiments. For the effect of this vaccine on the virus shedding, reduction in oropharyngeal virus shedding of contact chickens from the high HIT group was observed compared to that from the control group when the challenging dose was 10^6 EID_{50}. However, when combined with the data from two experiments, there was no significant difference between vaccinated (both in high HIT and low HIT group) and non-vaccinated chickens in the Wilcoxon rank test. Vaccination with inactivated virus vaccine did provide certain protection for those individuals obtaining high antibody response, but this protection did not stop virus shedding completely. This result may be biased by the fact that we have limited data for this group, and the infectious period was very short. The effects of the virus shedding on transmission were further analyzed using transmission models and statistical analysis.

In the statistical analysis, the inoculated dose was included as a modifying factor to obtain an overall estimation of the vaccination effect. Even though when challenging with less virus dose there were fewer infectious individuals, a shorter infectious period and lower total shedding amount were observed; still, the infection of contact chickens was observed, showing highly increased HI titers at the end of the experimental period (Table S1, except for the bird NO. 33). This suggests that vaccinated chickens can still be infected by the 10^6 EID_{50} viruses,

and the transmission rate is reduced by a high level of antibodies. But to achieve an optimal transmission model reflecting the transmission process with different viral doses and shedding level, as occurs in the field, we combined the two independent experiments together for analysis of the vaccination factor solely so that it would give a better estimation of the transmission rate parameters. The combined datasets showed that both virus shedding level and transmission rate were not significantly reduced by vaccination.

We applied the GLM to analyze our transmission experiments, as this is feasible for a heterogeneous population, providing higher power to find a difference in transmission between two treatment groups (Velthuis et al. 2007). After observing no differences between the control and low HIT group, we combined the parameters of these two groups (C&L). We also found no significant differences between the high HIT group and the combined (C&L) ($p = 0.594$). We thus accepted the null hypothesis that transmission rate parameters of vaccinated and unvaccinated chicken were not significantly different. As we only had a few groups and no large ones, we did a power analysis to evaluate the confidence of the conclusion drawn from this sample size. With an expected R value of 0.6 as an indication for successful vaccination, the power in our experiment was 78%, which is somewhat less than 80% due to the uneven sample sizes of the vaccinated and unvaccinated groups, and the lower number of individuals in the high HIT group. However, our results strongly suggest that even successful vaccination (in terms of achieving high titers $> 2^3$) cannot stop the transmission of H9N2 among vaccinated chickens.

In contrast to our results for H9N2, previous research showed a significant reduction in the transmission of some H5 and H7 HPAI subtype avian influenza in chickens after vaccination (Van der Goot et al. 2005, Bouma et al. 2009). In addition, research by van der Goot et al., (Van der Goot et al. 2007) showed a considerable reduction in transmission for H5N1. H9N2 vaccination was found to provide protective efficacy against disease for H9N2 avian influenza virus in ducks after intravenous injection of the virus. (Teng et al. 2015). However, in those vaccination and challenge experiments, the virus could still be detected (at low levels) in nasal or cloacal swabs during the experiments (Choi et al. 2008, Kim et al. 2017, Gan et al. 2019). For example, in the study suggesting that inactivated H9N2 vaccines containing at least 250 HAU/dose will minimize virus shedding in SPF chickens (Kilany et al. 2016), in 20%-50% chickens, 250 HAU/dose vaccination was detected with 2.3-2.48 EID_{50}/ml virus shedding. However, the transmission resulting from this virus shedding was not subsequently estimated. For the vaccines against HPAI in poultry, good protection against clinical signs and mortality was achieved (Swayne et al. 2016), whereas another study identified that a single vaccination dose hardly reduced transmission of the H5N1 virus in ducks, one week after vaccination (van der Goot et al. 2007). Because no clinical signs and mortality were observed in chickens infected with the LPAI H9N2 virus, the individual protection of this vaccine was evaluated based on virus shedding. In our experiment, the inactivated H9N2 vaccine did induce an immune response, as we measured an increase of antibodies in the serum. With reduced virus shedding, the transmission still continued in the population. Further research is required to provide more insights into the immune response and the relationship between vaccination and transmission of LPAI viruses.

In our research combining an experimental transmission study with mathematical modelling, we show that inactivated H9N2 vaccine is able to provide individual protection but unable to stop the transmission of the H9N2 AIV in chickens. The power of the statistical test used was 78%. Transmission modelling can give a statistical estimation of the vaccine effects on virus transmission in certain populations, thereby providing guidance for control strategies.

Acknowledgements

We are grateful for the fund and facility supports from Zejun Li's lab group at Shanghai Veterinary Research Institute (SHVIR), China. Hongrui Cui is enrolled through the Wageningen Institute of Animal Sciences (WIAS) and Production Ecology and Resource Conservation (PE&RC) graduate schools in the 2016 Sino-Dutch joint PhD program between the Chinese Academy of Agricultural Sciences (CAAS) and Wageningen University & Research.

Funding

This work was supported by the National Key Research and Development Program of China (2016YFD0500204) and the Chinese Academy of Agricultural Sciences of Technology Innovation Project.

Conflict of interest statement

There is no conflict of interest for all the authors in this research.

Supplementary materials

Table S2.1 HI titers of the anti-serum and group information for two experiments.

	Treatment	High-HIT group (Bird NO.)	HI titer after vaccination (HI log2)							Low-HIT group (Bird NO.)	HI titer after vaccination (HI log2)							Control group (Bird NO.)	HI titer after vaccination (HI log2)						
		d.p.i	0	1	3	5	7	9	14	d.p.i	0	1	3	5	7	9	14	d.p.i	0	1	3	5	7	9	14
Exp 1	Inoculated	(742)	6	5	5	5	10	7	die	(683)	2	\	1	1	7	12	9	(701)	1	\	\	\	\	\	7
		(745)	6	3	2	2	6	7	9	(684)	1	1	1	1	7	11	10	(702)	0	\	1	1	\	4	9
		(62)	8	7	7	6	6	9	10	(685)	1	\	1	\	11	12	11	(703)	0	\	1	1	5	9	10
		(64)	7	7	6	6	6	7	8	(692)	1	\	1	1	8	11	10	(704)	1	\	\	1	8	7	10
		(65)	6	5	4	4	5	8	9	(693)	2	\	1	1	1	5	12	(705)	0	\	\	\	7	8	8
		\	\	\	\	\	\	\	\	(694)	1	\	\	2	9	11	11	(707)	0	\	\	1	7	7	8
	Contact	(66)	7	\	7	6	5	5	7	(695)	1	\	1	\	\	1	12	(708)	0	\	\	\	2	9	11
		(67)	8	\	8	6	5	5	7	(696)	2	\	1	\	\	3	11	(709)	1	\	\	\	\	5	10
		(69)	6	\	6	4	3	3	9	(697)	2	\	1	1	3	7	11	(710)	0	\	\	\	\	3	11
		(70)	6	\	6	3	4	7	10	(698)	1	\	1	\	6	5	6	(717)	0	\	\	\	\	\	7
		(75)	6	\	6	1	1	7	10	(699)	1	\	1	\	\	\	8	(718)	0	\	\	\	\	6	7
		\	\	\	\	\	\	\	\	(700)	1	\	1	1	\	\	7	(719)	0	\	\	\	3	8	\
Exp 2	Inoculated	(31)	7	\	\	\	5	\	9	(35)	3	\	\	\	6	\	8	(935)	0	\	\	\	5	\	6
		(33)	10	\	\	\	9	\	10	(352)	2	\	\	\	5	\	7	(936)	0	\	\	\	4	\	4
		(36)	6	\	\	\	9	\	10	(353)	2	\	\	\	4	\	7	(938)	0	\	\	\	7	\	8
		(52)	4	\	\	\	8	\	7	(354)	2	\	\	\	8	\	7	(939)	0	\	\	\	5	\	6
		\	\	\	\	\	\	\	\	(355)	2	\	\	\	6	\	6	(942)	0	\	\	\	6	\	7
		\	\	\	\	\	\	\	\	(356)	3	\	\	\	7	\	8	\	\	\	\	\	\	\	\
	Contact	(37)	10	\	\	\	5	\	8	(57)	2	\	\	\	1	\	7	(941)	0	\	\	\	2	\	8
		(38)	6	\	\	\	6	\	11	(60)	1	\	\	\	1	\	7	(943)	0	\	\	\	3	\	7
		(34)	3	\	\	\	2	\	6	(333)	2	\	\	\	1	\	7	(944)	0	\	\	\	1	\	7
		(53)	5	\	\	\	4	\	9	(343)	2	\	\	\	1	\	7	(945)	0	\	\	\	1	\	6
		\	\	\	\	\	\	\	\	(348)	1	\	\	\	1	\	6	(947)	0	\	\	\	\	\	6
		\	\	\	\	\	\	\	\	(360)	2	\	\	\	2	\	8	\	\	\	\	\	\	\	\

NOTE: Exp 1, Experiment 1 used 10^7 EID_{50} H9N2 (A2093) virus for inoculation; Exp 2, Experiment 2 used 10^6 EID_{50} H9N2 (A2093) virus for inoculation. \, indicated no data collected.

Table S2.2 Viral titers in the oropharyngeal and cloaca swabs in Experiment 1.

Group		Challenge strain	Antigen	NO.	Virus titer test by egg inoculation-Oropharynx Days post inhalation (EID_{50}/100ul)						Virus titer test by egg inoculation-Cloaca Days post inhalation (EID_{50}/100ul)					
					D1	D3	D5	D7	D9	D14	D1	D3	D5	D7	D9	D14
High-titer Group	Inoculated	H9N2 (A2093)	H9N2 (A2093)	742	2.50	3.75	0	0	0	0	0	0	1.25	0	0	0
				745	3.50	4.75	4.75	1.25	0	0	0	0	0	0	1.25	0
				62	0.98	2.50	3.50	1.75	0	0	0	0	0.98	0	0	0
				64	1.50	4.25	3.50	0	0	0	0	0	0	0	0	0
				65	4.25	4.50	3.75	0	0	0	0	0	0	0	0	0
	Contact	H9N2 (A2093)	H9N2 (A2093)	66		0	0	2.50	4.25	1.25		0	0	0	0	0
				67		0.98	0	3.50	3.50	0		0	0	0.98	0	0
				69		0	2.50	2.75	3.50	0		0	0	0	0	0
				70		1.75	3.25	2.50	0	0		0	0	0	0	0
				75		3.50	3.50	1.25	1.75	0		0	0	0.98	0	1.25
Low-titer Group	Inoculated	H9N2 (A2093)	H9N2 (A2093)	683	3.75	4.25	3.75	0	0	0	0	0	0	0	0	0
				684	3.75	3.75	3.25	0	0	0	0	0	0	0	0	0
				685	3.50	4.75	0.98	0	0	0	0	0	0.98	0	0	0
				692	4.25	3.50	2.25	0	0.98	0	0	0	1.50	0	0	0
				693	3.50	4.50	5.25	3.25	1.50	0	0	0	0	0	0	0
				694	3.50	4.25	2.75	0	0	0	0	0	0	0	0.98	0
	Contact	H9N2 (A2093)	H9N2 (A2093)	695		0	1.75	4.50	3.75	0		0	0	0	0	0
				696		2.75	3.50	4.50	2.75	0		0	0	0	0	0
				697		3.50	3.75	3.25	0.00	0		0	0.98	1.25	0.98	0
				698		2.25	3.25	3.50	3.75	0.98		0	0	0	0	0
				699		0	1.75	2.50	3.25	0		0	0	0	0	0
				700		0	3.75	3.50	3.75	0		0	0.98	0	0	0
Control	Inoculated	H9N2 (A2093)	H9N2 (A2093)	701	3.50	5.25	4.75	3.50	0	0	0	0	0	1.25	0	0
				702	3.50	3.50	4.25	3.50	0	0	0	1.25	0.98	1.25	0	0
				703	3.50	4.75	4.75	0	0	0	0	0	0	0.98	0	0
				704	4.25	3.50	4.50	0	0	0	0	0	1.50	0.98	0	0
				705	3.75	3.50	5.50	0	0	0	0	0	0	1.50	0	0
				707	4.25	3.50	3.25	0	0	0	0	0	2.25	0	0.98	0
	Contact	H9N2 (A2093)	H9N2 (A2093)	708		1.75	3.25	0	0.98	0		0	0	2.75	0	0
				709		0	2.50	3.75	4.50	0		0	0	0	0	0
				710		0	1.75	1.75	3.50	0.98		0	0	0	1.75	0
				717		0	0	2.5	4.25	0.98		0	0	0	0	0
				718		0	2.50	3.25	3.75	0		0	0	0	0	0
				719		0	3.50	4.25	3.75	0		0	1.25	2.25	1.25	0

Table S2.3 Viral titers in the oropharyngeal and cloaca swabs in Experiment 2.

Group		Challenge strain	Antigen	NO.	Virus titer transformed from Ct value-Oropharynx Days post inhalation (EID_{50}/100ul)											Virus titer transformed from Ct value-Cloaca Days post inhalation (EID_{50}/100ul)										
					D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D14	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D14
High-titer Group	Inoculated	H9N2 (A2093)	H9N2 (A2093)	31	1.87	1.87	2.19	0.55	0	2.11	0	0	0	0	0	1.80	1.83	1.82	0	0	0	0	0	0	0	0
				33	1.89	1.83	1.95	0.47	0.20	0.27	0	0	0	0	0	1.91	1.84	1.84	0	0	0	0	0	0	0	0
				36	3.34	1.88	2.37	1.80	0	0.35	0	0	0	0	0	1.73	1.83	1.72	0	0	0	0	0	0	0	0
				52	1.91	1.91	2.60	2.40	1.24	1.22	0.14	0	0	0	0	1.78	1.78	0	0	0	0	0	0	0	0	0
	Contact	H9N2 (A2093)	H9N2 (A2093)	37		1.83	0	0	0	2.18	0.57	0	0	0	0		1.81	0	0	0	0	0	0	0	0	0
				38		1.51	0	1.05	0.19	1.74	0.78	0	0	0	0		1.39	0	0	0	0.02	0	0	0	0	0
				34		1.72	0	0	0	1.25	0.72	0	0	0	0		1.84	0	0	0	0	0	0	0	0	0
				53		1.65	0	0.54	0	2.60	1.39	0	0	0	0		1.56	0	0	0	0	0	0	0	0	0
Low-titer Group	Inoculated	H9N2 (A2093)	H9N2 (A2093)	35	0	1.68	1.68	2.07	2.20	1.79	0.31	0	0	0	0	0	1.65	1.75	1.72	0	0.02	0	0	0	0	0
				352	0.39	1.79	1.83	1.88	2.05	2.15	2.27	0.67	0	0	0	0	1.82	1.80	1.84	0	0	0	0.24	0	0	0
				353	0	1.69	1.77	1.76	0	0.68	0	0	0	0	0	0	0.96	1.71	1.77	0	0	0	0	0	0	0
				354	0.55	1.68	1.71	2.50	0.99	1.02	0.44	0	0	0	0	0	1.64	1.70	1.71	0	0	0	0	0	0	0
				355	1.22	1.59	1.75	1.72	0	1.28	1.56	0.11	0	0.58	0	0	1.70	1.80	1.74	0	0.13	0	0	0.37	0	0
				356	1.90	1.67	2.07	1.79	0	0.24	0.29	0	0	0	0	1.70	1.67	1.78	1.64	0	0	0	0	0	0	0
	Contact	H9N2 (A2093)	H9N2 (A2093)	57		1.89	1.87	1.65	0	0.92	0.90	1.88	1.57	1.50	0		1.83	1.73	1.69	0	0	0	0	0	0	0
				60		1.84	1.75	1.78	3.60	2.03	2.09	0	0	0	0		1.87	1.70	1.73	0	0.28	1.73	0	0	0	0
				333		1.88	1.57	1.78	0.04	3.01	2.87	1.94	1.38	0.13	0		1.83	1.70	1.74	0	0	0	0	0	0	0
				343		1.88	1.70	1.71	1.12	2.70	1.45	1.81	0.42	0	0		1.90	1.70	1.70	0	0	0	0	0	0	0
				348		1.89	1.75	1.76	0.67	2.41	1.87	1.73	1.86	0.61	0		1.87	1.81	1.79	0	0	0	0	0	0	0
				360		1.87	1.61	1.83	1.63	1.74	1.60	1.11	0.20	0	0		1.84	1.75	1.79	0	0	0	0	0	0	0
Control	Inoculated	H9N2 (A2093)	H9N2 (A2093)	935	1.22*	1.55	2.87	1.84	2.39	2.04	1.04	0	0	0	0	0	1.20	1.55	1.79	0	0	1.44	0	0	0	0
				936	0.77	1.79	1.63	2.06	2.45	1.73	1.78	0	0	0	0	0	1.58	1.58	1.75	1.32	0.54	0	0	0	0	0
				938	0.02	2.69	1.73	1.74	1.07	0.07	0	0	0	0	0	0	2.92	1.57	1.72	0	0	0	0	0	0	0
				939	0	2.51	1.63	1.74	0.42	1.00	0.98	0	0	0	0	0	3.26	1.62	1.86	0	0	0	0	0	0	0
				942	0	3.19	1.67	1.84	1.40	0	0.29	0	0	0	0	0	3.25	1.72	1.82	0	0	0	0.23	0	0	0
	Contact	H9N2 (A2093)	H9N2 (A2093)	941		1.51	1.88	1.81	2.04	2.77	2.11	1.16	1.38	1.10	0		1.44	1.74	1.78	0	0	0	0	0	0	0
				943		2.68	1.74	1.75	0.85	2.01	2.71	1.61	0.25	0.82	0		1.93	1.83	1.78	0	0	0	0	0	0	0
				944		1.90	1.83	1.87	1.96	3.12	2.64	1.19	1.17	0.23	0		1.97	1.74	1.58	0	0	0	0	0	0	0
				945		2.22	1.66	1.76	0.81	2.88	2.49	2.05	0.79	0.33	0		1.60	1.67	1.70	0	0	0	0	0	0	0
				947		1.62	1.74	1.74	0.87	2.30	2.83	0.09	0	0	0		1.63	1.62	1.82	0	0.21	0	0	0	0	0

Note: The cutoff value for the positive individual is 1.97 due the detection method of real-time PCR. *The first day has no infectious, but on day2 there were 5 infected. It could be possible the environment is poluted by the virus when doing inoculation.

Table S2.4 Datasets of the three groups in the two experiments for estimation in SIR model and GLM model.

Group	C_H	C_L	DS	DE	S	I	C	N	dt	offset	exp
Control	0	0	1	3	6	6	1	12	2	0	1
Control	0	0	3	5	5	7	4	12	2	0.154	1
Control	0	0	5	7	1	11	1	12	2	0.606	1
High	1	0	1	3	5	5	3	10	2	0	1
High	1	0	3	5	2	8	1	10	2	0.47	1
High	1	0	5	7	1	8	1	10	2	0.47	1
Low	0	1	1	3	6	6	3	12	2	0	1
Low	0	1	3	5	3	9	3	12	2	0.405	1
Control	0	0	2	3	3	5	0	10	1	-0.69	0
Control	0	0	3	4	3	3	0	10	1	-1.2	0
Control	0	0	4	5	3	4	1	10	1	-0.92	0
Control	0	0	5	6	2	5	2	10	1	-0.69	0
High	1	0	1	2	4	1	0	8	1	-2.08	0
High	1	0	2	3	4	1	0	8	1	-2.08	0
High	1	0	3	4	4	3	0	8	1	-0.98	0
High	1	0	4	5	4	2	0	8	1	-1.39	0
High	1	0	5	6	4	1	2	8	1	-2.08	0
High	1	0	6	7	2	3	0	8	1	-0.98	0
Low	0	1	3	4	6	1	0	12	1	-2.48	0
Low	0	1	4	5	6	3	1	12	1	-1.39	0
Low	0	1	5	6	5	3	3	12	1	-1.39	0
Low	0	1	6	7	2	5	0	12	1	-0.88	0
Low	0	1	7	8	2	3	0	12	1	-1.39	0

P. Novack

Molecular characterization of antigenic drift of avian H9N2 viruses in southern China

Abstract

The H9N2 subtype avian influenza virus (AIV) has become endemic in poultry globally, however due to its low pathogenicity, it is not under primary surveillance and control in many countries. Recent reports of human infection caused by H9N2 AIV has increased public concern. This study investigated pathogenic and antigenic analysis H9N2 AIV isolated from local markets in nine provinces in southern China from 2013 to 2018. We detected an increasing annual isolation ratio of H9N2 AIV. The Neighbor-joining tree of HA genes suggests that isolated strains were rooted in BJ94 lineage but have evolved into new subgroups (II & III) part from the existing subgroup I. The estimated mutation rate of the subgroup III strains was 6.23×10^{-3} substitutions/site/year, which was 1.5-fold faster than that of the average H9N2 HA rate (3.95×10^{-3} substitutions/site/year). Based on the antigenic distances of isolated strains to vaccine strain (F98 in subgroup I) in HI assay, subgroup II and III strains resulted in two clear antigenic clusters (cluster 2&3) away from the F98 in cluster 1. New antigenic properties of subgroup III viruses were identified at eleven amino acid positions in the HA protein sequence, which are examples of antigenic drift and potentially change the immunogenic properties of the H9N2 AIV. Our phylogenetic and antigenic analysis over 2013-2018 shows the evolutionary change of the HA ORF of the H9N2 strains in local markets in China, providing new insights in the antigenic drift.

IMPORTANCE The H9N2 LPAI virus has become endemic in poultry globally. In several Asian countries, vaccination against H9N2 AIV was permitted to reduce economic losses in the poultry industry. However, surveillance programs initiated after the introduction of vaccination identified the persistence of H9N2 AIV in poultry (especially in chicken in South Korea and China). Recent reports of human infection caused by H9N2 AIV has increased public concern. Surveillance of H9N2 circulating in poultry in the fields or markets was essential to update the vaccination strategies. This study investigated phylogenetic and antigenic analysis on H9N2 AIVs isolated from local markets in nine provinces in southern China from 2013 to 2018. The discoveries of the potential antigenic sites on the HA gene provide a timely reference for discovering the evolution of H9N2 under selection pressure from vaccination and updating vaccination strategy in poultry.

3.1 Introduction

Currently, H9N2 avian influenza virus (AIV) is one of the predominant subtypes (together with H5 and H7 subtype AIVs) circulating in both wild birds and poultry (Alexander 2000, Perez et al. 2003). Ever since the first isolation reported in the USA in 1966, the H9N2 subtype AIV has become endemic in many countries across different continents, including North America, Africa, Oceania, Asia and Europe (Homme et al. 1970, Peacock et al. 2019). Despite the direct economic loss in poultry industry over the past decade, H9N2 AIV also has a potential threat to public health by providing inner genes for reassortment. Recent reports of human infection cases caused by H9N2 AIV has increased public concerns (Butt et al. 2005, Wan et al. 2011, Huang et al. 2015, Pan et al. 2018).

Surveillance of H9N2 AIV is carried out in many countries, especially China and other Asian countries in which the live poultry markets (LPM) are a potential location for human-poultry transmission or reassortment (Liu et al. 2003, Nguyen et al. 2005). Genetic monitoring information shows two main phylogenic branches, the Eurasian branch and the American branch. The Eurasian branch consists of at least three stable poultry lineages, G1 (A/quail/Hong Kong/G1/1997), BJ94 (A/chicken/Beijing/1/94) and Y439/Korean lineage (A/chicken/Hong Kong/Y439/1997) (Guo et al. 2000, Lu et al. 2005). However, research shows that the dominant H9N2 AIV lineage differs depending on which endemic region is considered. The BJ94-like lineage is suggested to be dominant in poultry in mainland China (Lu et al. 2005). Li et al. reported that HA genes of some H9N2 isolated from chicken and other hosts between 2009-2011 were mainly derived from Y280-like lineage (Li et al. 2012). In addition, Yang et al. reported G9-like H9N2 viruses as being mainly isolated in mainland China (Yang et al. 2019). The Y280 and G9 lineage developed from the early BJ94 lineage, which has been detected throughout China since 1994 (Peacock et al. 2019).

In addition to monitoring programs, some countries have also implemented poultry vaccination to prevent H9N2 AIV, which has become endemic (Choi et al. 2008, Zhang et al. 2008, Gu et al. 2017, Dharmayanti et al. 2020). However, due to the rapid mutation of RNA virus, AIVs undergo antigenic drift under the immune pressure from the large-scale and long-term use of vaccines. Lessons learned from the failure of vaccine in prohibiting virus shedding and transmission of AIV (Lee et al. 2004, Park et al. 2011) show the need for correct and regular updates of vaccine strains, based on the genetic and antigenic properties. For example, the A/Chicken/Shanghai/F/98 strain was found to be entirely different from the G1 lineage directly related to human infection in Hong Kong, and this was then selected for a commercial poultry vaccine used in mainland China (Lu et al. 2003, Shi et al. 2005). In this way, monitoring programs providing information on the genotypic characters of H9N2 AIV that relate to potential antigenic changes contribute to better management. Besides, the continuous circulation of H9N2 in vaccinated poultry not only hints at an antigenic drift and/or clade replacement by vaccine, but also introduces a doubt whether the inactivated vaccine is effective at stopping transmission of LPAI H9N2 in poultry. Our previous research (**Chapter 2**) indicated the high possibility of transmission undergoing in vaccinated chicken with inactivated vaccine. This, consequently, might lead to genetic changes towards antigenic mutations. Research that combines genetic mutation with antigenic changes is needed to better understand the molecular determinants of H9 antigenicity.

Since 2003, monitoring programs of LPAI H9N2 in southern China were conducted by the Research Team of the Etiologic Ecology of Animal Influenza and Avian Emerging Viral Disease in SHVRI. Swab samples from domestic poultry (chicken, duck, pigeon, goose) were collected annually in the local markets in southern China. The most recent samples collected from 2013 to 2018 showed an increasing annual isolation ratio of H9N2 AIV in southern China. The phylogenetic and antigenic analyses of the H9N2 isolates were combined to identify a common clustering change. The most recent isolates clustered together but were far from the vaccine strains. Isolated strains forming a novel antigenic group in antigenic assay also clustered in a new subgroup in the phylogenetic tree. The evolution rate of strains in the new antigenic group was estimated to be higher than the average rate of H9N2 in China. Moreover, amino acid changes on HA protein indicating potential antigenic sites provides new evidence for the molecular evolution of H9N2.

3.2 Materials and methods

Ethics statement

All animal experiments were conducted in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China and the Netherlands. The protocols (SHVRI-SZ-20190730-01 and SHVRI-SZ-20190906-02) used in the study were approved by the Animal Care and Use Committee of Shanghai Veterinary Research Institute (SHVRI).

Samples collection and virus isolation

Between 2013 to 2018, a total of 13981 oropharyngeal swabs and cloaca swabs were collected from chickens, ducks, geese and pigeons in local markets from nine provinces in China (Guangzhou, Fujian, Jiangxi, Zhejiang, Shanghai, Anhui, Jiangsu, Shandong, Hunan). Swab samples were inoculated in the allantoic cavity of 10-day-old specific-pathogen-free (SPF) chicken egg embryos. The harvested allantoic fluids were tested by Hemagglutination Assay (HA). Total viral RNAs of HA-positive allantoic fluids were extracted to detect influenza viruses by amplifying the M gene in Polymerase chain reaction (PCR). Hemagglutination Inhibition (HI) assays were then used as anti-serums specific for H9 subtypes influenza viruses.

Phylogenic analysis, mutation rate and gene diversity of HA gene

The viral RNA was extracted and reverse transcription was performed with Uni12 primer as previously described (Hoffmann et al. 2001, Beato et al. 2014). The eight full-length segment nucleotide sequences of H9N2 collected from 2013 to 2018 in China were submitted to the GIASAID database (https://platform.gisaid.org/epi3/cfrontend#80368). For the strains isolated in 2018, only the HA and NA gene sequences were available at the time of performing the study. Complete HA sequences of H9N2 isolated strains were edited using EditSeq in Lasergene 7.0. Based on previous research (Teng et al. 2016), representative strains of H9N2 in China and from other Asian and American countries were selected for neighbor-joining (NJ) tree using MEGAx. The TN93 (Tamura-Nei, 93) +G (0.39) model was used with 1000 bootstraps.

To generate a time-scale tree of the whole lineage containing the H9N2 strains isolated from this lab (Zehender et al. 2012), we included all G9 lineage strains from the data set in previous research (Yang et al. 2019) plus a few additional representatives for the lineage

indications from mainland China. The exact date information was adjusted. We then constructed time-scaled phylogenies using a Bayesian Markov chain Monte Carlo (MCMC) framework implemented in the BEAST (v1.10.2) (Suchard et al. 2018). Maximum clade credibility (MCC) trees were reconstructed with 10% burn-in, and the posterior distribution of relevant parameters was assessed in FigTree (v 1.4.4). The Skyride plot was generated in Tracer with an estimation for the mean of mutation rate (substitution/site/year).

Antigenic assay

According to the phylogenetic tree of the HA gene, 29 H9N2 viruses were isolated from 2013 to 2018; we selected five early isolates (A/chicken/Hunan/B445/2011, A/chicken/Jiangsu/A2093/2011, A/Chicken/Shanghai/441/2009, A/chicken/Hunan/2106/2009), A/Chicken/Shanghai/F/1998) for the antigenic analysis. Briefly, specific antisera with each virus isolate were produced in SPF Leghorn chicken with adjuvant MONTANIDE ISA 71 VG (SEPPIC). The hemagglutination inhibition (HI) assay was conducted to test cross-activity between virus and anti-serum as described prescribed by the OIE (Stear 2005). The test was performed in duplicate and by two independent persons and medium value was calculated (Supplementary Table S3.1). This dataset of HI assay was used to calculate antigenic distances between the different H9N2 isolates by using the method as described previously (Sitaras et al. 2014) in R studio. Scatter plot of all the H9N2 isolates with their distance to the vaccine strain (F98) was programmed in R studio.

Molecular evolution analysis

To investigate the amino acid mutations that played an important role in the appearance of a new antigenic cluster, the HA protein sequences of selected 2013-2018 H9N2 isolated viruses and six previous H9N2 viruses were aligned and compared. Based on the new clustering in the antigenic assay, we searched for specific molecular changes on the amino acid sequences which were different between the antigenic clusters. To achieve the 3D position of the identified molecular changes on the sites of HA protein, the HA1 structure of H9 was simulated. The A/swine/Hong Kong/9/98(H9N2) (1JSD) (Ha et al. 2002) was used as a template with sequence identity as 86.21. The HA structure model was built with 0.96 GMQE (Global Model Quality Estimation) and 0.23 QMEAN (Studer et al. 2019).

We then studied the real-time dynamic changing of these potential antigenic sites by generating phylogenetic tree of the genotype at every site on one practical online database (https://nextstrain.org) (Hadfield et al. 2018). To zoom in on the evolutionary situation in China, we filtered the H9N2 sequence data from avian influenza in China from 1968 to 2020. We coloured the genotype and selected the interesting potential antigenic site on HA protein, so that the distribution and diversity of the amino acids at that site would display in a time-scale tree. In this way, the molecular evolution of one amino acid site locating on HA protein was traced based on real-time H9N2 data all over China.

3.3 Results

Epidemiology information of samples from southern China from 2013 to 2018

In order to monitor the prevalence of H9N2 AIV in southern China, we collected oropharynx and cloaca samples of poultry from LPMs in nine cities of different provinces in southern China. From 2013 to 2018, a total of 13981samples were collected from chickens (9363 samples), ducks (4505 samples), geese (81 samples) and pigeons (32 samples) in different LPMs (Table 3.1). The total isolation ratio of the H9N2 subtype accounted for the majority of the 1549 avian influenza-positive samples, with a ratio of 78.18% (1211 /1549) over the whole period. The separation rate of H9N2 AIV in 2013, 2014, 2015, 2016, 2017 and 2018 were 2.67% (104/3895), 6.10 % (225/3689), 15.69 % (339/2161), 15.25 % (329/2158), 10.44 % (188/1801), and 9.39% (26/277) respectively (Figure 3.1a, Table 3.1). From a regional perspective, the H9N2 AIV separation rate is higher in the three provinces and cities: Guangzhou, 11.23%; Shanghai, 10.88 %; Jiangxi 10.79 % (Figure 3.1b).

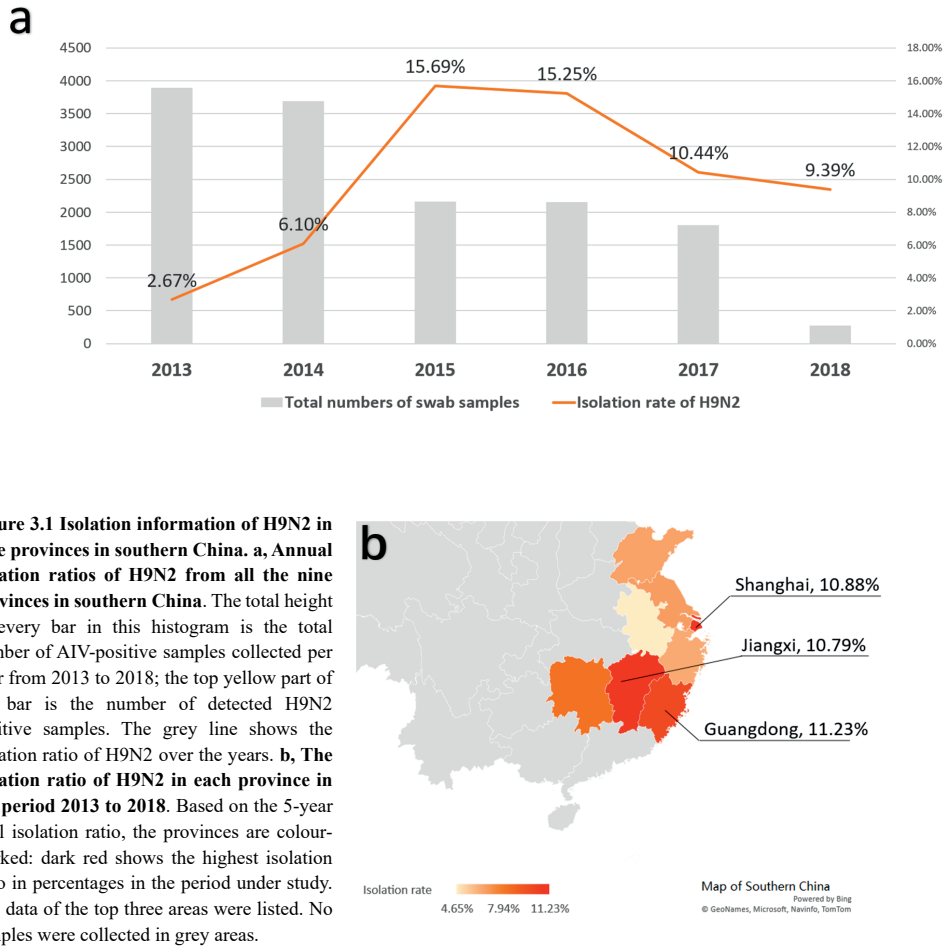


Figure 3.1 Isolation information of H9N2 in nine provinces in southern China. a, Annual isolation ratios of H9N2 from all the nine provinces in southern China. The total height of every bar in this histogram is the total number of AIV-positive samples collected per year from 2013 to 2018; the top yellow part of the bar is the number of detected H9N2 positive samples. The grey line shows the isolation ratio of H9N2 over the years. **b, The isolation ratio of H9N2 in each province in the period 2013 to 2018**. Based on the 5-year total isolation ratio, the provinces are colour-marked: dark red shows the highest isolation ratio in percentages in the period under study. The data of the top three areas were listed. No samples were collected in grey areas.

Table 3.1 AIV isolation data in the field markets of southern China from 2013 to 2018.

City	Isolation Year																	
	2013			2014			2015			2016			2017			2018		
	Total [1]	AIV[2]	H9N2[3]	Total	AIV	H9N2	Total	AIV	H9N2	Total	AIV	H9N2	Total	AIV	H9N2	Total	AIV	H9N2
Guangzhou	1232	75	59	861	79	47	632	133	120	606	181	171	302	14	11	NA.	NA.	NA.
Fujian	478	16	11	339	46	24	184	42	36	140	25	22	156	29	25	95	21	20
Jiangxi	NA.	NA.	NA.	386	55	30	244	32	26	300	30	25	256	47	47	NA.	NA.	NA.
Zhejiang	154	2	2	158	10	4	130	27	22	NA.	NA.	NA.	NA.	NA.	NA.	NA.	NA.	NA.
Shanghai	288	24	21	273	35	25	61	3	2	32*	32	17	155	24	23	NA.	NA.	NA.
Anhui	446	6	6	359	36	19	330	48	31	360	0	0	313	37	28	NA.	NA.	NA.
Jiangsu	337	0	0	516	30	18	281	72	54	305	31	26	419	48	37	158	1	1
Shandong	514	1	0	459	42	33	21*	19	15	207	32	31	NA.	NA.	NA.	NA.	NA.	NA.
Hunan	446	11	5	338	47	25	278	35	33	208	38	37	200	27	17	24	6	5
Total	3895	135	104	3689	380	225	2161	411	339	2158	369	329	1801	226	188	277	28	26

[1], Number of the total swab samples collected from duck/chicken in the market;

[2], Total number of the AIV positive samples;

[3], Total number of the H9N2 positive samples; NA. no data.* samples from sick poultry with flu-like symptoms and tested positive in HA assay.

The phylogenic tree of HA genes

In order to analyze the genetic evolution of the HA gene of the H9N2 isolates, 39 representative isolates were selected to perform HA gene sequencing and further analysis based on geographical and host differences. These included seven viruses isolated in 2013, six in 2014, five in 2015, seven in 2016, seven in 2017 and seven in 2018. Phylogenetic analysis showed that the HA genes of the isolates from 2013 to 2018 were all distributed in subgroup II or subgroup III, except for A/chicken/Guangdong/D2703/2013 and A/chicken/Fujian/F1132/2015 and the commercial vaccine strain A/chicken/Shanghai/F/1998 (F/98) which were co-located in subgroup I (Figure 3.2a). Among them, the virus strains located in subgroup II included one isolate (14.3%) in 2013, three isolates (50.0%) in 2014, one isolate (20.0%) in 2015, one isolate (14.3%) in 2016, and three isolates (42.8%) in 2017. The subgroup II lineage was represented by A/Chicken/Zhejiang/HJ/2007, belonging to the G57 genotype as described previously (Pu et al. 2015). In addition, a new branch of evolution, subgroup III, began to appear after 2013, including five virus (71.4%) isolated in 2013, four (66.7%) in 2014, three (60.0%) in 2015, six (85.7%) in 2016, four (57.1%) 2017, and seven (100.0%) in 2018.

In order to estimate the time when subgroup I, subgroup II and subgroup III viruses started to appear, the most recent common ancestor (tMRCA) was estimated based on the HA genes of all H9N2 isolates in the time-scaled phylogenic tree (data was not shown). Subgroup I lineage viruses first emerged in August 1992, with 95% HPD interval from September 1991 to May 1993. The tMRCA of subgroup II viruses was September 1996 (Jul 1995– Nov 1996). The most recently isolated H9N2 strains from 2018 were in Subgroup III, of which the tMRCA was estimated as September 2010 [95% HPD interval (Jan 2010 – May 2011)].

New antigenic cluster of H9N2 emerged

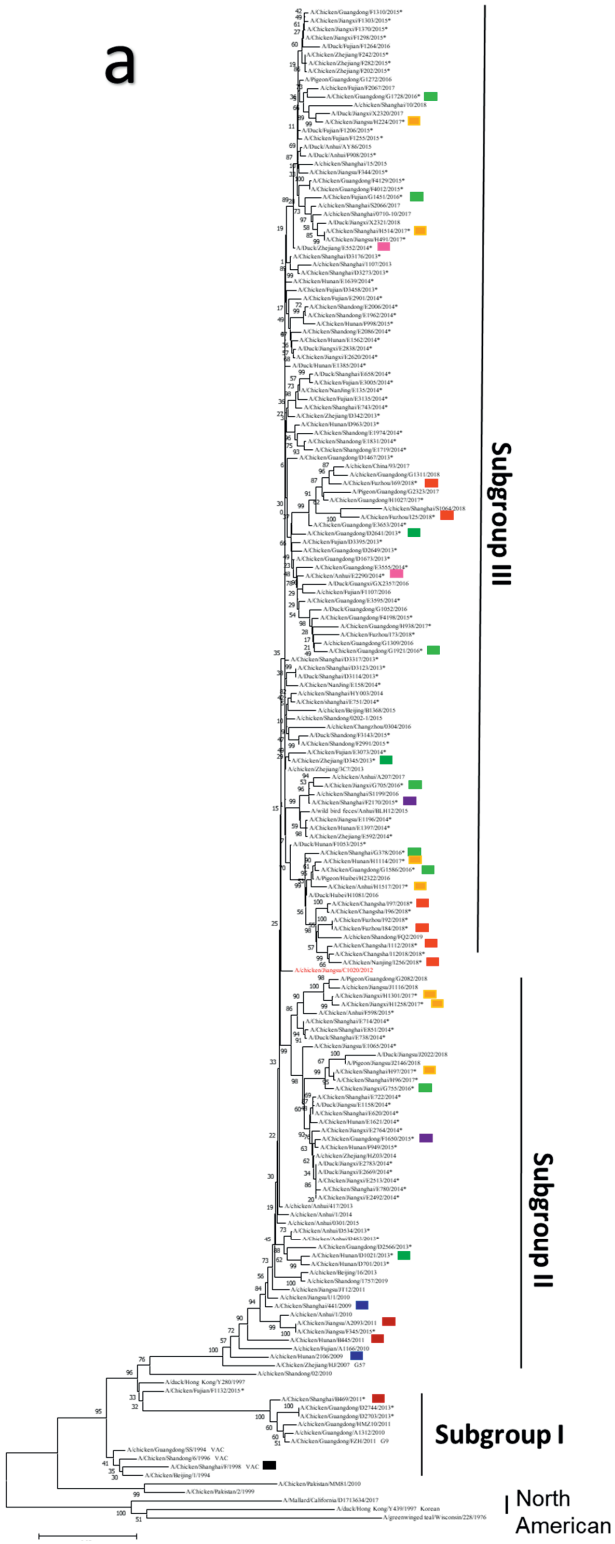
In order to monitor the antigenic map of the H9N2 isolated viruses, 29 representative isolates were selected for crossing HI tests, including three viruses isolated in 2013, three in 2014, two in 2015, seven in 2016, seven in 2017 and seven in 2018 (Figure 3.2a). A vaccine strain F/98 (Sun et al. 2010), and A/chicken/Shanghai/441/2009 (Teng et al. 2016) were used as represented viruses of antigenic cluster 1 and antigenic cluster 2, respectively. Three other earlyviruses (A/chicken/Hunan/2106/2009, A/chicken/Jiangsu/A2093/2011, A/chicken/Hunan/B445/2011) were used in this assay. The antigenic distances of the selected

H9N2 strains were shown in Supplementary Table S3.2 The antigenic distances of selected H9N2 strains to vaccine strain (F98) was visualized in Figure 3.2b. Partial H9N2 isolates from 2013 to 2017 gathered in antigenic cluster 2, including two (66.7%) viruses isolated in 2013, two (66.7%) in 2014, one (50.0%) in 2015, six (85.7%) in 2016 and four (57.1%) in 2017 (Figure 3.2b). A new antigenic cluster (cluster 3) started to appear after 2013 (Figure 2b), consisting of 1 (33.3%) virus isolated in 2013, one (33.3%) in 2014, one (50%) in 2015, one (14.3%) in 2016 and three (42.9%) in 2017. Moreover, all seven (100%) H9N2 AIVs isolated in 2018 belonged to antigenic cluster 3.

Antigenic cluster 3 viruses evolved the fastest

In order to study evolutionary rates of HA gene for H9N2 viruses over time, each antigenic cluster viruses were analyzed by calculating the mutation rate of HA genes. The average evolution rate of the HA gene of H9N2 AIVs from 1994 to 2018 was estimated, with 3.95×10^{-3} (95% HPD interval [3.61E-3, 4.32E-3]) substitutions/site/year over a 25-year period (Figure 3.3a). The HA gene of antigenic cluster 1 virus (subgroup I) has a slower evolution rate (0.86×10^{-3} substitutions/site/year , 95% HPD interval [0.36E-3, 1.42E-3]), with only 18.75% of the average evolution rate of the HA gene of H9N2 AIVs. In contrast, the HA genes of antigenic cluster 3 viruses (subgroup III) evolved most rapidly with 6.23×10^{-3} substitutions/site/year, 1.5-fold faster than that of the average mutation rate of HA genes for H9N2 viruses (Figure 3.3a).

To investigate whether this evolutionary rate of HA genes influenced its gene diversity, the HA gene diversity of H9N2 viruses was visualized in the Bayesian Skyride plot (BSP), including constant population size (Figure 3.3b). The population size of H9N2 HA reached a peak in 2001. From 2001 to 2013, the population sizes were stable. However, after 2013, the population size decreased rapidly.


a
Subgroup III
Subgroup II
Subgroup I
North American

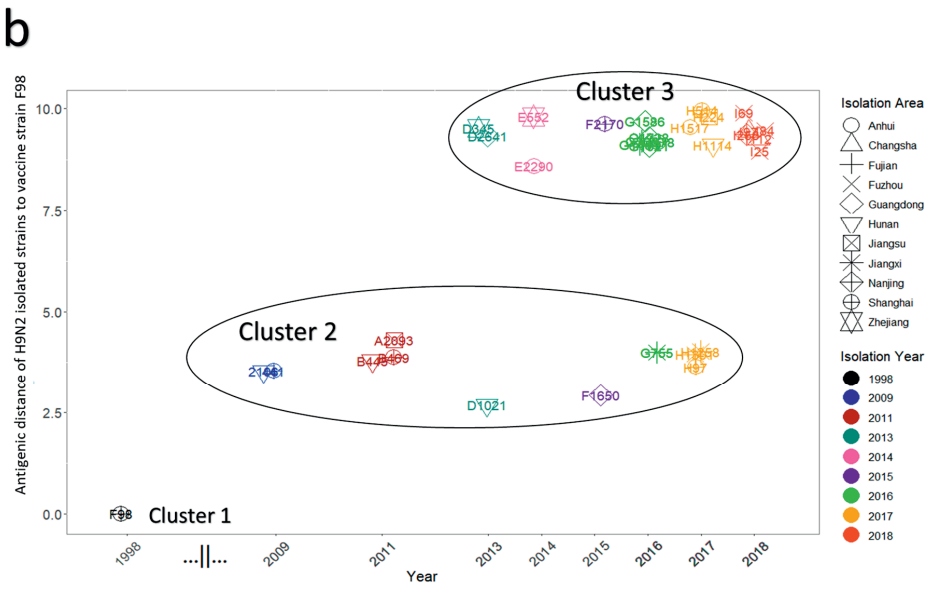


Figure 3.2 Neighbour-joining tree of the HA gene (a) and antigenic distance matrix (b) of isolated H9N2 strains. H9N2 strains isolated in the various years were selected for antigenic analysis and represented in different colours each year. * marked an outlier strain (A/chicken/Shanghai/B469/2011) in subgroup I lineage but presents large antigenic distance to F98 strain in cluster 1.

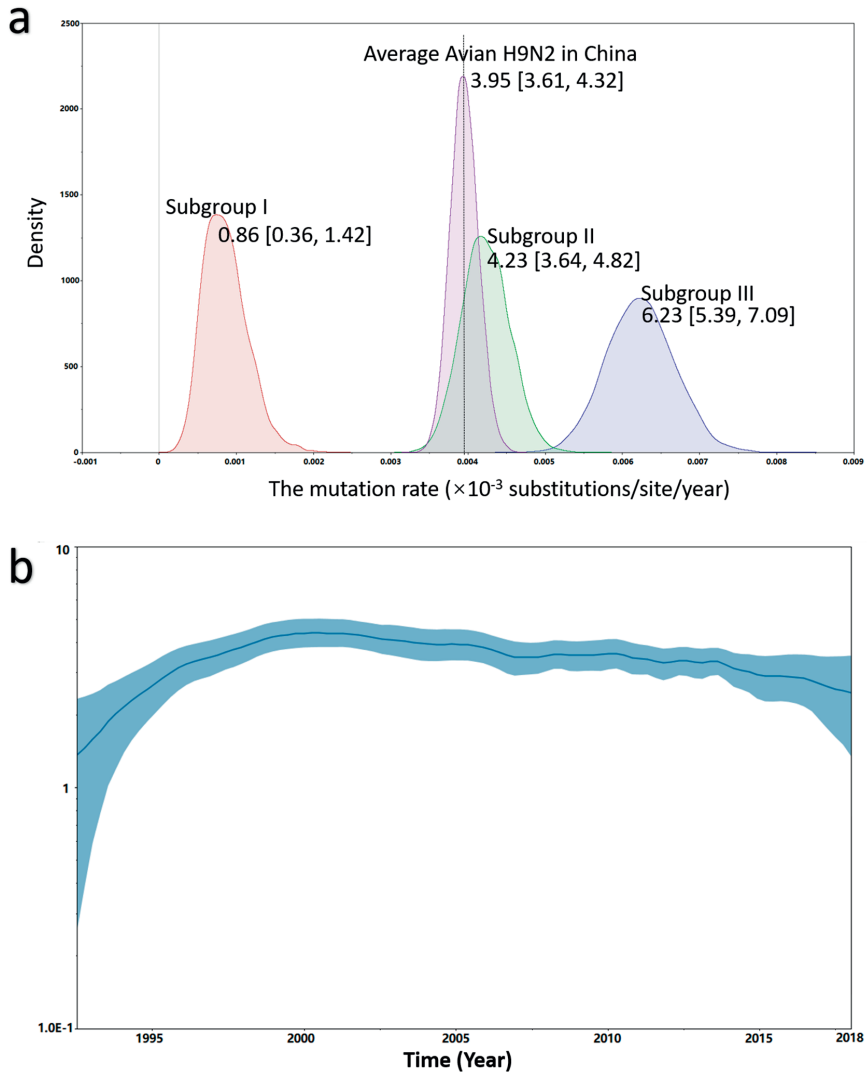


Figure 3.3 Mutation rate and diversity of H9N2 HA estimated in BEAST. a, The mutation rate (substitutions/site/year) of the HA ORF of the main clades of H9N2. All the 126 lab isolated H9N2 strains were analyzed. The values show the mean rate with a 95% HPD interval. **b, Bayesian Skyride plot (BSP).** The y-axis shows the constant population size (log value). The marginal density of the meanRate data estimated by Bayesian Skyride are displayed by KDE in tracer.

Potential antigenic related amino acid residues

The homology of HA protein from different antigenic clusters was analyzed to analyze the potential amino acid residues resulting in the antigen drift. Eleven amino acid residues were conserved in vaccine cluster viruses (4 classical vaccine strains used in China), but mutated into the same amino acid residues both in cluster 2 and 3 viruses, including M96L, T205A, D208E, Q226L, N273K, S274R, N275S, V276T, R285K, P306S and A325S (H3 numbering), indicating that these residues in the HA may contribute to the antigen change between vaccine strains and cluster 2&3 (Table 3.2, Figure 3.4a).

Investigation of the key amino acids characterizing antigenic properties of strains in cluster 2 showed ten mutated amino acid residues in HA protein between cluster 2 viruses and cluster 3 viruses, including N/H57Q, G81E, S/N133D, K137T, D145G, Q156G, N159G, A160D/N/E, V/A190T, T192R (Table 3.2, Figure 3.4b). In a further analysis, G81E, 137K, 145D, 156Q, 159N, A160D/N/E and T192R, were conserved in both cluster 1 and cluster 2 viruses, but mutated in the cluster 3 viruses. These results indicate that these seven amino residues may be important for the antigenic property of cluster 3 viruses, and influence the immune efficiency of inactivated H9N2 AIV vaccine.

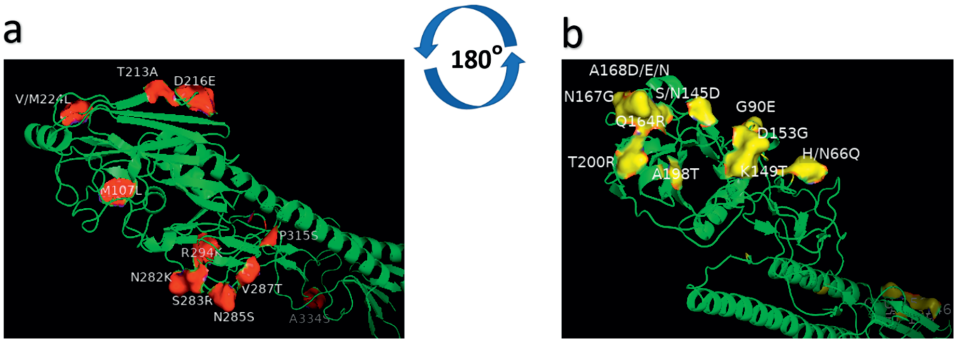


Figure 3.4 The mutated amino acid residues on the HA1 protein (H9 protein structure). The model was built based on the template A/swine/Hong Kong/9/98 and the modelled strain was A/Chicken/Fuzhou/I69/2018. **a, Potential residues in the HA contributing to the antigen change between vaccine strains (subgroup I) and circulating strains (subgroup II and III).** Residues (in red) (M96L, T205A, D208E, Q226L, N273K, S274R, N275S, V276T, R285K, P306S and A325S in H3 numbering) are different from the vaccine group (cluster 1) to cluster 2 and 3 (circulating strains in subgroup II and III). **b, Potential residues in the HA contributing to the antigen change between subgroup II and subgroup III.** Residues (in yellow) (N/H57Q, G81E, S/N133D, K137T, D145G, Q156G, N159G, A160D/N/E, V/A190T and T192R in H3 numbering) differ between cluster 2 and cluster 3.

Table 3.2 Specific amino acid mutations on HA of the representative H9N2 isolated strains.

		Potential antigenic sites																												RBS		
SubGroup	H3 numbering >>	57	81	96	133	135	137	145	156	158	159	160	183	188	189	190	192	193	199	205	208	216	273	274	275	276	285	306	325	226	227	228
	H9 numbering[1] >>	66*	90*	107#	145*	147	149*	153*	164*	166	167*	168*	191	196	197	198*	200*	201	207	213#	216#	224#	282#	283#	285#	287#	294#	315#	334#	234	235	236
	HA1 residues[2] >>	48	72	89	127	129	131	135	146	148	149	150	173	178	179	180	182	183	189	195	198	206	264	265	267	269	276	297	316	216	217	218
Cluster 1	NJ/99-vac	H	G	M	N	T	K	D	Q	S	N	A	N	D	T	T	T	N	D	T	D	M	N	S	N	V	R	P	A	Q	Q	G
	SD/96-vac	H	G	M	S	T	K	D	Q	D	N	A	N	D	T	A	T	N	D	T	D	V	N	S	N	V	R	P	A	Q	Q	G
	GD/94-vac	H	G	M	S	T	K	D	Q	S	N	A	N	D	T	A	T	N	D	T	D	V	N	S	N	V	R	P	A	Q	Q	G
	F/98-vac	H	G	M	S	T	K	D	Q	N	N	A	N	D	T	A	T	N	D	T	D	M	N	S	N	V	R	P	A	Q	Q	G
Cluster 2	A2093	H	G	L	S	T	K	D	Q	N	N	A	N	D	T	V	T	N	D	A	E	L	K	R	S	T	K	S	S	L	Q	G
	441	H	G	L	S	T	K	D	Q	N	N	A	N	D	T	V	T	N	D	A	E	L	K	R	N	T	K	S	S	L	Q	G
	2106	H	G	L	S	T	K	D	Q	N	N	A	N	E	T	A	T	N	D	A	E	L	K	R	S	T	K	S	S	L	Q	G
	B445	H	G	L	S	T	K	D	Q	N	N	A	N	D	T	A	T	N	D	A	E	L	K	R	S	T	K	S	S	L	Q	G
	B469	R	E	V	S	T	K	D	Q	N	N	N	N	D	T	A	T	N	D	A	D	L	K	S	N	T	R	P	A	L	Q	G
	E278	N	G	L	N	T	K	D	Q	N	N	A	N	D	T	A	T	N	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	F1650	H	G	L	S	T	K	D	Q	N	N	A	N	E	T	V	T	N	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	G755	N	G	L	S	T	K	D	Q	N	N	A	N	E	T	V	T	N	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	H97	N	G	L	S	T	K	D	Q	N	N	A	N	E	T	A	T	N	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	H1258	H	G	L	S	T	K	D	Q	N	N	A	N	D	T	V	T	N	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	H1301	H	G	L	S	T	K	D	Q	N	N	A	N	D	T	A	T	N	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	H1021	H	G	L	S	T	K	D	Q	D	N	A	N	D	T	V	T	N	D	A	E	L	K	R	S	T	K	S	S	L	M	G
Cluster 3	D2641	Q	E	L	N	T	T	G	R	N	G	D	N	D	T	T	R	D	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	D345	Q	E	L	D	T	T	G	R	N	G	D	N	D	T	T	R	N	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	E2290	Q	E	L	D	T	T	G	R	N	G	N	N	D	T	T	R	D	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	E552	Q	E	L	D	T	T	G	R	N	G	D	N	D	D	T	R	N	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	F2170	Q	E	L	D	T	T	G	R	N	G	D	N	D	T	T	R	N	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	G378	Q	E	L	D	T	T	G	R	N	G	E	N	D	D	T	R	G	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	G705	Q	E	L	D	T	T	G	R	N	G	E	N	D	T	T	R	D	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	G1451	Q	E	L	D	T	T	G	R	N	G	N	N	D	D	T	R	N	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	G1586	Q	E	L	D	T	T	G	R	N	G	E	N	D	V	T	R	G	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	G1728	Q	E	L	D	T	T	G	R	D	G	N	N	D	D	T	R	N	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	G1921	Q	E	L	D	T	T	G	R	D	G	N	N	D	T	T	R	G	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	H224	Q	E	L	D	T	T	G	R	D	G	N	N	D	D	T	R	N	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	H514	Q	E	L	D	T	T	G	R	D	G	N	N	D	D	T	R	S	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	H1114	Q	E	L	D	T	T	G	R	N	G	E	N	D	D	T	R	G	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	H1517	Q	E	L	D	T	T	G	R	N	G	E	N	D	D	T	R	G	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	I25	Q	E	L	D	T	N	G	R	D	G	N	N	D	T	T	R	N	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	I69	Q	E	L	D	T	T	G	R	N	G	N	N	D	D	T	R	N	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	I84	Q	E	L	D	T	T	G	R	N	G	E	N	D	E	T	R	R	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	I97	Q	E	L	D	T	T	G	R	N	G	E	N	D	D	T	R	G	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	I112	Q	E	L	D	T	T	G	R	N	G	E	N	D	D	T	R	G	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	I118	Q	E	L	D	T	T	G	R	N	G	E	N	D	D	T	R	G	D	A	E	L	K	R	S	T	K	S	S	L	M	G
	I256	Q	E	L	D	T	T	G	R	N	G	E	N	D	D	T	R	G	D	A	E	L	K	R	S	T	K	S	S	L	M	G

* key locations identified in this research that mutations contribute the antigenic distance between cluster 1 and 2;

the locations holding mutations contributes the antigenic distance between vaccine strains and isolates, potential location under selection from vaccines.

[1], The whole length of amino acid sequence of HA protein;

[2], The HA1 protein sequence without 10 restudies at the beginning of HA protein.

Evolution of the potential antigenic related amino acid residues

To gain information on the evolution of potential antigenic amino acid residues on HA from H9N2 over time in nature, we analyzed HA proteins of H9N2 isolates and other viruses submitted on the NCBI. Of the 11 potential antigenic amino acids characteristic in cluster 1, 10 were dominant patterns in China, including M96L, T205A, D208E, Q226L, N273K, N275R, V276T, R285K, P306S and A325S in HA protein (H3 numbering). Currently, S274R is the predominant amino acid for H9N2 HA, mixed with some 274S (Figure 3.5). Moreover, all mutations contributing to the antigenic change from cluster 1 to cluster 2 were equally distributed for the natural H9N2 AIVs, including N/H57Q, G81E, S/N133D, K137T, D145G, Q156G, N159G, A160D/N/E, V/A190T and T192R (Figure 3.6).

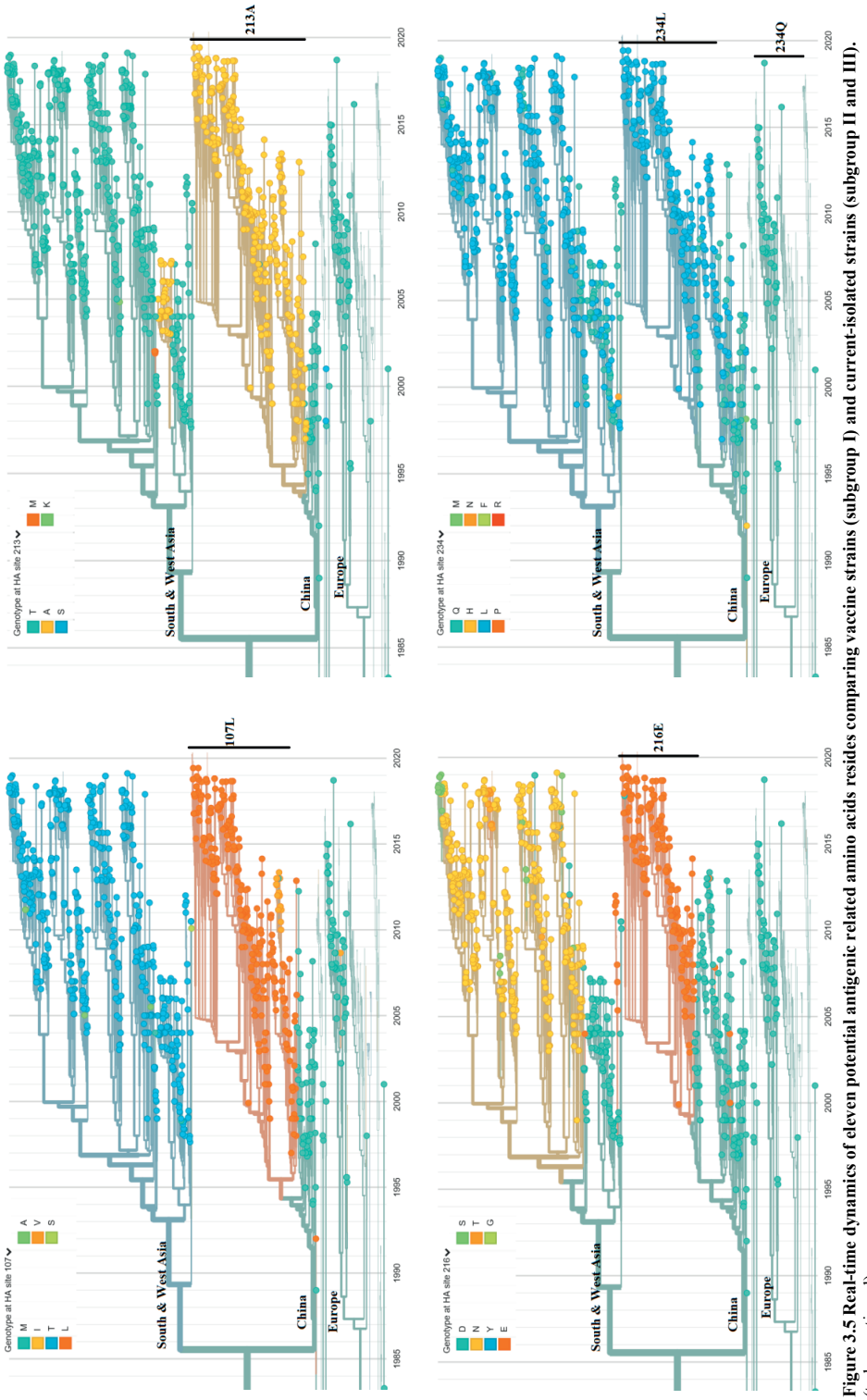
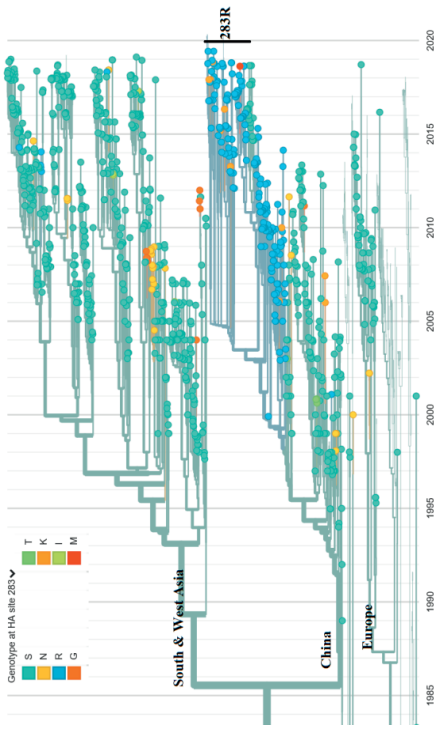
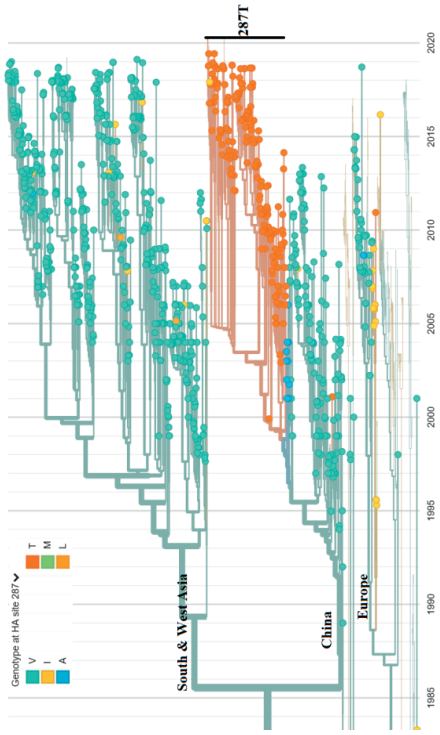
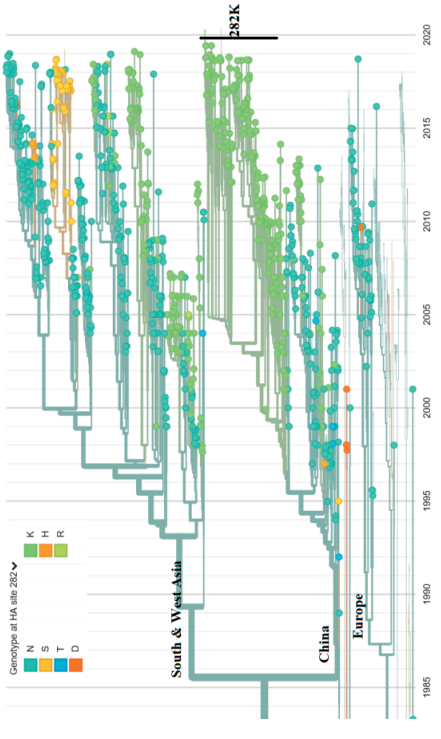


Figure 3.5 Real-time dynamics of eleven potential antigenic related amino acids resides comparing vaccine strains (subgroup I) and current-isolated strains (subgroup II and III).
(to be continued)







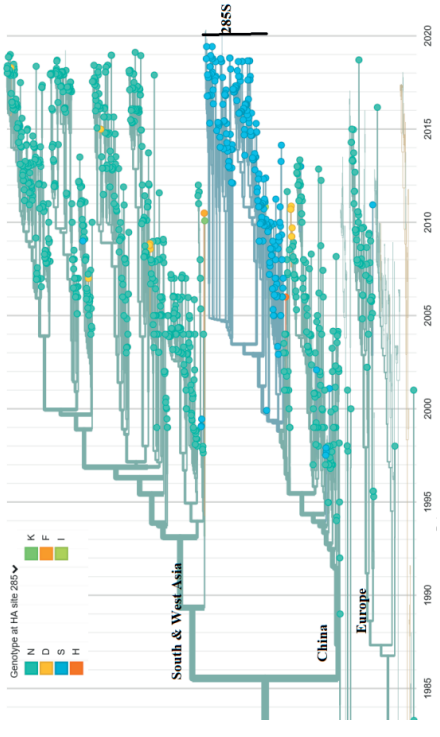


Figure 3.5 Real-time dynamics of eleven potential antigenic related amino acids resides comparing vaccine strains (subgroup I) and current-isolated strains (subgroup II and III).
(to be continued)

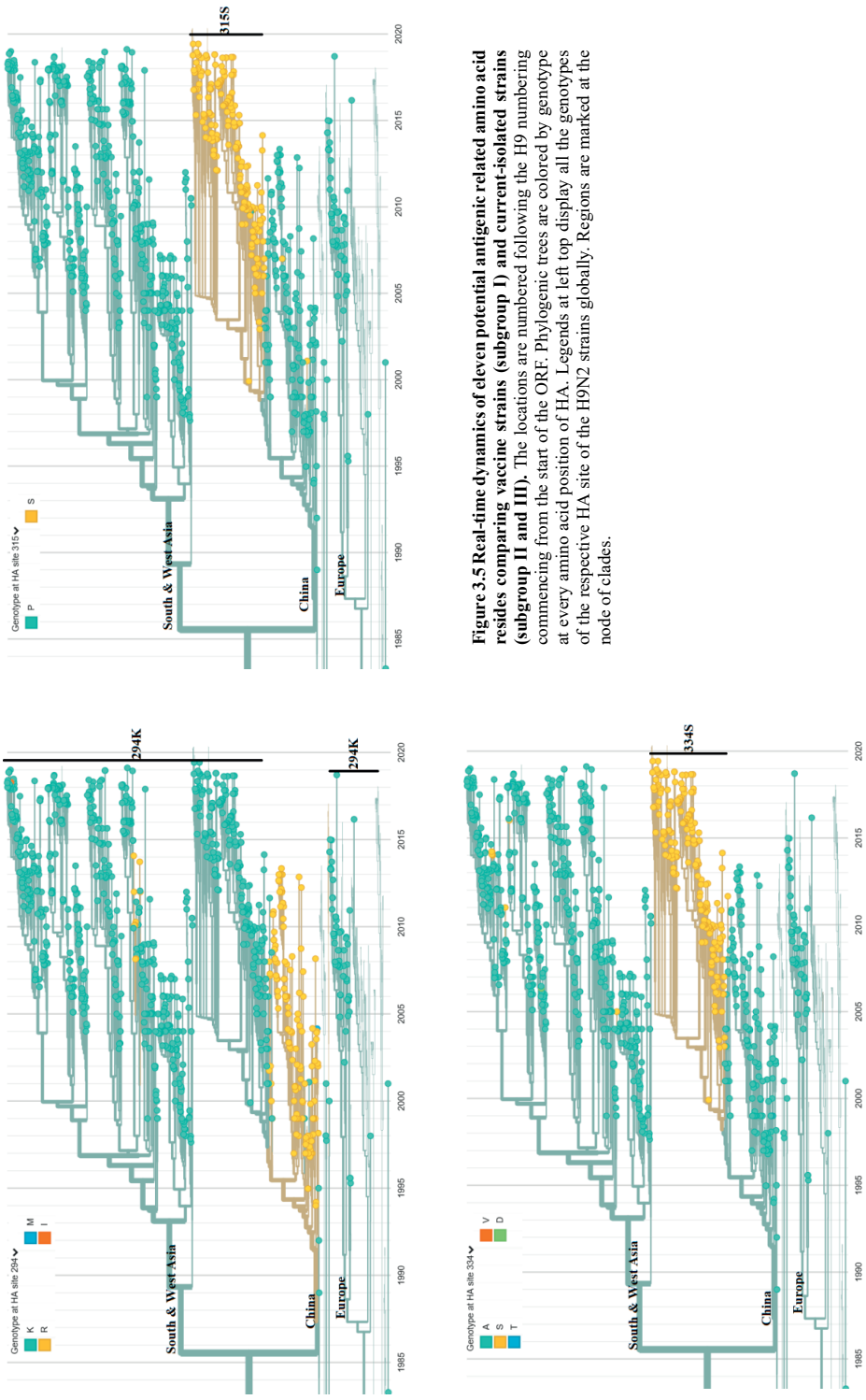


Figure 3.5 Real-time dynamics of eleven potential antigenic related amino acid resides comparing vaccine strains (subgroup I) and current-isolated strains (subgroup II and III). The locations are numbered following the H9 numbering commencing from the start of the ORF. Phylogenic trees are colored by genotype at every amino acid position of HA. Legends at left top display all the genotypes of the respective HA site of the H9N2 strains globally. Regions are marked at the node of clades.

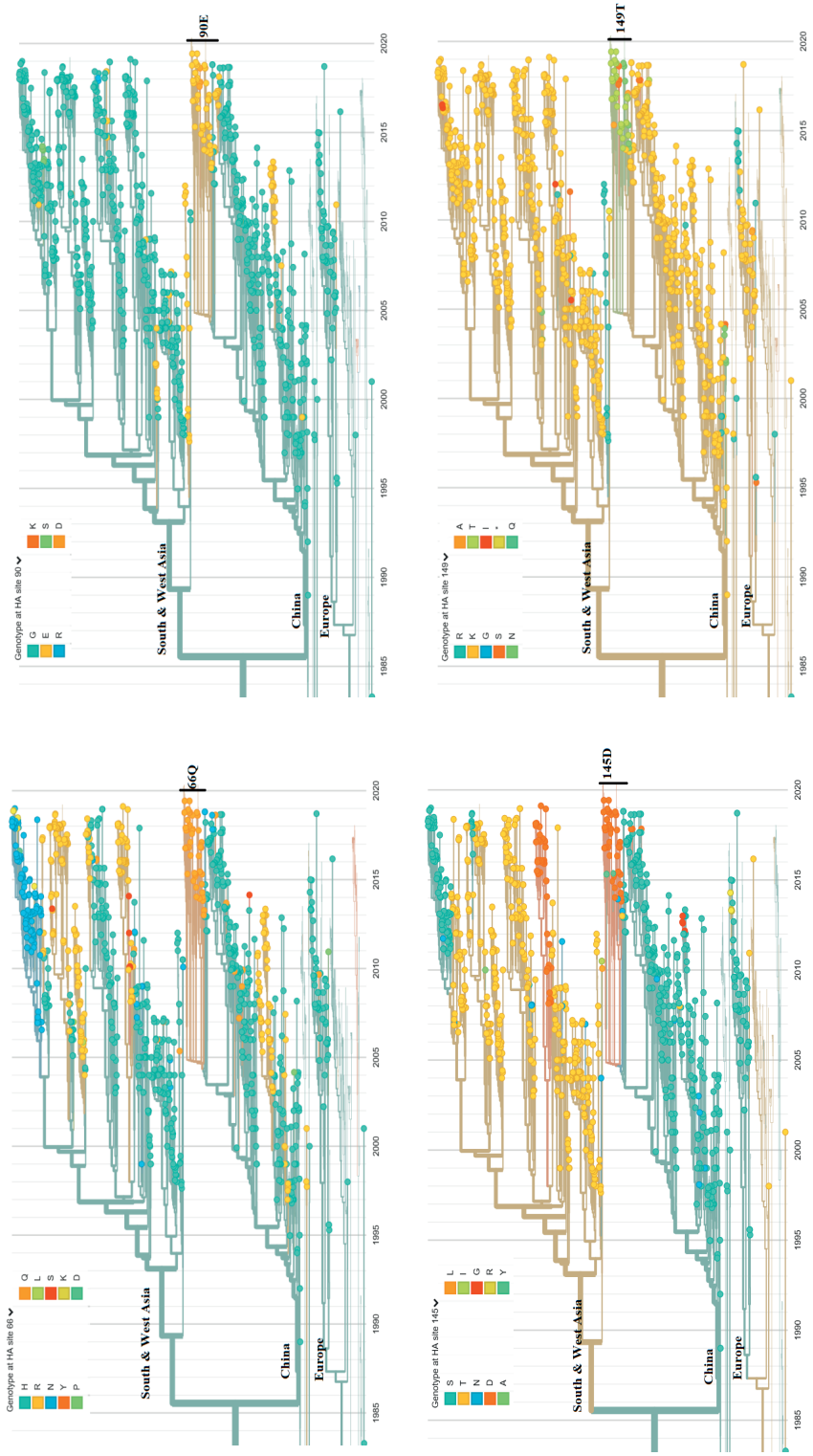


Figure 3.6 Real-time dynamics of ten potential antigenic-related amino acids resides comparing cluster 2 (subgroup II) and cluster 3 (subgroup III).
(to be continued)

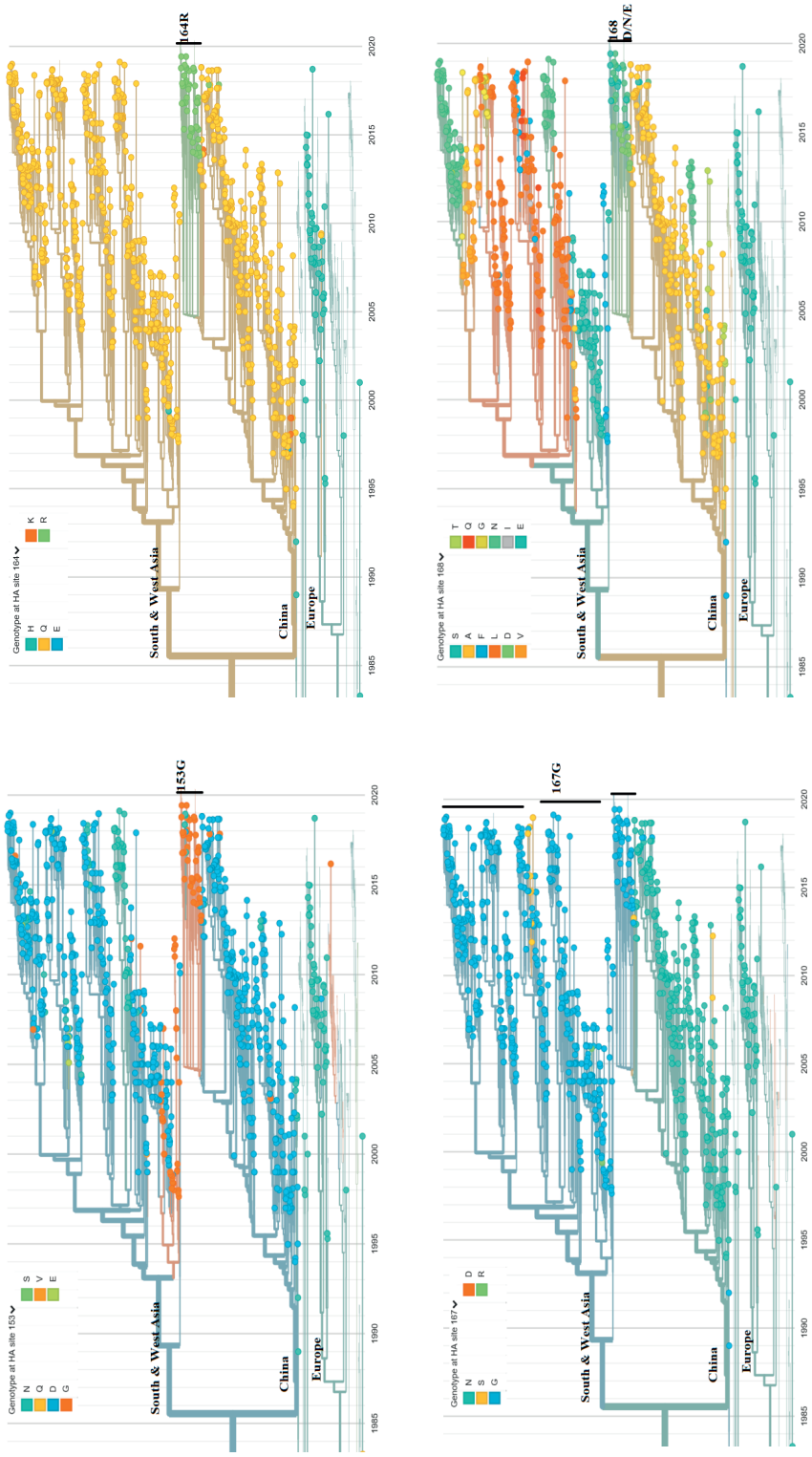


Figure 3.6 Real-time dynamics of ten potential antigenic-related amino acids resides comparing cluster 2 (subgroup II) and cluster 3 (subgroup III).
(to be continued)

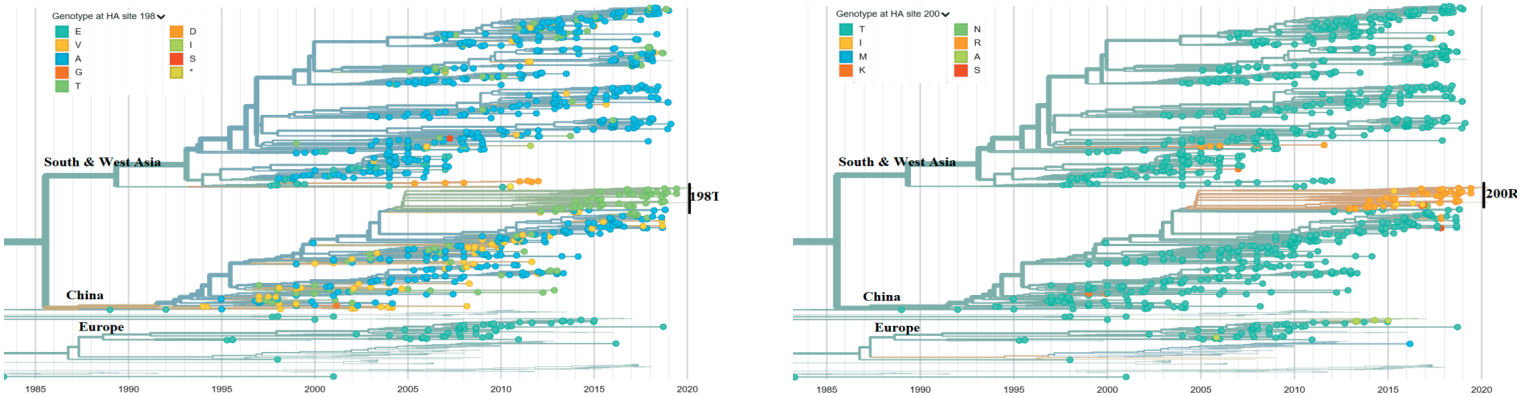


Figure 3.6 Real-time dynamics of ten potential antigenic-related amino acids resides comparing cluster 2 (subgroup II) and cluster 3 (subgroup III). The locations are numbered following the H9 numbering starting from the start of the ORF. Phylogenic trees are coloured by genotype at every HA site and legends at the left top display all the genotypes of respective HA site of the H9N2 strains globally. Regions are marked at the node of clades. Data and analysis from Nextstrain: Hadfield et al., Bioinformatics (2018). Showing 1092 of 1332 genomes sampled between Jan 1976 and Jun 2019. Filtered to Avian (1270) from Africa (115), China (358), Europe (55), South Asia (126), Southeast Asia (93), West Asia (395).

3.4 Discussion

The H9N2 virus is a predominant subtype of AIV circulating in Asia and other countries all over the world (Kim et al. 2006, Naeem et al. 2007, Kim et al. 2013, Lin et al. 2017, Jonas et al. 2018). It not only causes large economic losses in poultry, but it also provides internal genes for novel subtype influenza viruses such as H7N9, H10N8 and H5N1 that threaten human health (Li et al. 2014, Pu et al. 2015). Live poultry markets (LPMs) are considered as key public place of the transmission and circulation of influenza virus in poultry or from poultry to humans (Liu et al. 2015, Ma et al. 2018, Wang et al. 2020).

Our analysis of surveys of LPMs conducted in China from 2013 to 2018 demonstrate that H9N2 AIVs are still prevalent as described previously (Teng et al. 2016). An increasing trend of the annual isolation ratio was observed in poultry markets in southern China. To be noticed, the total sampling number in surveillance fluctuated annually. Small sampling numbers (samples from Shanghai in 2015 and samples from Fujian, Table 3.1) and a biased sampling (e.g. samples from Shandong in 2015 and samples from Shanghai in 2016 were from sick poultry, Table 3.1) increased the margin of error in the estimation of annual isolation ratio of H9N2 in southern China. In a previous survey, a fluctuating annual isolation ratio of H9N2 avian influenza virus was observed in 2013 (14.20%) and 2014 (11.90%) compared to 2009 (15.18%) and 2012 (8.20%) (Teng et al. 2016). The common reduced annual isolation ratio of H9N2 AIV in China detected in surveillance was also influenced by national or local policy on LPMs. The sale of live poultry in LPMs was prohibited in China in 2013 and 2014 due to the outbreak of H7N9 in humans in 2013 (Wu et al. 2014). This prohibition largely reduced the isolation ratio of all the AIVs in LPMS in southern China. However, the H9N2 AIV was still the predominant subtype LPAIV circulating in poultry in southern China. Based on our analysis, the overall isolation ratio of H9N2 was about 70% in total AIV positive samples in southern China between 2013 and 2018. Together with the continuous outbreaks in domestic poultry in other countries (Banet-Noach et al. 2007, Jeevan et al. 2019, Peacock et al. 2019), follow-up monitors and controls on H9N2 AIV are necessary even after long term marketing prohibitions and vaccination programs. This study thus updates currently available monitoring information of H9N2 AIV in the field in southern China from 2013 to 2018.

Together with the high isolation ratio and long-term endemic circulation, H9N2 AIVs in mainland China are undergoing a rapid antigenic drift under selection pressure. The hypothesis that the vaccination triggered rapid evolution leading to antigenic drift was reported in Korea only four years after the national vaccination program was applied in poultry in 2007 (Park et al. 2011). Similarly, this antigenic drift on H9 HA was identified from H9N2 viruses isolated from chickens and reported in many studies (Okamatsu et al. 2008, Sun et al. 2012). These called for caution regarding immunization failure resulting from antigenic drift and explained the prevalence of H9N2 AIV. However, it remained unclear how and how rapidly the antigenic changes on HA gene of H9N2 strains from the market occurred. To analyze this, we combined phylogenetic and antigenic analysis based on the current genetic data.

Phylogenic analysis of 126 H9N2 strains isolated from LRMs in southern China from 2013 to 2018, provided sufficient circulating and evolutionary information of H9N2 in local poultry. Consistent with previous studies indicating G9 (A/Chicken/Hong Kong/G9/1997) lineage (h9.4.2) (Jiang et al. 2012) being the dominant H9N2 in mainland China, the latest isolates were

also G9 lineage, but far removed from the original representative strains, BJ94 (A/Chicken/Beijing/1/1994) and Y280 (A/duck/Hong Kong/Y280/97), and the vaccine strains in the 1990s (Wu et al. 2010, Yang et al. 2019, Yang et al. 2019). In addition, the most recent isolated strains antigenically evolved into a new group from 2013. Phylogenetic analysis showed the estimated time for the emergence of the new antigenic cluster was between 2010 - 2011. This bias may be due to the lack of precise time data from isolated strains and the small sampling of virus strains selected in the antigenic assay. However, a rapid and clear subdivision of the isolated H9N2 strains (belonging to subgroup II in the current G9 lineage) matched the two new antigenic groups. This guaranteed the confidence interval when we estimated the mutation rate of H9N2 strains in new antigenic group (cluster2). Without considering the antigenic difference, the average mutation rate of HA gene from H9N2 AIV in poultry in China was estimated as 3.95×10^{-3} substitutions/site/year. This mutation rate increased almost 1.5-fold (6.23×10^{-3} substitutions/site/year) when antigenic differences were involved.

Noteworthy is that this is the first time the mutation rate of H9 HA (ORF region) using the sequences from the influenza database has been estimated. These rates are similar to the estimations using the same method for H3 (HA1 domain) in humans (5.7×10^{-3} substitutions/site/year) (Fitch et al. 1997). For better comparison, the mean H9 mutation rate (HA1 domain) of the antigenic cluster 2 was 7.43×10^{-3} substitutions/site/year, still slightly higher than the H3 viruses circulating in humans. This directly indicated that H9N2 AIV did not evolve slowly due to the immunity in vaccinated poultry population, but actually much faster than those found in human seasonal strains. This may be due to the short lifespan of poultry and inefficiencies in the vaccination in poultry that contributed to more transmission into new susceptants. However, more research is needed to better understand the reasons triggering the mutation rate in addition to selection pressure resulting from vaccination.

We found 21 amino acid mutations on H9 antigenic sites (in region 66-334 on H9 protein sequence) specific to strains in antigenic cluster 2, different from those in cluster 1 as well as three vaccine strains. Referring to the global online database of AIV H9N2 in NextStrain, all these mutations evolved after 2013 and became dominant in southern China. This explains why the isolation ratios in LRMs from 2013 to 2018 did not decrease even though vaccines had been used long-term in poultry, with reports that the H9N2 AIV continues to circulate in vaccinated chickens (Pu et al. 2015, Su et al. 2015).

We built the 3D structure of the HA1 protein to predict key potential antigenic sites dominating the subgroup of current H9N2 isolated from field markets in southern China. The four surface locations (145, 168, 198 and 235) at the top of the HA head also show high entropy in real-time tracing (Rhodes et al. 2010). This indicates high diversity of genotypes in these four locations. Overall, mutations at these potential antigenic sites occurred in China (mainly) and in South Asia, whereas in China, new genotypes showed the tendency to become dominant. In detail, 145D became the new genotype in China and South Asia as early as 2003. Mutations at 198 and 235 emerged and became dominant in China after 2012. To date, location 168 was more diverse with mutations from A (Alanine) to other amino acids after 2005, but no globally dominant genotype emerged. The 226L (234 in this H9 numbering) identified as an important host-receptor binding site (Wan et al. 2007) had been the stable dominant on HA of the H9N2 AIV circulating in China. However, at location 235, we identified that the change from Q (Glutarnine) to M (Methionine) occurred in 2013, which was consistent with the online data of

the H9N2 AIV all over China, however, the 235Q was the stable dominant in the European area where no H9N2 vaccine had been applied. The genotypes of the surface locations were conserved in Europe but became dominant in China after 2013. However, there may be data bias given that many European countries did not monitor LPAI H9N2 in poultry.

In summary, the time-scale phylogenetic analysis in our study directly shows the evolutionary mutation rate of the HA ORF of the H9N2 strains from LRMs in southern China between 2013-2018. The currently isolated H9N2 strain forms a new antigenic group to those from vaccine strains, and are also clearly divided into new subgroups in phylogenic trees. The declining diversity, together with the high mutation rate of H9N2 strains in the new antigenic group indicates that vaccination triggers the evolution of H9N2 into new antigenic groups. Moreover, the novel mutation sites on HA1 (66-334 region of H9 protein sequence) identified from recent isolates were potential molecular characteristics of the upcoming dominant H9N2 strains. The identified antigenic sites and the 66-334 region related to responding to selective pressure provide good references for potential approaches on rapid detection of new antigenic clustering of H9N2 AIV. Our analysis has not only updated the AIV H9N2 genomic database but has also showed the value of monitoring to provide molecular evolutionary information.

Acknowledgements

We would like to thank the GISAID Initiative and the GISRS Network for critical surveillance efforts and open data sharing. Sequence data and virus strains used in the antigenic analysis were provided by Zejun Li's lab group at the Shanghai Veterinary Research Institute (SHVIR), China.

Hongrui Cui is enrolled in the Wageningen Institute of Animal Sciences (WIAS) graduate school and the Production Ecology and Resource Conservation (PE&RC) as part of the 2016 Sino-Dutch joint PhD program between the Chinese Academy of Agricultural Sciences (CAAS) and Wageningen University & Research.

Funding

This work was supported by the National Key Research and Development Program of China (2016YFD0500204) and the Chinese Academy of Agricultural Sciences of Technology Innovation Project.

Conflict of interest statement

There is no conflict of interest for all the authors in this research.

Supplementary materials

Table S3.1 Selected H9N2 strains and HI titers of Cross-antigenic Assay.

NO.	Abbr.	Isolate Date	Antigen	I256	I112	I84	I97	I69	I25	H514	H224	H1114	H1517	G1728	G705	G1451	G1586	G378	G1921	F2170	E552	E2290	D345	D2641	H1301	H1258	H97	G755	F1650	D1021	A2093	E278	B445	B469	441	2106	F98
1	I256	2018-10	A/Chicken/Nanjing/I256/2018	2048	256	512	1024	2048	256	4096	2048	1024	4096	256	1024	4096	1024	1024	512	256	512	256	512	1024	256	128	256	1024	512	1024	16	8	256	64	64	256	32
3	I112	2018-10-15	A/Chicken/Changsha/I112/2018	512	128	128	256	512	64	512	512	128	512	32	128	1024	256	128	64	64	128	128	128	1024	64	64	32	128	128	256	8	8	64	8	16	32	32
4	I84	2018-11	A/Chicken/Fuzhou/I84/2018	512	32	1024	128	512	128	512	256	64	1024	64	256	1024	256	128	128	128	128	128	256	128	128	128	128	256	64	128	8	8	32	8	32	64	32
5	I97	2018-10-15	A/Chicken/Changsha/I97/2018	1024	128	128	512	1024	64	1024	512	256	1024	64	256	2048	256	256	64	128	256	128	256	128	64	64	64	256	128	256	16	8	32	8	16	64	32
6	I69	2018-11	A/Chicken/Fuzhou/I69/2018	256	128	128	256	4096	512	1024	1024	64	512	512	256	2048	256	128	256	128	512	256	512	512	64	128	64	512	128	1024	32	8	128	16	32	64	128
7	I25	2018-11-07	A/Chicken/Fuzhou/I25/2018	256	128	256	128	4096	4096	1024	2048	64	512	512	1024	2048	1024	128	2048	256	1024	256	1024	1024	256	128	64	1024	128	1024	16	8	128	16	32	128	128
8	H514	2017-10-24	A/Chicken/Shanghai/H514/2017	1024	128	256	512	2048	256	2048	1024	256	1024	256	256	4096	512	256	256	128	512	512	256	1024	256	128	256	1024	128	1024	16	32	256	32	64	256	64
9	H224	2017-10	A/Chicken/Jiangsu/H224/2017	128	32	64	128	2048	512	1024	1024	64	512	256	256	2048	128	64	128	128	512	128	256	256	64	128	64	512	64	512	16	16	64	16	16	64	128
10	H1114	2017-11-21	A/Chicken/Hunan/H1 114/2017	4096	512	512	2048	4096	256	2048	2048	2048	4096	256	1024	4096	1024	2048	512	512	1024	512	1024	2048	128	128	512	1024	512	1024	64	64	256	32	64	256	64
11	H1517	2017-10-31	A/Chicken/Anhui/H1 517/2017	2048	256	512	1024	2048	256	2048	512	512	4096	128	1024	4096	1024	2048	512	256	512	512	512	1024	128	64	256	1024	256	512	16	64	512	64	64	256	16
12	G1728	2016-11-01	A/Chicken/Guangdong/G1 728/2016	256	32	64	128	4096	512	1024	1024	64	512	256	512	4096	128	128	128	128	512	256	512	256	128	128	128	512	64	512	16	16	64	16	32	64	128
13	G705	2016-11-01	A/Chicken/Jiangxi/G705/2016	512	64	128	256	2048	256	512	512	64	512	128	8192	2048	512	256	128	256	256	512	256	2048	64	128	128	512	256	256	4	8	64	8	32	128	32
14	G1451	2016-07-08	A/Chicken/Fujian/G1 451/2016	512	64	128	512	4096	512	2048	1024	128	1024	128	512	8192	256	128	256	256	512	128	512	512	512	256	256	1024	256	2048	16	32	256	64	64	256	256
15	G1586	2016-11-01	A/Chicken/Guangdong/G1 586/2016	1024	256	256	512	2048	512	2048	512	256	2048	128	1024	2048	2048	512	2048	256	512	256	1024	1024	128	64	128	512	256	1024	16	16	128	16	32	128	256
16	G378	2016-09-01	A/Chicken/Shanghai/G378/2016	2048	512	512	1024	4096	256	2048	1024	1024	4096	256	1024	4096	1024	2048	512	256	512	512	1024	1024	128	128	256	2048	512	1024	8	64	256	32	64	256	32
17	G1921	2016-11-01	A/Chicken/Guangdong/G1 921/2016	512	128	256	256	1024	256	2048	256	128	1024	64	512	2048	512	256	2048	128	512	512	512	256	256	256	128	1024	128	1024	16	8	128	32	32	128	256
18	F2170	2015-09-01	A/Chicken/Shanghai/F2170/2015	256	64	256	256	4096	1024	1024	1024	64	1024	512	2048	4096	512	128	1024	512	1024	1024	1024	2048	128	128	128	1024	256	2048	16	32	128	8	32	256	512
19	E552	2014-09-01	A/Duck/Zhejiang/E552/2014	1024	256	512	1024	4096	2048	2048	2048	256	2048	1024	2048	8192	1024	512	512	512	1024	1024	1024	4096	256	256	512	2048	512	2048	16	128	512	64	64	512	512
20	E2290	2014-09-01	A/Chicken/Anhui/E2290/2014	64	16	32	64	1024	64	512	128	16	64	16	4096	1024	512	16	16	16	256	4096	256	4096	32	64	128	1024	256	2048	4	32	512	32	256	512	128
21	D345	2013-10-01	A/Chicken/Zhejiang/D345/2013	256	64	128	128	4096	1024	1024	1024	64	1024	256	1024	4096	512	64	1024	256	512	1024	1024	2048	128	128	64	512	256	2048	16	32	64	16	32	128	256
22	D2641	2013-10-01	A/Chicken/Guangdong/D2 641/2013	128	32	64	128	2048	128	512	128	32	64	32	4096	1024	512	32	32	16	256	4096	256	4096	64	128	128	1024	256	2048	8	32	512	32	256	512	256
23	H1301	2017-10-31	A/Chicken/Jiangxi/H1 301/2017	128	32	32	32	256	64	128	32	8	64	32	32	1024	32	32	64	16	64	256	64	256	1024	1024	512	2048	1024	4096	128	256	1024	256	512	1024	1024
24	H1258	2017-11-01	A/Chicken/Jiangxi/H1 258/2017	32	16	32	16	128	16	64	32	8	16	16	16	512	16	8	32	8	32	128	64	128	1024	512	256	2048	512	2048	128	128	512	128	256	1024	1024
25	H97	2017-10-15	A/Chicken/Shanghai/H97/2017	64	16	32	32	128	32	128	32	8	32	16	32	512	32	16	64	8	32	128	64	128	512	512	512	2048	1024	4096	128	256	1024	256	512	1024	1024
26	G755	2016-11-01	A/Chicken/Jiangxi/G755/2016	128	32	32	32	256	64	128	64	8	64	16	64	1024	32	16	64	32	64	256	64	256	1024	1024	512	4096	1024	4096	64	256	1024	256	512	1024	2048
27	F1650	2015-09-06	A/Chicken/Guangdong/F1 650/2015	16	16	16	16	64	32	64	16	2	16	16	16	256	16	8	32	16	32	128	32	128	512	512	256	2048	512	2048	64	128	512	128	256	512	1024
28	D1021	2013-10-01	A/Chicken/Hunan/D1 021/2013	32	32	32	16	128	32	64	32	4	16	16	32	256	32	16	64	16	32	256	32	128	512	512	256	2048	512	4096	128	128	1024	64	256	1024	1024
29	A2093	2011-07-03	A/chicken/Jiangsu/A2 093/2011	32	128	64	32	128	32	256	64	2	128	16	128	256	32	64	128	128	64	512	64	256	512	256	128	4096	512	4096	256	128	256	64	512	1024	2048
30	E278	2014-09-01	A/Chicken/NeiMenggu/E278/2014	64	16	32	32	256	64	128	32	2	64	32	32	1024	32	32	64	16	64	256	64	128	2048	512	1024	2048	2048	8192	64	2048	4096	512	1024	2048	4096
31	B445	2011-07-03	A/chicken/Hunan/B445/201 1#	128	32	32	32	512	64	256	64	8	128	16	64	2048	64	32	128	8	64	512	256	256	2048	1024	1024	4096	2048	8192	512	1024	2048	1024	2048	4096	4096
31	B469	2011-07-03	A/chicken/Shanghai/B469/201 1#	64	64	32	256	64	256	64	8	128	16	64	2048	64	32	64	16	128	256	256	128	2048	1024	1024	2048	1024	8192	128	1024	2048	1024	1024	1024	2048	2048
32	441	2009-07-01	A/Chicken/Shanghai/441/2009#	64	64	64	32	256	64	256	32	4	128	8	32	2048	64	32	128	16	128	512	256	256	4096	1024	1024	4096	2048	8192	256	2048	4096	1024	2048	4096	2048
33	2106	2009-07-01	A/chicken/Hunan/2106/2009#	128	64	128	64	512	128	256	64	8	128	16	128	2048	64	64	128	32	128	512	256	256	2048	1024	1024	4096	2048	8192	512	2048	4096	1024	2048	4096	2048
34	F98	1998-06-20	A/Chicken/Shanghai/F/1998	8	4	4	4	8	8	8	8	1	8	1	8	64	8	8	8	8	8	64	8	16	64	256	32	256	64	1024	16	32	256	16	64	256	1024

NOTE: The bold line grouped the virus strains basing on the time-scaled tree. The background color depth was based on the value of HI titers, red was the high HI titer, indicating close antigenicity; light yellow was low HI titers indicating big distance inactivity.

Table S3.2 The antigenic distances.

Strains	I256	I112	I84	I97	I69	I25	H514	H224	H1114	H1517	G1728	G705	G1451	G1586	G378	G1921	F2170	E552	E2290	D345	D2641	H1301	H1258	H97	G755	F1650	D1021	A2093	E278	B445	B469	441	2106	F98*
I256	0.00	2.56	5.13	1.39	5.92	7.88	4.47	5.31	1.54	2.41	6.92	7.70	6.00	5.05	2.42	7.17	4.83	5.44	8.27	5.43	7.02	9.08	9.35	8.23	8.85	8.76	9.32	8.87	8.87	8.94	8.97	9.08	9.05	9.37
I112	2.56	0.00	5.31	2.55	5.57	7.33	4.25	5.14	2.76	2.41	6.30	7.84	5.98	4.36	2.59	6.50	4.76	5.24	8.29	4.65	6.96	8.98	9.32	8.32	8.39	8.66	9.17	8.71	8.79	8.90	8.92	8.98	8.94	9.27
I84	5.13	5.31	0.00	5.20	6.45	7.08	5.44	6.01	5.73	4.52	6.32	7.82	6.33	5.64	5.23	6.83	5.31	5.92	8.32	5.71	7.24	9.11	9.42	8.61	9.03	9.06	9.44	9.02	8.89	9.03	9.02	9.14	9.12	9.47
I97	1.39	2.55	5.20	0.00	5.35	7.50	3.96	4.67	2.21	2.45	6.09	7.54	5.11	4.89	2.85	7.16	4.13	4.81	8.11	5.02	6.49	9.16	9.44	8.25	8.86	8.85	9.36	8.99	8.95	9.01	9.07	9.20	9.13	9.44
I69	5.92	5.57	6.45	5.35	0.00	5.16	4.32	2.85	6.02	5.37	3.91	6.85	3.92	5.34	6.10	6.08	3.70	2.84	7.57	3.09	5.93	9.61	9.97	9.28	9.53	9.57	9.69	9.52	9.40	9.57	9.54	9.66	9.65	9.90
I25	7.88	7.33	7.08	7.50	5.16	0.00	6.65	4.24	8.00	7.49	4.29	7.55	6.34	6.01	7.95	5.03	5.64	4.41	8.07	4.91	6.77	8.78	9.00	8.78	8.68	8.90	8.85	8.85	8.74	8.84	8.83	8.93	8.89	8.95
H514	4.47	4.25	5.44	3.96	4.32	6.65	0.00	4.44	5.23	3.65	5.47	7.63	3.94	4.03	4.79	5.35	4.26	3.90	8.04	4.12	6.68	9.54	9.98	9.08	9.44	9.58	9.82	9.53	9.39	9.53	9.48	9.66	9.64	9.96
H224	5.31	5.14	6.01	4.67	2.85	4.24	4.44	0.00	5.44	5.32	3.24	7.44	4.34	5.32	6.03	6.06	3.39	2.27	8.24	3.62	6.22	9.51	9.84	9.04	9.39	9.49	9.69	9.42	9.34	9.52	9.48	9.64	9.60	9.80
H1114	1.54	2.76	5.73	2.21	6.02	8.00	5.23	5.44	0.00	3.31	7.15	7.87	6.45	5.67	2.99	7.42	5.22	5.67	8.17	5.60	7.13	8.87	9.08	8.03	8.57	8.50	9.04	8.64	8.69	8.76	8.78	8.87	8.84	9.11
H1517	2.41	2.41	4.52	2.45	5.37	7.49	3.65	5.32	3.31	0.00	6.40	7.71	5.37	4.28	1.65	6.56	4.41	5.10	8.27	4.59	6.96	9.23	9.58	8.53	8.92	8.97	9.48	9.11	9.02	9.10	9.12	9.28	9.23	9.54
G1728	6.92	6.30	6.32	6.09	3.91	4.29	5.47	3.24	7.15	6.40	0.00	7.34	4.65	5.93	7.12	6.43	4.02	3.37	7.98	4.35	5.88	9.20	9.38	8.75	8.89	9.12	9.21	9.18	9.02	9.14	9.17	9.31	9.22	9.29
G705	7.70	7.84	7.82	7.54	6.85	7.55	7.63	7.44	7.87	7.71	7.34	0.00	7.52	6.65	7.69	7.85	6.72	7.12	5.31	7.26	4.41	9.14	9.31	8.97	9.03	8.95	9.07	9.08	8.90	8.90	9.00	8.90	8.92	9.21
G1451	6.00	5.98	6.33	5.11	3.92	6.34	3.94	4.34	6.45	5.37	4.65	7.52	0.00	6.12	6.24	7.06	4.19	4.35	8.06	4.66	6.39	8.57	8.97	7.99	8.62	8.63	8.75	8.81	8.52	8.65	8.50	8.76	8.68	9.12
G1586	5.05	4.36	5.64	4.89	5.34	6.01	4.03	5.32	5.67	4.28	5.93	6.65	6.12	0.00	5.11	4.38	4.68	4.63	7.33	3.70	5.80	9.44	9.85	9.04	9.29	9.29	9.58	9.38	9.22	9.30	9.34	9.43	9.41	9.69
G378	2.42	2.59	5.23	2.85	6.10	7.95	4.79	6.03	2.99	1.65	7.12	7.69	6.24	5.11	0.00	7.23	5.39	5.94	8.19	5.57	7.24	8.93	9.19	8.18	8.49	8.57	9.14	8.76	8.71	8.73	8.80	8.91	8.87	9.18
G1921	7.17	6.50	6.83	7.16	6.08	5.03	5.35	6.06	7.42	6.56	6.43	7.85	7.06	4.38	7.23	0.00	5.80	5.03	8.25	4.37	7.53	8.90	9.18	9.02	8.94	9.03	9.03	8.91	8.86	9.01	8.94	9.04	9.04	9.08
F2170	4.83	4.76	5.31	4.13	3.70	5.64	4.26	3.39	5.22	4.41	4.02	6.72	4.19	4.68	5.39	5.80	0.00	2.68	8.12	3.15	5.88	9.50	9.81	8.95	9.15	9.34	9.57	9.32	9.31	9.48	9.48	9.60	9.52	9.62
E552	5.44	5.24	5.92	4.81	2.84	4.41	3.90	2.27	5.67	5.10	3.37	7.12	4.35	4.63	5.94	5.03	2.68	0.00	7.67	2.74	5.79	9.47	9.86	9.04	9.37	9.48	9.60	9.45	9.28	9.43	9.42	9.58	9.54	9.80
E2290	8.27	8.29	8.32	8.11	7.57	8.07	8.04	8.24	8.17	8.27	7.98	5.31	8.06	7.33	8.19	8.25	8.12	7.67	0.00	7.72	3.50	8.60	8.80	8.49	8.26	8.40	8.15	8.48	8.38	8.20	8.42	8.12	8.17	8.59
D345	5.43	4.65	5.71	5.02	3.09	4.91	4.12	3.62	5.60	4.59	4.35	7.26	4.66	3.70	5.57	4.37	3.15	2.74	7.72	0.00	5.99	9.14	9.59	8.76	9.08	9.06	9.23	9.10	8.96	9.16	9.01	9.19	9.15	9.52
D2641	7.02	6.96	7.24	6.49	5.93	6.77	6.68	6.22	7.13	6.96	5.88	4.41	6.39	5.80	7.24	7.53	5.88	5.79	3.50	5.99	0.00	9.28	9.50	8.81	8.89	8.99	8.99	9.19	9.01	8.94	9.13	9.01	8.98	9.33
H1301	9.08	8.98	9.11	9.16	9.61	8.78	9.54	9.51	8.87	9.23	9.20	9.14	8.57	9.44	8.93	8.90	9.50	9.47	8.60	9.14	9.28	0.00	3.38	2.88	4.18	2.68	2.70	4.51	2.49	2.50	2.41	2.45	2.39	3.94
H1258	9.35	9.32	9.42	9.44	9.97	9.00	9.98	9.84	9.08	9.58	9.38	9.31	8.97	9.85	9.19	9.18	9.81	9.86	8.80	9.59	9.50	3.38	0.00	3.41	3.63	3.34	3.10	4.23	4.46	4.11	3.18	3.45	3.39	4.01
H97	8.23	8.32	8.61	8.25	9.28	8.78	9.08	9.04	8.03	8.53	8.75	8.97	7.99	9.04	8.18	9.02	8.95	9.04	8.49	8.76	8.81	2.88	3.41	0.00	4.28	2.03	2.39	4.41	2.75	2.60	2.29	2.82	2.77	3.62
G755	8.85	8.39	9.03	8.86	9.53	8.68	9.44	9.39	8.57	8.92	8.89	9.03	8.62	9.29	8.49	8.94	9.15	9.37	8.26	9.08	8.89	4.18	3.63	4.28	0.00	3.60	3.68	3.63	4.86	4.61	4.36	3.67	3.54	3.99
F1650	8.76	8.66	9.06	8.85	9.57	8.90	9.58	9.49	8.50	8.97	9.12	8.95	8.63	9.29	8.57	9.03	9.34	9.48	8.40	9.06	8.99	2.68	3.34	2.03	3.60	0.00	2.11	3.71	2.34	2.09	2.68	2.07	2.07	2.92
D1021	9.32	9.17	9.44	9.36	9.69	8.85	9.82	9.69	9.04	9.48	9.21	9.07	8.75	9.58	9.14	9.03	9.57	9.60	8.15	9.23	8.99	2.70	3.10	2.39	3.68	2.11	0.00	3.85	2.76	2.50	2.32	2.35	2.31	2.67
A2093	8.87	8.71	9.02	8.99	9.52	8.85	9.53	9.42	8.64	9.11	9.18	9.08	8.81	9.38	8.76	8.91	9.32	9.45	8.48	9.10	9.19	4.51	4.23	4.41	3.63	3.71	3.85	0.00	4.58	4.59	3.58	2.69	2.59	4.30
E278	8.87	8.79	8.89	8.95	9.40	8.74	9.39	9.34	8.69	9.02	9.02	8.90	8.52	9.22	8.71	8.86	9.31	9.28	8.38	8.96	9.01	2.49	4.46	2.75	4.86	2.34	2.76	4.58	0.00	0.92	2.78	2.59	2.66	3.84
B445	8.94	8.90	9.03	9.01	9.57	8.84	9.53	9.52	8.76	9.10	9.14	8.90	8.65	9.30	8.73	9.01	9.48	9.43	8.20	9.16	8.94	2.50	4.11	2.60	4.61	2.09	2.50	4.59	0.92	0.00	2.88	2.60	2.60	3.79
B469	8.97	8.92	9.02	9.07	9.54	8.83	9.48	9.48	8.78	9.12	9.17	9.00	8.50	9.34	8.80	8.94	9.48	9.42	8.42	9.01	9.13	2.41	3.18	2.29	4.36	2.68	2.32	3.58	2.78	2.88	0.00	1.72	1.84	3.86
441	9.08	8.98	9.14	9.20	9.66	8.93	9.66	9.64	8.87	9.28	9.31	8.90	8.76	9.43	8.91	9.04	9.60	9.58	8.12	9.19	9.01	2.45	3.45	2.82	3.67	2.07	2.35	2.69	2.59	2.60	1.72	0.00	0.64	3.53
2106	9.05	8.94	9.12	9.13	9.65	8.89	9.64	9.60	8.84	9.23	9.22	8.92	8.68	9.41	8.87	9.04	9.52	9.54	8.17	9.15	8.98	2.39	3.39	2.77	3.54	2.07	2.31	2.59	2.66	2.60	1.84	0.64	0.00	3.52
F98*	9.37	9.27	9.47	9.44	9.90	8.95	9.96	9.80	9.11	9.54	9.29	9.21	9.12	9.69	9.18	9.08	9.62	9.80	8.59	9.52	9.33	3.94	4.01	3.62	3.99	2.92	2.67	4.30	3.84	3.79	3.86	3.53	3.52	0.00

* the vaccine strain.

Identifying evolutionary changes of H9N2 in transmission with/without immune pressure using Next Generation Sequencing

Abstract

The continued in-field circulation of LPAI H9N2 is a major concern for public health. Human infection with H9N2 from direct contact with birds has given rise to increasing concerns about the rapid and unclear evolutionary changes of LPAI H9N2 replication in different hosts. We simulated the continuous replication of an avian H9N2 strain via serial passaging in different hosts *in vivo* and cell lines *in vitro*, with and without homologous vaccination. The HA genes from these virus progenies were sequenced at Illumina MiSeq platform using Next Generation Sequencing (NGS) to observe evolutionary changes, e.g. genetic diversity, general substitution frequency, genetic distance, and dN/dS ratios. In the chicks' respiratory system *in vivo*, the average substitution frequency was approximately 2.38×10^{-3} s/n/g, whereas *in vitro* it was 6.77×10^{-4} s/n/g. With higher substitution frequency, the genetic distances of parent virus from the offspring passaged *in vivo* were significantly lower than those *in vitro.* Moreover, we observed codon usage bias towards Adenine (A) and Cytosine (C) in synonymous mutations in progeny viruses after serial passaging in all the hosts/ cells, except for vaccinated chicken. Furthermore, we identified a total of six non-synonymous mutations which were potential mutations related to antigenic changes due to their locations on HA protein. Our methods and observations of the molecular evolution of the H9N2 AIV under continuous selective pressure provide references for evolutionary research on other low pathogenetic RNA viruses. In the long term, our findings may provide guidance for the role of vaccination in prevention strategies on different hosts.

4.1 Introduction

The circulation of low pathogenic avian influenza virus (LPAIV) H9N2 in poultry and wild birds is under continual surveillance in many countries around the world (Peacock et al. 2019). The viral strains transmitted by wild-domestic birds have been suggested to be selective for the determination of certain viral genetic factors (Bergervoet et al. 2019). LPAIV H9N2 was found not to be as threatening as HPAIV H5/H7 in birds, due to the low mortality. However, H9N2 is sporadically introduced to humans via close contact with infected poultry (Guo et al. 1999, Guo et al. 2002). Although the flu-like illness caused by H9N2 in humans is not as severe as H5 and seasonal H3, the adaption of H9N2 subtype to wide ranges of avian and mammalian hosts (Liu et al. 2013) may be a cause for future concerns.

During prolonged circulation, H9N2 subtype AIVs acquire diverse mutations functionally related to increased virulence (Sang et al. 2015), cross-species-transmission (Lina et al. 2019), or immune-escape (Peacock et al. 2017). The long-term circulation of H9N2 in poultry and the transmission between wild birds and domestic poultry may form a potential threat as an incubator of new-genetic type virus. In countries vaccinating poultry for H9N2 influenza, research indicates that antigenic selective pressure from vaccines has potentially directed the evolution of H9N2 (Lee et al. 2016, Meng et al. 2016, Umar et al. 2016). Most of this research was based on the phylogenic and bioinformatic analysis estimated from the current gene bank of influenza viruses (Blackburne et al. 2008). The frequent updates of vaccines against seasonal flu are reported in human-initiated studies on the relationship of vaccination and evolution of human influenza viruses, however the evolutionary mechanism is still unclear (Lam et al. 2017). Due to the enormous population of wild and domestic birds, we need a greater understanding of the potential threat of infection from the infectious circle of LPAI H9N2 in vaccinated poultry, wild birds, and humans because the virus population co-evolves with the host population (Domingo et al. 2007). The continuous circulation within and between the populations of birds (wild and domestic) and mammalian hosts gives the H9N2 viruses the time and platform to evolve either a new antigenic character or host-adapted traits.

The haemagglutinin (HA) of the influenza virus is the key gene for evolutionary changes related to antigenic or host-adapted properties because of its functions on membrane-fusion and receptor-binding. During fusion, the HA precursor (HA0) is cleaved into two polypeptides, HA1 and HA2 (White et al. 1982). Of these, HA1 is the crucial domain for antibody-mediated immunity and receptor-binding, and therefore the substitutions or amino acid changes on HA1 are more influenced by selective pressure (Skehel et al. 2000, Hay et al. 2001) from innate or acquired immunity. Benjamin P. et al. suggests that the selective pressure was largely found in locations or near locations that coded for host-adaption and antigenicity of the virus (Blackburne et al. 2008). However, their work was based on different evolutionary models, not on experimental results. The Next Generation Sequencing (NGS) has enabled experimental researchers to identify the substitutions at every location on the gene, showing the genetic diversity of the quasi-species of the virus. Together with bioinformatic knowledge and methods, it is now possible to explore the molecular evolution patterns of the virus under specific selective pressure from the host. Due to the rapid replication with low fidelity and large population size, we hypothesize that when the mutations of LPAI H9N2 accumulate in the serial passage in a certain host population under specific selective pressure, these evolutionary

changes can be observed and quantified based on the previously mentioned bioinformatic techniques.

To investigate this, we simulated continuous selective pressure with and without antigenic selection by serial passaging a virus strain in hosts with and without homologous vaccination. A field isolated avian H9N2 strain (A/chicken/Jiangsu/A2093/2011) was purified using reverse genetics technique and then serial passaged in different hosts *in vivo* and *in vitro*. The progeny viruses were harvested, and the viral RNA was extracted for amplification of HA genes using a high-fidelity polymerase. All the HA genes from different virus progenies were sequenced on the Illumina MiSeq platform. Based on these NGS data, we calculated the substitution frequency at every location on the HA open reading frame (ORF). The calculation of relative substitution rate and genetic distance was under the threshold of 0.006, considering NGS system error. Substitutions with a frequency over 0.01 (the threshold for significant mutation after selection) were observed and counted. Based on the consensus sequence of parent A2093-H9N2 strain and the standard codon table, codon usage preference for synonymous mutations and the dN/dS ratio were calculated to observe the mutation patterns under selective pressure. This observation of the molecular evolution of the H9N2 AIV under continuous selective pressure provides information for research on other low pathogenetic RNA viruses. In addition, our study may provide guidance for the role of vaccination in prevention strategies on different hosts.

4.2 Materials and methods

Ethics statement

The animal experiment and procedures followed the recommendations made by the Shanghai Veterinary Research Institute (SHVRI), CAAS (ID: SHVRI-PO-2014-0098). The project license was approved by the Animal Association of Science and Technology Commission of Shanghai Municipality, China (Permit Number: 2013-11). All the experimental animals were treated based on animal welfare requirements. Euthanasia was done humanely before the animals were sacrificed. Manure was removed at the end of the experiment. Infectious materials were professionally treated to achieve sterilization.

Viruses and reverse genetic platform

The reverse genetic systems for A2093 (A/chicken/Jiangsu/A2093/2011, H9N2) (Teng et al. 2016) viruses were constructed with pBD bidirectional expression vector. The viruses were generated by reverse genetics as previously reported (Hoffmann et al. 2000, Hatta et al. 2001). The reverse genetics system was used to generate stable-replicating A2093 virus stock as the parent virus for serial passaging. Transfection of this parent A2093 virus stock was performed on the 293T cell line, then seeded and harvested in chicken egg embryo (CEE) allantoic fluid, and separately stored in 300ul at -80 °C. Repeated freeze-thaw was avoided.

Primary cell, cell lines, and animals

The 293T cell line (a stable clone derivative of the human embryonic kidney (HEK) 293 cell line) was cultured with DMEM (Dulbecco's Modified Eagle's Medium, high glucose Gibco™) containing 10% fetal bovine serum (FBS, Gibco™) and 2mM L-glutamine (Gibco™). The MDCK (Madin-Darby Canine Kidney) cell line was cultured in DMEM containing 5% FBS. And the Vero cell line (derived from the kidney of an African green monkey,

Cercopithecus aethiops) was in the same basic medium but containing 10% FBS. The A549 cell line (a human epithelial cell line derived from a lung carcinoma tissue) was cultured with F-12K Medium (Gibco™) containing 10% FBS. All cell lines were cultured with 100 units/ml penicillin and 100 μg/ml streptomycin and incubated at 37°C in a humidified incubator with 5% CO_2.

Chicken and duck embryonic fibroblasts (CEF and DEF) were made from 10-day old specific-pathogen-free (SPF) chicken embryos (Merial, Shanghai, China) and 12-day old Pekin duck embryos (Chinese Academy of Agriculture, Beijing, China). The CEF and DEF cells were seeded at a density of approximately 1×10^6 cells/T75 culture flask overnight in MEM (Minimum Essential Medium, Gibco™) with 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C, 5% CO_2. The non-adherent cells were removed by washing the monolayers with sterile Phosphate-Buffered Saline (PBS, Gibco™) after overnight culturing.

SPF chicken was hatched from SPF embryonated chicken eggs (ECEs) in a forced-air incubator at 37°C, 55-65% humidity (Hatchery 2017). Chickens less than 10-days old were housed and raised in air re-circulating isolators at a temperature between 30-33°C. Chickens older than 10-days were raised at room temperature controlled by the central air conditioning at 23-25°C with 40-60% humidity.

Serial passaging without antigenic pressure

MDCK, A549, Vero cell lines, CEF, DEF primary cells *in vitro* and 1-7 day old chicks (chicks for short) *in vivo* were used as the hosts for serial passaging. No specific immunity was available in these host environments, simulating the selective pressure without specific antigenic selective pressure.

In vitro, infections and passaging on cell lines and primary cells were carried out simultaneously following the same procedure. In detail, three T25-flasks of 80% monolayer cells were seeded 12 hours before the infection. Parent virus was inoculated with cells at an MOI of 0.001 for one hour at 37°C, 5% CO_2. Cells were washed three times with Serum-Free Media (SFM, Thermo Fisher Scientific) and then maintained with SFM for at least 24h at 37°C, 5% CO_2. The HA titer of supernatant and cell conditions were checked every 6 hours till the HA titer was over 16 HA Units and more than 70% cell lysis was observed. The progeny viruses were incubated with the corresponding cells right after being harvested from the previous generation.

In vivo, three chicks were infected by injecting 10^6 EID_{50} parent viruses into the air sac. Infected chicks were housed in an air re-circulating isolator with a temperature between 30-33°C with sufficient clean water. Chicks that survived 12 hours after injection were maintained till 72 h.p.i. and then sacrificed for lung, trachea, and air sac tissues. Progeny viruses from different tissues were harvested separately by grinding finely with PBS (containing 10% penicillin-streptomycin) and centrifuging at 12,000 g for 10 min. Serial passaging was carried out with three groups of chicks for the virus from lung, trachea, and air sac tissues, respectively. Harvested viruses were filtered with the 0.45um filter and injected via the air sac in all the chicks, but the tissues to harvest were specific to each group. An HA assay was performed for every generation prior to injection.

Serial passaging under antigenic pressure

To produce the anti-sera for serial passaging under antigenic pressure *in vitro*, wild-type H9N2-A2093/2011 strain (A2093) was inactivated by incubating virus stock with 1:2000 β-propiolactone for 12h at 4℃. The residual β-propiolactone was degraded at 37℃ for 2h. The complete inactivation was determined by two passages of inoculation in CEEs for at least 72h prior to HA assay, in which a negative of each passage was the only criterion for successful inactivation. The HA titer of inactivated A2093 was tested before emulsification to guarantee good antigenicity. The inactivated A2093 and adjuvant Montanide VG71 ($0.85 g/cm^3$) was mixed at volume ratio 3:7. Three-week-old white leghorns were vaccinated with this virus emulsion by intramuscular injection in the legs. Three weeks after vaccination, the HI titer (HIT) of the sera was tested every two days for a total of three times, with the same HIT as 2^9 HI units. When the HIT was stable in assay, we collected the sera and stored it at -20℃ before use.

In vitro, an optimum serum dilution, a dilution that leads to ≈ 1000 × drop in the $TCID_{50}$ or EID_{50} titer of the parent virus, was determined as 1:10 following the lab protocol from Wageningen Bioveterinary Research (WBVR). Parent virus (A2093) was diluted to 24 HA Units and mixed with an equal volume of established optimum dilution of homologous polyclonal sera (1:10 for the first several generations) in sterile tubes, and incubated for 2hrs at 37 °C. After incubation, a negative control was 1000 × diluted with PBS, then all the strains with and without serum were inoculated into two 9-day-old SPF ECEs with 200μL. ECEs were inoculated at 37℃ for 48 hrs and HA titers were tested before harvesting. Progeny viruses were collected from the allantoic fluid with smaller HA titers; if the titers were the same, we mixed the allantoic fluid at parallel volume for one virus stock.

In vivo, vaccinated and SPF chickens with a similar health condition were used for the serial passaging via intranasal infection to simulate the natural infection route. Vaccination was carried out with the same inactivated vaccine, and the HI titer of above 2^6 was guaranteed before the passaging. The well-vaccinated SPF chickens were infected intranasally with 10^7 EID_{50} viruses, and serial passaging was performed by contact infection. Thus, the first generation was infected by intranasal drops, and the second was infected via contact by being hosted in one small isolator for sufficient interaction. Oropharynx swabs were collected from 2 d.p.i - 4 d.p.i (during the detoxification period) to harvest the progeny viruses for the corresponding generation. The first chicken was removed and sacrificed for blood and lung tissue at 5 d.p.i, and new “susceptible” chickens were added for the next generation of infection. Successful infection was guaranteed by checking the HI titer of the anti-serum and the possible viral titer in lung tissue. Oropharynx swabs were soaked and vibrated in PBS (containing 10% penicillin-streptomycin) thoroughly. Viruses of every generation in the supernatant were collected and filtered with a 0.45um filter.

Samples preparation for NGS

For the Next Generation Sequencing (NGS), we selected the parent virus strain (A2093-H9N2) and the corresponding progeny viruses serially passaged in different hosts. The middle and the last generation were chosen to reveal a brief variation tendency of the mutation rates during a period of continuous passaging in the host. The vRNA was extracted from 140μl supernatant from cell passaging, or from tissue homogenates using a QIAGEN Viral RNA Isolation Kit. The isolated RNAs were all eluted into 30μl diethylpyrocarbonate-treated water.

Two-step RT-PCR was employed to amplify each viral gene segment. The first-strand cDNA was transcripted by using Transcriptor High Fidelity cDNA Synthesis Kit (ROCHE, Cat. No. 05091284001) with universal primer (5'-AGCAAAAGCAGG-3') for influenza A virus, in a final volume of 20μl following the manufacturer's protocol.

Illumina MiSeq platform was applied to sequence the PCR products of gene HA. To access high-quality reads and better coverage, PCR products were restrained to 500 base pairs (bp). Accordingly, there were 4 fragments of the HA gene, for which the primers for PCR were listed in Supplementary Table S4.1. Amplicons of around 500bp were gel electrophoresis without degradation and were subsequently gel-purified by using a DNA Gel Extraction Kit (Axygen, Hangzhou, China). The concentration and quality were estimated by using NanoDrop 2000C. The concentration ranges of the PCR products were 15ng/ul-150ng/ul, 260/280 was limited to 1.6-2.0.

Balanced mixtures of 30ng per PCR product were constructed into DNA libraries by using VAHTS Universal DNA Library Prep Kit for Illumina. Random six-nucleotide indexes (Supplementary Table S4.2), specific to viruses from different serial passaging lines, were constructed and linked to the upstream of the forward primers. One DNA library was sequenced as one NGS sample for the separate run of sequencing. Sorting of different originated viruses was based on the indexes and mapping was approached by combining the separate gene fragments according to the reference sequences.

NGS data analysis

The libraries were sequenced using Illumina MiSeq at 2 × 150 bp (4.5Gb for 10-15M read pairs) configuration (Illumina, San Diego, CA, United States) at high coverage (average >10000 per nucleotide position) by GENEWIZ (Suzhou, China). Quality control-passed sequence reads were mapped to the reference sequence of the A2093 virus. The primer sequences were removed from the reads, then the overlapping region of two PCR fragments was avoided by mapping the reads till the median location (the NGS coverage region in supplementary Table S4.1). From the mapped data, we obtained the reads counts (sequencing depth) of each nucleotide type (A/T/C/G) at every location on the whole length of the HA gene, N_{ij} (i is the position of the substitution on the gene; j refers to one nucleotide type, A/T/C/G). In this way, the frequency of each nucleotide type at the location can be approached as $f_{ij} = N_{ij}/ C_i$ (C_i is the sequencing depth at location i). Based on the reference sequence, a total substitution frequency at every location was available as

$f_i = \sum_j^3 f_{ij}$ (large dataset, not shown in this thesis).

Under the assumption that the sequencing errors that occurred at every location of the gene are binomially independent, the probability of observing a substitution follows the Poisson distribution. Binning the f_i with a range of 0.001, we identified whether the validated $f_i > 0.005$ (threshold analysis was based on the parent A2093 data) was available bin 0.005 - 0.006 in all the progeny viruses. Therefore, $f_i \geq 0.6\%$ were filtered as minority single base substitutions (SBS). The geometric mean of minority SBS indicated the mutation rate of these "heat-spots" on the HA gene. This was calculated by:

$F_g = (\prod_i^n f_i)^{\frac{1}{n}}$;
n represents the nucleotide number of the locations with $f_i \geq 0.6\%$.

The general mutation rate over the certain domain of the HA gene (F_g per nt) was, however, calculated by:

F_g per nt = $\frac{1}{N}\sum_i^N f_i$;
N representing the nucleotide numbers of the sequence region of the HA gene.

The genetic distance between the two virus stocks A and B were calculated with a population-wide measure:

$d = \sqrt{\frac{1}{N}\sum_i^n (f_{iA} - f_{iB})^2}$ (Morelli et al. 2013),

where N was the nucleotide numbers of the sequence region of the HA gene. We calculated the distance of recombinant viruses (f_{iA}) to that of wt A2093 (f_{i_wt}). Calculations were programmed in R Studio.

For the calculation of transition/transversion and non-synonymous mutations, we extended the threshold of f_i to $\geq$ 1%, including a frequency of 0.5% or more as detection limit for reliable recognition of variants in the viral (Van den Hoecke et al. 2015) and the background error from PCR and sequencing. For transition/transversion definition, one SBS with $f_i \geq 0.01$ observed at one location was counted as one occurrence of transition [Ts, interchanges of two-ring purines (A /G) or one-ring pyrimidines (C/ T)] or transversion (Tv, interchanges of purine for pyrimidine bases) based on the consensus sequence. The sum of all the f_i of transition over the whole genome was defined as the probability of the transition after selection (P_{Ts}). The same is true for the possibility of transversion (P_{Tv}). Simultaneously, the total numbers of Ts and Tv were summed to indicate the diversity of one viral population (one virus stock). The numbers of synonymous and non-synonymous substitutions were calculated based on the amino acid sequence of HA gene from parent strain (A2093). Multiple t-test Discovery was determined using the Two-stage linear step-up procedure (Benjamini, Krieger and Yekutieli) (Benjamini et al. 2006), with Q = 1%. Each row was analyzed individually using GraphPad Prism, without assuming a consistent SD.

dN/dS calculation for NGS datasets

The dN/dS ratio is defined as the number of nonsynonymous substitutions per non-synonymous site (P_N) to the number of synonymous substitutions per synonymous site (P_S). In our study, the non-synonymous site (N) and synonymous site (S) was based on the reference nucleotide sequence of A2093, and the other calculations were based on previous studies (Nei et al. 1986, Orton 2014). We first estimated the numbers of non-synonymous (n) and synonymous (s) of the 64 nucleotide-triplet based on the standard genetic code (Supplementary Table S4.3) based on previously described methods (Nei et al. 1986). We considered mutations changing the triplet into stopping-codon as neither non-synonymous nor synonymous. Based

on the amino acid sequence HA, the estimated non-synonymous (N) and synonymous (S) numbers over the whole amino acid sequence were calculated as $N = \sum_1^r n_r$, $S = \sum_1^r s_r$ ($r \in (1: 560)$, indicating amino acid sites of HA protein sequence).

To obtain the discovered non-synonymous (N_d) and synonymous (S_d) numbers, the nucleotide sequence of the ORF region ($i \in (55: 1680)$) was displayed as a triplet chain and mapped to an HA amino acid sequence. Substitutions ($f_i \geq 0.01$) were observed at every location over the ORF region and then mapped to triplets to translate into amino acid residues of the amino acid sequence. Based on the standard codon table, we counted the observed substitutions causing non-synonymous/synonymous mutations. The proportion of synonymous mutations (P_S) and non-synonymous mutations (P_N) were estimated by $P_S = S_d/S$, $P_N = N_d/N$. The dN/dS ratio over the gene was calculated under the condition that $P_S < 0.75$, $P_N < 0.75$.

$$d_N = -\frac{3}{4}\ln\left(1 - \frac{4P_N}{3}\right)$$

$$d_S = -\frac{3}{4}\ln\left(1 - \frac{4P_S}{3}\right)$$

dN/dS ratio: $\frac{dN}{dS} = \frac{d_N}{d_S}$

4.3 Results

Evolutionary patterns of progeny viruses from serial passaging

A2093 virus stock from the reverse genetic system was passaged on MDCK and Vero cells for three generations and on CEF and DEF cells for ten generations. *In vivo,* we obtained progeny viruses from the lung, trachea, and air sac from the 1st, 5th, and 10th generations of the host. Figure 4.1 displays all the serial passaging processes and the samples used for NGS. If failure occurred in harvesting the progeny viruses from the cells or host tissues in between the serial passaging, then the inoculation of the per-generation virus was performed to continue the passaging.

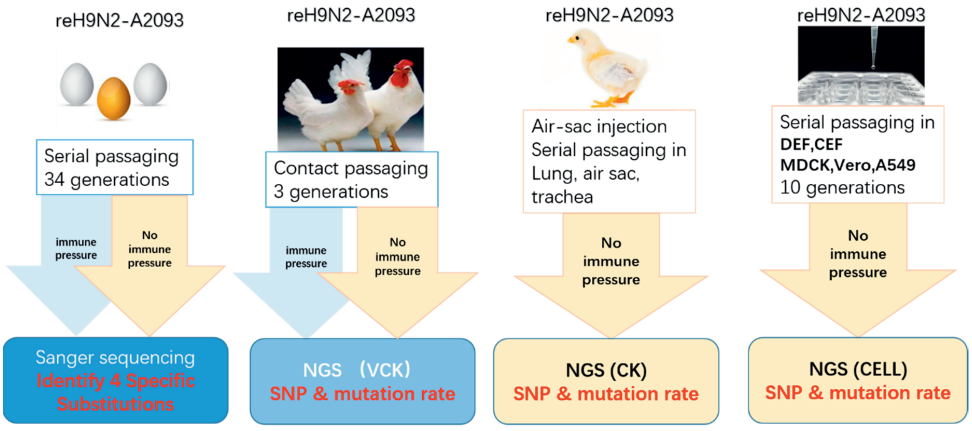


Figure 4.1 Flow of serial passaging in vivo and in vitro.

Based on the NGS data with the 0.006 cutoff, the estimated general mutation frequency of the parent virus A2093 was 3.18×10^{-5} s/n/g (substitutions per nucleotide per host generation) for the HA gene. Comparing to the parent virus, the general mutation frequency of the 1st generation progeny viruses from chick air sac was 2.3 times higher, and that from DEF cells was 1.8 times lower (Table 4.1). As the serial passaging continued, the mutation frequency decreased per host generation (g) due to the selection from the hosts.

Table 4.1 The evolutionary parameters calculated from substitution frequency in NGS data.

Host	Factor	G*	Abbri.	SBS Count	SNP average frequency ratio	Ts	P_{Ts}	Tv	P_{Tv}	Genetic distance (d)	d per generation	F_g[1]	F_g (s/n)[2]	F_g (s/n/g)[3]	dN/dS	Non-synonymous percentage
parent virus	na.	0	Parent -v	4	0.0123	4	0.05	0	0.00	0	0	1.28E-02	3.18E-05	3.18E-05	na.	25.00%
chick	airsac	1	CK-A1	61	0.0529	52	2.98	9	0.25	1.19E-02	1.19E-02	1.25E-02	3.35E-03	3.35E-03	0.14	36.07%
chick	airsac	5	CK-A5	4	0.0102	1	0.01	3	0.03	4.55E-03	9.11E-04	1.01E-02	1.54E-03	3.08E-04	na.	100.00%
chick	airsac	10	CK-A10	7	0.0145	5	0.07	2	0.03	3.97E-03	3.97E-04	9.54E-03	1.31E-03	1.31E-04	1.89	85.71%
chick	lung	1	CK-L1	38	0.0203	31	0.68	7	0.09	5.28E-03	5.28E-03	1.00E-02	1.93E-03	1.93E-03	0.10	28.95%
chick	lung	5	CK-L5	8	0.0233	3	0.13	5	0.05	5.31E-03	1.06E-03	9.92E-03	1.61E-03	3.22E-04	0.52	62.50%
chick	lung	10	CK-L10	6	0.0425	5	0.20	1	0.06	5.48E-03	5.48E-04	9.77E-03	1.38E-03	1.38E-04	0.31	50.00%
chick	trachea	1	CK-T1	43	0.0131	39	0.52	4	0.05	4.87E-03	4.87E-03	9.94E-03	1.86E-03	1.86E-03	0.16	37.21%
chick	trachea	5	CK-T5	2	0.0106	1	0.01	1	0.01	4.54E-03	9.07E-04	1.04E-02	1.48E-03	2.97E-04	na.	100.00%
cells	Vero	3	Vero-3	6	0.0383	4	0.20	2	0.03	5.13E-03	1.71E-03	8.47E-03	1.20E-03	4.00E-04	0.63	66.67%
cells	A549	3	A549-3	45	0.0244	40	0.90	5	0.20	6.84E-03	2.28E-03	1.00E-02	1.59E-03	5.31E-04	0.16	37.78%
cells	MDCK	3	MDCK-3	6	0.0140	3	0.04	3	0.04	2.99E-03	9.98E-04	8.38E-03	8.95E-04	2.98E-04	1.57	83.33%
cells	CEF	1	CEF-1	11	0.0571	8	0.55	3	0.08	1.10E-02	1.10E-02	8.72E-03	1.35E-03	1.35E-03	3.16	90.91%
cells	CEF	3	CEF-3	10	0.0840	7	0.74	3	0.10	1.48E-02	4.94E-03	8.94E-03	1.48E-03	4.93E-04	2.84	90.00%
cells	CEF	10	CEF-10	4	0.0488	3	0.18	1	0.01	2.22E-02	2.22E-03	8.64E-03	1.49E-03	1.49E-04	0.94	75.00%
cells	DEF	1	DEF-1	3	0.0128	1	0.02	2	0.02	2.78E-03	2.78E-03	8.24E-03	8.05E-04	8.05E-04	0.63	66.67%
cells	DEF	3	DEF-3	4	0.0151	3	0.05	1	0.01	3.20E-03	1.07E-03	8.41E-03	1.03E-03	3.44E-04	0.94	75.00%
cells	DEF	10	DEF-10	10	0.1022	4	0.14	6	0.88	1.51E-02	1.51E-03	9.16E-03	1.54E-03	1.54E-04	0.73	70.00%
chicken	Vaccinated	3	VAC-3A	8	0.0216	5	0.14	3	0.04	3.19E-03	1.06E-03	8.50E-03	5.78E-04	1.93E-04	0.52	62.50%
chicken	Vaccinated	3	VAC-3B	6	0.0113	3	0.03	3	0.03	2.33E-03	7.75E-04	8.11E-03	5.52E-04	1.84E-04	1.57	83.33%
chicken	Non-Vaccinated	3	NV-3A	38	0.0140	31	0.43	7	0.11	3.42E-03	1.14E-03	9.22E-03	9.45E-04	3.15E-04	0.14	31.58%
chicken	Non-Vaccinated	3	NV-3B	7	0.0147	5	0.08	2	0.02	2.47E-03	8.23E-04	8.21E-03	6.08E-04	2.03E-04	0.79	71.43%

*G, indicates generation of the infected host.

[1], select Fi>0.006 locations, geometric mean of these locations;

[2], sum Fi (>0.006)/length(region), s/n represents substitution per nucleotide;

[3], sum Fi (>0.006)/length(region)/5, s/n/g represents substitution per nucleotide per generation in host.

Substitution changes of progeny viruses after serial passaging in different hosts were visualized as the genetic distances compared to the parent virus A2093. The genetic distances of progeny viruses decreased during the serial passaging in one host. Progeny viruses replicating in different chick tissues *in vivo* had similar genetic distances. However, the *in vivo* genetic distances were significantly lower than those from *in vitro*, showing an opposing tendency: a larger decrease of distance between 1st and 10th generations in CEF and DEF passaging; a slight increase of distance was observed in chick-respiratory passaging lines (Figure 4.2).

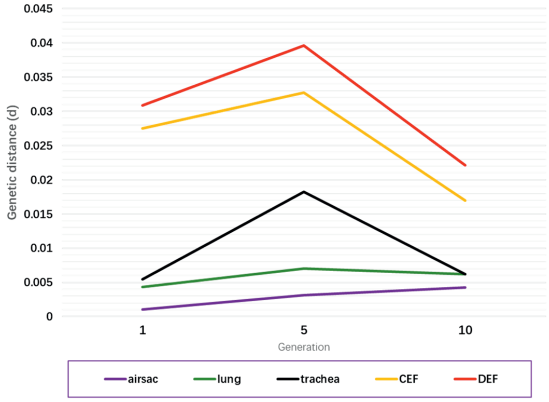


Figure 4.2. Genetic distance (d) of the progeny viruses in chick-respiratory passaging line and in vitro passaging line. The x-axis displayed the generations of progeny viruses; the progeny viruses in chick-respiratory passaging line were displayed as purple (from air sac), green (from lung) and black (from trachea); the progeny viruses in vitro were displayed as orange (in CEF) and red (in DEF).

Mutation characters of progeny viruses from serial passaging

For the nucleotide changes observed under the cutoff of $f_i \geq 1\%$, we discovered substitutions distributed at the head and tail of HA (Supplementary Figure S4.1). The total SNP numbers were statistically higher for viruses passaged in chicks *in vivo* compared to those passaged *in vitro*. These high SNP numbers were observed *in vivo* for the 1st generation, but decreased while passaging. Progeny viruses from serial passaging on DEF and CEF cells showed an opposing tendency in the substitution numbers (SNP count in Table 4.1). Comparing the progeny viruses from the 3rd generation of incubation passaging on cell lines (Vero, A549 and MDCK) and those of contact-infection passaging (vaccinated and SPF chicken), the substitution number on the Vero cell-line was the smallest. No differences were observed for substitution number of progeny viruses passaged in A549 and MDCK *in vitro*, nor for SPF chickens.

The substitutions causing synonymous and non-synonymous mutations based on the standard codons were calculated (Supplementary Figure S4.1, numbering in amino acid sequencing with nucleotides in triplets). In general, substitutions towards Adenine (A) happened in all the progeny viruses. We further calculated the numbers of four types of nucleotides of the ORF region on the HA gene from progeny viruses. Subtracting the corresponding numbers from the parent virus, we obtained the relative numbers of nucleotides changed after replicating in hosts (Figure 4.3). When considering the codon usage bias in synonymous mutations, Adenine (A) and Cytosine (C) were found to be preference nucleotides for progeny viruses after serial passaging in all the hosts except for vaccinated chicken (Figure 4.3). In contrast, Cytosine (C) was preference nucleotide for progeny viruses after passaging in the A549 cell lines and the SPF chicken without vaccination. Among the 21 amino acids residues using the standard codons (Table 4.2), more than half [Threonine (T), Leucine (L), Serine (S), Valine (V), Glycine (G), Glutamic acid (E), Tyrosine (Y), Arginine (R), Asparagine (N), Histidine (H) and Isoleucine (I)] showed codon usage bias in progeny viruses after serial passaging in chicks and cells.

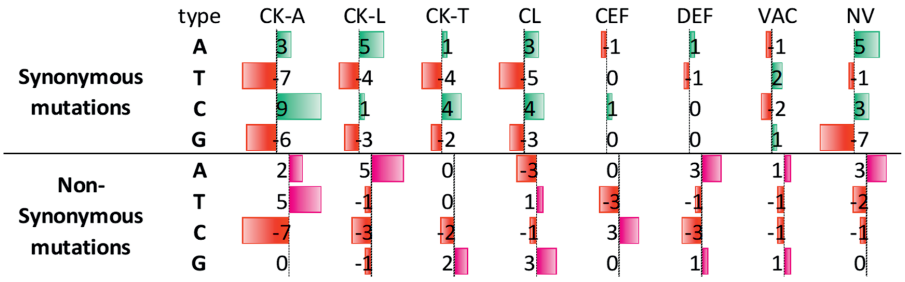


Figure 4.3 Substitutions at codon of progeny viruses from different serial passaging lines. a, Substitution preference that causing synonymous and non-synonymous mutations. Red bars towards left indicating the decreased number of certain nucleotides which substituted by others; green bars towards right indicating the substitutions towards certain nucleotides causing synonymous mutations on HA; Cherry bar towards right indicating the substitutions towards certain nucleotide causing non-synonymous mutations on HA. A/T/C/G are the four nucleotide types, CK-A, is the virus replicating in chick air sac; CK-L represent viruses replicating in chick lung; CK-T represent the viruses from chick trachea; CL indicating the cell lines; CEF and DEF are the primary cells; VAC, represented viruses from vaccinated chicken, NV, represented viruses from SPF chicken without vaccination. Blue indicating the substitutions causing non-synonymous mutations; green indicating the synonymous mutations.

The viral population harvested from the vaccinated chicken was less than the other serial passaging lines (data not shown); therefore, it showed no bias in codon usage compared to the parent strain. In addition, codon usage shifting to Cysteine (C) or Glutamine (Q) were only observed in non-vaccinated adult chickens but not in chicks or cell lines.

Table 4.2 Substitutions on synonyms discovered in progeny viruses and the codon usage table of HA gene.

Amino Acid	Single letter code	DNA codons	Host for serial passaging [1]							Influenza A virus "HA" 3679 CDS's (2081925 codons) [2]			H9N2 "HA" CDS's (561 codons) [3]		
			CK-A	CK-L	CK-T	CL	CE	VAC	NV	Fraction	Frequency /1000	Number	Fraction	Frequency/ 1000	Number
Threonine	T	ACA	▼-1	▼-1	▼-1	▼-2	▼-1	▼-1	▬0	0.43	25.9	54022	0.53	35.7	20
		ACG	▬0	▲1	▲1	▲1	▬0	▲1	▬0	0.1	5.8	11994	0.05	3.6	2
		ACC	▲1	▼-1	▬0	▲1	▲1	▬0	▬0	0.14	8.3	17334	0.13	8.9	5
		ACT	▬0	▲1	▬0	▬0	▬0	▬0	▬0	0.33	19.7	41051	0.29	19.6	11
Leucine	L	TTG	▼-2	▬0	▬0	▬0	▬0	▬0	▼-2	0.2	16.3	33979	0.14	12.5	7
		TTA	▼-2	▼-1	▼-1	▼-1	▬0	▬0	▼-1	0.1	8.1	16769	0.08	7.1	4
		CTG	▲2	▬0	▬0	▬0	▬0	▬0	▲2	0.25	20.2	42026	0.22	19.6	11
		CTA	▬0	▲1	▲1	▲1	▬0	▬0	▬0	0.19	15.8	32796	0.3	26.7	15
		CTT	▬0	▬0	▬0	▬0	▬0	▬0	▬0	0.17	13.8	28633	0.12	10.7	6
		CTC	▲2	▬0	▬0	▬0	▬0	▬0	▲1	0.09	6.9	14356	0.14	12.5	7
Serine	S	TCA	▼-2	▼-1	▼-1	▼-1	▲1	▬0	▬0	0.28	21.3	44271	0.33	25	14
		AGT	▼-1	▼-1	▼-1	▼-1	▬0	▬0	▬0	0.18	13.5	28064	0.16	12.5	7
		TCT	▬0	▼-1	▬0	▼-1	▬0	▬0	▲1	0.14	10.8	22519	0.14	10.7	6
		TCC	▬0	▬0	▬0	▲1	▼-1	▬0	▼-1	0.13	9.6	20030	0.14	10.7	6
		TCG	▲2	▲2	▲1	▲1	▬0	▬0	▬0	0.03	2.4	4977	0.05	3.6	2
		AGC	▲1	▲1	▲1	▲1	▬0	▬0	▬0	0.24	18	37445	0.19	14.3	8
Valine	V	GTC	▼-1	▼-2	▼-1	▼-1	▬0	▬0	▬0	0.19	10.1	21089	0.18	10.7	6
		GTG	▼-1	▼-1	▼-1	▼-1	▬0	▬0	▼-1	0.25	13.3	27623	0.3	17.8	10
		GTT	▲1	▲1	▲1	▲1	▼-1	▬0	▲1	0.29	15.5	32270	0.18	10.7	6
		GTA	▲1	▲2	▲1	▲1	▲1	▬0	▬0	0.27	14.7	30566	0.33	19.6	11
Glycine	G	GGC	▼-1	▼-1	▼-1	▼-1	▬0	▬0	▬0	0.13	9.8	20414	0.09	7.1	4
		GGG	▼-1	▼-1	▼-1	▬0	▬0	▬0	▼-1	0.28	21.3	44354	0.3	23.2	13
		GGA	▲1	▬0	▬0	▬0	▬0	▬0	▲1	0.4	30.8	64182	0.41	32.1	18
		GGT	▲1	▲2	▲2	▲1	▬0	▬0	▬0	0.19	14.5	30101	0.2	16	9
Alanine	A	GCA	▬0	▲1	▬0	▬0	▬0	▬0	▲1	0.43	22.8	47389	0.45	25	14
		GCG	▬0	▬0	▬0	▬0	▬0	▬0	▼-1	0.08	4.1	8500	0.03	1.8	1
		GCC	▬0	▼-1	▬0	▬0	▬0	▬0	▬0	0.23	12	25063	0.23	12.5	7
		GCT	▬0	▬0	▬0	▬0	▬0	▬0	▬0	0.27	14.5	30202	0.29	16	9
Lysine	K	AAA	▬0	▬0	▼-1	▲1	▬0	▬0	▲1	0.7	42.8	89167	0.71	39.2	22
		AAG	▬0	▬0	▲1	▼-1	▬0	▬0	▼-1	0.3	18.3	37998	0.29	16	9
Phenylalanine	F	TTC	▬0	▬0	▬0	▼-1	▬0	▬0	▲1	0.58	21.1	43872	0.67	17.8	10
		TTT	▬0	▬0	▬0	▲1	▬0	▬0	▼-1	0.42	15.4	31981	0.33	8.9	5
Glutamic acid	E	GAG	▼-4	▼-3	▼-3	▼-3	▬0	▬0	▲1	0.39	22.9	47685	0.32	17.8	10
		GAA	▲4	▲3	▲3	▲3	▬0	▬0	▼-1	0.61	35.8	74525	0.68	37.4	21
Tyrosine	Y	TAT	▼-1	▼-2	▼-2	▼-2	▬0	▬0	▲1	0.52	19.6	40879	0.52	21.4	12
		TAC	▲1	▲2	▲2	▲2	▬0	▬0	▼-1	0.48	18.3	38104	0.48	19.6	11
Arginine	R	AGG	▼-2	▼-1	▼-1	▼-1	▬0	▬0	▼-2	0.32	14.5	30183	0.29	14.3	8
		CGG	▬0	▬0	▬0	▬0	▬0	▬0	▼-1	0.06	2.8	5876	0.04	1.8	1
		AGA	▲2	▲1	▲1	▲1	▬0	▬0	▲2	0.52	23.4	48765	0.61	30.3	17
		CGA	▬0	▬0	▬0	▬0	▬0	▬0	▲1	0.06	2.7	5591	0.04	1.8	1
		CGT	▬0	▬0	▬0	▬0	▬0	▬0	▬0	0.01	0.3	625	0.04	1.8	1
		CGC	▬0	▬0	▬0	▬0	▬0	▬0	▬0	0.02	1	2093	0	0	0
Asparagine	N	AAT	▼-2	▼-1	▼-1	▼-1	▬0	▲1	▬0	0.56	43.8	91161	0.67	49.9	28
		AAC	▲2	▲1	▲1	▲1	▬0	▼-1	▬0	0.44	34.1	71048	0.33	25	14
Histidine	H	CAT	▬0	▼-1	▼-1	▼-1	▬0	▬0	▲1	0.55	11.9	24867	0.5	10.7	6
		CAC	▬0	▲1	▲1	▲1	▬0	▬0	▼-1	0.45	9.8	20453	0.5	10.7	6
Isoleucine	I	ATT	▼-1	▼-1	▼-1	▼-1	▬0	▬0	▬0	0.34	25.5	53145	0.39	26.7	15
		ATC	▲1	▲1	▲1	▬0	▬0	▼-1	▼-1	0.3	22.6	47008	0.29	19.6	11
		ATA	▬0	▬0	▬0	▲1	▬0	▲1	▲1	0.35	26.3	54858	0.32	21.4	12
Cysteine	C	TGT	▬0	▬0	▬0	▬0	▬0	▬0	▼-2	0.48	14.1	29282	0.67	17.8	10
		TGC	▬0	▬0	▬0	▬0	▬0	▬0	▲2	0.52	15.5	32341	0.33	8.9	5
Glutamine	Q	CAA	▬0	▬0	▬0	▬0	▬0	▬0	▲1	0.67	25.1	52305	0.52	19.6	11
		CAG	▬0	▬0	▬0	▬0	▬0	▬0	▼-1	0.33	12.5	25979	0.48	17.8	10
	.	TAA	▬0	▬0	▼-1	▬0	▬0	▬0	▬0	0.31	0.6	1155	1	1.8	1
		TAG	▬0	▬0	▬0	▬0	▬0	▬0	▬0	0.09	0.2	317	0	0	0
		TGA	▬0	▬0	▲1	▬0	▬0	▬0	▬0	0.6	1.1	2250	0	0	0

[1], ▼-1, negative numbers indicated the decreased numbers of DNA codons on HA gene in progeny viruses; ▲1, positive numbers indicated the increased numbers of DNA codons on HA gene in progeny viruses; ▬0, indicated the no mutations occurred in DNA codons on HA gene in progeny viruses; CK-A, is the virus replicating in chick air sac; CK-L represent viruses replicating in chick lung; CK-T represent the viruses from chick trachea; CL indicating the cell lines; CE are the primary cells; VAC, represented viruses from vaccinated chicken, NV, represented viruses from SPF chicken without vaccination.

[2], The codon usage table of Influenza A virus HA gene, 3679 CDS's (2081925 codons), data was from Codon Usage Database;

[3], The codon usage table of H9N2 HA gene, A/chicken/Guangxi/KMI/99 (561 codons), data was from Codon Usage Database;

Influence of selective pressures

Selective pressure on a protein-coding gene was estimated by the dN/dS substitution ratio which was not influenced by the percentage of non-synonymous mutations (Table 4.1). The dN/dS substitution ratios of progeny viruses decreased during serial passaging (Table 4.1), and there was no accumulated effect of the continuous selective pressure. The dN/dS substitution ratios also showed high variance between individuals *in vivo*. Whereas, after three generations of serial passaging, the dN/dS ratio of virus replicating in vaccinated chickens was >1, the same high ratios were observed in MDCK and CEF *in vitro*. In contrast, the dN/dS ratio of viruses replicating in Vero (0.629), A549 cell line (0.162), and in DEF (mean and sd 0.769 ± 0.160) were all <1, indicating less selective pressure from these cells. No mutations were observed on the consensus sequences of the HA gene in the last generation of progeny viruses from the host without antigenic pressure. Therefore, we did not observe host-adapted mutations in the ten generations of successful replications in the host.

Similarly, A2093 virus stock was passaged under antigenic pressure *in vitro* for 34 generations. We found five non-synonymous mutations (S145N, N201K, R205I, T206N and D207N, H9 numbering) on the consensus sequences of the HA gene. Together with these five non-synonymous mutations detected at the 30th to 34th generations, another non-synonymous mutation (N191H) was detected at the 34th generation (Table 4.3). S145N and T206N observed under antigenic and non-antigenic selection were considered to be related to host-adaption, whereas N191H, N201K, R205I, and D207N observed only in viruses under antigenic selection were potential immune-escape mutations. No mutations on the consensus sequences were observed in the viruses passaged under the antigenic pressure in vaccinated chickens, partly due to the small amount of progeny viruses harvested.

Table 4.3 Substitutions introducing non-synonymous mutations.

Substitutions	Mutations	EID_{50}/ml	$TCID_{50}$/ml	Anti-selection	Non-anti selection
wt-A2093	none	8.50	4.67	na.	na.
G434A	S145N	9.25	5.00	+/+	+/-
C617A	T206N	na.	na.	+/-	+/-
A571C	N191H	7.75	4.50	+/-	-/-
T603A	N201K	3.50	1.50	+/-	-/-
G614T	R205I	8.50	4.50	+/-	-/-
G619A	D207N	7.50	5.00	+/+	-/-

Note: Substitutions introducing non-synonymous mutations in serial passaging with homogeneous antisera in chicken embryo allantoic fluid. na. No estimated data.

4.4 Discussion

Like many other RNA viruses, the influenza virus functions as a prolonged troublemaker largely due to its high mutation rate, which in turn provides genetic resources for rapid evolution towards between-host adaption or immune escape. Should these two processes occur, a pandemic could be just around the corner. The evolution of influenza viruses is the result of the interaction of substitutions attributed to polymerase error and selective pressure from the host environment, including the host's immune status. To reveal the mutation pattern of influenza A viruses, previous studies report a range of mutation rates from approximately 10^{-5} to 10^{-6} mutations per nucleotide per cellular infection (Suárez et al. 1992, Nobusawa et al. 2006, Sanjuán et al. 2010). More recent research found an overall mutation rate as about 10^{-4} substitutions per nucleotide per strand copied (s/n/r) in linear replication mode (Pauly et al. 2017). Not only do the estimated mutation rates differ based on the methods used, but also because properties differ from gene segments due to the segmented genome characteristics of influenza A viruses. It has been suggested that the positive selection by the host immune system is the reason for a high evolutionary rate at surface genes (HA and NA genes) (Fitch et al. 1991, Ina et al. 1994, Cox et al. 1995). However, few studies focus on the interaction of mutation frequency and selective pressure.

To better understand the influence of host selective pressure on the evolutionary patterns of the influenza A virus, we studied the mutations in the HA gene after several generations of serial passaging in different hosts. Before the virus strain underwent any selective pressure, the estimated general substitution frequency of the parent virus A2093 was 3.18×10^{-5} s/n/g for the HA gene, about ten-fold lower than the progeny viruses from all the passages. The estimation was based on the substitution frequency from the next generation sequencing involving rarer, more deleterious mutations (Acevedo et al. 2014, Geller et al. 2016). This estimated value provided a reference for the progeny viruses following selective pressures. In comparison, we observed high substitution rates after a single replication in the chicks and in the different cell lines. In the chicks' respiratory system *in vivo*, the average substitution frequency was approximately 2.38×10^{-3} s/n/g, whereas *in vitro* it was 6.77×10^{-4} s/n/g; more mutations occurred *in vivo*. We then identified a decreasing tendency during serial passaging, in contrast to our hypothesis that the mutation rate accumulated for generations of viruses. Under the condition that the absolute substitution frequency was stable due to the polymerase error rate, the observed substitution decreased as a result of the selective pressure on the population of progeny virus. However, the higher substitutions *in vivo* suggest an influence of the host on the replication process of the virus. Marine Combe et al. reported that "virus yield in the very first infection cycle correlates with the short-term ability of the virus to create diversity" (Combe et al. 2015). That is, the observed genetic diversity and the calculated substitution frequency and genetic distance were determined by the number of viral particles in one infectious unit and their success in the replication procedure. All the evolutionary parameters in chicks and cell lines in this study reflect the average effect of total cell-bottleneck *in vivo* or *in vitro* as a selective pressure.

The replication cycle of the influenza virus depends on host machinery and cellular components for protein synthesis. Therefore, codon usage in influenza virus in its hosts could be expected to affect viral replication (Wong et al. 2010). In general, we observed codon usage

bias in synonymous mutations: Adenine (A) and Cytosine (C) were preference nucleotides for progeny viruses after serial passaging in all the hosts except for vaccinated chicken. Given the dN/dS ratio of virus replicating in vaccinated chickens was above one, the progeny viruses we harvested from vaccinated chicken were heavily selected by the immune system triggered by the homogenous vaccine. This antigenic neutralization on the virus did not happen at the replication stage (within the cell); selective pressure of vaccine-induced immunity was in addition to the natural selective pressure from the host. This explains why the vaccine-induced immunity dramatically reduced the population size and diversity of the influenza virus population, but still triggered the evolution towards antigenic drift. In other words, the interaction of the high replication error and natural selection pressure generated a virus population with genetic variants as a genetic pool. Then the vaccine-induced immunity pressure caused an additional antigenic-originated selection on the excreted viruses which were then exposed to the antibodies. Either a large virus population or long-term circulation increase the possibility of the occurrence of antigenic drift from this additional antigenic-originated selection.

In our study, we identified a total of six non-synonymous mutations: S145N, N201K, R205I, T206N D207N and N191H. Earlier experimental research using a monoclonal antibody (Mab) identified amino acid sites 145, 201 and 207 as HA protein epitopes of the H9N2 subtype AIV (CHEN et al. 2012, Wan et al. 2014). The 145S was the same in vaccine strains (A/Chicken/Shandong/6/96, A/Chicken/Shanghai/F/98) as in the A2093 strain. Along with the evolution of HA gene, the mutation S145N introduced glycosylation may have played a role in antigenic variation in a previous study (Ping et al. 2008). Moreover, in our previous research (**Chapter 3**), the mutation at site 145 evolved from N (Asparagine) towards D (Aspartic acid) in the H9N2 viruses isolated in the field between 2013-2018, forming a new antigenic cluster compared to those from the vaccine strains in the 1990s. The other sites 191, 205, 206 were all near the antigenic position at HA1, and mutations at these sites were also reported related to antigenic changes (Okamatsu et al. 2008, Bahari et al. 2015). However, N191H, N201K, R205I, and D207N were found in a small proportion among natural mutations in mainland China (Nextstrain 2021), suggesting a long evolution in nature in the endemic population of H9N2. Experimental research to identify these antigenic sites predicted the potential mutations related to antigenic shift.

Worth noting is the significant difference between the natural evolution and the experimental evolution of our research. The virus population was forced to undergo continuous selective pressure in the experiment via inoculation, however, in the natural process, selective pressure on one virus unit would stop if no successful new infection occurred. This failure of new infection could be due to host-specific differences. However, no failure of infection was reported in the serial passaging experiment. This also explains why fewer differences were observed in the evolutionary patterns of viruses from vaccinated chicken. The dN/dS ratios of progeny viruses at 1st generation indicate a higher host-specific selection *in vivo* than *in vitro* in general. However, the higher selection in the human lung cancer cell line (A549) than in the Vero cell line was highly influenced by the success of viral infection *in vitro*.

In summary, we observed evolutionary changes of viruses replicating in different hosts or cell lines with and without antigenic selection. Within ten generations, the general substitution frequency and the genetic distances of progeny viruses decreased due to selective pressure

during serial passaging in one host. Progeny viruses passaged (within ten generations) in the Vero cell line had the least number of substitutions. Even though no differences were observed for the progeny viruses passaged substitution numbers *in vitro* and *in vivo*, the codon usage (cytosine as preferred nucleotides) was observed on progeny viruses after passaging in the A549 cell lines and the SPF chicken without vaccination. After three generations of serial passaging, the dN/dS ratio of virus replication in vaccinated chickens was above one, indicating more selective pressure compared to that of the cell lines. Moreover, we identified a total of six non-synonymous mutations as potential immune-escape mutations under the antigenic pressure *in vitro* after more than 30 generations of serial passaging. This not only provides predictable information for antigenic drift, but also suggests that selective pressure introduced by the vaccine is more antigenic-destinated than host-specific selective pressure.

Acknowledgements

We are grateful for the fund and facility support from Zejun Li's lab group at the Shanghai Veterinary Research Institute (SHVIR), China. Hongrui Cui is enrolled at the graduate schools Wageningen Institute of Animal Sciences (WIAS) and Production Ecology and Resource Conservation (PE&RC) as part of the 2016 Sino-Dutch joint PhD program between the Chinese Academy of Agricultural Sciences (CAAS) and Wageningen UR.

Funding

This work was supported by the National Key Research and Development Program of China (2016YFD0500204) and the Chinese Academy of Agricultural Sciences of Technology Innovation Project.

Conflict of interest statement

There is no conflict of interest.

Supplementary materials

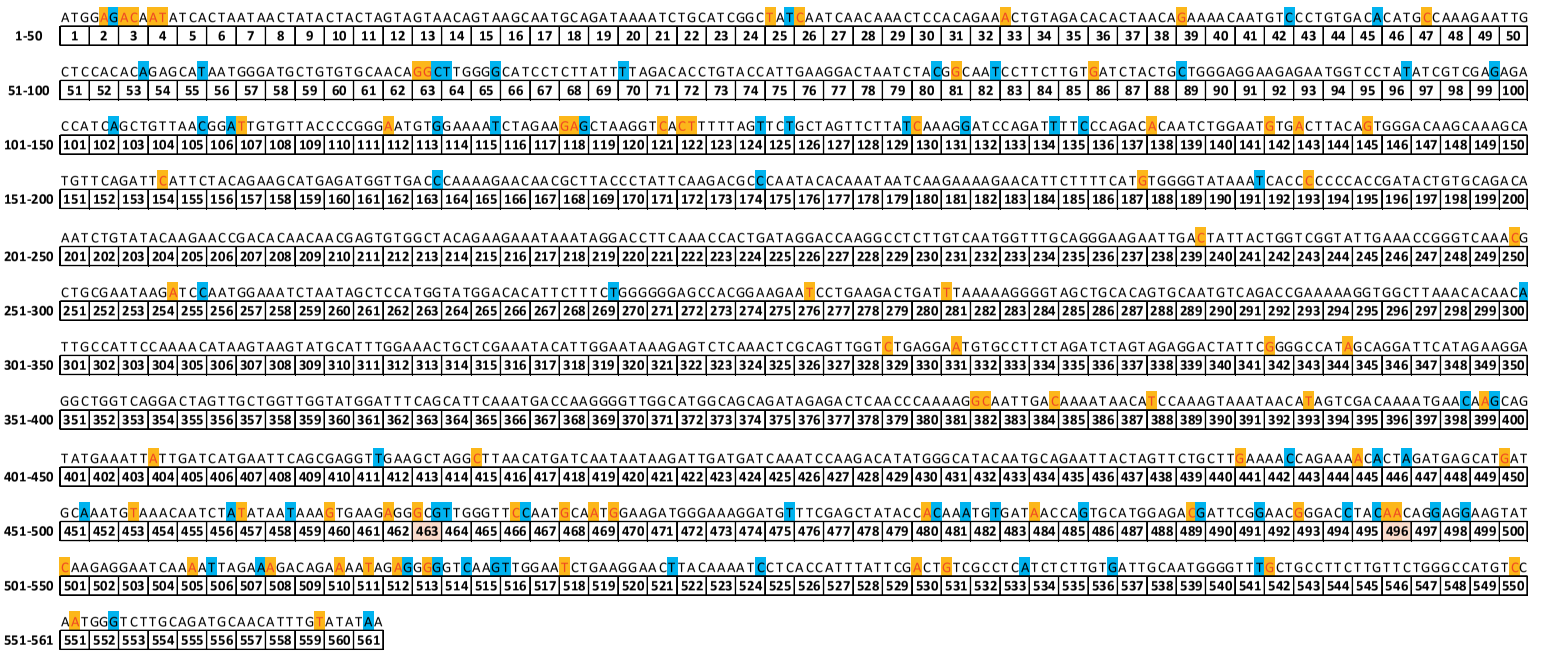


Figure S4.1 Substitutions resulting synonymous/non-synonymous mutations on HA gene. Nucleotide sequence of HA gene with codon numbers at the amino acid sequence. The nucleotide in yellow was the substitutions causing non-synonymous mutations; in blue causing synonymous mutations.

Table S4.1 PCR primers for 4 fragments of HA gene.

	FRAGMENT 1	FRAGMENT 2	FRAGMENT 3	FRAGMENT 4
Forward (5'-3')	AGCAAAAGCAGGGGAATT	ACAATCTGGAATGTGACTTA	GCACAGTGCAATGTCAGA	CCAAGACATATGGGCATACA
Reverse (5'-3')	AACATGCTTTGCTTGTCC	GTTGTGTTTAAGCCACCTT	CATCATGCTCATCTAGTG	TAGAAACAAGGGTGTTTTTG
PCR coverage	1-487	445-932	890-1384	1311-1740
NGS coverage	34-460	461-912	913-1364	1365-1716

Table S4.2 Progeny virus samples for Next Generation Sequencing.

NGS run	Host	Factor	Ggeneration	Abbri.	5' Index
1	chick	airsac	G1	RA1	cttagt
1	chick	airsac	G5	RA5	ctgcat
1	chick	airsac	G10	RA10	ctcact
1	chick	lung	G1	RL1	gaacac
1	chick	lung	G5	RL5	gacgac
1	chick	lung	G10	RL10	tactag
1	chick	trachea	G1	RT1	tattat
1	chick	trachea	G5	RT5	acgata
2	cells	Vero	G3	Rv3	cttagt
2	cells	A549	G3	Ra549_3	ctgcat
2	cells	MDCK	G3	Rmdck_3	ctcact
2	cells	CEF	G1	Rcef_1	gaacac
2	cells	CEF	G3	Rcef_3	gacgac
2	cells	CEF	G10	Rcef_10	tactag
2	cells	DEF	G1	Rdef_1	tattat
2	cells	DEF	G3	Rdef_3	acgata
2	cells	DEF	G10	Rdef_10	actgcc
3	chicken	vaccinated	G3	vac-G3A	accatg
3	chicken	vaccinated	G3	vac-G3B	ctgata
3	chicken	SPF	G3	SPF-G3A	ctcgaa
3	chicken	SPF	G5	SPF_G5A	acatag
3	parent - r	na.	G0	R0	aacaag

Table S4.3 The numbers of non-synonymous (n) and synonymous (s) of the 64 nucleotide-triplet based on the standard genetic code.

Triplet	TTT	TTC	TTA	TTG	CTT	CTC	CTA	CTG	ATT	ATC	ATA	ATG	GTT	GTC	GTA	GTG
amino acid	F	F	L	L	L	L	L	L	I	I	I	M	V	V	V	V
n	2.67	2.67	2.33	2.33	2	2	1.67	1.67	2.33	2.33	2.33	3	2	2	2	2
s	0.33	0.33	0.67	0.67	1	1	1.33	1.33	0.67	0.67	0.67	0	1	1	1	1

Table S4.3 continued

Triplet	TCT	TCC	TCA	TCG	CCT	CCC	CCA	CCG	ACT	ACC	ACA	ACG	GCT	GCC	GCA	GCG
amino acid	S	S	S	S	P	P	P	P	T	T	T	T	A	A	A	A
n	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
s	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

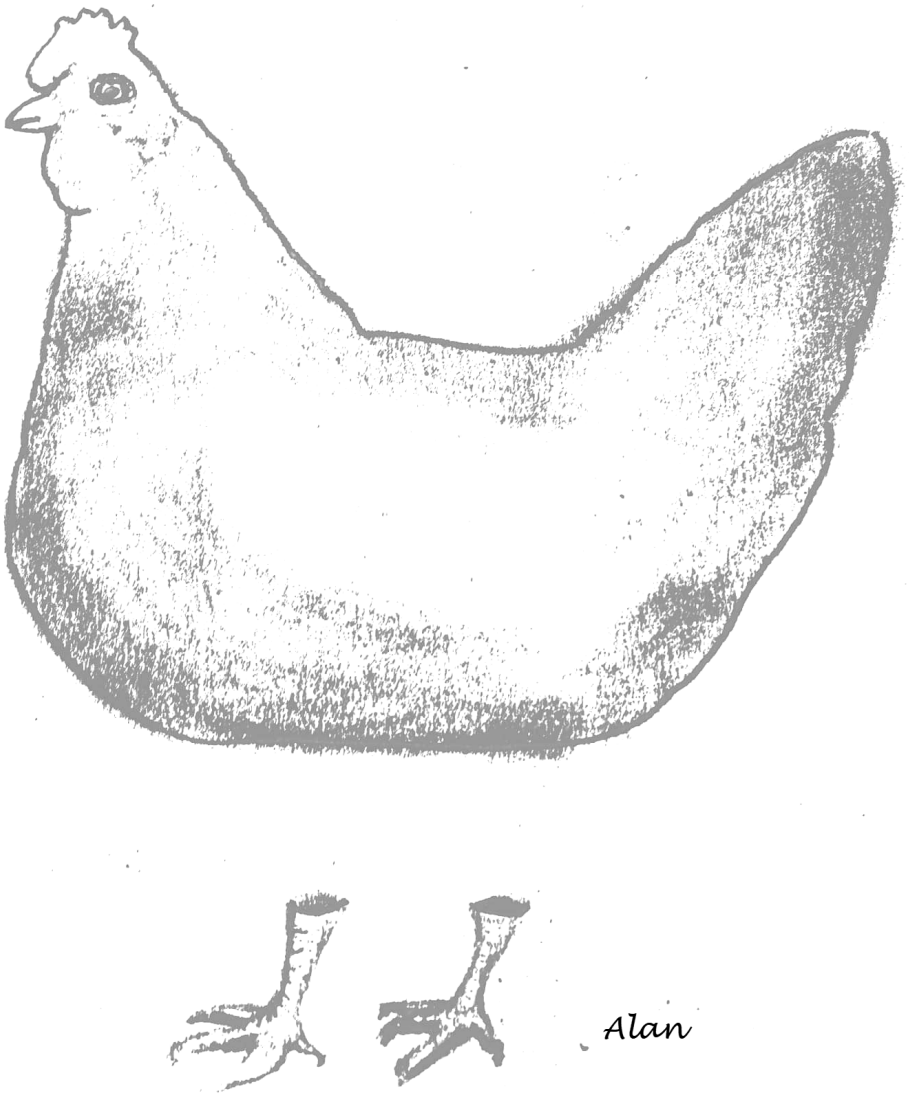
Table S4.3 continued

Triplet	TAT	TAC	TAA	TAG	CAT	CAC	CAA	CAG	AAT	AAC	AAA	AAG	GAT	GAC	GAA	GAG
amino acid	Y	Y	*	*	H	H	Q	Q	N	N	K	K	D	D	E	E
n	2	2	3	3	2.67	2.67	2.67	2.67	2.67	2.67	2.67	2.67	2.67	2.67	2.67	2.67
s	1	1	0	0	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33

Table S4.3 continued

Triplet	TGT	TGC	TGA	TGG	CGT	CGC	CGA	CGG	AGT	AGC	AGA	AGG	GGT	GGC	GGA	GGG
amino acid	C	C	*	W	R	R	R	R	S	S	R	R	G	G	G	G
n	2.50	2.50	2.33	2.33	2	2	1.50	1.67	2.67	2.67	2.17	2.33	2	2	2	1.33
s	0.50	0.50	0.67	0.67	1	1	1.50	1.33	0.33	0.33	0.83	0.67	1	1	1	1.67

NOTE: n, expected non-synonymous numbers for each codon; s, expected synonymous numbers for each codon. Method in (https://bioinformatics.cvr.ac.uk/calculating-dnds-for-ngs-datasets/).



Alan

The PB1 gene from H9N2 avian influenza virus showed high compatibility and increased mutation rate after reassorting with a human H1N1 influenza virus

Abstract

Reassortment between human and avian influenza A viruses may result in novel viruses with new characteristics that may threaten human health when causing a next flu pandemic. A particular risk may be the posed by avian influenza viruses of subtype H9N2 that are currently massively circulating in domestic poultry in Asia and have been shown to infect humans. In this study, we investigate the characteristics and compatibility of a human H1N1 virus with avian H9N2 derived genes. Reassortant viruses were generated by reverse genetics in which genes of the human WSN-H1N1 virus (A/WSN/1933) were replaced by genes of the avian A2093-H9N2 virus (A/chicken/Jiangsu/A2093/2011). We replaced both the Hemagglutinin (HA) and Neuraminidase (NA) genes in combination with one of the genes involved in the viral ribonucleoprotein (RNP) complex (either PB2, PB1, PA or NP). Reassortant viruses were able to replicate in MDCK and DF1 cells and in mice. In particular, for the PB1 reassortant virus increase virulence for mice was measured by increased body weight loss of mice after infection. The reassortant viruses were passaged for five generations in MDCK cells and mice. Analysis of the virus populations by next-generation sequencing showed a higher substitution rate for the PB1-reassortant virus. In addition, we show that the avian PB1 gene increased the polymerase activity of the RNP complex using luciferase reporter assays. The higher polymerase activity, and increased mutation frequency measured for the PB1 reassortant virus suggests that the avian PB1 gene may drive the evolution and adaptation of novel reassortant viruses to the human host. This study provides novel insights in the characteristics of novel viruses that may arise by reassortment of human and avian influenza viruses. Surveillance for infections with H9N2 viruses and the emergence of novel reassortant viruses in humans is important for pandemic preparedness.

5.1 Introduction

Highly pathogenicity avian influenza viruses (HPAIVs), specifically the H7 and H5 subtypes, are a continuous threat to the poultry industry, as these viruses can cause up to 100% mortality in poultry. In contrast, low pathogenicity avian influenza viruses (LPAIVs) typically cause only mild or no clinical symptoms in poultry. Since the first identification of H9N2 isolated from quail in 1988 (Perez et al. 2003), H9N2 AIV has been isolated from poultry (Lee et al. 2000, Liu et al. 2004, Perk et al. 2006, Lee et al. 2007) and wild birds (Kawaoka et al. 1988, Jackwood et al. 2007) across Europe, Asia and North America. Since the 1990s, the widespread of H9N2 AIV in poultry has led to human infections in China (Peiris et al. 1999, Butt et al. 2005), Bangladesh (Chakraborty et al. 2011), Pakistan (Ali et al. 2019) and Oman (Alexander 2007). Three stable poultry lineages are recognized with the representative viruses, A/quail/Hong Kong/G1/1997 (G1), A/chicken/Beijing/1/94 (BJ94, also known variously as the Y280 or G9 lineage) and A/chicken/Hong Kong/Y439/1997 (Y439, also known as the Korean lineage) (Guo et al. 2000). In 1998, the first human infection with H9N2 AIV was reported in Hong Kong (Peiris et al. 1999), and in the same year, five human patients were confirmed infected with H9N2 AIV in Southern China (Guo et al. 1999). Since that, a total of 70 laboratory-confirmed human cases have been reported in southern China, other Asian countries and Africa based on the records of WHO till December 2020 (Butt et al. 2005, James et al. 2019, Potdar et al. 2019, WHO 2020). The human clinical cases with H9N2 AIV indicated that certain strains, e.g. G1 and BJ94 (Y280 or G9) have a preference for the human-like α-2,6-linked sialic acid (SA) receptor due to some specific gene mutations in the HA (Hemagglutinin) gene (Butt et al. 2005, Huang et al. 2015, Peacock et al. 2017). Further evolution of the H9N2 AIV may lead to adaptation of the virus to humans; however, no human-to-human transmission has been reported yet (Zhou et al. 2018).

The evolution of H9N2 AIV may be enhanced by the co-circulation with other LPAI or HPAI viruses in poultry due to reassortment events in which gene segments are exchanged between viruses. Evidence for this is provided by phylogenetic analysis of the (A/Quail/Hong Kong/G1/97) G1-like H9N2 AIV (Guan et al. 2000). Reassortment events, in particular for the PB2 (Polymerase basic protein 2), HA, NP (Nucleoprotein), and NA (Neuraminidase) genes, resulted in the rapid evolution of the H9N2 AIV, adapting the wild bird viruses to poultry. As H9N2 AIVs were shown to have the potential to infect humans, reassortment with seasonal human influenza viruses may also occur during co-infections. The exchange of gene segments between different viral strains can cause sudden changes in pathogenicity, virulence or transmission ability (Scholtissek 1995, Holmes et al. 2005, Ince et al. 2013). This can give rise to novel influenza viruses that are adapted to the human host. Experimental studies using a ferret model showed that reassortant viruses harbouring H9N2 AIV surface genes and seasonal human H3N2 influenza virus internal genes are efficiently transmitted after adaptation by serial passaging (Sorrell et al. 2009). In addition, human infections were reported with other zoonotic subtypes of avian influenza viruses, such as H5Nx, H7N9, and H10N8 (Lam et al. 2015). Genetic analysis of these zoonotic viruses revealed that they arose by re-assortment events with H9N2 AIVs, in which they obtained internal genes of H9N2 AIVs (Gu et al. 2014, Hao et al. 2019). Several mutations were identified in internal genes of avian H9N2 strains that enhance transmission from poultry to mammals or increase the virulence of the viruses (Best et al. 2000, Li et al. 2014), such as the mutation 627E/701D in the PB2 gene.

Besides re-assortment events, the high mutation rate of influenza viruses also contributes to their fast evolution. Replication of the RNA genome is mediated by the virus-encoded RNA polymerase, which is highly error-prone. This results in virus population, or quasi-species, with high genetic diversity. This diversity allows a viral population to rapidly adapt to dynamic environments, thereby allowing instance escape from the host immune response or vaccination. In particular genetic changes in the two surface proteins, HA and NA, can lead to changes in the antigenicity of the virus (Lindstrom et al. 2004). The viral RNA polymerase complex is composed of the PB2, PB1 (Polymerase basic protein 1) and PA (Polymerase acidic protein) (Fodor 2013), packaged by the NP protein (a single-strand RNA-binding nucleoprotein) (Biswas et al. 1998) to generate the viral ribonucleoprotein complexes (RNPs). The RNPs provide the minimal set of proteins required for the transcription and replication of viral RNAs (Andino et al. 1993, De la Luna et al. 1993). The polymerase proteins were previously reported to affect the replication and virulence of influenza viruses (Watanabe et al. 2009). The PB2 protein was found to influence virulence (Hatta et al. 2001, Graef et al. 2010) as well as host preference (Shinya et al. 2004, Labadie et al. 2007). A study on single gene reassortment identified a critical role for PB1 (in addition to HA and NA) gene in the high virulence of the 1918 pandemic influenza virus (Pappas et al. 2008). Reassortment events between human and avian influenza viruses in combination with rapid evolution and adaptation due to error-prone replication may lead to a novel human pandemic. In this study, we investigate the replication capacity of the human WSN-H1N1 virus in combination with avian polymerase genes derived from the A2093-H9N2 virus. We show that the PB1 gene of the H9N2 AIV is able to increase polymerase activity and the error rate of the reassortant human viruses. The avian PB1 gene thus may thereby drive the evolution of novel human reassortant viruses. As the H9N2 AIV is currently still circulating intensively in poultry populations in Asia, and human H9N2 infections have been observed, the emergence of novel reassortant viruses in humans must be carefully monitored for pandemic preparedness.

5.2 Materials and methods

Ethics statement

The animal experiment and procedures were in accordance with the recommendations made by the Shanghai Veterinary Research Institute, CAAS (ID: SHVRI-PO-2014-0098). The project license was approved by the Animal Association of Science and Technology Commission of Shanghai Municipality, China (Permit Number: 2013-11). All the animal experiments were designed to involve as least number of animals as possible. Animals involved were guaranteed sufficient food, water and contact space. Animals to be sacrificed for tissues were under euthanasia. All the virus (LPAI H9N2) related materials and experiments were carried out in biosafety level 2 (BSL-2) conditions.

Viruses and reverse genetic platform

The reverse genetic systems for WSN (A/WSN/1933, H1N1, GenBank: LC333185.1) and A2093 (A/chicken/Jiangsu/A2093/2011, H9N2, GenBank: KP865958.1) (Teng et al. 2016) viruses have been constructed using the pBD bidirectional expression vector. The viruses were generated by reverse genetics, as previously reported (Hoffmann et al. 2000, Hatta et al. 2001). The reverse genetics system was used to generate stable-replicating reassortant viruses

containing segments of A2093-H9N2 in the WSN-H1N1 virus background. One of the internal genes of WSN/H1N1 (PB2, PB1, PA or NP) and the surface genes (both HA and NA) were replaced by that of the avian A2093-H9N2 virus to obtain PB2-reassortant, PB1-reassortant, PA-reassortant and NP-reassortant. The wild type (wt) A2093-H9N2 and WSN-H1N1 were also rescued in this system for control.

Cell lines and animals

Madin-Darby Canine Kidney (MDCK) cells and avian DF-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose Gibco™) containing 5 % fetal bovine serum (FBS, Gibco™) and 1% penicillin-streptomycin. The 293T cells were cultured with DMEM containing 10% FBS and 2mM L-glutamine (Gibco™). All cell lines were incubated at 37°C in a humidified incubator with 5% CO_2.

Specific-pathogen-free (SPF) 4- to 6-week-old female BALB/c mice were purchased from Vital River Laboratories, Beijing, China. They were housed in isolators with air re-circulation system. The room housing isolators was controlled by the central air conditioning to 23-25 °C with 40-60% humidity. Mice were housed under optimal light conditions and feed and water were provided ad libitum. Sawdust and corn cob beddings were provided in the isolators. Bedding materials, food and water were refreshed every week. Virus infection experiments were carried out in high containment facilities, each isolator had a separate air-circulation purification system. The mice were euthanized at the end of the experiment. Manure was removed at the end of the experiment.

Activity of the reassortant polymerase in vitro

A minigenome assay was performed to compare the activities of viral RNP complexes with one of the polymerase-related genes from avian influenza H9N2 (A2093) following the manufacturer's instructions of Dual-Luciferase Reporter 1000 Assay Systems (Promega Corporation, Wisconsin, USA). Briefly, we constructed eight eukaryotic expression plasmids with genes from virus strains A2093-H9N2 and WSN-H1N1, in the backbone of the pCAGGS expression construct: A2093-PB2, A2093-PB1, A2093-PA, and A2093-NP and WSN-PB2, WSN-PB1, WSN-PA, and WSN-NP.

Then 1×10^5 293T cells were cultured in 12-well plates together with the four protein expression plasmids (pCAGGS PB2, pCAGGS PB1, pCAGGS PA, and pCAGGS NP [0.5 g of each]) for each of the 8 RNP combinations of A2093-H9N2 and WSN-H1N1 virus proteins. The open reading frame (ORF) of Firefly Luciferase was inserted in the packaging signals of NA gene (N' 183 base pairs (bp) and C' 157bp) of the PR8 strain (A/Puerto Rico/8/1934), was placed under the control of the pPol I promoter, and was inserted into a multiple cloning site (MCS) of pUC18 plasmid (Takara Bio, Dalian, China). At 48 hours post-transfection, the Relative light units (RLU) of Firefly Luciferase were measured on a GloMax 96 microplate luminometer (Promega) according to the manufacturer's instructions. As an internal control for the dual-luciferase assay, Renilla (20ng each, Promega) was used. The results were presented as the mean of three independent parallel experiments, with the standard deviation.

Replication of H1N1/H9N2 reassortant viruses in MDCK cells and mice

The growth kinetics of the rescued viruses were estimated by infection of mammalian MDCK cells and avian DF-1 cells at 0.001 multiplicity of infection (MOI). The 70%-80% monolayer cells were prepared in three T25 flasks 12 hours before infection. One flask of cells was used for counting the number of cells. The other two are for parallel control. A total of 1ml virus solution was incubated on the cells for 1 hour at 37℃, 5% CO_2 incubator. After removing the virus solution, 5ml fresh SFM (Serum-Free Cell Culture, Gibco) was added. Cell supernatant samples (300µl in volume) were collected at 12, 24, 36, 48, 60 and 72 hours post-infection to determine the virus titer.

The replication of the reassortant viruses in mice was estimated. For each infection group, three 4-week-old female BALB/c mice were infected intranasally with 10^6 PFU virus stocks in PBS (Phosphate-buffered saline). These mice were sacrificed for the tracheal and lung tissues at 4 d.p.i. Clear supernatants of homogenates were used to evaluate the viral titer. Viral titers of these samples were determined in plaque-forming units per millilitre (PFU/ml) by plaque assays on MDCK cells as previously described (Tobita et al. 1975). Here we applied the immunohistochemical staining method with influenza A virus nucleoprotein monoclonal antibody (clone FluA-NP 2C9) to count the plaques. Three independent experiments were carried out. Mean with standard error (sd) were used for data analysis.

Based on the replication ability and viral titer of parent virus strains, the virulence of the virus was further evaluated by observing the bodyweight loss of infected mice. For each infection group, five 4-week-old female BALB/c mice were infected intranasally with 10^6 PFU virus stocks in PBS (Phosphate-buffered saline). As control, five 4-week-old female BALB/c mice were inoculated with the same amount of PBS. Five mice were numbered, the body weight and health condition were observed and record every 24 hours for 14 days post-infection (d.p.i). The percentage of the body weight change after infection was calculated comparing to the original body weight measured before infection (0 d.p.i). The average percentage body weight loss of five mice of every group was calculated. Multiple t-test with a = 0.05 were performed for significant differences.

Serial passaging of H1N1/H9N2 reassortant viruses in cells and mice

Reassortant viruses, wt WSN-H1N1 and A2093-H9N2 were passaged in MDCK cells at an MOI of 0.001. In detail, three T25-flasks of 80% monolayer cells were seeded 12 hours before the infection. Parent virus was inoculated with cells at an MOI of 0.001 for one hour at 37°C, 5% CO_2. Cells were washed three times with Serum-Free Media (SFM, Thermo Fisher Scientific) and then maintained with SFM for at least 24h at 37°C, 5% CO_2. The HA titer of supernatant and cell conditions were checked every 6 hours till the HA titer was over 16 HA Units and more than 70% cell lysis was observed. The progeny viruses were incubated for a new generation host circle with the same amount of MDCK cells right after being harvested from the previous generation.

In vivo, reassortant WSN-H1N1/A2093-H9N2 viruses and the corresponding wt WSN-H1N1 and A2093-H9N2) were passaged in 4-week-old BALB/c mice. Three mice were intranasally inoculated with 10^6 PFU parent virus stocks and sacrificed for the tracheal and lung tissues at 4 d.p.i. The homogenates of the two tissues from 3 mice were mixed separately and subsequently used for infection (intranasally) of two groups of mice. One group was for serial

passaging in mouse lung (3 mice), and the other group was for serial passaging in mouse trachea (3 mice). During the serial passaging, the age and health condition of the mice were maintained the same as possible.

The above serial passaging procedures were repeated five times for five generations, and two independent parallel passaging experiments were performed.

Sample preparation for NGS

The reassortant WSN-H1N1/A2093-H9N2 viruses and the wt strains obtained after serially passaging in MDCK cells and mice (lung and turbinate, specifically) were prepared for Next Generation Sequencing (NGS). The initial virus stock and the last generation (P5) were selected for NGS sequencing. The details of sequenced viruses are listed in Table S5.1, and the experimental workflow of amplicon sequencing is shown in Supplementary Figure S5.1 (Additional file1.pptx). All samples were analyzed in duplicate, starting from two independent RNA isolations.

Viral RNA (vRNA) was extracted from 140 μl supernatants from cell passaging or tissues' homogenates by using QIAGEN Viral RNA Isolation Kit following the manufacture's instructions. The isolated RNAs were all eluted into 30μl diethylpyrocarbonate-treated water. Two-step RT-PCR was employed to amplify each viral gene segment. The first-strand cDNA was transcripted by using Transcriptor High Fidelity cDNA Synthesis Kit (ROCHE, Cat. No. 05 091 284 001) with universal primer (5'-AGCAAAAGCAGG-3') for influenza A virus in a final volume of 20μl as manufacturer's protocol. The HA gene was amplified using primer-pairs to obtain amplicons of approximately 500bp with PCR. Accordingly, there were four fragments of HA gene, two fragments of M and NS genes, respectively. Six-nucleotide indexes were added to the forward primers for PCR to allow bar-coding of the samples (Supplementary Table S5.1). All the primers for PCR are listed in Supplementary Table S5.2. In detail, the PCR amplification was carried out with Phanta Max Super-Fidelity DNA Polymerase (Vazyme Biotech Co., Ltd) in a 50μl system, using 2μl cDNA template. The amplification program consisted of a 3-min period at 95℃ and was followed by 35 cycles with the following conditions: 95℃ for 30sec, 55℃ for 30 sec, and 72℃ for 40sec, and ended with one cycle at 72℃ for 5 min. The amplicons were purified in 1% agarose gel electrophoresis and then purified using a DNA Gel Extraction Kit (Axygen, Hangzhou, China). The concentration and quality were estimated by using NanoDrop 2000C. The concentration ranges of the PCR products were 15ng/ul-150ng/ul, and the 260/280 absorption was between 1.6-2.0. All bar-coded PCR products of the same gene fragment were mixed in equal quantities (30ng) to construct one DNA library for NGS analysis. The DNA libraries were generated using VAHTS Universal DNA Library Prep Kit for Illumina.

Analysis of NGS data

The libraries were sequenced using Illumina MiSeq at 2 × 150 bp (4.5 Gb for 10-15M read pairs) configuration (Illumina, San Diego, CA, United States) at high coverage (average >10000 per nucleotide position) by GENEWIZ (Suzhou, China). Quality control-passed sequence reads were mapped using the WSN and A2093 virus reference sequences and used to detect minority variants. The primer sequences were removed from the reads, then the overlapping region of two PCR fragments was avoided by mapping the reads to the median location. From the mapped

data, we obtained the reads counts (sequencing depth) of each nucleotide type (A/T/C/G) at every location of the whole length of HA gene, N_{ij} (i is the position of the substitution on the gene; j refers to one nucleotide type, A/T/C/G). Then the frequency of each nucleotide type at the location can be approached as $f_{ij} = N_{ij}/ C_i$ (C_i is the sequencing depth at location i). Based on the reference sequence, total substitution frequency at every location was available as $f_i = \sum_j^3 f_{ij}$. Under the assumption that the sequencing errors that occurred at every location of the gene are identically independent distributed with a binomial distribution, the probability of observing a substitution follows approximately a Poisson distribution because these probabilities are low. Binning the f_i with a range of 0.001, we identified the low-frequency sequencing errors occur in bins before bin 0.005 - 0.006 with the threshold >0.05 (threshold analysis was based on the parent A2093 data). Therefore, $f_i \geq 0.6\%$ were filtered as minority single base substitutions (SBS). The geometric mean of minority SBS indicating the mutation rate of these "hot-spots" on HA gene. This was calculated by the geometric mean:

$F_g = (\prod_i^n f_i)^{\frac{1}{n}}$, n represented the nucleotide number of the locations with $f_i \geq 0.6\%$.

However, the general mutation rate over a certain domain of the HA gene (F_g per nt) was calculated by the arithmetic mean:

F_g per nt = $\frac{1}{N}\sum_i^N f_i$, N was the nucleotide numbers of the sequence region of HA gene.

The genetic distance between two virus stocks A and B was calculated with a population-wide measure:

$d = \sqrt{\frac{1}{N}\sum_i^n (f_{iA} - f_{iB})^2}$ (Morelli et al. 2013), N was the nucleotide numbers of the sequence region of HA gene.

In this research, we calculated the distance of reassortant viruses (f_{iA}) to that of parent A2093-H9N2 (f_{iB}). Calculations were programmed in R Studio (Team 2013).

For the calculation of transition/transversion and non-synonymous mutations, we extended the threshold of f_i to $\geq$ 1%, as the detection limit for reliable recognition of variants in the viral (Van den Hoecke et al. 2015) and the background error from PCR and sequencing. For transition/transversion definition, one SBS with $f_i \geq 0.01$ observed at one location was counted as one occurrence of transition (Ts) or transversion (Tv) basing on the consensus sequence. Sum of all the f_i of transition or transversion over the whole genome was the probability that transition and transversion were observed after selection, as P_{Ts} and P_{Tv}. Simultaneously, the total numbers of Ts and Tv were summed up, which indicating the diversity of one viral population (one virus stock).

Two parallel independent NGS runs (run1, run2) were performed to analyze the reproducibility of the results. NGS data from these two runs of sequencing were compared. Multiple t-test Discovery was applied using the Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with Q = 1%. Each row was analyzed individually, without assuming a consistent SD by using GraphPad Prism.

5.3 Results

Polymerase activity of reassortant viruses in vitro

The polymerase activity of the avian A2093-H9N2 influenza virus was compared to that of the human WSN-H1N1 influenza virus using an *in vitro* Luciferase reporter assay. In addition, reassortant H1N1 and H9N2 polymerases were tested in which either the PB2, PB1, PA or NP genes were exchanged between the two viruses. The activity of the recombined polymerases was compared to that of the WSN-H1N1 and A2093-H9N2 polymerases (Figure 5.1). Compared to the WSN-H1N1 polymerase, the reassortant polymerase containing the PB1 gene of the avian A2093-H9N2 virus significantly increased the activity of polymerase complex *in vitro* (mean diff. -7.245, 95% CI of diff. -12.76 to -1.734). Replacement of the PB2 or PA genes dramatically decreased the polymerase activity (mean diff. were 22.57 and 20.07 respectively), whereas replacement of NP did not significantly affect the activity (mean diff. -0.09848, 95% CI of diff. -5.610 to 5.413).

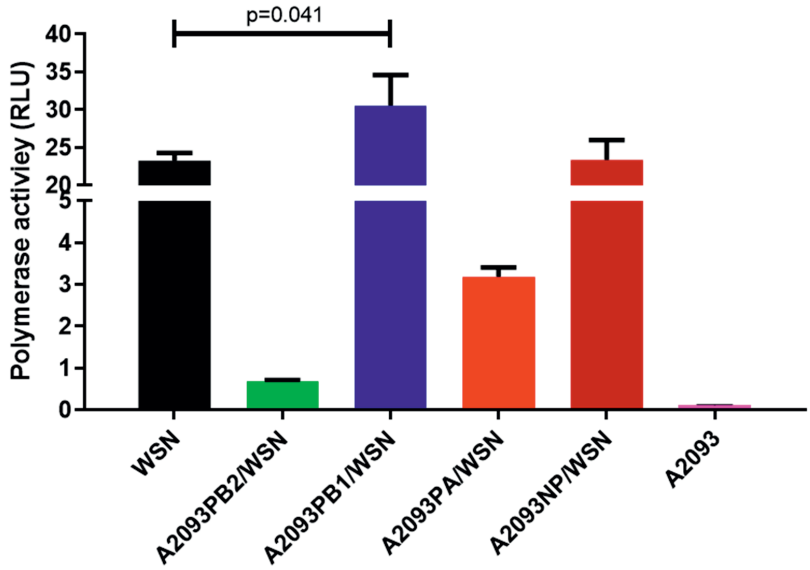


Figure 5.1 Viral RNPs functional assay of recombinant polymerase complex. The polymerase activities of the human WSN-H1N1 RNP, the recombinant RNPs containing PB2, PB1, PA or NP genes derived from the avian A2093-H9N2 RNP, and the avian H2093 RNP were measured using the Dual-Luciferase Assay System. Firefly luciferase activity was measured to determine polymerase activity, and Renilla luciferase activity was used as an internal control for monitoring transfection efficiency. Experiments were repeated three times, mean and SD were applied. Multiple t-test with a= 0.05. *significant difference.

Replication of the reassortant viruses in cells

Reassortant WSN-H1N1 viruses were generated, in which both the HA and NA genes were replaced by that of A2093-H9N2, in combination with one of the polymerase-related genes PB2, PB1, PA or NP. The replication of these viruses was studied after infection of mammalian MDCK cell and duck DF1 cells, as shown in Figure 5.2. The viral titers of wild-type and reassortant viruses were evaluated using plaque assays and HA titers were determined (Table 5.1). In MDCK cells, the replication of the PB1-reassortant viruses was similar to that of wt

WSN-H1N1 virus. The peak viral titers were obtained after 24 hrs, 2.83 $\times 10^6$ ($\pm 1.17 \times 10^6$) PFU/ml for wt WSN-H1N1, and 5.69 $\times 10^6$ ($\pm 1.10 \times 10^6$) PFU/ml for the PB1-reassortant virus. For the other reassortant viruses, delayed replication kinetic peak and lower titers were observed: 2.21 $\times 10^4$ ($\pm 1.23 \times 10^4$) PFU/ml for PB2-reassortant to 1.28 $\times 10^6$ ($\pm 1.25 \times 10^6$) PFU/ml for PA-reassortant. On DF1 cells titers of around 10^2 - 10^5 PFU/ml were obtained for the reassortant viruses, whereas a peak titer of 2.09 (± 1.2) $\times 10^5$ PFU/ml was observed for wt WSN-H1N1 virus at 24 hpi. On DF1 cells, lower peak viral titers were observed compared to MDCK cells. Due to inefficient replication on DF1 cells, MDCK cells were selected for subsequent serial passaging experiments with the reassortant viruses.

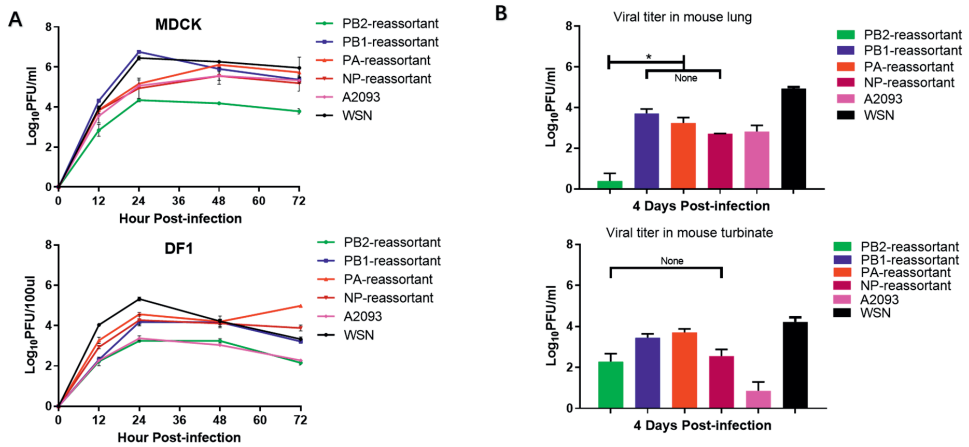


Figure 5.2 Replication of reassortant viruses in cell lines (A) and in mouse (B). A, Virus replication of human wt WSN1-H1N1, avian wt 2093-H9N2 and reassortant viruses containing the avian PB2, PB1, PA or NP segments. Virus replication was measured by determining plaque-forming units at several hours post-infection of MDCK and DF1 cells. **B,** Virus replication of human wt WSN1-H1N1, avian wt 2093-H9N2 and reassortant viruses containing the avian PB2, PB1, PA or NP segments. Virus replication in mouse turbinal tissue and lung was measured at 4 days post-infection using plaque assays. Plaque assays were repeated three times, the mean and sd of the PFU in the logarithm of 10 were applied. Multiple t-test with a = 0.05. * significant difference.

Replication and virulence of reassortant viruses in mice

Virus replication for the reassortant viruses was studied by infection of mice. At four days post infection, virus titers in the mouse lung and nasal turbinate were measured (Figure 5.2B). The human-originated WSN-H1N1 virus replicated more efficiently in mice than the avian A2093 virus, likely due to its adaptation to replication in mammals. The replication of the reassortant viruses containing either the A2093-H9N2 PB2 or NP genes was reduced compared to wt WSN-H1N1 virus. However, virus replication of the WSN-H1N1 was not significantly affected by replacement with the avian PB1 and PA genes. That is, these two reassortant viruses were able to replicate in both turbinate and lung up to 10^4 PFU/ml. The A2093-H9N2 showed a slight preference for replication in the lung compared to turbinate. The reassortant viruses were able to replicate to titers of 10^3 PFU/ml (without significant differences between the viruses), except for PB2-reassortant virus for which significantly lower titers were obtained. These results show that the lowest levels of replication were observed for the PB2-reassortant virus in both cell lines and mice, whereas the other reassortant viruses replicate to similar levels.

To study the virulence of the reassortant viruses, we inoculated eight mice with the reassortant viruses that were found to efficiently replicate in mice (PB1, PA and NP-reassortant viruses). Subsequently, the body-weight loss of five mice was measured 14 days after infection as a marker for virulence. This showed that the PB1-reassortant virus caused a significant bodyweight drop (Figure 5.3) in infected mice, similar to the wt WSN-H1H1 virus at the 5th, 6th and 7th d.p.i. After inoculation with the PB1-reassortant virus, significant body weight loss was observed with decreasing trend on 5th, 6th and a slightly increasing trend on 7th and 8th d.p.i. The increasing trend of WSN-H1N1 strain was observed after 10 d.p.i. No weight loss was observed for the NP-reassortant and PA-reassortant viruses, however, the PA-reassortant virus was detected at a low level in the lung and turbinate at 4 d.p.i (Figure 5.2B). In conclusion, the PB1-reassortant virus was able to replicate in mice and resulted in significant body weight loss.

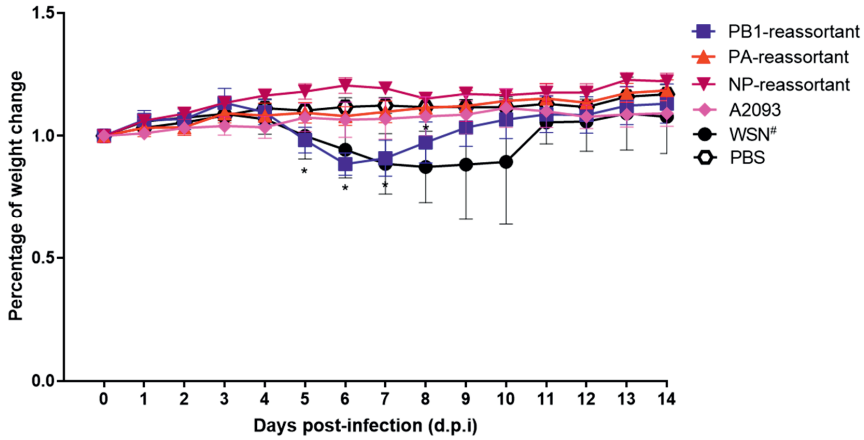


Figure 5.3 Virulence of reassortant viruses in mice. Virulence of the PB1-, PA- and NP-reassortant viruses was measured by infection of five mice, after which body-weight loss was measured for 14 days after infection. For reference, the human wt WSN-H1N1 and avian wt A2093-H9N2 viruses are shown. The percentage of body weight loss is plotted, and significant differences (Multiple t-test with a = 0.05) are marked (*). The percentage weight changes in body weight (n=5) are expressed as the mean with SD in each group. # One mouse died on day 8, 9 and 11 d.p.i.

Serial passaging of reassortant viruses

To study the replication and evolution of the viruses during prolonged passaging, the reassortant viruses and wt A2093-H9N2 virus were passaged in both MDCK cells and in mice. As a result of low replication for the PB2-reassortant virus in both cell lines and mice, we were unable to obtain the progeny viruses of this reassortant strain. However, five generations of progeny viruses of the PB1, PA and NP-reassortant viruses and wt A2093-H9N2, WSN-H1N1 virus were collected from serial passaging on MDCK cells.

These reassortant viruses and the A2093-H9N2 virus were also successfully passaged for five generations in mice. Viruses harvested from individual mouse lung or turbinate were pooled and inoculated into the next group of mice. We were able to continue passaging for five generations in mice lung; however, for turbinate only one generation was successful.

The viral titers of the parent virus before inoculation and the 5th generation of progeny viruses are listed in Table 5.1. For the following analysis, the PB2-reassortant strain was not included due to unsuccessful serial passaging. There was no significant difference in the titers obtained for the other viruses, suggesting a similar population size for subsequent analysis of viral sequences.

Table 5.1 The viral titers of viruses before and after 5-generation's serial passaging.

Rescued viruses	Parent Virus HA titer (log2)	Parent Virus (generation 0) (log10 copies/ml)#	5th Generation from MDCK cell line (log10 copies/ml)	5th Generation from Mouse lung (log10 copies/ml)
A2093HANAPB2/WSN	10	8.05±0.02	2.16±0.17	2.56±0.82
A2093HANAPB1/WSN	6	7.09±0.04	7.59±0.01	6.59±0.48
A2093HANAPA/WSN	10	8.33±0.06	5.48±0.00	7.31±0.28
A2093HANANP/WSN	9	8.06±0.20	2.33±0.05	6.73±0.27
A2093	11	7.76±0.02	6.60±0.00	6.16±0.03
WSN	7	7.87±0.04	6.74±0.00	7.86±0.30

Q-PCR were repeated twice, mean and sd were calculated to estimate the viral particles. It was suggested that the copy number of viral particles were 20–60 times of one infectious virus. (Yoshikawa T, Matsuo K, Matsuo K, Suzuki Y, Nomoto A, Tamura SI, Kurata T, Sata T. Total viral genome copies and virus-Ig complexes after infection with influenza virus in the nasal secretions of immunized mice. J Gen Virol. 2004 Aug;85(Pt 8):2339-2346. doi: 10.1099/vir.0.79892-0. PMID: 15269375.)

General mutation frequency and hot-spots on HA gene

We analyzed the mutation rate of the reassortant viruses during serial passaging in both MDCK cells and mouse lung. The virus population was analyzed by Illumina sequencing, the reads were mapped onto the HA ORF region of the reference sequence. Comparing the mutation rates ($F_{g/nt,}$ mutation/site/infection cycle) calculated from the HA1 and HA2 region (Table 5.2), after five passages in mouse lungs, showed that the net mutation rates (after selection) of the PB1- and PA-reassortant viruses were almost 2-3 times higher than that of A2093-H9N2 and WSN-H1N1 strains. The genetic distance in the HA sequences of the viruses after five passages in MDCK cells and mouse lung was calculated (shown in Figure 5.4A). This analysis showed that for the PB1- and PA-reassortant viruses, the genetic distance in the HA sequences significantly increased compared to that of the wt A2093-H9N2 virus after passaging in mouse lung. No significant difference was observed for the NP-reassortant after passaging in mouse lung. For none of the viruses, a significant difference in the genetic distance was observed after passaging in MDCK cells. The genetic distances of the HA sequences observed during passaging of the PB1- and PA-reassortant viruses in mouse lung were also larger than measured during passaging of the WSN-H1N1 virus. Differences were observed in the substitution frequency measured for different domains in the HA gene (Table 5.2). The region from 350 to 950 on the HA nucleotide sequence (marked as $HA1^b$ in Table 5.2) includes the 130 helix, RBS and 220 loop domains of the HA head. The mutation frequency in this functional HA1 domain was compared to that of the complete HA gene, and that of the HA2 domain (region 1015-1615 for A2093, region 1030-1630 for WSN). The general mutation rates ($F_{g/nt,}$ mutation/site/infection cycle) over the HA1 domain was 2.1 times (Std. Error = 9.425E-02) higher than that over the whole HA domain (in linear regression with $F_{g/nt}$ ($HA1^b$) = 2.13 $F_{g/nt}$ (whole HA), R^2 = 0.967, p-value = 3.938e-14). The plot of the dataset containing $F_{g/nt}$ of

different HA domains was visualized in R, and exported on Supplementary Figure S5.2. The general mutation rates over the HA2 domain displayed no linear relationship with that over the whole HA regions (Table 5.2), and the general mutation rates of the HA2 domain were lower than the HA1 domain for all the progeny viruses. These results suggest that the HA1 region, for with higher substitution frequency was measured, reflects a mutation "hot-spot" on the HA gene. This is likely due to increased selective pressure on this functional HA domain.

Mutation preference of reassortant viruses

We analyzed the substitutions observed in the HA ORF in further detail with a threshold of $f_i \geq 0.01$ for significant SBS after selection. The total number of SBS, which reflects the diversity of the gene was calculated, as well as the number of transitions and transversion and the number of synonymous and non-synonymous mutations (Table 5.3). Few significant SBS were observed in the HA gene after serial passaging in MDCK cells. The total number of SBS detected in the HA ORF was highest for the PB1-reassortant virus after passaging in mice lungs. Therefore, the gene diversity of the HA gene of PB1-reassortant virus was higher than estimates for the other reassortant viruses after five passages in mouse lungs.

Substitution frequency (f_i) for A (Adenine) towards G (Guanine) was higher than that for other types of substitutions on viruses passaged in both MDCK cells and mouse lungs. Only for the PB1-reassortant virus, the estimated possibility of transversions (P_{Tv}) was higher than that of transitions (Table 5.3). There was a positive correlation between a higher transition/transversion ratio and a higher non-synonymous mutations ratio for all reassortant viruses. Most of the significant SBS we observed in mouse lung passages were non-synonymous mutations (Figure 5.4B). The probability of SBS resulting in a non-synonymous mutation in HA was significantly higher for the PA- and PB1-reassortant viruses after five passages in mouse lungs.

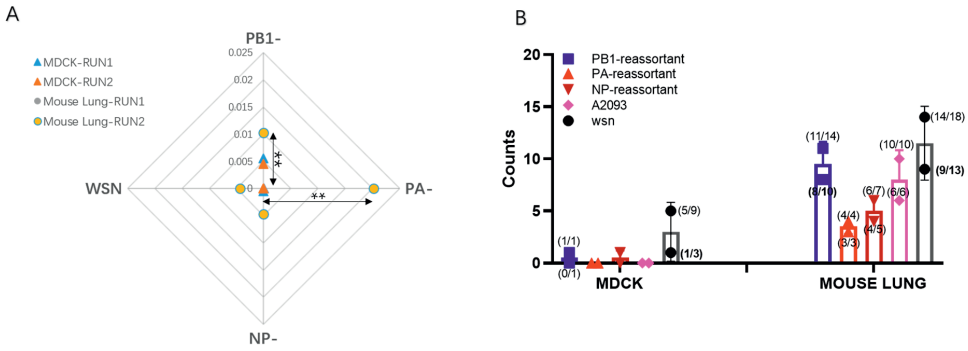


Figure 5.4 Genetic variation in the HA sequence after passaging of reassortant viruses. A. Genetic distance of HA sequences. The genetic distance of the HA sequences of the PB1-, PA- and NP-reassortant viruses after five passages in MDCK cells (triangles) and mouse lung (circles) is plotted relative to the wt A2093-H9N2 virus. The genetic distance of the WSN-H1N1 after five passages is plotted relative to the wt WSN-H1N1 virus. Two independent experiments were performed in cells and mice that were analyzed by NGS and are plotted separately, as RUN1 and RUN2. Further details on the NGS results are shown in supplementary Table S5.3. ** significant difference in the genetic distance compared to the WSN-H1N1 strain.
B, Non-synonymous numbers over open read frame (ORF) of HA nucleotide sequence. The number of substitutions (SBS) is plotted for the PB1, PA and NP reassortant viruses and the wt A2093-H9N2 and WSN-H1N1 viruses after passaging in MDCK cells and mice lungs. Numbers in the bracket indicated non-synonymous numbers with F_i >0.01, and the total SBS number separating by "/".

5.4 Discussion

Influenza viruses are characterized by a rapid mutation caused by error-prone viral RNA-polymerase enzyme on the negative-RNA genome during the replication. In addition, the segmented genome of influenza viruses allows these viruses to obtain novel genetic information by reassortment with other influenza strains. Error-prone RNA polymerase activity may be beneficial for the virus by providing diverse gene mutations that may allow rapid adaptation to a new host. Reassortment may also enable the virus to acquire features from other influenza viruses within short infection circles, thereby possibly rapidly adapted to new host populations and became endemic or pandemic. Many influenza pandemics in history were caused by reassortant viruses originating from mammalian-adapted viruses that obtained genes from avian influenza viruses. For instance, the H1N1 virus causing the 1918 pandemic was a reassortant virus, containing HA from H1 subtype human-adapted strain and NA and other gene segments from avian influenza viruses (Worobey et al. 2014). Also, the viruses causing the 1957 pandemic (Kilbourne 2006) and the 2009 pandemic (Garten et al. 2009, Smith et al. 2009) were reassortant viruses.

Considering the pandemic threat of avian influenza viruses, it is important to provide more insight in the characteristics of human influenza viruses after reassortment with avian influenza viruses. To approach this, we simulated the reassortment of human H1N1 virus (inner genes) and H9N2 AIVs (both HA and NA genes, and one of the polymerase genes). The surface genes were derived from the H9N2 AIV, and the reassortant viruses thus were not adapted to the mammalian receptor. The reassortant viruses in this research were used as a model to study the early stage of evolution of avian-human reassortant influenza viruses. We showed that the RNP complex with the PB1 gene from A2093-H9N2 in the background of the WSN-H1N1 virus significantly promoted the activity of RNPs complex in a Dual-Luciferase Assay System. In MDCK cells, the PB1-reassortant virus was found to replicate with similar efficiency as wt WSN-H1N1 virus. In DF1 cells, the replication of the PB1-reassortant was reduced compared to wt WSN-H1N1 virus. The other reassortant viruses replicated less efficiently in both MDCK and DF1 cells compared to wt WSN-H1N1 virus. The reassortant viruses were able to replicate efficiently in the mouse turbinate and lung, to similar levels as wt WSN-H1N1 virus. Except for the PB2-reassortant virus that showed significantly reduced replication in the mouse lung. The bodyweight of the mice was measured as an indication of virulence (replication ability) of the viruses. Most interestingly, we observed increased virulence (replication ability) in mice for the PB1-reassortant virus. Due to the low pathogenicity of the virus, no mortality other than a decrease in body weight was observed in the infected mice. A previous study detected a high polymerase activity of the combination of mammalian PB2 gene and avian PB1 gene in human cells (Li et al. 2009). The involvement of the avian PB1 gene in mammalian-adapted virus might obtain a higher virulence in the new host by generating adaptive mutations under new selection pressure. To obtain more information on the replication and evolution of the WSN-H1N1 virus containing inner gene segments of the H9N2 AIV, we performed serial passaging of the reassortant viruses in MDCK cells and in mice.

In this study, the “mutation rate” was calculated during serial passaging of the reassortant viruses. The mutation rate is, therefore, a combination of initial errors made during RNA replication, combined with the effects of host selection (Sanjuán et al. 2016). Mutations in HA may lead to changes of antibody or receptor binding and may be preferentially selected (Suzuki

et al. 1999, Hay et al. 2001). For all reassortant viruses in this study, we observed a higher mutation rate (mutation/site/infection cycle) in mouse lungs compared to MDCK cells. This difference was likely caused by the increased selection pressure mediated by the mouse immune system. The mutation rate measured on the HA gene was more than two-fold increased for the PA-reassortant virus compared to wt WSN-H1N1 and A2093-H9N2 viruses, and more than 1.5 times for the PB1-reassortant virus. Previous studies suggested the mutation rate of influenza A viruses ranged from 7.1×10^{-6} to 4.5×10^{-5} substitutions per nucleotide per cell infection cycle (s/n/c) of the whole genome (Sanjuán et al. 2010, Pauly et al. 2017). In this study, we measured mutation rates of 5.0×10^{-4} and 6.2×10^{-4} mutation/site/infection cycle on the HA gene for the PB1 and PA-reassortant virus, which is higher than previously reported. We showed that polymerase activity was increased for the PB1-reassortant virus, which may have resulted in an increased error rate during RNA replication. However, decreased polymerase activity was measured for the PA-reassortant. The higher mutation rate observed, therefore, may also result from the strong selection pressure on the reassortant viruses due to their novel genetic composition. We analyzed the HA sequence in this study, and higher selection pressure may be expected for virus surface protein (Webster et al. 1992). Furthermore, the mutation rate may not only depend on the gene segment analyzed but also on the virus subtype as was reported previously (Webster et al. 1992, Nobusawa et al. 2006). Finally, differences in the analysis methods may have contributed to variation in the error rates reported (Parvin et al. 1986, Pauly et al. 2017). The number/ratio of non-synonymous mutations is an indicator of the selection pressure on the virus (Hu et al. 2008). With similar high SBS numbers, the PB1-reassortant virus showed the lowest percentages of non-synonymous changes, whereas, in the PA-reassortant virus, only non-synonymous changes were found. This high percentage of non-synonymous mutations in the PA-reassortant virus was also reflected in a high relative genetic distance, suggesting there is a strong positive selection on the PA-reassortant virus. The serial passaging experiments, in which five host-infection circles were observed, showed that the substitution rates of both the PA and PB1-reassortant viruses were increased compared to the other reassortant viruses. This suggests that reassortant viruses obtaining the PB1 gene from avian H9N2 are more likely to rapidly adapt to new hosts. This is in accordance with a previous study which showed that virus replication was more efficient when PB1 was derived from an avian virus, regardless of the origin of the other proteins (Naffakh et al. 2000). Furthermore, we identified a mutation hot-spot in the HA gene that is located near the antigenic and receptor binding sites (Ha et al. 2001, Wan et al. 2014, Peacock et al. 2016). We measured a significantly increased substitution frequency for the 350-950 domain of HA1 compared to the complete ORF region. Our results are consistent with previous studies, which showed that the head domain of HA evolves faster than the stalk domain (Kirkpatrick et al. 2018). This domain included the 130 helix and 220 loop structure of the HA head which are exposed to the surface and therefore can be easily captured by the host immune system (Raymond et al. 2018). Together with the receptor-binding function, the highly mutable HA1 domain might compromise viral replicative fitness, which means the globular head of HA are highly tolerant of mutations (Doud et al. 2016). We further indicated that the mutation patterns could be highly influenced

by the reassortant viral vRdRp complex, especially in reassortment between human and avian viruses. However, further research will be required to provide more insight in the intracellular mechanism at the molecular level.

Reassortment events between human and avian influenza viruses in combination with rapid evolution and adaptation due to error-prone replication may lead to a novel human pandemic. The H9N2 AIV is currently the most frequently detected subtype (particularly in live bird markets) and has become endemic in poultry across Asia since the 1990s (Alexander 2007). Several studies provided evidence of interspecies transmission of the H9N2 AIV from poultry to mammals, such as swine (Cong et al. 2008, Yu et al. 2008). Swine may represent a "mixing vessel" for influenza viruses as they are susceptible for infected with swine, human and avian influenza viruses (Ma et al. 2009). An experimental study showed the replication of the H9N2 AIV (A/guinea fowl/Hong Kong/WF10/99, A/guinea fowl/Hong Kong/NT184/03) in mice without adaptation (Choi et al. 2004), likely because of its properties of internal genes related to polymerase function. As human infections with avian H9N2 viruses have been reported (Guo et al. 1999, Butt et al. 2005), there is a high probability of reassortment with human influenza viruses. In this study, we showed that reassortment between a human H1N1 virus and the H9N2 AIV might potentially result in a novel virus that can readily adapt to humans: the reassortant virus with the avian PB1 gene showed increased polymerase activity, better replication in mouse lung and high mutation rate at HA gene, in particular in the HA1 domain related to receptor binding and immunogenicity. Therefore, human infections with H9N2 AIVs and the possible emergence of reassortant influenza viruses carrying avian H9N2 polymerase genes must be carefully monitored for pandemic preparedness.

Acknowledgements

We are grateful for the fund and facility supports from Zejun Li's lab group in Shanghai Veterinary Research Institute (SHVIR), China. Hongrui Cui is enrolled through the graduate schools Wageningen Institute of Animal Sciences (WIAS) and Production Ecology and Resource Conservation (PE&RC) in the 2016 Sino-Dutch joint PhD program between the Chinese Academy of Agricultural Sciences (CAAS) and Wageningen University & Research.

Funding

This work was supported by the National Key Research and Development Program of China (2016YFD0500204) and the Chinese Academy of Agricultural Sciences of Technology Innovation Project. Nancy Beerens was funded by the Dutch Ministry of Agriculture, Nature and Food Quality (project WOT-01-003-087 and KB-37-003-015).

Conflict of interest statement

There is no conflict of interest for all the authors in this research.

Supplementary materials

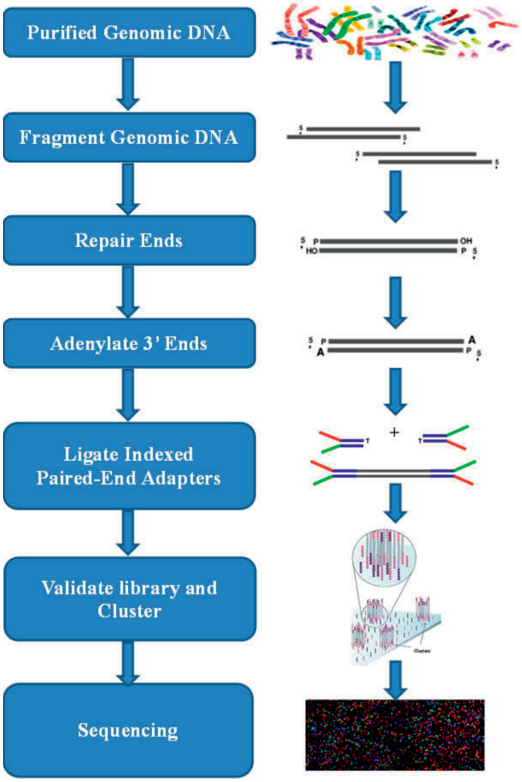


Figure S5.1 The experimental workflow of amplicon sequencing.

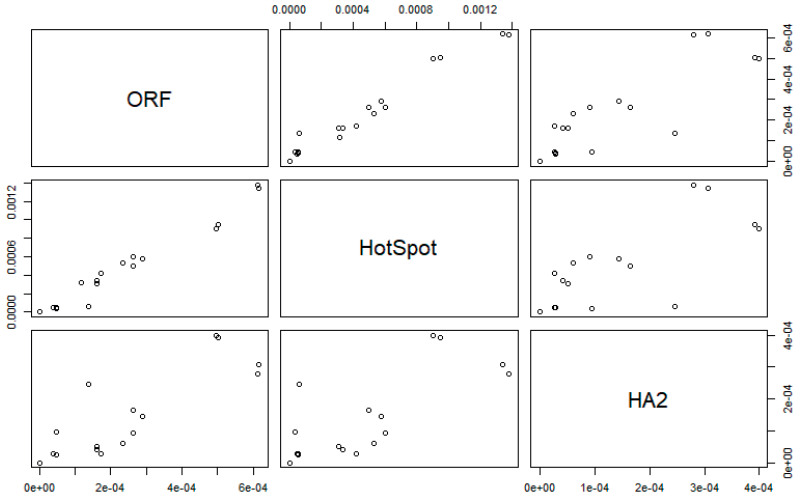


Figure S5.2 The plot of the dataset containing $F_{g/nt}$ of different HA domains.

Table S5.1 Specific indexes (Barcodes) for reassortant viruses from different hosts.

Host	Recombinant viruses	Barcode	Generation selected
Parent	A2093HANAPB2/WSN	ACAGCA	0
	A2093HANAPB1/WSN	ACCTCC	0
	A2093HANAPA/WSN	ACGCTC	0
	A2093HANANP/WSN	ACGTAT	0
	A2093	ACTATG	0
	WSN	AGAGTC	0
MDCK	A2093HANAPB2/WSN	AACGTG	5
	A2093HANAPB1/WSN	GAACAG	5
	A2093HANAPA/WSN	ATGCCT	5
	A2093HANANP/WSN	AGTGGT	5
	A2093	ACCACT	5
	WSN	ACATTG	5
Mouse lung	A2093HANAPB2/WSN	AGATCG	5
	A2093HANAPB1/WSN	AGTCAC	5
	A2093HANAPA/WSN	ATTGAG	5
	A2093HANANP/WSN	GACTAG	5
	A2093	CACTTC	5
	WSN	CATACC	5

Table S5.2 Primer sequence for segment amplification of HA gene.

Gene	PCR Primer Pairs	Sequence Information (5'-3')	Gene Regions
A2093-HA	A2093-HA-14F[a]	GAATTTCACAACCACTCAAGAT	14-483
	A2093-HA-483R[b]	TGCTTTGCTTGTCCCACTGTAA	
	A2093-HA-452F	GGAATGTGACTTACAGTGGGAC	452-922
	A2093-HA-922R	AGCCACCTTTTTCGGTCTGACA	
	A2093-HA-890F	GCACAGTGCAATGTCAGACCGA	890-1388
	A2093-HA-1388R	TTTGCATCATGCTCATCTAGTG	
	A2093-HA-1365F	AACACTAGATGAGCATGATG	1365-1735
	A2093-HA-1735R	ACAAGGGTGTTTTTGCCAAT	

[a], forward sequence; [b], reverse sequence

Table S5.3 The genetic distances of HA from reassortant viruses and WSN strain.

	PB1-	PA-	NP-	WSN
MDCK-RUN1	0.005576924	4.76E-05	0.000502871	0
MDCK-RUN2	0.00450567	0	0	0
Mouse Lung-RUN1	0.0101668	0.02007605	0.0048566	0.004104706
Mouse Lung-RUN2	0.01026862	0.02019742	0.00468139	0.004346665

Table 5.2 The geometric mean of substitution frequency and general mutation rate of viruses from 5th generation serial passaging.

Virus strain	Origination of the 5th generation	HA[a] (RUN1, RUN2)		HA1[b] (RUN1, RUN2)		HA2[c] (RUN1, RUN2)	
		F_g	$F_{g/nt}$ #	F_g	$F_{g/nt}$	F_g	$F_{g/nt}$
A2093	MDCK	1.13E-02, 0	3.69E-05, 0	9.38E-03, 0	4.94E-05, 0	1.75E-02, 0	2.91E-05, 0
WSN		1.84E-02, 1.32E-02	4.77E-05, 1.37E-04	2.10E-02, 1.63E-02	3.49E-05, 5.82E-05	1.67E-02, 1.53E-02	9.56E-05, 2.47E-04
A2093HANAPB1/WSN		2.35E-02, 3.55E-02	1.71E-04, 1.16E-04	3.27E-02, 3.55E-02	4.16E-04, 3.14E-04	1.58E-02, 0	2.63E-05, 0
A2093HANAPA/WSN		1.20E-02, 0	3.85E-05, 0	1.03E-02, 0	5.31E-05, 0	1.75E-02, 0	2.91E-05, 0
A2093HANANP/WSN		1.25E-02, na.	4.82E-05, na.	9.99E-03, na.	5.21E-05, na.	1.57E-02, 0	2.61E-05, 0
A2093	MOUSE LUNG	1.43E-02, 2.24E-02	2.62E-04, 2.34E-04	1.89E-02, 3.72E-02	6.00E-04, 5.32E-04	8.93E-03, 1.22E-02	9.18E-05, 6.19E-05
WSN		1.38E-02, 1.63E-02	2.90E-04, 2.62E-04	1.64E-02, 2.34E-02	5.75E-04, 4.94E-04	1.17E-02, 1.31E-02	1.45E-04, 1.65E-04
A2093HANAPB1/WSN		1.82E-02, 2.49E-02	5.03E-04, 4.98E-04	1.77E-02, 3.44E-02	9.49E-04, 9.04E-04	2.06E-02, 2.12E-02	3.91E-04, 3.99E-04
A2093HANAPA/WSN		3.96E-02, 2.91E-02	6.17E-04, 6.13E-04	4.91E-02, 2.43E-02	1.34E-03, 1.38E-03	4.66E-02, 3.93E-02	3.06E-04, 2.80E-04
A2093HANANP/WSN		1.30E-02, 1.34E-02	1.60E-04, 1.61E-04	1.35E-02, 1.35E-02	3.36E-04, 3.07E-04	8.27E-03, 9.55E-03	4.15E-05, 5.10E-05

The cutoff value for the mutation rate calculation was fi >0.006, which was generated basing on the NGS data from the parent virus as background control. The value 0 indicated no locations has the fi > 0.006; the na. indicated no NGS data available. # Fg/nt, mutation/site/infection cycle.

[a], the H9 HA1&2 of A2093 is 55-1680 of ORF region, the H1 HA1&2 of WSN is 52-1695;

[b], the hot-spot region is 350-950 for both;

[c], HA 2 for WSN strain is 1030-1630, for A 2093 strain is 1015-1615.

Table 5.3 Transition/transversion and non-synonymous mutations of viruses passaged for five generations on MDCK or mouse lung.

Virus strain	Origination of the 5th generation	SBS Count		Ts		Tv		PT_S		PTv		Ratio of P_{Ts} and P_{Tv}		Non-synonymous count		Non-synonymous percentage[#]	
		run1	run2	run1	run2	run1	run2	run1	run2	run1	run2	run1	run2	run1	run2	run1	run2
A2093	MDCK	0	0	0	0	0	0	0	0	0	0	na.	na.	0	0	na.	na.
WSN	MDCK	3	9	3	9	0	0	0.069	0.199	0	0	na.	na.	1	5	33.33%	55.56%
A2093HANAPB1/WSN	MDCK	1	1	0	1	1	0	0	0.181	0.225	0	0	na.	1	0	100.00%	0.00%
A2093HANAPA/WSN	MDCK	0	0	0	0	0	0	0	0	0	0	na.	na.	0	0	na.	na,
A2093HANANP/WSN	MDCK	2	na.	1	na.	1	na.	0.017	na.	0.492	na.	0.035	na.	1	na.	50.00%	na.
A2093	MOUSE LUNG	10	6	5	4	5	2	0.245	0.228	0.109	0.074	2.249	3.092	10	6	100.00%	100.00%
WSN	MOUSE LUNG	18	13	11	13	7	0	0.330	0.362	0.069	0	4.769	na.	14	9	77.78%	69.23%
A2093HANAPB1/WSN	MOUSE LUNG	14	10	7	7	7	3	0.255	0.249	0.811	0.771	0.315	0.323	11	8	78.57%	80.00%
A2093HANAPA/WSN	MOUSE LUNG	4	3	3	3	1	0	0.945	0.949	0.014	0	67.138	na.	4	3	100.00%	100.00%
A2093HANANP/WSN	MOUSE LUNG	7	5	2	2	5	3	0.099	0.093	0.092	0.068	1.070	1.375	6	4	85.71%	80.00%

NOTE: The cutoff value of fi >0.01 guaranteed that the observed transitions or transversions were not from system error of sequencing and PCR progress. The value 0 indicated no locations has the fi > 0.006; na. indicated no NGS data or calculation unavailable. #, questionable results of progeny viruses in MDCK passaging line because the observed numbers of SBS were too less for statistical calculation

General discussions

Introduction

The low pathogenic avian influenza virus (LPAIV) H9N2 is one of the three main avian influenza viruses (AIVs) threatening the poultry industry and public health (together with the H5 and H7 subtype AIVs). In some Asian countries, the H9N2 LPAIV has become endemic in poultry (Choi et al. 2005, Karimi-Madab et al. 2010, Kim et al. 2013, Peacock et al. 2019), and vaccination with an inactivated virus vaccine was generally applied in poultry (Li et al. 2005, Choi et al. 2008). However, H9N2 LPAIV still persisted in countries with a national vaccination program for the poultry industry (Zhang et al. 2008). In this thesis, I showed that transmission of H9N2 LPAIV in the chicken populations vaccinated with the inactivated virus as the vaccine does still occur. Under this situation, I will discuss the outcome of my studies focusing on 1) the performance of the inactivated virus vaccine in the prevention of LPAIV H9N2 in poultry, 2) the evolutionary changes in the HA gene of the H9N2 strains in populations with and without antigenic pressure from vaccination, 3) the involvement of the genes for the viral polymerase and for the non-structural proteins from H9N2 avian strains are involved in reassortments with human strains (H1N1 in this research), and 4) the advantages and disadvantages of the methods used in this research.

6.1. The high isolation rate of LPAIV H9N2 after preventative vaccination with the inactivated virus vaccine

Inactivated virus vaccines against influenza for use in both animals and humans are commercially available all over the world (Swayne 2012, WHO 2019, Daly et al. 2021). The vaccine strains for human seasonal flu are updated regularly based on surveillance programs. Hence, the implementation of seasonal flu vaccines has shown success in reducing the risk of severe clinical illness by 40% to 60% (CDC 2019). Vaccination with an inactivated virus vaccine was applied by several countries to control avian influenza to prevent disease and mortality in poultry during outbreaks of high pathogenicity avian influenza (HPAI) (Swayne et al. 2000, Sims 2007, Villarreal 2007). Some countries, which suffered from outbreaks of endemic LPAIV H9N2 in poultry, also applied vaccination in the poultry industry to reduce the economic losses associated with such outbreaks (Naeem et al. 2006, Banet-Noach et al. 2007, Lee et al. 2013, Bahari et al. 2015, Lau et al. 2016). The vaccination policy was set up nationally or locally, using an "autogenous vaccine" based on the virus strains circulating in the field at that moment in time (Capua et al. 2003). But after long-term usage, the vaccine against the LPAIV H9N2 virus was reported to drive the viral evolution rather than efficiently controlling viral transmission (Li et al. 2005, Lee et al. 2016). The neglected transmission and the vaccine triggered evolution potentially resulted in asymptomatic replication in vaccinated poultry and enzootic infection cases in poultry workers (Capua et al. 2008, Swayne et al. 2008, Takano et al. 2009, Kilany et al. 2010). The shortcomings of vaccination in LPAIV H9N2 were noticed due to an unexpected high isolation rate of these viruses in clinal samples in the countries with a vaccination policy for poultry in place (VASFI et al. 2002, Kwon et al. 2006).

In China, the H9N2 subtype AIV has become stably established in the wild bird population and is endemic in poultry enterprises. The H9N2 vaccine has been allowed to be used in poultry in China since 1998, but reports from surveillance in different regions in China still showed a high isolation rate of 92.5% H9N2 AIV in chicken from poultry farms experiencing severe drops in egg production (Bi et al. 2010). In these farms, the possibility of incorrect vaccination procedures or antigenic drift in the circulating virus leading to reduced protection had to be considered even though the vaccination data was not specified in that report.

We confirmed the high incidence of H9N2 in local markets in southern China in 2013-2018 in **Chapter 3**. Even though the sampling numbers varied over the years, the increasing trend was significant. However, from 2009 to 2012, a descending isolation rate of H9N2 had been displayed in previous research (Teng et al. 2016). Hence, the combined dataset showed a valley in isolation percentages of the H9N2 subtype among the AIV positive samples from 2012 to 2014 (Figure 6.1).

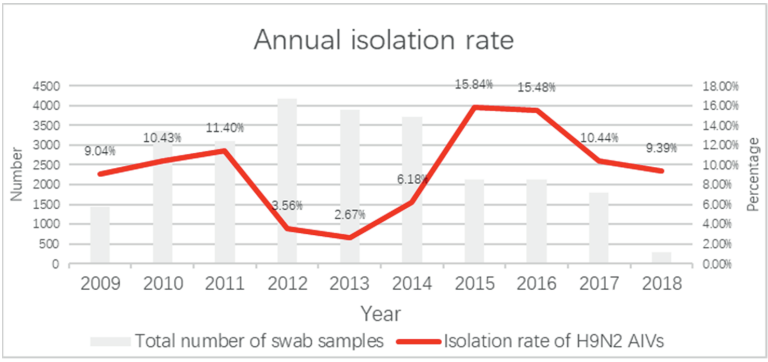


Figure 6.1 The annual isolation rate of LPAIV H9N2 in southern China. This graph combines data obtained from 2009-2012 (Teng et al. 2016) and 2013-2018 [this thesis]. The bars indicate the percentages H9N2, i.e. number of positive samples among the total number of swab samples collected from the markets.

Control measures were taken against an outbreak of a novel reassortant virus (H7N9) (Chen et al. 2013, Gao et al. 2013) in 2013 may partly explain the reduction in the isolation rate of the H9N2 AIV in poultry. This human-infecting virus originated from a reassortment of H7N3, H7N9, and LPAIV H9N2 that had spread widely in poultry in the Yangtze River delta (Jonges et al. 2013). Because the infection risks for humans via contact with birds in live poultry markets (LPMs) was very high (Liao et al. 2009, Cowling et al. 2013, Lam et al. 2013), the municipal governments in southern China temporarily closed (or gradually replaced) LPMs in 2013 (on April 6 for Shanghai) (He et al. 2014) and carried out initial disinfections (Yuan et al. 2015). When these measures were lifted, the annual isolation rate of H9N2 increased rapidly back to more than 10%, as described in **Chapter 3**. A similar tendency was shown incomparable research performed in Hong Kong (Kung et al. 2003, Lau et al. 2007, Leung et al. 2012). Although the formal LPMs were not re-opened officially, the live poultry trade in local markets in southern China re-emerged after the outbreak. The coverage of vaccination in this was not clear and also unknown for individual poultry due to the unclear background of these birds, which were either from farms or from peoples' backyards. Research indicated that the isolation rate of H9N2 in free-range poultry (chicken, duck, swan, goose, parrot, pigeon that were not vaccinated (Wang et al. 2013)) showed a total isolation rate of 3.2% (Zhu et al. 2018) when sampled from eight cities in four provinces between January 2014 and October 2015. Hence, the transmission of LPAIV in poultry in China was not reduced permanently, with the depopulation strategy of LPMs (Yuan et al. 2014).

6.2 The role of inactivated virus vaccine in prevention of avian influenza in poultry

The limited effect of vaccination in farms and the high frequency of free-range poultry accompanied by the high prevalence in wild birds may all contribute to the problems seen with a too low level of herd immunity against endemic H9N2. Others suggested before that antigenic drift may have led to a failure of the vaccine when there is transmission in the vaccinated

population. And in return, the weakness of the herd immunity may have allowed the continuous transmission of H9N2, which would then further evolve under antigenic pressure from the vaccination, e.g. (Lee et al. 2016, Su et al. 2020). This pressure acts as a selection factor that triggers further antigenic drift, allowing immune escape mutants to be generated instead of reducing the viral population size efficiently. Considering the limited success of vaccination, we questioned the effect of the inactivated virus vaccine on stopping the transmission of LPAIV H9N2. In our research, we were able to present estimated transmission parameters for vaccinated chicken, indicating that the vaccine could not prevent transmission of the homologous virus (**Chapter 2**). So, in addition to immune evasion through antigenic drift and possible incorrect procedures during vaccination, we have clear indications that the transmission of even the homologous H9N2 AIV will not be stopped by the vaccination. The transmission experiment in **Chapter 2** well displayed the potential risks for a population with a strong protection level (antisera HIT 2^{4-10} after one shot vaccination) or weak protection (antisera HIT no more than 2^3 after one shot vaccination). A good antibody level can reduce virus shedding by individuals to a great extent, but even then, transmission still happens (R>1).

Since the inactivated virus vaccine was not able to stop the transmission of the virus, it is not the optimal measure for virus control. Because in **Chapter 2,** we observed shedding virus and the transmission in vaccinated chicken who achieved antibody (HI titer ranged from 8 to 1024 HIU) in serum. The inactivated vaccine is injected, leading mainly to IgG production in the blood and extracellular fluid (Janeway Jr et al. 2001), while vaccines enhancing adaptive humoral immune defence at mucosal surfaces (estimated by secretory IgA) (Holmgren et al. 2005) may be more effective against LPAIV. Because the entrance and shedding of the virus were mostly via the aerodigestive and the urogenital tracts, which are both covered with mucous membranes (Holmgren et al. 2005). A response of humoral mucosal immune (Brandtzaeg 1995) was achieved by adjuvanted inactivated virus vaccine with cholera toxin or other mucosal adjuvants that induced interferon (IFN)-γ–producing CD4+ T cells (Mestecky et al. 2009). The delivery method of the vaccine may also be important. The live influenza vaccine delivered by an intranasal spray (FluMist) licensed for the actual influenza season in the United States (Belshe et al. 2004) induced an immune response that resembled more closely the natural immunity than the injected vaccine (Cox et al. 2004).

Besides humoral mucosal immunity, cell-mediated immunity was also reported to play an important part in the clearance of the virus (Borrow 1997, La Gruta et al. 2014). Manman Dai and co-authors (Dai et al. 2021) found that similar antibody levels were induced by the H9N2 AIV inactivated virus vaccine as by a natural H9N2 AIV infection. For vaccinated chicken, these researchers discovered an increase in CD4+ T cells instead of the $CD8^+$ T cells, and therefore an up-regulation of Th2 cytokines in peripheral blood lymphocytes (PBL). Considering Th1 cytokines promote the cellular immune reaction, whereas the Th2 cytokines enhance humoral immune response (Sobue et al. 2001), the imbalance in activities of Th1 and Th2 cells also suggested the insufficient cell-mediated response via an inactivated vaccine. Manman Dai and co-authors also indicated that (Dai et al. 2021), the absence of cytotoxicity-associated gene expression and the deficiency of CD8+ T cell response after vaccination was explained as the potential key reason for the limited protection by the inactivated virus vaccine.

In addition, the influence of maternally derived antibodies (MDA) is also a key point to consider in vaccination protocols for poultry. It was reported that the induction of antibody titers

by the vaccine was severely inhibited by maternal immunity in 14-day-old progeny chickens (Maas et al. 2011). However, the influence of MDA on mucosal immunity is not clear to date. Further research is needed to clarify the mechanism of interference MDA with mucosal immunity, systemic immunity, as well as cell-mediated immunity. Last but not least, novel adjuvants introducing mucosal immunity, but inhibiting interference by MDA, are promising in the development of vaccines for poultry.

6.3 Optimization of transmission fitness by LPAIV H9N2

For the occurrence of transmission, the virus needs to finish the process of entering a host, replicating its genome in the host, repackaging into a complete virus and then disseminating to other host cells and tissues, and finally passing through a portal of exit to be transmitted to a new host (Louten 2016). Because the virus depends on the host organism to finish the genomic replication and reassembly (Al-Kobaisi 2007, Louten 2016), sufficient transmission to the next organism is an important part of the viral replication cycle. Undoubtedly, allowing transmission lies with the virus' ability to overcome multiple barriers, from cell-to-cell and tissue-to-tissue within the host organism and organism-to-organism (within one individual host), and even across species (from individual host to another one). And this transmissibility of the virus as an infectious agent is the basic reproduction rate (R0) in epidemiology and can be estimated by mathematical models. Quantitatively, the R0 >1 indicates successful transmission in the host population. All these steps determine the concept and the model to describe viral transmission.

At the organism-to-organism level, the virus transmits as a population of genotypes (quasi-species), which contains enormous infectious virus particles. Successful transmission at this population-level initiates with shedding, maintaining infectious status, and reaching new susceptible host organisms. For a successful transmission, the influenza virus should firstly finish virus replication (by using host cytoplasmic products) and within-host transmission, which may lead to illness/death of the individual host organism. Once the virus can overcome cell-to-cell (or tissue-to-tissue) barriers and spreads in the host body, ultimately a spillover will lead to transmission between hosts (interspecies or cross-species) (Fermin 2018). The threat of avian influenza virus for humans is its transmission from animal to human or even from human to human, together with its pathogenicity causing disease or even mortality in both animal and human hosts.

6.3.1 Low transmission rate

The basic reproductive number (R_0) used in **Chapter 2** provides a quantitative estimate of the transmission efficiency of the virus (Heffernan et al. 2005). For influenza viruses, the R_0 is relatively low. Comparing to viruses with an $R_0 \geq 5$, such as Measles (R_0 valued 12-18), Mumps (R_0 valued 4-7), Rubella (R_0 valued 3-9) (Winter et al. 2018) and Poliovirus (R_0 valued 5-7) (Fine 1993), the R_0 values reported for influenza viruses in humans vary between 1.27 and 1.8 (Fraser et al. 2009, Yang et al. 2009). The pathogens with high R_0 values were found to be antigenically stable. In contrast, the antigenicity of influenza virus with low R_0 is highly diverse with constant antigenic drift (Rodpothong et al. 2012), which introduced problems in the prevention and control of influenza via vaccination (Boni 2008). In this thesis, H9N2 LPAIV is observed to have a low transmission rate but a high antigenic diversity.

As to the "low transmission rate", the R_0 of H9N2 in the human population cannot be determined, due to the finding that H9N2 does not transmit from human to human but from poultry to human. However, the high possibility of contact between humans and poultry, together with the high mutation rate of the virus, is a "breeding ground" for the evolution of H9N2 LPAIV strains with more frequent cross-species transmission. **Chapter 2** estimated the R_0 as 5.0-6.5 in unvaccinated chicken. In previous research on the transmission dynamics of avian influenza in poultry during an outbreak, the value of R_0 was estimated as 6.5 (95% confidence interval [CI], 3.1-9.9) for HPAI H7N7 (Stegeman et al. 2004), and 5.5 (95% posterior credible interval: 3.4–18.3) for LPAIV (H7N3 and H7N1) in turkey (Comin et al. 2011).

Control measures are able to reduce the R_0 in different ways. The control measures used in the outbreak of HPAI H7N7 in the Netherlands (in 2003) decreased the R_0 (between-flock) from 6.5 before notification to 1.2 (95% CI, 0.6–1.9) after notification in one outbreak region (Stegeman et al. 2004). Vaccination was also reported to reduce the R0 value by reducing the excretion of the virus (Karunakaran et al. 1987, Boyle et al. 2000, Swayne et al. 2001, Tollis et al. 2002, Di Trani et al. 2003). Another study, accessing a triple reassortant H1N1 strain (A/Sw/IA/00239/04), observed a statistically significant reduction of R_0 (95%CI) from 10.66 (6.57-16.46) in non-vaccinated pigs to 1 (0.39-2.09) in the vaccinated groups ($p < 0.05$) (Romagosa et al. 2011). However, the effectiveness of vaccination varied largely among species. There was also research that reported that vaccination of golden pheasants did not affect the excretion of H7N7 and had no influence on its transmission while providing clinical protection (van der Goot et al. 2007).

In the results in **Chapter 2** we presented the impact of the inactivated virus vaccine on vaccinated chicken. Upon infection, the viral dose for inoculation had a greater impact on the virus shedding rather than vaccination. Vaccination reduced the individual virus shedding of vaccinated chicken but was not sufficient enough to stop the transmission. Besides, in the combined dataset in **Chapter 2,** no significant difference in the virus shedding between vaccinated (combined chicken with high and low antibody levels) and non-vaccinated chicken. This is in contrast to previous research on the HPAI H5/H7subtype virus showing a significant reduction in transmission in chicken after vaccination (Van der Goot et al. 2005, Van der Goot et al. 2007, Bouma et al. 2009). The differences in pathogenicity and receptor tropism between HPAIV and LPAIV (Post et al. 2012, Böttcher-Friebertshäuser et al. 2014) needs to be concerned when to compare their transmission patterns in the vaccinated population. Because those biological differences of antigens influence the immune response of the individual host, which leads to different levels of virus shedding or recovery process.

Additionally, in the published vaccination and challenging experiments, the virus could still be detected (at low levels) in nasal or cloacal swabs during the experiments (Choi et al. 2008, Kim et al. 2017, Gan et al. 2019). For example, in the research suggesting that inactivated H9N2 vaccines containing at least a 250 HAU/dose will minimize virus shedding in SPF chickens (Kilany et al. 2016), 20%-50% of the vaccinated chicken were still shedding 2.3-2.48 EID_{50}/ml

of virus. However, the change of subsequent transmission of this shedded virus was not estimated in that study. In our experiments, the inactivated H9N2 vaccine did induce an immune response, as we measured an efficient increase of antibody levels in the serum. However, despite reduced virus shedding, the transmission still went on in the population, although at a lower rate.

6.3.2 A new antigenic group

With the concern that the inactivated virus vaccine cannot stop the transmission of LPAIV H9N2 in poultry, **Chapter 3** displayed the phylogenic and antigenic patterns of H9N2 viruses isolated from local markets in southern China during 2013-2018. These strains clustered in a new antigenic group apart from the isolates collected during 2009-2012 (Teng et al. 2016). In addition, many new antigenic characteristics different from the commercial vaccine strains [A/chicken/Shandong/6/96 (SD696), A/chicken/Guangdong/SS/94 (SS) and A/chicken /Shandong/F/98 (F98)] were identified in other studies (Li et al. 2005, Huang et al. 2010, Sun et al. 2010, Xia et al. 2017). To be noticed, the identification of a new antigenic strain was seen almost every four or five years during the surveillance. From the Bayesian Skyride plot (BSP) in **Chapter 3**, the current H9N2 isolates in the new antigenic group root back to Sep 2010 (95% HPD interval: Jan 2010 – May 2011). In the long-term period, a slight reduction in genetic diversity was observed between 2000 to 2005 and from 2013 to 2018. The decline in genetic diversity in the period of 2000 to 2005 might be induced by the implementation of the vaccine in 1998, but overall, the reduction in antigenic diversity of H9N2 LPAIV was less than expected.

In theory, continuous circulation, despite a vaccine-induced drop in the transmission rate, provides an opportunity for virus evolution. The genomic changes that happen during replication will experience selective pressure from host barriers and antiviral immunity. In the field, sub-optimal and inappropriate use of vaccines may also add antigenic selection pressure and drive virus evolution. An example is the failure to control LPAIV H5N2 in Mexico, where this strain was sustained for over ten years despite with prophylactic use of vaccines (Lee et al. 2004). The findings in this thesis also make it clear that intensive surveillance on the H9N2 strain for the genetic and antigenic changes remains necessary, even after the use of vaccination. Furthermore, the high genetic diversity of the H9N2 provides a pool of genetic material that may occasionally lead to new human infections (Butt et al. 2005, Khan et al. 2015, Pan et al. 2018).

6.4 Evolutionary changes of H9N2 virus under continuous transmission

The two most common evolutionary mechanisms used by influenza viruses are characterized by substitutions caused by the error-prone RdRp and gene segment reassortments (Webster 1999). Mutations, including substitutions, insertions, and deletions, contribute to the diverse variants in the quasi-species of influenza viruses. A faster substitution rate, therefore, might promote the diversity of the virus within a fixed period (Thomas et al. 2010). The substitutions provide “materials/possibility” for evolution, and the selection pressure from the host guides the destination. It has been proven that different gene segments showed different evolutionary patterns (e.g., substitution frequency) due to the selective pressures they are faced with. For example, the surface proteins encoded by the HA and NA genes of the influenza virus

were suggested to undergo strong selection pressure from neutralizing antibodies (Domingo et al. 2008). Because parts of the viral surface protein are the first determinants with antigenic information for the host immune system. The observed mutations are, therefore, a result of initial errors made during RNA replication, combined with the effects of selection (Sanjuán et al. 2016). Mutations in HA, for instance, may lead to reduced antibody recognition or may affect receptor binding and may therefore be preferentially selected for or against (Suzuki et al. 1999, Hay et al. 2001). Knowing the fact that LPAI H9N2 is continuously circulating in poultry even after vaccination, we focused on the mutational changes in the HA gene in both vaccinated and naïve chicken (**Chapter 4**).

6.4.1 The mutation rate after selection

In this project, I used two datasets to observe the mutation rate of H9 HA. The first was to use the HA sequences from H9N2 isolated from the field during surveillances or outbreaks, which allowed me to describe the mutation situation in the field. The second was to use the next generation sequencing (NGS) date from serial passaging experiments, comparing vaccinated and non-vaccinated individuals with and without specific immune pressure.

Based on the first dataset, the average net mutation rate (after selection) of the HA gene (H9 HA ORF region) from H9N2 AIV in poultry in China was estimated as 3.95×10^{-3} substitutions/site/year in **Chapter 3.** The net mutation rate of the H9N2 strains from the new antigenic group (antigenic cluster 2) to which the recently isolated H9N2 strains in China belong was almost 1.5-times higher (6.23×10^{-3} substitutions/site/year) than that of the average level of H9N2. These rates were similar to the estimations for H3 (HA1 domain only) in humans (5.7×10^{-3} substitutions/site/year) (Fitch et al. 1997). The HA1 domain is a high substitution region because it contains receptor binding sites and antigenic sites as the head part of viral surface protein. Besides, the net mutation rates of different domains on the HA gene are different. For a better comparison, the net mutation rate of the H9 (HA1 domain only) of antigenic cluster 2 showed a mean value of 7.43×10^{-3} substitutions/site/year. So, indeed higher than the H3 viruses circulating in humans. This directly indicated that H9N2 AIV did not evolve anomalously slow because of immunity in vaccinated poultry but actually faster and with higher rates than human seasonal flu strains. This might be explained by the short lifespan of poultry where natural immunity hardly plays a role, in combination with inefficient vaccination that does not prevent transmission to new susceptible hosts.

In this research, before the virus strain underwent any immune-related selective pressure, the estimated general substitution frequency of the parent A2093 virus was 3.18×10^{-5} s/n/g (substitutions per nt per host generation) for the HA gene, about ten-fold lower than for the progeny viruses passaged in vaccinated chicken. These mutations were caused by error-prone viral RNA-polymerase enzyme on the negative-RNA genome. The estimation was based on the substitution frequency found in NGS data, probably displaying also rarer, more deleterious mutations (Acevedo et al. 2014, Geller et al. 2016). This estimated value provided a reference for the progeny viruses generated under selective pressure. In comparison, in the chick respiratory system *in vivo,* the average substitution frequency was approximately 2.38×10^{-3} s/n/g, whereas *in vitro* it was 6.77×10^{-4} s/n/g. There were more mutations with high substitution frequency detected in vivo in **Chapter 4**. The same virus stock (quasi-species) presented different substitution frequencies after replicating in hosts or cell lines, indicating the different

selective pressure from hosts/cells. These different selections from hosts/cells might also influence other evolutionary changes for progeny viruses comparing to the parent virus stock (quasi-species), e.g., the mutation type in the following section.

6.4.2 Codon usage and non-synonymous mutations differ under different selection

The replication cycle of the influenza virus depends on cellular components for protein synthesis. Therefore codon usage in influenza viruses could be expected to affect viral replication (Wong et al. 2010). In this research, the codon usage bias in synonymous mutations was analyzed in progeny viruses (**Chapter 4 and 5**). Cytosine (C) was a preferred nucleotide for progeny viruses after passage in A549 cell lines and non-vaccinated SPF chicken.

The ratio of non-synonymous over synonymous [N/S] mutations is an indicator for the selection pressure acting on the virus (Hu et al. 2008). For A2093 replicating in different hosts/tissues, limited data were obtained that indicated a higher non-synonymous/synonymous ratio in mouse *in vivo*. Considering the HA genes were from the avian-originated H9N2 strain, the cellular environment of the mouse *in vivo* was the novel host environment possessing higher selective pressure for these viruses in serial passaging experiments. As to the reassortant viruses, with a similar large number of single base substitutions (SBS), the PB1-reassortant virus showed the lowest percentage of non-synonymous changes, whereas in the PA-reassortant virus only non-synonymous changes were found. This high percentage of non-synonymous mutations in the PA-reassortant virus was also reflected in a high relative genetic distance, suggesting there is a strong positive selection on the PA-reassortant virus. The serial passaging experiments, in which five host-infection circles were observed, showed that the substitution rates of both the PA and PB1-reassortant viruses were increased. This suggests that reassortant viruses obtaining the PB1 gene from avian H9N2 are more likely to rapidly adapt to new hosts. This following a previous study that showed that virus replication was more efficient when PB1 was derived from an avian virus, regardless of the origin of the other proteins (Naffakh et al. 2000).

Considering the antigenic selection from vaccination, the progeny viruses we harvested from vaccinated chicken were heavily selected by the immune system triggered by the homogenous vaccine. And this antigenic neutralization on the virus did not happen at the replication stage, selective pressure of vaccine-introduced immunity was an extra selection in addition to the natural selective pressure from the host. That explained why the vaccine-introduced immunity dramatically reduced the population size and diversity of the influenza virus but still triggered the evolution towards antigenic shift. The interaction of the high replication error and natural selective pressure generating genetic pools with priority went through the cell bottlenecks in the host, and then the vaccine-introduced immunity pressure performed the antigenic-originated selection. Whereas the emerge of antigenic drift of the influenza virus needed a big host population and long-term circulation.

Under selection from the homological antisera, antigenically related mutations were identified in **Chapter 4**. There were five non-synonymous mutations, S145N, N201K, R205I, T206N, and D207N, on the HA gene at 30^{th} to 34^{th} generation and one non-synonymous mutation (N191H) at 34^{th} generation. Hereinto, the site 145 (CHEN et al. 2012), 201, and 207 (Wan et al. 2014) were identified as HA protein epitope of the H9N2 subtype AIV by using a monoclonal antibody (Mab) in experimental research. And S145 was the same in the vaccine

strain used in that research (A/Chicken/Shandong/6/96) and A2093. The mutation S145N introduced glycosylation might play a role in antigenic variation (Ping et al. 2008). Moreover, in **Chapter 2**, the mutation at site 145 already evolved from N (Asparagine) towards D (Aspartic acid) in the H9N2 viruses isolated during 2013-2018, forming a new antigenic cluster apart from the vaccine strains in the 1990s. The other sites 191, 205, 206 were all near the antigenic position at HA1, and mutations at these sites were reported related to antigenic changes (Okamatsu et al. 2008, Bahari et al. 2015) as well. However, N191H, N201K, R205I, and D207N were in a small proportion among natural mutations in mainland China (Nextstrain 2021), suggesting a long evolution in nature in the endemic population of H9N2. Experimental research of identifying these antigenic sites predicted the potential mutations related to antigenic shift.

6.5 Reassortment of LPAIV provide a genetic advantage at the evolution

Many influenza pandemics in history were caused by reassortant viruses originating from mammalian-adapted viruses that obtained genes from avian influenza viruses. For instance, the H1N1 virus causing the 1918 pandemic was a reassortant containing HA from an H1 subtype human-adapted strain, while NA and other gene segments came from avian influenza viruses (Worobey et al. 2014). Also, the viruses causing the 1957 pandemic (Kilbourne 2006) and the 2009 pandemic (Garten et al. 2009, Smith et al. 2009) were reassortants.

The rapid antigenic drift and widespread distribution in poultry throughout China have led to reassortment and generation of diverse genotypes (Zhu et al. 2018) for influenza viruses. The high reassortment of the inner genes from H9N2 leads to a question of what (or if) the reassortment might increase the fitness for the new virus strain. On the one hand, the subsequent question was if the RNA-polymerase-related genes after reassortment may also influence the substitutions in the HA gene? Or further, if the reassortment enabled the virus to acquire features from other influenza viruses, thereby possibly gaining the ability to rapidly adapt to a new host population and become epidemic or pandemic.

In **Chapter 5**, the net mutation rate determined for the HA gene was more than two-fold increased for the PA-reassortant virus compared to the original WSN-H1N1 and the A2093-H9N2 viruses, and more than 1.5 times for the PB1-reassortant virus. Previous studies suggested the net mutation rate of influenza A viruses ranged from 7.1×10^{-6} to 4.5×10^{-5} substitutions per nucleotide per cell infection cycle (s/n/c) of the whole genome (Sanjuán et al. 2010, Pauly et al. 2017). In this study, we measured net mutation rates of 5.0×10^{-4} and 6.2×10^{-4} mutation/site/infection cycle on the HA gene for the PB1 and PA-reassortant viruses. The progeny viruses isolated from one (cell) infection cycle was described as one generation this study for comparison. The estimated net mutation rates of the HA gene for the PB1 and PA-reassortant viruses were higher than that of the whole genome in previous reports; however, the influences from the host and calculation methods deserved consideration.

Despite the high net mutation rates, the PB1 and PA gene from avian LPAIV H9N2 has good adaption in the human-H1N1 background. The reassortant viruses were able to replicate efficiently in the mouse turbinate and lung, to similar levels as wt WSN-H1N1 virus. Except for the PB2-reassortant virus that showed significantly reduced replication in the mouse lung. The bodyweight of the mice was measured as an indication of the virulence of the viruses. Most

interestingly, we observed increased virulence in mice for the PB1-reassortant virus based on a decrease in body weight were observed in the infected mice. Similar results were obtained in a previous study, indicating high polymerase activity detected from the combination of mammalian PB2 gene and avian PB1 gene in human cells (Li et al. 2009). Meanwhile, the internal genes of H9N2 viruses have exhibited high compatibility with surface genes from other avian influenza virus subtypes. For example, H5N1(Guan et al. 1999), H5N6 (Yang et al. 2017), H7N9 (Cui et al. 2014), and H10N8 viruses (Ye et al. 2016), which have caused the human infection, have all acquired internal genes from H9N2 viruses (Shi et al. 2014).

Reassortment events between human and avian influenza viruses in combination with rapid evolution and adaptation due to error-prone replication may lead to a novel human pandemic. Additionally, PB1 was also suggested to be an avian-human transmitted gene (Kawaoka et al. 1989), considering the evolutionary history of one single gene segment. The H9N2 AIV is currently the most frequently detected subtype (particularly in live bird markets) and has become endemic in poultry across Asia since the 1990s (Alexander 2007). Several studies provided evidence of interspecies transmission of the H9N2 AIV from poultry to mammals (Cong et al. 2008, Yu et al. 2008). Moreover, an experimental study showed the replication of the H9N2 AIV (A/guinea fowl/Hong Kong/WF10/99, A/guinea fowl/Hong Kong/NT184/03) in mice without adaptation (Choi et al. 2004), likely because of its properties of internal genes related to polymerase function. As human infections with H9N2 AIVs have been reported (Guo et al. 1999, Butt et al. 2005), there is a high probability of reassortment with human influenza viruses. The reassortment between a human H1N1 virus and the H9N2 AIV may potentially result in a novel virus that can readily adapt to humans: the reassortant virus with the avian PB1 gene showed increased polymerase activity, better replication in mouse lung and a high mutation rate at HA gene. Therefore, human infections with H9N2 AIVs and the possible emergence of reassortant influenza viruses carrying avian H9N2 polymerase genes must be carefully monitored for pandemic preparedness.

6.6 Lessons from the methodology

This experimental protocol was investigated based on the hypothesis that the viral evolutionary patterns might be influenced by cellular environments from hosts and the genetic characteristics of the virus itself. The former is the selection pressure forcing the direction of the viral evolution, and the changing viral genome keeps updating the “material” pool for higher survival possibilities. Experiments to explore the mechanism need to be considered at both the population level for evolution and the molecular level for the genetic changes. Therefore, based on the feasibility, an LPAIV H9N2 strain with a complete reverse genetic manipulation system was used for serial passaging in chick (White Leghorns, 1-7 days old), mouse (BALB/c, female, 3 weeks old), vaccinated and non-vaccinated chicken (White Leghorns, 4-5 weeks old) in vivo and MDCK, Vero, A549, CEF, DEF cells in vitro. But the virus, including the reassortant strains with the background of human H1N1, showed different replication abilities when passaging on these hosts. This was unpredictable and not pre-considered in the protocol, the same goes for the animal experiment itself. Besides, the sample preparation for Next Generation Sequencing (NGS) method was newly designed based on the platform (MiSeq) so that there was no specific reference for the analysis. We, therefore, only provided observations calculated based on the

concepts and methods used in evolutionary studies.

All these added difficulties at finishing the project efficiently, but I wish the drawbacks in this project would be side references for the following research. Therefore, the lessons were concluded in this discussion as well.

6.6.1 Simulated evolution experiment (serial passaging virus in different hosts)

There was a significant difference between the natural evolution and the simulated one in this research. Between the serial passaging, failure occurred in harvesting the progeny viruses from the cells or host tissues, then the inoculation of the per-generation virus was performed to continue the passaging. The virus population was forced to undergo continuous selective pressure in the experiment; however, in the natural process, selective pressure on one virus unit would stop if no successful new infection happened. And this failure of new infection could show host-specific differences. However, the passaging experiments did not show these host bottlenecks because of the infection dose and inoculation method. This also explained why fewer differences were observed in the evolutionary patterns of viruses from different host tissues. On the other hand, there was a bigger host population in the natural process, which allowed individual failure at infection because the transmission was going on at the level of the virus population. But the substitutions identified in NGS data were selected by the pressure, with which we were able to observe the evolutionary patterns of the 1st generation of virus in a host. The dN/dS ratios of progeny viruses at 1st generation indicating a higher host-specific selection in vivo than in vitro in general. But the higher selection in the human lung cancer cell line (A549) than the Vero cell line was highly influenced by the success of viral infection. This also alarmed that the long-term transmission in the field like this serial passaging providing the continuous selective pressure might help functional mutants survive and be detected, even though host-adapted mutations need more than ten generations of successful replications in the host in the lab. Due to the large population size of poultry in the field, host-adapted mutations were easier to select in high-density farms.

6.6.2 Applying of NGS in evolutionary analysis

Next-Generation Sequencing (NGS, or deep sequencing) is a massively parallel sequencing technology that allows the rapid parallel sequencing of complete virus genomes. One of the major platforms for NGS is provided by Illumina (MiSeq or HiSeq), which was used in this project following the MiSeq protocol, which enables up to 15 Gb of output with 25 million sequencing reads and 2 × 300 bp read lengths (https://www.illumina.com/systems/sequencing-platforms/miseq.html). NGS provides information on all genomes present in a virus population (quasi-species), whereas Sanger sequencing only sequences the major components of the sample, disregarding the minority data. For our purpose, it is important that NGS provide more reliable data than Sanger sequencing for low-frequency variants in a given region of interest (Jamuar et al. 2014). Considering a large number of samples from in vitro and in vivo experiments and the possibly low frequency of individual SNPs in the genome of viral quasi-species, NGS is the ideal technique to efficiently identify and calculate intrinsic and extrinsic mutation rates of AI viruses. However, the Illumina error is observable when examining viral populations at ultra-deep coverage. Besides, the sequencing of influenza viruses depended on the Reverse Transcriptase (RT) step, and the PCR process also added errors from enzyme

fidelity and numerous amplification cycles (Orton et al. 2015). To at most reduce the error from this system, we applied high fidelity enzymes at RT and PCR steps but did not measure the system error from the MiSeq platform. This was considered during the data analysis when a threshold was in need to select reliable substitutions. A high cutoff value of 0.01 was chosen for high confidence but resulted in fewer numbers of substitutions for statistical methods. The lesson was to set up an error test for the platform while sequencing specific samples. The cutoff value from the sample-specific test would help improve the precision of the genetic data.

6.7 Conclusions

This research showed that inactivated H9N2 vaccine was able to protect against disease but cannot stop the transmission of the H9N2 AIV in vaccinated chicken. The veil of vaccination provides a high possibility of long-term circulation in the host population under selection pressure. This pressure triggers evolutionary changes in circulating strains in the field, and similar effects were seen with virus strains generated in evolutionary experiments. With a high mutation rate, a virus population with diverse variants evolved that obtained characteristic mutations as a consequence of immune evasion and/or host-adaption. Besides, the avian PB1 gene was highly compatible within an artificial human (H1N1)-avian (H9N2) reassortant virus and promoted the adaptation of novel reassortant viruses to a mammal host. Taken all data together, the prevention of LPAIV H9N2 in poultry needs an updated strategy, combining timely surveillance, biosafety measures to reduce human infection with avian influenza virus, and the development of “new-types” of vaccines. Vaccine inducing broader immunity at mucosal tissues was suggested to improve vaccination effect on stopping the virus transmission in a high-density population.

Bibliography

Bibliography

Acevedo, A., Brodsky, L. and Andino, R. (2014). Mutational and fitness landscapes of an RNA virus revealed through population sequencing. Nature 505(7485): 686-690.

Agrawal, B. (2019). Heterologous Immunity: Role in Natural and Vaccine-Induced Resistance to Infections. Frontiers in Immunology 10: 2631-2631.

Air, G. M. and Laver, W. G. (1989). The neuraminidase of influenza virus. Proteins: Structure, Function, and Bioinformatics 6(4): 341-356.

Alexander, D. (2003). Report on avian influenza in the Eastern Hemisphere during 1997–2002. Avian Diseases 47(s3): 792-797.

Alexander, D. and Swayne, D. (1989). A laboratory manual for the isolation and identification of avian pathogens.

Alexander, D. J. (2000). A review of avian influenza in different bird species. Veterinary Microbiology 74(1-2): 3-13.

Alexander, D. J. (2007). An overview of the epidemiology of avian influenza. Vaccine 25(30): 5637-5644.

Alexander, D. J. (2007). Summary of avian influenza activity in Europe, Asia, Africa, and Australasia, 2002–2006. Avian Diseases 51(s1): 161-166.

Alexander, D. J. and Brown, I. H. (2009). History of highly pathogenic avian influenza. Revue scientifique et technique (International Office of Epizootics) 28(1): 19-38.

Alexander, D.J., Capua, I. and Koch, G. (2008). Highly pathogenic avian Influenza outbreaks in Europe, Asia, and Africa since 1959, excluding the Asian H5N1 virus outbreaks. Avian Influenza, 1, pp.217-237.

Ali, M., Yaqub, T., Mukhtar, N., Imran, M., Ghafoor, A., Shahid, M. F., Naeem, M., Iqbal, M., Smith, G. J. and Su, Y. C. (2019). Avian influenza A (H9N2) virus in poultry worker, Pakistan, 2015. Emerging Infectious Diseases 25(1): 136.

Andino, R. and Domingo, E. (2015). Viral quasispecies. Virology 479-480: 46-51.

Andino, R., Rieckhof, G. E., Achacoso, P. L. and Baltimore, D. (1993). Poliovirus RNA synthesis utilizes an RNP complex formed around the 5′-end of viral RNA. The EMBO Journal 12(9): 3587-3598.

Antigua, K. J. C., Choi, W. S., Baek, Y. H. and Song, M. S. (2019). The Emergence and Decennary Distribution of Clade 2.3.4.4 HPAI H5Nx. Microorganisms 7(6): 156.

Arafat, N., Eladl, A. H., Marghani, B. H., Saif, M. A. and El-Shafei, R. A. (2018). Enhanced infection of avian influenza virus H9N2 with infectious laryngeotracheitis vaccination in chickens. Veterinary Microbiology 219: 8-16.

Avian Influenza Community Reference Laboratory. Avian Influenza: The Intravenous Pathogenicity Test (IVPI) for Avian Influenza.

Ayora-Talavera, G., Shelton, H., Scull, M. A., Ren, J., Jones, I. M., Pickles, R. J. and Barclay, W. S. (2009). Mutations in H5N1 influenza virus hemagglutinin that confer binding to human tracheal airway epithelium. PloS One 4(11): e7836.

Bahari, P., Pourbakhsh, S. A., Shoushtari, H. and Bahmaninejad, M. A. (2015). Molecular characterization of H9N2 avian influenza viruses isolated from vaccinated broiler chickens in northeast Iran. Tropical Animal Health and Production 47(6): 1195-1201.

Baigent, S. J. and McCauley, J. W. (2003). Influenza type A in humans, mammals and birds: determinants of virus virulence, host-range and interspecies transmission. BioEssays 25(7): 657.

Banet-Noach, C., Perk, S., Simanov, L., Grebenyuk, N., Rozenblut, E., Pokamunski, S., Pirak, M., Tendler, Y. and Panshin, A. (2007). H9N2 influenza viruses from Israeli poultry: a five-year outbreak. Avian Diseases 51(s1): 290-296.

Bankowski, R. (1981). Proceedings of the First International Symposium on Avian Influenza, Beltsville, Maryland, USA, April 22-24, 1981. 1st International Symposium on Avian Influenza, Beltsville, Md.(USA), 1981, Carter Composition Corp.

Banks, J., Speidel, E., Harris, P. and Alexander, D. (2000). Phylogenetic analysis of influenza A viruses of H9 haemagglutinin subtype. Avian Pathology 29(4): 353-359.

Bean, W., Schell, M., Katz, J., Kawaoka, Y., Naeve, C., Gorman, O. and Webster, R. (1992). Evolution of the H3 influenza virus hemagglutinin from human and nonhuman hosts. Journal of Virology 66(2): 1129-1138.

Beato, M. S., Xu, Y., Long, L. P., Capua, I. and Wan, X. F. (2014). Antigenic and genetic evolution of low-pathogenicity avian influenza viruses of subtype H7N3 following heterologous vaccination. Clinical and Vaccine Immunology 21(5): 603-612.

Beerens, N., Heutink, R., Harders, F., Bossers, A., Koch, G. and Peeters, B. (2020). Emergence and selection of a highly pathogenic avian influenza H7N3 virus. Journal of Virology 94(8).

Belshe, R., Lee, M.-S., Walker, R. E., Stoddard, J. and Mendelman, P. M. (2004). Safety, immunogenicity and efficacy of intranasal, live attenuated influenza vaccine. Expert Review of Vaccines 3(6): 643-654.

Benjamini, Y., Krieger, A. M. and Yekutieli, D. (2006). Adaptive linear step-up procedures that control the false discovery rate. Biometrika 93(3): 491-507.

Bergervoet, S., Pritz-Verschuren, S., Gonzales, J., Bossers, A., Poen, M., Dutta, J., Khan, Z., Kriti, D., Bakel, H., Bouwstra, R., Fouchier, R. and Beerens, N. (2019). Circulation of low pathogenic avian influenza (LPAI) viruses in wild birds and poultry in the Netherlands, 2006-2016. Scientific Reports 9.

Best, S. M. and Kerr, P. J. (2000). Coevolution of host and virus: the pathogenesis of virulent and attenuated strains of myxoma virus in resistant and susceptible European rabbits. Virology 267(1): 36-48.

Bi, J., Deng, G., Dong, J., Kong, F., Li, X., Xu, Q., Zhang, M., Zhao, L. and Qiao, J. (2010). Phylogenetic and molecular characterization of H9N2 influenza isolates from chickens in Northern China from 2007–2009. PloS One 5(9): e13063.

Biswas, S. K., Boutz, P. L. and Nayak, D. P. (1998). Influenza virus nucleoprotein interacts with influenza virus polymerase proteins. Journal of Virology 72(7): 5493-5501.

Blackburne, B. P., Hay, A. J. and Goldstein, R. A. (2008). Changing selective pressure during antigenic changes in human influenza H3. PLoS Pathogens 4(5): e1000058.

Boni, M. F. (2008). Vaccination and antigenic drift in influenza. Vaccine 26: C8-C14.

Boni, M. F., de Jong, M. D., van Doorn, H. R. and Holmes, E. C. (2010). Guidelines for identifying homologous recombination events in influenza A virus. PloS One 5(5): e10434.

Borrow, P. (1997). Mechanisms of viral clearance and persistence. Journal of Viral Hepatitis 4:16-24.

Böttcher-Friebertshäuser, E., Garten, W., Matrosovich, M. and Klenk, H.D. (2014). The hemagglutinin: a determinant of pathogenicity. Influenza Pathogenesis and Control-Volume I, pp.3-34.

Bouma, A., Claassen, I., Natih, K., Klinkenberg, D., Donnelly, C. A., Koch, G. and Van Boven, M. (2009). Estimation of transmission parameters of H5N1 avian influenza virus in chickens. PLoS Pathogens 5(1).

Boyle, D., Selleck, P. and Heine, H. (2000). Vaccinating chickens against avian influenza with fowlpox recombinants expressing the H7 haemagglutinin. Australian Veterinary Journal 78(1): 44-48.

Brandtzaeg, P. (1995). The role of humoral mucosal immunity in the induction and maintenance of chronic airway infections. American Journal of Respiratory and Critical Care Medicine 151(6), pp.2081-2086.

Broom, D. M. (2017). Animal welfare in the European Union. Animal Welfare in the European Union.

Brown, I. H. (2010). Summary of avian influenza activity in Europe, Asia, and Africa, 2006-2009. Avian Diseases 54(1 Suppl): 187-193.

Butt, K., Smith, G. J., Chen, H., Zhang, L., Leung, Y. C., Xu, K., Lim, W., Webster, R. G., Yuen, K. and Peiris, J. M. (2005). Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. Journal of Clinical Microbiology 43(11): 5760-5767.

Capua, I. and Alexander, D. J. (2004). Human health implications of avian influenza viruses and paramyxoviruses. European Journal of Clinical Microbiology and Infectious Diseases 23(1): 1-6.

Capua, I. and Alexander, D. J. (2008). Avian influenza vaccines and vaccination in birds. Vaccine 26: D70-D73.

Capua, I., Cattoli, G. and Marangon, S. (2004). DIVA--a vaccination strategy enabling the detection of field exposure to avian influenza. Developments in Biologicals 119: 229-233.

Capua, I. and Marangon, S. (2003). The use of vaccination as an option for the control of avian influenza. Avian Pathology 32(4): 335-343.

Capua, I. and Marangon, S. (2006). Control of avian influenza in poultry. Emerging Infectious Diseases 12(9): 1319.

Capua, I., Marangon, S., Dalla Pozza, M., Terregino, C. and Cattoli, G. (2003). Avian influenza in Italy 1997–2001. Avian Diseases 47(s3): 839-843.

Cattaneo, R., Schmid, A., Eschle, D., Baczko, K., ter Meulen, V. and Billeter, M. A. (1988). Biased hypermutation and other genetic changes in defective measles viruses in human brain infections. Cell 55(2): 255-265.

CDC. (2019). The 2009 H1N1 Pandemic: A New Flu Virus Emerges. Pandemic Influenza, from https://www.cdc.gov/flu/pandemic-resources/2009-h1n1-pandemic.html.

CDC. (2019). Vaccine effectiveness: how well do the flu vaccines work. Questions & Answers. Assessed from https://www. cdc. gov/flu/vaccines-work/vaccineeffect. htm.

Chakraborty, A., Arifeen, S. and Streafield, P. (2011). Outbreak of mild respiratory disease caused by H5N1 and H9N2 infections among young children in Dhaka, Bangladesh, 2011. Health Sci Bull 9(2): 5-12.

Chang, H.P., Peng, L., Chen, L., Jiang, L.F., Zhang, Z.J., Xiong, C.L., Zhao, G.M., Chen, Y. and Jiang, Q.W. (2018). Avian influenza viruses (AIVs) H9N2 are in the course of reassorting into novel AIVs. Journal of Zhejiang University-SCIENCE B 19(5): 409-414.

Chare, E. R., Gould, E. A. and Holmes, E. C. (2003). Phylogenetic analysis reveals a low rate of homologous recombination in negative-sense RNA viruses. Journal of General Virology 84(10): 2691-2703.

Chen, H., Yuan, H., Gao, R., Zhang, J., Wang, D., Xiong, Y., Fan, G., Yang, F., Li, X. and Zhou, J. (2014). Clinical and epidemiological characteristics of a fatal case of avian influenza A H10N8 virus infection: a descriptive study. The Lancet 383(9918): 714-721.

Chen, L., Zheng, L., Zhao, J., Wang, C. and Wang, Z.. (2012). Characteristics of Pathogenic and Antigenic Variation of H9N2 Avian Influenza Viruses HA S145N Mutants in China. Chinese Journal of Animal and Veterinary Sciences 1.

Chen, R. and Holmes, E.C. (2006). Avian influenza virus exhibits rapid evolutionary dynamics. Molecular Biology and Evolution 23(12).

Chen, W., Calvo, P. A., Malide, D., Gibbs, J., Schubert, U., Bacik, I., Basta, S., O'Neill, R., Schickli, J. and Palese, P. (2001). A novel influenza A virus mitochondrial protein that induces cell death. Nature Medicine 7(12).

Chen, Y., Liang, W., Yang, S., Wu, N., Gao, H., Sheng, J., Yao, H., Wo, J., Fang, Q. and Cui, D. (2013). Human infections with the emerging avian influenza A H7N9 virus from wet market poultry: clinical analysis and characterisation of viral genome. The Lancet 381.

Choi, J. G., Lee, Y. J., Kim, Y. J., Lee, E. K., Jeong, O. M., Sung, H. W., Kim, J. H. and Kwon, J. H. (2008). An inactivated vaccine to control the current H9N2 low pathogenic avian influenza in Korea. Journal of Veterinary Science 9(1): 67-74.

Choi, Y. K., Ozaki, H., Webby, R. J., Webster, R. G., Peiris, J. S., Poon, L., Butt, C., Leung, Y. H. C. and Guan, Y. (2004). Continuing Evolution of H9N2 Influenza Viruses in Southeastern China. Journal of Virology 78(16).

Choi, Y. K., Seo, S. H., Kim, J. A., Webby, R. J. and Webster, R. G. (2005). Avian influenza viruses in Korean live poultry markets and their pathogenic potential. Virology 332(2).

Cohen, J. (2009). Pandemic influenza. Straight from the pig's mouth: swine research with swine influenzas. Science 325(5937).

Combe, M., Garijo, R., Geller, R., Cuevas, J. M. and Sanjuan, R. (2015). Single-Cell Analysis of RNA Virus Infection Identifies Multiple Genetically Diverse Viral Genomes within Single Infectious Units. Cell Host Microbe 18(4).

Combe, M. and Sanjuan, R. (2014). Variation in RNA virus mutation rates across host cells. PLoS Pathogens 10(1).

Comin, A., Klinkenberg, D., Marangon, S., Toffan, A. and Stegeman, A. (2011). Transmission dynamics of low pathogenicity avian influenza infections in Turkey flocks. PLoS One 6(10).

Cong, Y. L., Wang, C. F., Yan, C. M., Peng, J. S., Jiang, Z. L. and Liu, J. H. (2008). Swine infection with H9N2 influenza viruses in China in 2004. Virus Genes 36(3).

Cowling, B. J., Jin, L., Lau, E. H., Liao, Q., Wu, P., Jiang, H., Tsang, T. K., Zheng, J., Fang, V. J. and Chang, Z. (2013). Comparative epidemiology of human infections with avian influenza A H7N9 and H5N1 viruses in China: a population-based study of laboratory-confirmed cases. The Lancet 382(9887).

Cox, N. J. and Bender, C. A. (1995). The molecular epidemiology of influenza viruses. Seminars in Virology, Elsevier.

Cox, R., Brokstad, K. and Ogra, P. (2004). Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. Scandinavian Journal of Immunology 59(1): 1-15.

Cuevas, J. M., Geller, R., Garijo, R., Lopez-Aldeguer, J. and Sanjuan, R. (2015). Extremely High Mutation Rate of HIV-1 In Vivo. PLoS Biology 13(9).

Cui, L., Liu, D., Shi, W., Pan, J., Qi, X., Li, X., Guo, X., Zhou, M., Li, W. and Li, J. (2014). Dynamic reassortments and genetic heterogeneity of the human-infecting influenza A (H7N9) virus. Nature Communications 5(1): 1-9.

Dai, M., Li, S., Keyi, S., Sun, H., Zhao, L., Deshui, Y., Liao, J., Xu, C. and Liao, M. (2021). Comparative analysis of key immune protection factors in H9N2 avian influenza viruses infected and immunized specific pathogen–free chicken. Poultry Science 100(1): 39-46.

Dalgaard, P. (2008). Introductory statistics with R, Springer.

Daly, J. M. and Kembou-Ringert, J. E. (2021). Equine, Canine, and Swine Influenza (Orthomyxoviridae). Encyclopedia of Virology (Fourth Edition). D. H. Bamford and M. Zuckerman. Oxford, Academic Press: 287-293.

De Jong M.C. M. and Kimman T.G. (1994). Experimental quantification of vaccine-induced reduction in virus transmission. Vaccine, 12(8), 761.

De la Luna, S., Martín, J., Portela, A. and Ortín, J. (1993). Influenza virus naked RNA can be expressed upon transfection into cells co-expressing the three subunits of the polymerase and the nucleoprotein from simian virus 40 recombinant viruses. Journal of General Virology 74(3): 535-539.

Desvaux S., Grosbois V., Pham T. T. H., Dao D. T., Nguyen T. D., Fenwick S., Roger F., Ellis T., Peyre M. (2013). Evaluation of the vaccination efficacy against H5N1 in domestic poultry in the Red River Delta in Vietnam. Epidemiology and Infection 141(4): 776-788.

Dharmayanti, N.L.P.I., Indriani, R. and Nurjanah, D., 2020. Vaccine efficacy on the novel reassortant H9N2 virus in indonesia. Vaccines, 8(3), 449.

Di Trani, L., Cordioli, P., Falcone, E., Lombardi, G., Moreno, A., Sala, G. and Tollis, M. (2003). Standardization of an inactivated H7N1 avian influenza vaccine and efficacy against A/Chicken/Italy/13474/99 high-pathogenicity virus infection. Avian Diseases 47(s3): 1042-1046.

Diekmann, O., Heesterbeek, J. A. and Metz, J. A. (1990). On the definition and the computation of the basic reproduction ratio R0 in models for infectious diseases in heterogeneous populations. Journal of Mathematical Biology 28.4 (1990): 365.

Domingo, E., Parrish, C. R. and Holland, J. J. (2008). Origin and evolution of viruses, Elsevier.

Domingo, E. and Perales, C. (2007). Virus evolution. eLS.

Domingo, E., Sheldon, J. and Perales, C. (2012). Viral quasispecies evolution. Microbiology and Molecular Biology Reviews : MMBR 76(2): 159-216.

Dou, D., Revol, R., Östbye, H., Wang, H. and Daniels, R. (2018). Influenza A Virus Cell Entry, Replication, Virion Assembly and Movement. Frontiers in Immunology 9(1581).

Doud, M. B. and Bloom, J. D. (2016). Accurate measurement of the effects of all amino-acid mutations on influenza hemagglutinin. Viruses 8(6): 155.

Duan, L., Campitelli, L., Fan, X., Leung, Y., Vijaykrishna, D., Zhang, J., Donatelli, I., Delogu, M., Li, K. and Foni, E. (2007). Characterization of low-pathogenic H5 subtype influenza viruses from Eurasia: implications for the origin of highly pathogenic H5N1 viruses. Journal of Virology 81(14): 7529-7539.

Dugan, V. G., Chen, R., Spiro, D. J., Sengamalay, N., Zaborsky, J., Ghedin, E., Nolting, J., Swayne, D. E., Runstadler, J. A. and Happ, G. M. (2008). The evolutionary genetics and emergence of avian influenza viruses in wild birds. PLoS Pathogens 4(5).

Eisfeld, A. J., Neumann, G. and Kawaoka, Y. (2015). At the centre: influenza A virus ribonucleoproteins. Nature Reviews Microbiology 13(1): 28-41.

European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian influenza, Adlhoch Cornelia, Brouwer Adam, Kuiken Thijs, Mulatti Paolo, Smietanka Krzysztof, Staubach Christoph and Willeberg Preben (2018). Avian influenza overview November 2017-February 2018. EFSA Journal 16(3): e05240.

European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian Influenza, Adlhoch Cornelia, Fusaro Alice, Kuiken Thijs, Niqueux Eric, Staubach Christoph, Terregino Calogero, Guajardo Irene Muñoz and Baldinelli Francesca (2020). Avian influenza overview February - May 2020. EFSA journal. European Food Safety Authority 18(6): e06194-e06194.

European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian influenza, Adlhoch Cornelia, Fusaro Alice, Gonzales José L, Kuiken Thijs, Marangon Stefano, Niqueux Éric, Staubach Christoph and Terregino Calogero (2021). Avian influenza overview December 2020–February 2021. EFSA Journal 19(3): e06497.Feare, C. J. (2007). The role of wild birds in the spread of HPAI H5N1. Avian Diseases 51(1 Suppl): 440-447.

Fenner, F. and Maurin, J. (1976). The classification and nomenclature of viruses. Archives of Virology, 51(1-2) 141.

Fermin, G. (2018). Host range, host–virus interactions, and virus transmission. Viruses: 101-134.

Fine, P. E. (1993). Herd immunity: history, theory, practice. Epidemiologic Reviews 15(2): 265-302.

Fitch, W. M., Bush, R. M., Bender, C. A. and Cox, N. J. (1997). Long term trends in the evolution of H (3) HA1 human influenza type A. Proceedings of the National Academy of Sciences of the United States of America 94(15): 7712-7718.

Fitch, W. M., Leiter, J., Li, X. and Palese, P. (1991). Positive Darwinian evolution in human influenza A viruses. Proceedings of the National Academy of Sciences of the United States of America 88(10): 4270-4274.

Fodor, E. (2013). The RNA polymerase of influenza a virus: mechanisms of viral transcription and replication. Acta Virologica 57(2): 113.

Fodor, E. and Brownlee, G. G. (2002). Influenza virus replication. Perspectives in Medical Virology, Elsevier. 7: 1-29.

Foster, J. E. (2018). Chapter 8 - Viruses as pathogens: animal viruses affecting wild and domesticated species. Viruses; Tennant, P., Fermin, G. and Foster, J. E. Academic Press: 189-216.

Fraser, C., Donnelly, C. A., Cauchemez, S., Hanage, W. P., Van Kerkhove, M. D., Hollingsworth, T. D., Griffin, J., Baggaley, R. F., Jenkins, H. E. and Lyons, E. J. (2009). Pandemic potential of a strain of influenza A (H1N1): early findings. Science 324(5934).

Gan, L., Tian, Y., Zhao, Y., Shan, X. Q., Zhou, W., Xia, B. B., Chen, J., Wang, M. L. and Zhao, J. (2019). Enhancing immunogenicity and protective efficacy of inactivated avian influenza H9N2 vaccine with recombinant chicken IFN-alpha in chicken. Veterinary Microbiology 234: 77-82.

Gao, R., Cao, B., Hu, Y., Feng, Z., Wang, D., Hu, W., Chen, J., Jie, Z., Qiu, H. and Xu, K. (2013). Human infection with a novel avian-origin influenza A (H7N9) virus. New England Journal of Medicine 368(20): 1888.

Gao, Y., Zhang, Y., Shinya, K., Deng, G., Jiang, Y., Li, Z., Guan, Y., Tian, G., Li, Y., Shi, J., Liu, L., Zeng, X., Bu, Z., Xia, X., Kawaoka, Y. and Chen, H. (2009). Identification of amino acids in HA and PB2 critical for the transmission of H5N1 avian influenza viruses in a mammalian host. PLoS Pathogens 5(12): e1000709.

Garten, R. J., Davis, C. T., Russell, C. A., Shu, B., Lindstrom, S., Balish, A., Sessions, W. M., Xu, X., Skepner, E. and Deyde, V. (2009). Antigenic and genetic characteristics of swine-origin 2009 A (H1N1) influenza viruses circulating in humans. Science 325(5937): 197-201.

Gastaminza, P., Perales, B., Falcón, A. M. and Ortín, J. (2003). Mutations in the N-terminal region of influenza virus PB2 protein affect virus RNA replication but not transcription. Journal of Virology 77(9): 5098-5108.

Geller, R., Estada, Ú., Peris, J. B., Andreu, I., Bou, J.-V., Garijo, R., Cuevas, J. M., Sabariegos, R., Mas, A. and Sanjuán, R. (2016). Highly heterogeneous mutation rates in the hepatitis C virus genome. Nature Microbiology 1(7): 1-7.

Germeraad, E. A., Sanders, P., Hagenaars, T. J., de Jong, M. C. M., Beerens, N. and Gonzales, J. L. (2019). Virus shedding of avian influenza in poultry: a systematic review and meta-analysis. Viruses 11(9): 812.

Gonzales, J., Boender, G., Elbers, A., Stegeman, J. and de Koeijer, A. (2014). Risk based surveillance for early detection of low pathogenic avian influenza outbreaks in layer chickens. Preventive Veterinary Medicine 117(1): 251-259.

Graef, K. M., Vreede, F. T., Lau, Y.-F., McCall, A. W., Carr, S. M., Subbarao, K. and Fodor, E. (2010). The PB2 subunit of the influenza virus RNA polymerase affects virulence by interacting with the mitochondrial antiviral signaling protein and inhibiting expression of beta interferon. Journal of Virology 84(17): 8433-8445.

Gu, M., Chen, H., Li, Q., Huang, J., Zhao, M., Gu, X., Jiang, K., Wang, X., Peng, D. and Liu, X. (2014). Enzootic genotype S of H9N2 avian influenza viruses donates internal genes to emerging zoonotic influenza viruses in China. Veterinary Microbiology 174(3-4): 309-315.

Gu, M., Xu, L., Wang, X. and Liu, X. (2017). Current situation of H9N2 subtype avian influenza in China. Veterinary Research 48(1): 1-10.

Guan, Y., Shortridge, K. F., Krauss, S., Chin, P. S., Dyrting, K. C., Ellis, T. M., Webster, R. G. and Peiris, M. (2000). H9N2 influenza viruses possessing H5N1-like internal genomes continue to circulate in poultry in southeastern China. Journal of Virology 74(20): 9372.

Guan, Y., Shortridge, K. F., Krauss, S. and Webster, R. G. (1999). Molecular characterization of H9N2 influenza viruses: were they the donors of the “internal” genes of H5N1 viruses in Hong Kong? Proceedings of the National Academy of Sciences of the United States of America 96(16): 9363-9367.

Guo, Y., Dong, J., Wang, M., Zhang, Y., Guo, J. and Wu, K. (2001). Characterization of hemagglutinin gene of influenza A virus subtype H9N2. Chinese Medical Journal 114(1): 76-79.

Guo, Y., Krauss, S., Senne, D., Mo, I., Lo, K., Xiong, X., Norwood, M., Shortridge, K., Webster, R. and Guan, Y. (2000). Characterization of the pathogenicity of members of the newly established H9N2 influenza virus lineages in Asia. Virology 267(2): 279-288.

Guo, Y., Li, J. and Cheng, X. (1999). Discovery of men infected by avian influenza A (H9N2) virus. Chinese Journal of Experimental and Clinical Virology 13(2): 105-108.

Guo, Y., Xie, J., Wu, K., Dong, J., Wang, M., Zhang, Y., Guo, J., Chen, J., Chen, Z. and Li, Z. (2002). Characterization of genome of A/Guangzhou/333/99(H9N2) virus. Chinese Journal of Experimental and Clinical Virology 16(2): 142-145.

Ha, Y., Stevens, D. J., Skehel, J. J. and Wiley, D. C. (2001). X-ray structures of H5 avian and H9 swine influenza virus hemagglutinins bound to avian and human receptor analogs. Proceedings of the National Academy of Sciences of the United States of America 98(20).

Ha, Y., Stevens, D. J., Skehel, J. J. and Wiley, D. C. (2002). H5 avian and H9 swine influenza virus haemagglutinin structures: possible origin of influenza subtypes. The EMBO journal 21(5): 865.

Hadfield, J., Megill, C., Bell, S. M., Huddleston, J., Potter, B., Callender, C., Sagulenko, P., Bedford, T. and Neher, R. A. (2018). Nextstrain: real-time tracking of pathogen evolution. Bioinformatics 34(23): 4121.

Hallinan, N. (2011). Tree shape: phylogenies & macroevolution. Integrative Biology B 200.

Hampson, A.W. (2002). Influenza virus antigens and 'antigenic drift'. In Perspectives in Medical Virology (7) 49, Elsevier.

Han, L., He, W., Yan, H., Li, X., Wang, C., Shi, Q., Zhou, T. and Dong, G. (2019). The evolution and molecular characteristics of H9N2 avian influenza viruses in Jiangxi of China. Journal of Medical Virology 91(4): 711-716.

Hanada, K., Suzuki, Y. and Gojobori, T. (2004). A large variation in the rates of synonymous substitution for RNA viruses and its relationship to a diversity of viral infection and transmission modes. Molecular Biology and Evolution 21(6): 1074.

Hao, X., Wang, X., Hu, J., Gu, M., Wang, J., Deng, Y., Jiang, D., He, D., Xu, H. and Yang, Y. (2019). The PB2 and M genes of genotype S H9N2 virus contribute to the enhanced fitness of H5Nx and H7N9 avian influenza viruses in chickens. Virology 535: 218.

Harvey, W. T., Benton, D. J., Gregory, V., Hall, J. P., Daniels, R. S., Bedford, T., Haydon, D. T., Hay, A. J., McCauley, J. W. and Reeve, R. (2016). Identification of Low- and High-Impact Hemagglutinin Amino Acid Substitutions That Drive Antigenic Drift of Influenza A(H1N1) Viruses. PLoS Pathogens 12(4).

Hatchery, M. (2017). How to Hatch Chicken Eggs Using an Incubator. from https://blog.meyerhatchery.com/2017/06/how-to-hatch-chicken-eggs-using-an-incubator/.

Hatta, M., Gao, P., Halfmann, P. and Kawaoka, Y. (2001). Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science 293(5536): 1840.

Hay, A. J., Gregory, V., Douglas, A. R. and Lin, Y. P. (2001). The evolution of human influenza viruses. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences 356(1416): 1861.

He, Y., Liu, P., Tang, S., Chen, Y., Pei, E., Zhao, B., Ren, H., Li, J., Zhu, Y., Zhao, H., Pan, Q., Gu, B., Yuan, Z. and Wu, F. (2014). Live poultry market closure and control of avian influenza A(H7N9), Shanghai, China. Emerging Infectious Diseases 20(9): 1565.

Heffernan, J. M., Smith, R. J. and Wahl, L. M. (2005). Perspectives on the basic reproductive ratio. Journal of the Royal Society Interface 2(4): 281.

Hoffmann, E., Neumann, G., Kawaoka, Y., Hobom, G. and Webster, R. G. (2000). A DNA transfection system for generation of influenza A virus from eight plasmids. Proceedings of the National Academy of Sciences of the United States of America 97(11): 6108.

Hoffmann, E., Stech, J., Guan, Y., Webster, R. and Perez, D. (2001). Universal primer set for the full-length amplification of all influenza A viruses. Archives of Virology 146(12): 2275.

Holmes, E. C., Ghedin, E., Miller, N., Taylor, J., Bao, Y., George, K. S., Grenfell, B. T., Salzberg, S. L., Fraser, C. M. and Lipman, D. J. (2005). Whole-genome analysis of human influenza A virus reveals multiple persistent lineages and reassortment among recent H3N2 viruses. Plos Biology 3(9): e300.

Holmgren, J. and Czerkinsky, C. (2005). Mucosal immunity and vaccines. Nature Medicine 11(4): S45-S53.

Homme, P. and Easterday, B. (1970). Avian influenza virus infections. I. Characteristics of influenza A/Turkey/Wisconsin/1966 virus. Avian Diseases: 66-74.

Homme, P. J. and Easterday, B. C. (1970). Avian influenza virus infections. I. Characteristics of influenza A-turkey-Wisconsin-1966 virus. Avian Diseases:14(1): 66-74.

Hong, B. O., Dong, L. B., Zhang, Y., Dong, J., Zou, S. M., Gao, R. B., Wang, D. Y., Shu, Y. L. J. B. and Bes, E. S. (2014). H5N1 Avian Influenza Pre-pandemic Vaccine Strains in China. Biomedical and Environmental Sciences 27(10): 763-769.

Hsu, S. M. and Wang, C. Efficacy of Avian Influenza Vaccine in Poultry: A Meta-analysis. Avian Diseases, 54(4), 1197-1209.

Hu, T. and Banzhaf, W. (2008). Nonsynonymous to Synonymous Substitution Ratio Ka/ks: Measurement for Rate of Evolution in Evolutionary Computation. International Conference on Parallel Problem Solving from Nature, Springer.

Huang, Y., Hu, B., Wen, X., Cao, S., Gavrilov, B. K., Du, Q., Khan, M. I. and Zhang, X. (2010). Diversified reassortant H9N2 avian influenza viruses in chicken flocks in northern and eastern China. Virus Research 151(1): 26-32.

Huang, Y., Li, X., Zhang, H., Chen, B., Jiang, Y., Yang, L., Zhu, W., Hu, S., Zhou, S. and Tang, Y. (2015). Human infection with an avian influenza A (H9N2) virus in the middle region of China. Journal of Medical Virology 87(10): 1641-1648.

Hurt, A., Fouchier, R. A. and Vijaykrishna, D. (2017). Ecology and evolution of avian influenza viruses. Genetics and evolution of infectious diseases, Elsevier: 621-640.

Ina, Y. and Gojobori, T. (1994). Statistical analysis of nucleotide sequences of the hemagglutinin gene of human influenza A viruses. Proceedings of the National Academy of Sciences of the United States of America 91(18): 8388-8392.

Ince, W. L., Gueyembaye, A., Bennink, J. R. and Yewdell, J. W. (2013). Reassortment complements spontaneous mutation in influenza A virus NP and M1 genes to accelerate adaptation to a new host. Journal of Virology 87(8): 4330-4338.

Isidoro, M. and Melero, J. A. (2002). A model for the generation of multiple A to G transitions in the human respiratory syncytial virus genome: predicted RNA secondary structures as substrates for adenosine deaminases that act on RNA. Journal of General Virology 83(6): 1445-1455.

Jackwood, M. W. and Stallknecht, D. E. (2007). Molecular epidemiologic studies on North American H9 avian influenza virus isolates from waterfowl and shorebirds. Avian Diseases 51(1): 448-450.

Jagger, B., Wise, H., Kash, J., Walters, K.-A., Wills, N., Xiao, Y.L., Dunfee, R., Schwartzman, L., Ozinsky, A. and Bell, G. (2012). An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. Science 337(6091): 199-204.

James, J., Sealy, J. E. and Iqbal, M. (2019). A global perspective on H9N2 avian influenza virus. Viruses 11(7): 620.

Jamuar, S. S., Lam, A.T. N., Kircher, M., D'Gama, A. M., Wang, J., Barry, B. J., Zhang, X., Hill, R. S., Partlow, J. N. and Rozzo, A. (2014). Somatic mutations in cerebral cortical malformations. New England Journal of Medicine 371(8): 733-743.

Janeway Jr, C. A., Travers, P., Walport, M. and Shlomchik, M. J. (2001). The distribution and functions of immunoglobulin isotypes. Immunobiology: The Immune System in Health and Disease. 5th edition, Garland Science.

Jeevan, T., Darnell, D., Gradi, E. A., Benali, Y., Kara, R., Guetarni, D., Rubrum, A., Seiler, P. J., Crumpton, J. C. and Webby, R. J. (2019). A (H9N2) influenza viruses associated with chicken mortality in outbreaks in Algeria 2017. Influenza and Other Respiratory Viruses 13(6): 622-626.

Ji, K., Jiang, W. M., Liu, S., Chen, J. M., Chen, J., Hou, G. Y., Li, J. P. and Huang, B. X. (2010). Characterization of the hemagglutinin gene of subtype H9 avian influenza viruses isolated in 2007-2009 in China. Journal of Virological Methods 163(2): 186-189.

Jiang, W., Liu, S., Hou, G., Li, J., Zhuang, Q., Wang, S., Zhang, P. and Chen, J. (2012). Chinese and global distribution of H9 subtype avian influenza viruses. PloS One 7(12): e52671-e52671.

Jing, Z., Huiming, Y., Hongjun, X., Zengbin, M. and Guozhong, Z. (2017) Efficacy of an inactivated bivalent vaccine against the prevalent strains of Newcastle disease and H9N2 avian influenza. Virology Journal 14, 56 DOI: 10.1186/s12985-017-0723-7.

Jonas, M., Sahesti, A., Murwijati, T., Lestariningsih, C. L., Irine, I., Ayesda, C. S., Prihartini, W. and Mahardika, G. N. (2018). Identification of avian influenza virus subtype H9N2 in chicken farms in Indonesia. Preventive Veterinary Medicine 159: 99-105.

Jonges, M., Meijer, A., Fouchier, R., Koch, G., Li, J., Pan, J. C., Chen, H., Shu, Y. L. and Koopmans, M. (2013). Guiding outbreak management by the use of influenza A (H7Nx) virus sequence analysis. Eurosurveillance 18(16): 20460.

Joseph, U., Su, Y. C., Vijaykrishna, D. and Smith, G. J. (2017). The ecology and adaptive evolution of influenza A interspecies transmission. Influenza and Other Respiratory Viruses 11(1), 74-84.

Kaleta, E. F. and Rülke, C. P. A. (2009). The Beginning and Spread of Fowl Plague (H7 High Pathogenicity Avian Influenza) across Europe and Asia (1878–1955). Avian Influenza: 145

Karimi-Madab, M., Ansari-Lari, M., Asasi, K. and Nili, H. (2010). Risk factors for detection of bronchial casts, most frequently seen in endemic H9N2 avian influenza infection, in poultry flocks in Iran. Preventive Veterinary Medicine 95(3-4): 275-280.

Karlsson, E. A., Engel, G. A., Feeroz, M. M., San, S., Rompis, A., Lee, B. P. Y. H., Shaw, E., Oh, G., Schillaci, M. A., Grant, R., Heidrich, J., Schultz-Cherry, S. and Jones-Engel, L. (2012). Influenza virus infection in nonhuman primates. Emerging Infectious Diseases 18(10): 1672-1675.

Karunakaran, D., Newman, J., Halvorson, D. A. and Abraham, A. (1987). Evaluation of inactivated influenza vaccines in market turkeys. Avian Diseases: 498-503.

Kawaoka, Y., Chambers, T. M., Sladen, W. L. and Webster, R. G. (1988). Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? Virology 163(1): 247-250.

Kawaoka, Y., Krauss, S. and Webster, R. G. (1989). Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. Journal of Virology 63(11): 4603-4608.

Kawaoka, Y. and Webster, R. G. (1988). Molecular mechanism of acquisition of virulence in influenza virus in nature. Microbial Pathogenesis 5(5): 311-318.

Khan, S. U., Anderson, B. D., Heil, G. L., Liang, S. and Gray, G. C. (2015). A systematic review and meta-analysis of the seroprevalence of influenza A (H9N2) infection among humans. The Journal of Infectious Diseases 212(4): 562-569.

Kilany, W., Arafa, A., Erfan, A., Ahmed, M., Nawar, A., Selim, A., Khoulosy, S., Hassan, M., Aly, M. and Hafez, H. (2010). Isolation of highly pathogenic avian influenza H5N1 from table eggs after vaccinal break in commercial layer flock. Avian Diseases 54(3): 1115-1119.

Kilany, W. H., Ali, A., Bazid, A. H., El-Deeb, A. H., El-Abideen, M. A., Sayed, M. E. and El-Kady, M. F. (2016). A dose-response study of inactivated low pathogenic avian influenza H9N2 virus in specific-pathogen-free and commercial broiler chickens. Avian Diseases 60(1 Suppl): 256-261

Kilbourne, E. D. (2006). Influenza pandemics of the 20th century. Emerging Infectious Diseases 12(1): 9.

Kim, J. A., Cho, S. H., Kim, H. S. and Seo, S. H. (2006). H9N2 influenza viruses isolated from poultry in Korean live bird markets continuously evolve and cause the severe clinical signs in layers. Veterinary Microbiology 118(3-4): 169-176.

Kim, K.I., Choi, J.G., Kang, H.M., To, T. L., Nguyen, T. D., Song, B.M., Hong, M.S., Choi, K.S., Kye, S.J. and Kim, J.Y. (2013). Geographical distribution of low pathogenic avian influenza viruses of domestic poultry in Vietnam and their genetic relevance with Asian isolates. Poultry Science 92(8): 2012-2023.

Kim, S. M., Kim, Y.I., Park, S.J., Kim, E.H., Kwon, H.I., Si, Y.J., Lee, I.W., Song, M.S. and Choi, Y. K. (2017). Vaccine Efficacy of Inactivated, Chimeric Hemagglutinin H9/H5N2 Avian Influenza Virus and Its Suitability for the Marker Vaccine Strategy. Journal of Virology 91(6): e01693-01616.

Kirkpatrick, E., Qiu, X., Wilson, P. C., Bahl, J. and Krammer, F. (2018). The influenza virus hemagglutinin head evolves faster than the stalk domain. Scientific Reports 8(1): 10432.

Klimov, A., Balish, A., Veguilla, V., Sun, H., Schiffer, J., Lu, X., Katz, J. M. and Hancock, K. (2012). Influenza virus titration, antigenic characterization, and serological methods for antibody detection. Influenza Virus, Springer: 25-51.

Klinkenberg, D., De Bree, J., Laevens, H. and De Jong, M. C. M. (2002). Within- and between-pen transmission of Classical Swine Fever Virus: a new method to estimate the basic reproduction ratio from transmission experiments. Epidemiology and Infection 128(2): 293-299.

Koel, B. F., Burke, D. F., Bestebroer, T. M., Van Der Vliet, S., Zondag, G. C., Vervaet, G., Skepner, E., Lewis, N. S., Spronken, M. I. and Russell, C. A. (2013). Substitutions near the receptor binding site determine major antigenic change during influenza virus evolution. Science 342(6161): 976-979.

Kung, N., Guan, Y., Perkins, N., Bissett, L., Ellis, T., Sims, L., Morris, R., Shortridge, K. and Peiris, J. S. M. (2003). The impact of a monthly rest day on avian influenza virus isolation rates in retail live poultry markets in Hong Kong. Avian Diseases 47(s3): 1037-1041.

Kwon, H.J., Cho, S.H., Kim, M.C., Ahn, Y.J. and Kim, S.J. (2006). Molecular epizootiology of recurrent low pathogenic avian influenza by H9N2 subtype virus in Korea. Avian Pathology 35(4): 309-315.

La Gruta, N. L. and Turner, S. J. (2014). T cell mediated immunity to influenza: mechanisms of viral control. Trends in Immunology 35(8): 396-402.

Labadie, K., Afonso, E. D. S., Rameix-Welti, M.-A., Van Der Werf, S. and Naffakh, N. (2007). Host-range determinants on the PB2 protein of influenza A viruses control the interaction between the viral polymerase and nucleoprotein in human cells. Virology 362(2): 271-282.

Lam, H. C., Bi, X., Sreevatsan, S. and Boley, D. (2017). Evolution and Vaccination of Influenza Virus. Journal of Computational Biology : a journal of computational molecular cell biology 24(8): 787-798.

Lam, T. T. Y., Wang, J., Shen, Y., Zhou, B., Duan, L., Cheung, C. L., Ma, C., Lycett, S. J., Leung, C. Y. H. and Chen, X. (2013). The genesis and source of the H7N9 influenza viruses causing human infections in China. Nature 502(7470): 241-244.

Lam, T. T., Zhou, B., Wang, J., Chai, Y., Shen, Y., Chen, X., Ma, C., Hong, W., Chen, Y. and Zhang, Y. (2015). Dissemination, divergence and establishment of H7N9 influenza viruses in China. Nature 522(7554): 102.

Lamb, R. A. and Choppin, P. W. (1983). The gene structure and replication of influenza virus. Annual Review of Biochemistry 52(1): 467-506.

Lau, E. H., Leung, Y. C., Zhang, L. J., Cowling, B. J., Mak, S. P., Guan, Y., Leung, G. M. and Peiris, J. M. (2007). Effect of interventions on influenza A (H9N2) isolation in Hong Kong's live poultry markets, 1999–2005. Emerging Infectious Diseases 13(9), 1340.

Lau, S.Y., Joseph, S., Chan, K.H., Chen, H., Patteril, N. A. G., Elizabeth, S. K., Muhammed, R., Baskar, V., Lau, S. K. and Kinne, J. (2016). Complete genome sequence of influenza virus H9N2 associated with a fatal outbreak among chickens in Dubai. Genome Announcements 4(4).

Lauring, A. S., Frydman, J. and Andino, R. (2013). The role of mutational robustness in RNA virus evolution. Nature Reviews Microbiology 11(5): 327-336.

Lee, C. W., Senne, D. A. and Suarez, D. L. (2004). Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. Journal of Virology 78(15): 8372-8381.

Lee, C. W., Song, C. S., Lee, Y. J., Mo, I. P., Garcia, M., Suarez, D. L. and Kim, S. J. (2000). Sequence analysis of the hemagglutinin gene of H9N2 Korean avian influenza viruses and assessment of the pathogenic potential of isolate MS96. Avian Diseases 44(3): 527-535.

Lee, D. H., Fusaro, A., Song, C. S., Suarez, D. L. and Swayne, D. E. (2016). Poultry vaccination directed evolution of H9N2 low pathogenicity avian influenza viruses in Korea. Virology 488: 225-231.

Lee, D. H. and Song, C. S. (2013). H9N2 avian influenza virus in Korea: evolution and vaccination. Clinical and Experimental Vaccine Research 2(1): 26.

Lee, Y. J., Shin, J. Y., Song, M. S., Lee, Y. M., Choi, J. G., Lee, E. K., Jeong, O. M., Sung, H. W., Kim, J. H. and Kwon, Y. K. (2007). Continuing evolution of H9 influenza viruses in Korean poultry. Virology 359(2): 313-323.

Lee, Y. J., Shin, J. Y., Song, M. S., Lee, Y. M., Choi, J. G., Lee, E. K., Jeong, O. M., Sung, H. W., Kim, J. H., Kwon, Y. K., Kwon, J. H., Kim, C. J., Webby, R. J., Webster, R. G. and Choi, Y. K. (2007). Continuing evolution of H9 influenza viruses in Korean poultry. Virology 359(2): 313-323.

Leong, H. K., Goh, C. S., Chew, S. T., Lim, C. W., Lin, Y. N., Chang, S. F., Yap, H. H. and Chua, S. B. (2008). Prevention and control of avian influenza in Singapore. Annals Academy of Medicine Singapore 37(6): 504-509.

Leung, Y. C., Lau, E. H., Zhang, L. J., Guan, Y., Cowling, B. J. and Peiris, J. M. (2012). Avian influenza and ban on overnight poultry storage in live poultry markets, Hong Kong. Emerging Infectious Diseases 18(8): 1339.

Li, C., Yu, K., Tian, G., Yu, D., Liu, L., Jing, B., Ping, J. and Chen, H. (2005). Evolution of H9N2 influenza viruses from domestic poultry in Mainland China. Virology 340(1): 70-83.

Li, O. T., Chan, M. C., Leung, C. S., Chan, R. W., Guan, Y., Nicholls, J. M. and Poon, L. L. (2009). Full factorial analysis of mammalian and avian influenza polymerase subunits suggests a role of an efficient polymerase for virus adaptation. PloS One 4(5): e5658.

Li, S., Li, X., Zhong, S., Sun, H., Pan, J., Chen, S., Peng, D. and Liu, X. (2012). Genome sequencing and phylogenetic analysis of avian influenza viruses subtype H9N2. Chinese Journal of Virology 28(1): 7-14.

Li, X., Shi, J., Guo, J., Deng, G., Zhang, Q., Wang, J., He, X., Wang, K., Chen, J. and Li, Y. (2014). Genetics, receptor binding property, and transmissibility in mammals of naturally isolated H9N2 avian influenza viruses. PLoS Pathogens 10(11): e1004508.

Liao, Q., Lam, W. T., Leung, G. M., Jiang, C. and Fielding, R. (2009). Live poultry exposure, Guangzhou, China, 2006. Epidemics 1(4): 207-212.

Lin, R. W., Chen, G. W., Sung, H. H., Lin, R. J., Yen, L. C., Tseng, Y. L., Chang, Y. K., Lien, S. P., Shih, S. R. and Liao, C. L. (2019). Naturally occurring mutations in PB1 affect influenza A virus replication fidelity, virulence, and adaptability. Journal of Biomedical Science 26(1): 55.

Lin, T. N., Nonthabenjawan, N., Chaiyawong, S., Bunpapong, N., Boonyapisitsopa, S., Janetanakit, T., Mon, P. P., Mon, H. H., Oo, K. N. and Oo, S. M. (2017). Influenza A (H9N2) virus, Myanmar, 2014–2015. Emerging Infectious Diseases 23(6): 1041.

Lin, Y., Shaw, M., Gregory, V., Cameron, K., Lim, W., Klimov, A., Subbarao, K., Guan, Y., Krauss, S. and Shortridge, K. (2000). Avian-to-human transmission of H9N2 subtype influenza A viruses: relationship between H9N2 and H5N1 human isolates. Proceedings of the National Academy of Sciences of the United States of America 97(17): 9654-9658.

Lina, L., Saijuan, C., Chengyu, W., Yuefeng, L., Shishan, D., Ligong, C., Kangkang, G., Zhendong, G., Jiakai, L., Jianhui, Z., Qingping, L., Wenting, Z., Yu, S., Honglin, W., Tengfei, Z., Guoyuan, W., Jiping, Z., Chunmao, Z., Meilin, J., Yuwei, G., Huabin, S. and Zongzheng, Z. (2019). Adaptive amino acid substitutions enable transmission of an H9N2 avian influenza virus in guinea pigs. Scientific Reports 9(1): 19734.

Lindh, E., Ek-Kommonen, C., Väänänen, V.-M., Vaheri, A., Vapalahti, O. and Huovilainen, A. (2014). Molecular epidemiology of H9N2 influenza viruses in Northern Europe. Veterinary Microbiology 172(3-4): 548-554.

Lindstrom, S. E., Cox, N. J. and Klimov, A. (2004). Genetic analysis of human H2N2 and early H3N2 influenza viruses, 1957-1972: evidence for genetic divergence and multiple reassortment events. Virology 328(1): 101-119.

Liu, J., Xiao, H., Lei, F., Zhu, Q., Qin, K., Zhang, X. W., Zhang, X. L., Zhao, D., Wang, G. and Feng, Y. J. S. (2005). Highly pathogenic H5N1 influenza virus infection in migratory birds. Science 309(5738), 1206-1206.

Liu, J. H., Okazaki, K., Mweene, A., Shi, W. M., Wu, Q. M., Su, J. L., Zhang, G. Z., Bai, G. R. and Kida, H. (2004). Genetic conservation of hemagglutinin gene of H9 influenza virus in chicken population in Mainland China. Virus Genes 29(3): 329-334.

Liu, M., He, S., Walker, D., Zhou, N., Perez, D. R., Mo, B., Li, F., Huang, X., Webster, R. G. and Webby, R. J. (2003). The influenza virus gene pool in a poultry market in South central china. Virology 305(2): 267-275.

Liu, M., Li, X., Yuan, H., Zhou, J., Wu, J., Bo, H., Xia, W., Xiong, Y., Yang, L. and Gao, R. (2015). Genetic diversity of avian influenza A (H10N8) virus in live poultry markets and its association with human infections in China. Scientific Reports 5(1): 1-8.

Liu, Q., Lu, L., Sun, Z., Chen, G.-W., Wen, Y. and Jiang, S. (2013). Genomic signature and protein sequence analysis of a novel influenza A (H7N9) virus that causes an outbreak in humans in China. Microbes and Infection 15(6-7): 432-439.

Liu, Q., Zhou, B., Ma, W., Bawa, B., Ma, J., Wang, W., Lang, Y., Lyoo, Y., Halpin, R. A., Lin, X., Stockwell, T. B., Webby, R., Wentworth, D. E. and Richt, J. A. (2014). Analysis of recombinant H7N9 wild-type and mutant viruses in pigs shows that the Q226L mutation in HA is important for transmission. Journal of Virology 88(14): 8153-8165.

Liu, R., Zhao, B., Li, Y., Zhang, X., Chen, S. and Chen, T. (2018). Clinical and epidemiological characteristics of a young child infected with avian influenza A (H9N2) virus in China. Journal of International Medical Research 46(8): 3462-3467.

Liu, T. and Ye, Z. (2002). Restriction of viral replication by mutation of the influenza virus matrix protein. Journal of Virology 76(24): 13055-13061.

Lu, J. H., Liu, X. F., Shao, W. X., Liu, Y. L., Wei, D. P. and Liu, H. Q. (2005). Phylogenetic analysis of eight genes of H9N2 subtype influenza virus: a mainland China strain possessing early isolates' genes that have been circulating. Virus Genes 31(2): 163-169.

Lu, J., Liu, X., Shao, W., Zhang, P. and Wei, D. (2003). Genetic characterization of the entire genome of an H9N2 avian influenza virus A/Chicken/Shanghai/F/98. Acta Microbiologica Sinica 43(4): 434-441.

Lupiani, B. and Reddy, S. M. (2009). The history of avian influenza. Comparative Immunology, Microbiology and Infectious Diseases 32(4): 311-323.

Ma, M. J., Zhao, T., Chen, S. H., Xia, X., Yang, X. X., Wang, G. L., Fang, L. Q., Ma, G. Y., Wu, M. N. and Qian, Y. H. (2018). Avian influenza A virus infection among workers at live poultry markets, China, 2013–2016. Emerging Infectious Diseases 24(7): 1246.

Ma, W., Kahn, R. E. and Richt, J. A. (2009). The pig as a mixing vessel for influenza viruses: human and veterinary implications. Journal of Molecular and Genetic Medicine: an international journal of biomedical research 3(1): 158.

Maas, R., Rosema, S., van Zoelen, D. and Venema, S. (2011). Maternal immunity against avian influenza H5N1 in chickens: limited protection and interference with vaccine efficacy. Avian Pathology 40(1): 87-92.

Mamelund, S. E. (2008). Influenza, Historical. Medicine 54, 361-371.

Masel, J. (2011). Genetic drift. Current Biology 21(20): R837-R838.

Matrosovich, M. N., Matrosovich, T. Y., Gray, T., Roberts, N. A. and Klenk, H. D. (2004). Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium. Journal of Virology 78(22): 12665-12667.

McDonald, N. J., Smith, C. B. and Cox, N. J. (2007). Antigenic drift in the evolution of H1N1 influenza A viruses resulting from deletion of a single amino acid in the haemagglutinin gene. Journal of General Virology 88(12): 3209-3213.

Memorandum, W. (1980). A revision of the system of nomenclature for influenza viruses: a WHO memorandum. Bull World Health Organ 58(4): 585-591.

Meng, F., Xu, H., Zhang, W., Huang, D., Zhang, Z., Liu, X., Chang, W. and Qin, Z. (2016). Genetic evolution and substitution frequency of avian influenza virus HA gene in chicken H9N2 subtype in China in the last 20 years. Wei Sheng Wu Xue Bao 56(1): 35-43.

Mestecky, J., Moldoveanu, Z., Smith, P. D., Hel, Z. and Alexander, R. C. (2009). Mucosal immunology of the genital and gastrointestinal tracts and HIV-1 infection. Journal of Reproductive Immunology 83(1-2): 196-200.

Monne, I., Fusaro, A., Nelson, M. I., Bonfanti, L., Mulatti, P., Hughes, J., Murcia, P. R., Schivo, A., Valastro, V. and Moreno, A. (2014). Emergence of a highly pathogenic avian influenza virus from a low-pathogenic progenitor. Journal of Virology 88(8): 4375-4388.

Morelli, M. J., Wright, C. F., Knowles, N. J., Juleff, N., Paton, D. J., King, D. P. and Haydon, D. T. (2013). Evolution of foot-and-mouth disease virus intra-sample sequence diversity during serial transmission in bovine hosts. Veterinary Research 44(1): 12.

Mouritz A. A. St. M. (Arthur Albert St. M.) (1921). The Flu: a brief history of influenza in U.S. America, Europe, Hawaii. Hawaii, Advertiser Publishing Co., Ltd., Honolulu.

Muramoto, Y., Noda, T., Kawakami, E., Akkina, R. and Kawaoka, Y. (2013). Identification of novel influenza A virus proteins translated from PA mRNA. Journal of Virology 87(5): 2455-2462.

Murphy, D. G., Dimock, K. and Kang, C. Y. (1991). Numerous transitions in human parainfluenza virus 3 RNA recovered from persistently infected cells. Virology 181(2): 760-763.

Naeem, K. and Siddique, N. (2006). Use of strategic vaccination for the control of avian influenza in Pakistan. Developments in Biologicals 124: 145-150.

Naeem, K., Siddique, N., Ayaz, M. and Jalalee, M. (2007). Avian influenza in Pakistan: outbreaks of low-and high-pathogenicity avian influenza in Pakistan during 2003–2006. Avian Diseases 51(s1): 189-193.

Naeve, C., Hinshaw, V. and Webster, R. (1984). Mutations in the hemagglutinin receptor-binding site can change the biological properties of an influenza virus. Journal of Virology 51(2): 567-569.

Naffakh, N., Massin, P., Escriou, N., Crescenzo-Chaigne, B. and van der Werf, S. (2000). Genetic analysis of the compatibility between polymerase proteins from human and avian strains of influenza A viruses. Microbiology 81(5): 1283-1291.

Nei, M. and Gojobori, T. (1986). Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Molecular Biology and Evolution 3(5): 418-426.

Nelson, M. I., Simonsen, L., Viboud, C., Miller, M. A. and Holmes, E. C. (2007). Phylogenetic analysis reveals the global migration of seasonal influenza A viruses. PLoS Pathogens 3(9): e131.

Nextstrain. (2021). Real-time tracking of influenza A/H9N2 virus evolution. from https://nextstrain.org/flu/avian/h9n2/ha?c=gt-HA_207&gmax=659&gmin=574.

Nguyen, D. C., Uyeki, T. M., Jadhao, S., Maines, T., Shaw, M., Matsuoka, Y., Smith, C., Rowe, T., Lu, X. and Hall, H. (2005). Isolation and characterization of avian influenza viruses, including highly pathogenic H5N1, from poultry in live bird markets in Hanoi, Vietnam, in 2001. Journal of Virology 79(7): 4201-4212.

Nobusawa, E. and Sato, K. (2006). Comparison of the mutation rates of human influenza A and B viruses. Journal of Virology 80(7): 3675-3678.

OIE (2019). INFECTION WITH AVIAN INFLUENZA VIRUSES. 2019 © OIE - Terrestrial Animal Health Article 10.14.11.

Okamatsu, M., Sakoda, Y., Kishida, N., Isoda, N. and Kida, H. (2008). Antigenic structure of the hemagglutinin of H9N2 influenza viruses. Archives of Virology 153(12): 2189.

Olsen, B., Munster, V. J., Wallensten, A., Waldenström, J., Osterhaus, A. D. M. E. and Fouchier, R. A. M. (2006). Global Patterns of Influenza A Virus in Wild Birds. Science 312(5772): 384-388.

Orton, R. (2014). Calculating dN/dS for NGS datasets. from https://bioinformatics.cvr.ac.uk/calculating-dnds-for-ngs-datasets/.

Orton, R. J., Wright, C. F., Morelli, M. J., King, D. J., Paton, D. J., King, D. P. and Haydon, D. T. (2015). Distinguishing low frequency mutations from RT-PCR and sequence errors in viral deep sequencing data. BMC Genomics 16(1): 229.

Otte, J., Hinrichs, J., Rushton, J., Roland-Holst, D. and Zilberman, D. (2008). Impacts of avian influenza virus on animal production in developing countries. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 3(080): 18.

Ozawa, M., Basnet, S., Burley, L. M., Neumann, G., Hatta, M. and Kawaoka, Y. (2011). Impact of amino acid mutations in PB2, PB1-F2, and NS1 on the replication and pathogenicity of pandemic (H1N1) 2009 influenza viruses. Journal of Virology 85(9): 4596-4601.

Palese, P. and Schulman, J. L. (1976). Mapping of the influenza virus genome: identification of the hemagglutinin and the neuraminidase genes. Proceedings of the National Academy of Sciences of the United States of America 73(6): 2142-2146.

Pan, Y., Cui, S., Sun, Y., Zhang, X., Ma, C., Shi, W., Peng, X., Lu, G., Zhang, D. and Liu, Y. (2018). Human infection with H9N2 avian influenza in northern China. Clinical Microbiology and Infection 24(3): 321-323.

Pappas, C., Aguilar, P. V., Basler, C. F., Solórzano, A., Zeng, H., Perrone, L. A., Palese, P., García-Sastre, A., Katz, J. M. and Tumpey, T. M. (2008). Single gene reassortants identify a critical role for PB1, HA, and NA in the high virulence of the 1918 pandemic influenza virus. Proceedings of the National Academy of Sciences of the United States of America 105(8): 3064-3069.

Park, K. J., Kwon, H.-i., Song, M.-S., Pascua, P. N. Q., Baek, Y. H., Lee, J. H., Jang, H.-L., Lim, J.-Y., Mo, I.-P. and Moon, H.-J. (2011). Rapid evolution of low-pathogenic H9N2 avian influenza viruses following poultry vaccination programmes. Journal of General Virology 92(1): 36-50.

Parvin, J. D., Moscona, A., Pan, W., Leider, J. and Palese, P. (1986). Measurement of the mutation rates of animal viruses: influenza A virus and poliovirus type 1. Journal of Virology 59(2): 377-383.

Pasick, J., Handel, K., Robinson, J., Copps, J., Ridd, D., Hills, K., Kehler, H., Cottam-Birt, C., Neufeld, J. and Berhane, Y. (2005). Intersegmental recombination between the haemagglutinin and matrix genes was responsible for the emergence of a highly pathogenic H7N3 avian influenza virus in British Columbia. Journal of General Virology 86(3): 727-731.

Pauly, M. D., Procario, M. and Lauring, A. S. (2017). The mutation rates and mutational bias of influenza A virus. bioRxiv: 110197.

Pauly, M. D., Procario, M. C. and Lauring, A. S. (2017). A novel twelve class fluctuation test reveals higher than expected mutation rates for influenza A viruses. Elife 6: e26437.

Peacock, T., Reddy, K., James, J., Adamiak, B., Barclay, W., Shelton, H. and Iqbal, M. (2016). Antigenic mapping of an H9N2 avian influenza virus reveals two discrete antigenic sites and a novel mechanism of immune escape. Scientific Reports 6(1): 1-12.

Peacock, T. P., Benton, D. J., James, J., Sadeyen, J.-R., Chang, P., Sealy, J. E., Bryant, J. E., Martin, S. R., Shelton, H., Barclay, W. S. and Iqbal, M. (2017). Immune Escape Variants of H9N2 Influenza Viruses Containing Deletions at the Hemagglutinin Receptor Binding Site Retain Fitness In Vivo and Display Enhanced Zoonotic Characteristics. Journal of Virology 91(14): e00218-00217.

Peacock, T. P., Benton, D. J., Sadeyen, J.-R., Chang, P., Sealy, J. E., Bryant, J. E., Martin, S. R., Shelton, H., McCauley, J. W. and Barclay, W. S. (2017). Variability in H9N2 haemagglutinin receptor-binding preference and the pH of fusion. Emerging Microbes & Infections 6(1): 1-7.

Peacock, T. T. P., James, J., Sealy, J. E. and Iqbal, M. (2019). A global perspective on H9N2 avian influenza virus. Viruses 11(7): 620.

Peiris, M., Yuen, K., Leung, C., Chan, K., Ip, P., Lai, R., Orr, W. and Shortridge, K. (1999). Human infection with influenza H9N2. The Lancet 354(9182): 916-917.

Peiris, M., Yuen, K. Y., Leung, C. W., Chan, K. H., Ip, P. L., Lai, R. W., Orr, W. K. and Shortridge, K. F. J. L. (1999). Human infection with influenza H9N2. The Lancet 354(9182): 916.

Perez, D. R., Lim, W., Seiler, J. P., Yi, G., Peiris, M., Shortridge, K. F. and Webster, R. G. (2003). Role of quail in the interspecies transmission of H9 influenza A viruses: molecular changes on HA that correspond to adaptation from ducks to chickens. Journal of Virology 77(5): 3148.

Perk, S., Panshin, A., Shihmanter, E., Gissin, I., Pokamunski, S., Pirak, M., Lipkind, M., Schudel, A. and Lombard, M. (2006). Ecology and molecular epidemiology of H9N2 avian influenza viruses isolated in Israel during 2000-2004 epizootic. Developments in Biologicals 124(124): 201-209.

Perroncito, E. (1878). Epizoozia tifoide nei gallinacei. Annali Accademia Agricoltura Torino 21: 87-126.

Peyre, M., Samaha, H., Yilma Jobre Makonnen, A. S., Abd-Elnabi, A., Galal, S., Ettel, T., Dauphin, G., Lubroth, J., Roger, F. and Domenech, J. (2009). Avian influenza vaccination in Egypt: limitations of the current strategy. Journal of Molecular and Genetic Medicine: an international journal of biomedical research 3(2): 198.

Ping, J., Li, C., Deng, G., Jiang, Y., Tian, G., Zhang, S., Bu, Z. and Chen, H. (2008). Single-amino-acid mutation in the HA alters the recognition of H9N2 influenza virus by a monoclonal antibody. Biochemical and Biophysical Research Communications 371(1): 168-171.

Ping, J., Lopes, T. J., Nidom, C. A., Ghedin, E., Macken, C. A., Fitch, A., Imai, M., Maher, E. A., Neumann, G. and Kawaoka, Y. (2015). Development of high-yield influenza A virus vaccine viruses. Nature Communications 6: 8148.

Poland, G. A. (2018). Influenza vaccine failure: failure to protect or failure to understand? Expert Review of Vaccines 17(6): 495-502.

Poore, J. and Nemecek, T. (2018). Reducing food's environmental impacts through producers and consumers. Science 360(6392): 987-992.

Post, J., Burt, D. W., Cornelissen, J. B., Broks, V., van Zoelen, D., Peeters, B. and Rebel, J. M. (2012). Systemic virus distribution and host responses in brain and intestine of chickens infected with low pathogenic or high pathogenic avian influenza virus. Virology Journal 9(1): 1-14.

Potdar, V., Hinge, D., Satav, A., Simões, E. A., Yadav, P. D. and Chadha, M. S. (2019). Laboratory-confirmed avian influenza A (H9N2) virus infection, India, 2019. Emerging Infectious Diseases 25(12): 2328.

Pu, J., Wang, S., Yin, Y., Zhang, G., Carter, R. A., Wang, J., Xu, G., Sun, H., Wang, M. and Wen, C. (2015). Evolution of the H9N2 influenza genotype that facilitated the genesis of the novel H7N9 virus. Proceedings of the National Academy of Sciences of the United States of America 112(2): 548-553.

Pu, J., Yin, Y., Liu, J., Wang, X., Zhou, Y., Wang, Z., Sun, Y., Sun, H., Li, F. and Song, J. (2021). Reassortment with dominant chicken H9N2 influenza virus contributed to the fifth H7N9 virus human epidemic. Journal of Virology 95(11): e01578-01520.

Pusch, E. A. and Suarez, D. L. (2018). The multifaceted zoonotic risk of H9N2 avian influenza. Veterinary Sciences 5(4): 82.

Qi, W., Zhou, X., Shi, W., Huang, L., Xia, W., Liu, D., Li, H., Chen, S., Lei, F. and Cao, L. (2014). Genesis of the novel human-infecting influenza A (H10N8) virus and potential genetic diversity of the virus in poultry, China. Eurosurveillance 19(25): 20841.

Raymond, D. D., Bajic, G., Ferdman, J., Suphaphiphat, P., Settembre, E. C., Moody, M. A., Schmidt, A. G. and Harrison, S. C. (2018). Conserved epitope on influenza-virus hemagglutinin head defined by a vaccine-induced antibody. Proceedings of the National Academy of Sciences of the United States of America 115(1): 168-173.

Reed, L. J. and Muench, H. (1938). A simple method of estimating fifty per cent endpoints. American Journal of Epidemiology 27(3): 493-497.

Rhodes, C. J. and Demetrius, L. (2010). Evolutionary entropy determines invasion success in emergent epidemics. PLoS One 5(9): e12951.

Ritchey, M. B., Palese, P. and Schulman, J. L. (1976). Mapping of the influenza virus genome. III. Identification of genes coding for nucleoprotein, membrane protein, and nonstructural protein. Journal of Virology 20(1): 307-313.

Rodpothong, P. and Auewarakul, P. (2012). Viral evolution and transmission effectiveness. World Journal of Virology 1(5): 131-134.

Romagosa, A., Allerson, M., Gramer, M., Joo, H. S., Deen, J., Detmer, S. and Torremorell, M. (2011). Vaccination of influenza a virus decreases transmission rates in pigs. Veterinary Research 42(1): 120.

Salzberg, S. L., Kingsford, C., Cattoli, G., Spiro, D. J., Janies, D. A., Aly, M. M., Brown, I. H., Couacy-Hymann, E., De Mia, G. M., Dung do, H., Guercio, A., Joannis, T., Maken Ali, A. S., Osmani, A., Padalino, I., Saad, M. D., Savic, V., Sengamalay, N. A., Yingst, S., Zaborsky, J., Zorman-Rojs, O., Ghedin, E. and Capua, I. (2007). Genome analysis linking recent European and African influenza (H5N1) viruses. Emerging Infectious Diseases 13(5): 713-718.

Sang, X., Wang, A., Chai, T., He, X., Ding, J., Gao, X., Li, Y., Zhang, K., Ren, Z. and Li, L. (2015). Rapid emergence of a PB2-E627K substitution confers a virulent phenotype to an H9N2 avian influenza virus during adaption in mice. Archives of Virology 160(5): 1267-1277.

Sanjuán, R. and Domingo-Calap, P. (2016). Mechanisms of viral mutation. Cellular and Molecular Life Sciences 73(23): 4433-4448.

Sanjuán, R., Nebot, M. R., Chirico, N., Mansky, L. M. and Belshaw, R. (2010). Viral mutation rates. Journal of Virology 84(19): 9733-9748.

Schäffr, J. R., Kawaoka, Y., Bean, W. J., Süss, J., Senne, D. and Webster, R. G. (1993). Origin of the pandemic 1957 H2 influenza A virus and the persistence of its possible progenitors in the avian reservoir. Virology 194(2): 781-788.

Scholtissek, C. (1995). Molecular evolution of influenza viruses. Virus Genes 11(2-3): 209-215.

Scholtissek, C., Rohde, W. v., Von Hoyningen, V. and Rott, R. (1978). On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology 87(1): 13-20.

Selman, M., Dankar, S. K., Forbes, N. E., Jia, J.-J. and Brown, E. G. (2012). Adaptive mutation in influenza A virus non-structural gene is linked to host switching and induces a novel protein by alternative splicing. Emerging Microbes & Infections 1(1): 1-10.

Sharma, S. and Thomas, P. G. (2014). The two faces of heterologous immunity: protection or immunopathology. Journal of Leukocyte Biology 95(3): 405-416.

Shi, H., Lu, J., Chen, S., Sun, L. and Liu, X. (2005). Generation of A/Chicken/Shanghai/F/98 (H9N2) Avian influenza virus from eight plasmids. Acta Microbiologica Sinica 45(3): 373-376.

Shi, W., Li, W., Li, X., Haywood, J., Ma, J., Gao, G. F. and Liu, D. (2014). Phylogenetics of varied subtypes of avian influenza viruses in China: potential threat to humans. Protein & Cell 5(4): 253-257.

Shinya, K., Hamm, S., Hatta, M., Ito, H., Ito, T. and Kawaoka, Y. (2004). PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice. Virology 320(2): 258-266.

Shope, R. E. (1931). The etiology of swine influenza. Science 73(1886): 214-215.

Shope, R. E. (1936). The incidence of neutralizing antibodies for swine influenza virus in the sera of human beings of different ages. The Journal of Experimental Medicine 63(5): 669-684.

Short, K. R., Richard, M., Verhagen, J. H., van Riel, D., Schrauwen, E. J. A., van den Brand, J. M. A., Mänz, B., Bodewes, R. and Herfst, S. (2015). One health, multiple challenges: The inter-species transmission of influenza A virus. One Health 1: 1-13.

Shortridge, K. (1992). Pandemic influenza: a zoonosis? in Seminars in respiratory infections. (Vol. 7, No. 1, pp. 11-25).

Sims, L. (2007). Experience in control of avian influenza in Asia. Developments in Biologicals 130: 39-43.

Sitaras, I., Kalthoff, D., Beer, M., Peeters, B. and de Jong, M. C. M. (2014). Immune escape mutants of highly pathogenic avian influenza H5N1 selected using polyclonal sera: identification of key amino acids in the HA protein. PLoS One 9(2): e84628.

Sitaras, I., Rousou, X., Kalthoff, D., Beer, M., Peeters, B. and de Jong, M. C. M. (2016). Role of vaccination-induced immunity and antigenic distance in the transmission dynamics of highly pathogenic avian influenza H5N1. Journal of The Royal Society Interface 13(114): 20150976.

Sitaras, I., Spackman, E., de Jong, M. C. M. and Parris, D. J. (2020). Selection and antigenic characterization of immune-escape mutants of H7N2 low pathogenic avian influenza virus using homologous polyclonal sera. Virus Research 290: 198188.

Skehel, J. J. and Wiley, D. C. (2000). Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annual Review of Biochemistry 69(1): 531-569.

Smith, G. J., Vijaykrishna, D., Bahl, J., Lycett, S. J., Worobey, M., Pybus, O. G., Ma, S. K., Cheung, C. L., Raghwani, J. and Bhatt, S. (2009). Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 459(7250): 1122-1125.

Smith, G. J. D., Fan, X. H., Wang, J., Li, K. S., Qin, K., Zhang, J. X., Vijaykrishna, D., Cheung, C. L., Huang, K. and Rayner, J. M. (2006). Emergence and predominance of an H5N1 influenza variant in China. Proceedings of the National Academy of Sciences of the United States of America 103(45): 16936-16941.

Sobue, S., Nomura, T., Ishikawa, T., Ito, S., Saso, K., Ohara, H., Joh, T., Itoh, M. and Kakumu, S. (2001). Th1/Th2 cytokine profiles and their relationship to clinical features in patients with chronic hepatitis C virus infection. Journal of Gastroenterology 36(8): 544-551.

Sonnberg, S., Webby, R. J. and Webster, R. G. (2013). Natural history of highly pathogenic avian influenza H5N1. Virus Research 178(1): 63-77.

Sorrell, E. M., Wan, H., Araya, Y., Song, H., Perez, D. R. and Palese, P. (2009). Minimal Molecular Constraints for Respiratory Droplet Transmission of an Avian-Human H9N2 Influenza A Virus. Proceedings of the National Academy of Sciences of the United States of America 106(18): 7565-7570.

Spackman, E. and Swayne, D. E. (2013). Vaccination of gallinaceous poultry for H5N1 highly pathogenic avian influenza: Current questions and new technology. Virus Research 178(1): 121-132.

Spackman, E. (2008). A Brief Introduction to the Avian Influenza Virus. Avian Influenza Virus 436(5): 1-6.

Stafseth, H. (1958). Advances in knowledge of poultry diseases over the past fifty years. Poultry Science 37(4): 741-775.

Stear, M. J. (2005). OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Mammals, Birds and Bees) 5th Edn. Volumes 1 & 2. World Organization for Animal Health 2004. Parasitology 130(6): 727-727.

Stegeman, A., Bouma, A., Elbers, A. R., de Jong, M. C. M., Nodelijk, G., de Klerk, F., Koch, G. and van Boven, M. (2004). Avian influenza A virus (H7N7) epidemic in The Netherlands in 2003: course of the epidemic and effectiveness of control measures. The Journal of Infectious Diseases 190(12): 2088-2095.

Stubbs, E. (1965). Fowl plague. Diseases of Poultry: 813-822.

Studer, G., Rempfer, C., Waterhouse, A. M., Gumienny, R., Haas, J. and Schwede, T. (2019). QMEANDisCo—distance constraints applied on model quality estimation. Bioinformatics 36(6): 1765-1771.

Su, H., Zhao, Y., Zheng, L., Wang, S., Shi, H. and Liu, X. (2020). Effect of the selection pressure of vaccine antibodies on evolution of H9N2 avian influenza virus in chickens. AMB Express 10(1): 98.

Su, S., Bi, Y., Wong, G., Gray, G. C., Gao, G. F. and Li, S. (2015). Epidemiology, evolution, and recent outbreaks of avian influenza virus in China. Journal of Virology 89(17): 8671-8676.

Suarez, D. L., Senne, D. A., Banks, J., Brown, I. H., Essen, S. C., Lee, C.-W., Manvell, R. J., Mathieu-Benson, C., Moreno, V. and Pedersen, J. C. (2004). Recombination resulting in virulence shift in avian influenza outbreak, Chile. Emerging Infectious Diseases 10(4): 693.

Suárez, P., Valcarcel, J. and Ortin, J. (1992). Heterogeneity of the mutation rates of influenza A viruses: isolation of mutator mutants. Journal of Virology 66(4): 2491-2494.

Suchard, M. A., Lemey, P., Baele, G., Ayres, D. L., Drummond, A. J. and Rambaut, A. (2018). Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. Virus Evolution 4(1): vey016.

Sun, Y. and Liu, J. (2015). H9N2 influenza virus in China: a cause of concern. Protein & Cell 6(1): 18-25.

Sun, Y., Pu, J., Fan, L., Sun, H., Wang, J., Zhang, Y., Liu, L. and Liu, J. (2012). Evaluation of the protective efficacy of a commercial vaccine against different antigenic groups of H9N2 influenza viruses in chickens. Veterinary Microbiology 156(1-2): 193-199.

Sun, Y., Pu, J., Jiang, Z., Guan, T., Xia, Y., Xu, Q., Liu, L., Ma, B., Tian, F. and Brown, E. (2010). Genotypic evolution and antigenic drift of H9N2 influenza viruses in China from 1994 to 2008. Veterinary Microbiology 146(3-4): 215-225.

Suzuki, Y. and Gojobori, T. (1999). A method for detecting positive selection at single amino acid sites. Molecular Biology and Evolution 16(10): 1315-1328.

Swayne, D. and Suarez, D. (2000). Highly pathogenic avian influenza. Revue Scientifique et Technique-office International des Epizooties 19(2): 463-475.

Swayne, D. E. (2012). Impact of vaccines and vaccination on global control of avian influenza. Avian Diseases 56(4s1): 818-828.

Swayne, D. E., Beck, J. R., Perdue, M. L. and Beard, C. W. (2001). Efficacy of vaccines in chickens against highly pathogenic Hong Kong H5N1 avian influenza. Avian Diseases: 355-365.

Swayne, D. E. and Kapczynski, D. (2008). Strategies and challenges for eliciting immunity against avian influenza virus in birds. Immunological Reviews 225(1): 314-331.

Swayne, D. E. and Kapczynski, D. R. (2016). 15 Vaccines and vaccination for avian influenza in poultry. Animal Influenza: 378.

Swayne, D. E., Perdue, M. L., Beck, J. R., Garcia, M. and Suarez, D. L. (2000). Vaccines protect chickens against H5 highly pathogenic avian influenza in the face of genetic changes in field viruses over multiple years. Veterinary Microbiology 74(1): 165-172.

Takano, R., Nidom, C. A., Kiso, M., Muramoto, Y., Yamada, S., Sakai-Tagawa, Y., Macken, C. and Kawaoka, Y. (2009). Phylogenetic characterization of H5N1 avian influenza viruses isolated in Indonesia from 2003–2007. Virology 390(1): 13-21.

Taubenberger, J. K. and Morens, D. M. (2006). 1918 Influenza: the Mother of All Pandemics-Volume 12, Number 1—January 2006-Emerging Infectious Disease journal-CDC.

Taubenberger, J. K., Reid, A. H., Janczewski, T. A. and Fanning, T. G. (2001). Integrating historical, clinical and molecular genetic data in order to explain the origin and virulence of the 1918 Spanish influenza virus. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences 356(1416): 1829-1839.

Tavakkoli, H., Asasi, K. and Mohammadi, A. (2011). Effectiveness of two H9N2 low pathogenic avian influenza conventional inactivated oil emulsion vaccines on H9N2 viral replication and shedding in broiler chickens. Iranian Journal of Veterinary Research 12(3): 214-221.

Team, R. C. (2013). R: A language and environment for statistical computing.

Teng, Q., Shen, W., Liu, Q., Rong, G., Chen, L., Li, X., Chen, H., Yang, J. and Li, Z. (2015). Protective efficacy of an inactivated vaccine against H9N2 avian influenza virus in ducks. Virology Journal 12(1): 143.

Teng, Q., Xu, D., Shen, W., Liu, Q., Rong, G., Li, X., Yan, L., Yang, J., Chen, H., Yu, H., Ma, W. and Li, Z. (2016). A Single Mutation at Position 190 in Hemagglutinin Enhances Binding Affinity for Human Type Sialic Acid Receptor and Replication of H9N2 Avian Influenza Virus in Mice. Journal of Virology 90(21): 9806-9825.

Thomas, J. A., Welch, J. J., Lanfear, R. and Bromham, L. (2010). A Generation Time Effect on the Rate of Molecular Evolution in Invertebrates. Molecular Biology and Evolution 27(5): 1173-1180.

Tiensin, T., Nielen, M., Vernooij, H., Songserm, T., Kalpravidh, W., Chotiprasatintara, S., Chaisingh, A., Wongkasemjit, S., Chanachai, K., Thanapongtham, W., Srisuvan, T. and Stegeman, A. (2007). Transmission of the highly pathogenic avian influenza virus H5N1 within flocks during the 2004 epidemic in Thailand. The Journal of Infectious Diseases 196(11): 1679-1684.

Tobita, K., Sugiura, A., Enomoto, C. and Furuyama, M. (1975). Plaque assay and primary isolation of influenza A viruses in an established line of canine kidney cells (MDCK) in the presence of trypsin. Medical Microbiology and Immunology 162(1): 9-14.

Tollis, M. and Trani, L. D. (2002). Recent developments in avian influenza research: epidemiology and immunoprophylaxis. The Veterinary Journal 164(3): 202-215.

Umar, S., Delverdier, M., Delpont, M., Belkasmi, S. F., Teillaud, A., Bleuart, C., Pardo, I., El Houadfi, M., Guérin, J.-L. and Ducatez, M. F. (2018). Co-infection of turkeys with Escherichia coli (O78) and H6N1 avian influenza virus. Avian Pathology 47(3): 314-324.

Umar, S., Guerin, J. L. and Ducatez, M. F. (2017). Low pathogenic avian influenza and coinfecting pathogens: a review of experimental infections in avian models. Avian Diseases 61(1): 3-15.

Umar, S., Sarfraz, S., Mushtaq, A. and Attique, M. (2016). Emerging threat of H9N2 viruses in poultry of Pakistan and vaccination strategy. World's Poultry Science Journal 72(2): 343-352.

Uyeki, T. M. and Cox, N. J. (2013). Global concerns regarding novel influenza A (H7N9) virus infections. New England Journal of Medicine 368(20): 1862-1864.

Van den Hoecke, S., Verhelst, J., Vuylsteke, M. and Saelens, X. (2015). Analysis of the genetic diversity of influenza A viruses using next-generation DNA sequencing. BMC Genomics 16: 79.

Van der Goot, J., Koch, G., de Jong, M. C. M. and Van Boven, M. (2005). Quantification of the effect of vaccination on transmission of avian influenza (H7N7) in chickens. Proceedings of the National Academy of Sciences of the United States of America 102(50): 18141-18146.

Van der Goot, J., Van Boven, M., de Jong, M. C. M. and Koch, G. (2007). Effect of vaccination on transmission of HPAI H5N1: the effect of a single vaccination dose on transmission of highly pathogenic avian influenza H5N1 in Peking ducks. Avian Diseases 51(s1): 323-324.

Van der Goot, J. A., van Boven, M., Koch, G. and de Jong, M. C. M. (2007). Variable effect of vaccination against highly pathogenic avian influenza (H7N7) virus on disease and transmission in pheasants and teals. Vaccine 25(49): 8318-8325.

Vasfi, M. M. and Bozorg, M. M. (2002). Isolation of H9N2 subtype of avian influenza viruses during an outbreak in chickens in Iran. Iran Biomed Journal, 6(1), 13-17.

Velkers, F. C., Manders, T. T., Vernooij, J. C., Stahl, J., Slaterus, R. and Stegeman, J. A. (2020). Association of wild bird densities around poultry farms with the risk of highly pathogenic avian influenza virus subtype H5N8 outbreaks in the Netherlands, 2016. Transboundary and Emerging Diseases.

Velthuis, A. G. J., Bouma, A., Katsma, W. E. A., Nodelijk, G. and De Jong, M. C. M. (2007). Design and analysis of small-scale transmission experiments with animals. Epidemiology and Infection 135(2): 202-217.

Villarreal, C. (2007). Experience in control of avian influenza in the Americas. Developments in Biologicals 130: 53-60.

Voskarides, K., Christaki, E. and Nikolopoulos, G. K. (2018). Influenza Virus—Host Co-evolution. A Predator-Prey Relationship? Frontiers in Immunology 9(2017).

Wan, H. and Perez, D. R. (2007). Amino acid 226 in the hemagglutinin of H9N2 influenza viruses determines cell tropism and replication in human airway epithelial cells. Journal of Virology 81(10): 5181-5191.

Wan, X. F., Dong, L., Lan, Y., Long, L. P., Xu, C., Zou, S., Li, Z., Wen, L., Cai, Z. and Wang, W. (2011). Indications that live poultry markets are a major source of human H5N1 influenza virus infection in China. Journal of Virology 85(24): 13432-13438.

Wan, Z., Ye, J., Xu, L., Shao, H., Jin, W., Qian, K., Wan, H. and Qin, A. (2014). Antigenic mapping of the hemagglutinin of an H9N2 avian influenza virus reveals novel critical amino acid positions in antigenic sites. Journal of Virology 88(7): 3898-3901.

Wang, W., Artois, J., Wang, X., Kucharski, A. J., Pei, Y., Tong, X., Virlogeux, V., Wu, P., Cowling, B. J. and Gilbert, M. (2020). Effectiveness of live poultry market interventions on human infection with avian influenza A (H7N9) virus, China. Emerging Infectious Diseases 26(5): 891.

Wang, Y., Jiang, Z., Jin, Z., Tan, H. and Xu, B. (2013). Risk factors for infectious diseases in backyard poultry farms in the Poyang Lake area, China. PLoS One 8(6): e67366.

Watanabe, T., Watanabe, S., Shinya, K., Kim, J. H., Hatta, M. and Kawaoka, Y. (2009). Viral RNA polymerase complex promotes optimal growth of 1918 virus in the lower respiratory tract of ferrets. Proceedings of the National Academy of Sciences of the United States of America 106(2): 588-592.

Webster, R. G. (1999). Chapter 14 - Antigenic Variation in Influenza Viruses. Origin and Evolution of Viruses. E. Domingo, R. Webster and J. Holland. London, Academic Press: 377-390.

Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M. and Kawaoka, Y. (1992). Evolution and ecology of influenza A viruses. Microbiology and Molecular Biology Reviews 56(1): 152-179.

Wei, Y., Qi, L., Gao, H., Sun, H., Pu, J., Sun, Y. and Liu, J. J. S. R. (2016). Generation and protective efficacy of a cold-adapted attenuated avian H9N2 influenza vaccine. Scientific Reports 6, 1-10

Werrner, O. (1998). Avian influenza–situation in Germany 1995/1997. Proceedings of the Joint Fourth Annual Meetings of the National Newcastle Disease and Avian Influenza Laboratories of Countries of the European Union.–Brussels, Belgium.

Westgeest, K. B., Russell, C. A., Lin, X., Spronken, M. I., Bestebroer, T. M., Bahl, J., van Beek, R., Skepner, E., Halpin, R. A. and de Jong, J. C. (2014). Genomewide analysis of reassortment and evolution of human influenza A (H3N2) viruses circulating between 1968 and 2011. Journal of Virology 88(5): 2844-2857.

White, J., Kartenbeck, J. and Helenius, A. (1982). Membrane fusion activity of influenza virus. The EMBO Journal 1(2): 217-222.

WHO. (2018). Influenza (Seasonal). from https://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal).

WHO. (2019). Influenza Vaccines, from https://www.who.int/biologicals/vaccines/influenza/en/.

WHO. (2019). Influenza (seasonal). Retrieved 6 November 2018, from https://www.who.int/en/news-room/fact-sheets/detail/influenza-(seasonal).

WHO (2020). Influenza at the human-animal interface. Summary and assessment, 2020, from https://www.who.int/influenza/human_animal_interface/HAI_Risk_Assessment/en/.

Wilhelm, J. and Pingoud, A. (2003). Real-time polymerase chain reaction. Chembiochem 4(11): 1120-1128.

Winter, A. K., Pramanik, S., Lessler, J., Ferrari, M., Grenfell, B. T. and Metcalf, C. J. E. (2018). Rubella vaccination in India: identifying broad consequences of vaccine introduction and key knowledge gaps. Epidemiology and Infection 146(1): 65-77.

Wise, H. M., Foeglein, A., Sun, J., Dalton, R. M., Patel, S., Howard, W., Anderson, E. C., Barclay, W. S. and Digard, P. (2009). A complicated message: Identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA. Journal of Virology 83(16): 8021-8031.

Wise, H. M., Hutchinson, E. C., Jagger, B. W., Stuart, A. D., Kang, Z. H., Robb, N., Schwartzman, L. M., Kash, J. C., Fodor, E. and Firth, A. E. (2012). Identification of a novel splice variant form of the influenza A virus M2 ion channel with an antigenically distinct ectodomain. PLoS Pathogens 8(11): e1002998.

Wolff, T. and Veit, M. (2020). Influenza B, C and D Viruses. Reference Module in Life Sciences: B978-970-912-809633-809638.821505-809637.

Wong, E. H., Smith, D. K., Rabadan, R., Peiris, M., & Poon, L. L. (2010). Codon usage bias and the evolution of influenza A viruses. Codon Usage Biases of Influenza Virus. BMC Evolutionary Biology, 10(1), 1-14.

Woolson, R. (2007). Wilcoxon signed-rank test. Wiley Encyclopedia of Clinical Trials: 1-3.

Worobey, M., Han, G.Z. and Rambaut, A. (2014). Genesis and pathogenesis of the 1918 pandemic H1N1 influenza A virus. Proceedings of the National Academy of Sciences of the United States of America 111(22): 8107-8112.

Wu, P., Jiang, H., Wu, J. T., Chen, E., He, J., Zhou, H., Wei, L., Yang, J., Yang, B. and Qin, Y. (2014). Poultry market closures and human infection with influenza A (H7N9) virus, China, 2013–14. Emerging Infectious Diseases 20(11): 1891.

Wu, Y., Wu, Y., Tefsen, B., Shi, Y. and Gao, G. F. (2014). Bat-derived influenza-like viruses H17N10 and H18N11. Trends Microbiology 22(4): 183-191.

Wu, Z., Ji, J., Zuo, K., Xie, Q., Li, H., Liu, J., Chen, F., Xue, C., Ma, J. and Bi, Y. (2010). Cloning and phylogenetic analysis of hemagglutinin gene of H9N2 subtype avian influenza virus from different isolates in China during 2002 to 2009. Poultry Science 89(6): 1136-1143.

Xia, J., Cui, J.Q., He, X., Liu, Y.Y., Yao, K.C., Cao, S.J., Han, X.F. and Huang, Y. (2017). Genetic and antigenic evolution of H9N2 subtype avian influenza virus in domestic chickens in southwestern China, 2013–2016. PloS One 12(2): e0171564.

Xu, C., Fan, W., Wei, R. and Zhao, H. (2004). Isolation and identification of swine influenza recombinant A/Swine/Shandong/1/2003 (H9N2) virus. Microbes and Infection 6(10): 919-925.

Xu, K. M., Smith, G. J. D., Bahl, J., Duan, L., Tai, H., Vijaykrishna, D., Wang, J., Zhang, J. X., Li, K. S., Fan, X. H., Webster, R. G., Chen, H., Peiris, J. S. M. and Guan, Y. (2007). The genesis and evolution of H9N2 influenza viruses in poultry from southern China, 2000 to 2005. Journal of Virology 81(19): 10389-10401.

Yamayoshi, S., Watanabe, M., Goto, H. and Kawaoka, Y. (2016). Identification of a novel viral protein expressed from the PB2 segment of influenza A virus. Journal of Virology 90(1): 444-456.

Yang, J., Müller, N. F., Bouckaert, R., Xu, B. and Drummond, A. J. (2019). Bayesian phylodynamics of avian influenza A virus H9N2 in Asia with time-dependent predictors of migration. PLoS Computational Biology 15(8): e1007189-e1007189.

Yang, L., Zhu, W., Li, X., Bo, H., Zhang, Y., Zou, S., Gao, R., Dong, J., Zhao, X. and Chen, W. (2017). Genesis and dissemination of highly pathogenic H5N6 avian influenza viruses. Journal of Virology 91(5).

Yang, Y., Sugimoto, J. D., Halloran, M. E., Basta, N. E., Chao, D. L., Matrajt, L., Potter, G., Kenah, E. and Longini, I. M. (2009). The transmissibility and control of pandemic influenza A (H1N1) virus. Science 326(5953): 729-733.

Ye, G., Liang, C. H., Hua, D. G., Song, L. Y., Xiang, Y. G., Guang, C., Lan, C. H. and Ping, H. Y. (2016). Phylogenetic analysis and pathogenicity assessment of two strains of avian influenza virus subtype H9N2 isolated from migratory birds: high homology of internal genes with human H10N8 virus. Frontiers in Microbiology 7: 57.

Yoon, S. W., Webby, R. J. and Webster, R. G. (2014). Evolution and ecology of influenza A viruses. Influenza Pathogenesis and Control-Volume I: 359-375.

Yu, H., Hua, R. H., Wei, T. C., Zhou, Y. J., Tian, Z. J., Li, G. X., Liu, T. Q. and Tong, G. Z. (2008). Isolation and genetic characterization of avian origin H9N2 influenza viruses from pigs in China. Veterinary Microbiology 131(1-2): 82-92.

Yu, X., Jin, T., Cui, Y., Pu, X., Li, J., Xu, J., Liu, G., Jia, H., Liu, D. and Song, S. (2014). Influenza H7N9 and H9N2 viruses: coexistence in poultry linked to human H7N9 infection and genome characteristics. Journal of Virology 88(6): 3423-3431.

Yuan, J., Lau, E. H. Y., Li, K., Leung, Y. H. C., Yang, Z., Xie, C., Liu, Y., Liu, Y., Ma, X., Liu, J., Li, X., Chen, K., Luo, L., Di, B., Cowling, B. J., Tang, X., Leung, G. M., Wang, M. and Peiris, M. (2015). Effect of Live Poultry Market Closure on Avian Influenza A(H7N9) Virus Activity in Guangzhou, China, 2014. Emerging Infectious Diseases 21(10): 1784-1793.

Yuan, J., Tang, X., Yang, Z., Wang, M. and Zheng, B. (2014). Enhanced disinfection and regular closure of wet markets reduced the risk of avian influenza A virus transmission. Clinical Infectious Diseases 58(7): 1037-1038.

Zahn, R. C., Schelp, I., Utermohlen, O. and von Laer, D. (2007). A-to-G hypermutation in the genome of lymphocytic choriomeningitis virus. Journal of Virology 81(2), 457-464

Zehender, G., Pariani, E., Piralla, A., Lai, A., Gabanelli, E., Ranghiero, A., Ebranati, E., Amendola, A., Campanini, G. and Rovida, F. (2012). Reconstruction of the evolutionary dynamics of the A (H1N1) pdm09 influenza virus in Italy during the pandemic and post-pandemic phases. PLoS One 7(11): e47517.

Zhang, P., Tang, Y., Liu, X., Peng, D., Liu, W., Liu, H., Lu, S. and Liu, X. (2008). Characterization of H9N2 influenza viruses isolated from vaccinated flocks in an integrated broiler chicken operation in eastern China during a 5 year period (1998–2002). Journal of General Virology 89(12): 3102-3112.

Zhang, Y., Aevermann, B. D., Anderson, T. K., Burke, D. F., Dauphin, G., Gu, Z., He, S., Kumar, S., Larsen, C. N. and Lee, A. J. (2017). Influenza Research Database: An integrated bioinformatics resource for influenza virus research. Nucleic Acids Research 45(D1): D466-D474.

Zhang, Y., Yin, Y., Bi, Y., Wang, S., Xu, S., Wang, J., Zhou, S., Sun, T. and Yoon, K. J. (2012). Molecular and antigenic characterization of H9N2 avian influenza virus isolates from chicken flocks between 1998 and 2007 in China. Veterinary Microbiology 156(3-4): 285-293.

Zhen, L., Amber, F., Guishuang, L., Gane, K. S. W., Andrew, L. M., David, B., Alyson, A. K., David, J. K. and Alberto, J. L. (2014). Next-generation sequencing and bioinformatic approaches to detect and analyze influenza virus in ferrets. The Journal of Infection in Developing Countries 8(04).

Zhou, J., Wu, J., Zeng, X., Huang, G., Zou, L., Song, Y., Gopinath, D., Zhang, X., Kang, M., Lin, J., Cowling, B. J., Lindsley, W. G., Ke, C., Peiris, J. S. and Yen, H. L. (2016). Isolation of H5N6, H7N9 and H9N2 avian influenza A viruses from air sampled at live poultry markets in China, 2014 and 2015. Eurosurveillance 21(35).

Zhou, L., Chen, E., Bao, C., Xiang, N., Wu, J., Wu, S., Shi, J., Wang, X., Zheng, Y. and Zhang, Y. (2018). Clusters of human infection and human-to-human transmission of avian influenza A (H7N9) virus, 2013–2017. Emerging Infectious Diseases 24(2): 397.

Zhu, C., Hu, C., Gui, B., Chen, Q., Zhang, S. and He, G. (2018). Genetic characteristics of H9N2 avian influenza viruses isolated from free-range poultry in Eastern China, in 2014–2015. Poultry Science 97(11): 3793-3800.

Zhu, R., Xu, D., Yang, X., Zhang, J., Wang, S., Shi, H. and Liu, X. (2018). Genetic and biological characterization of H9N2 avian influenza viruses isolated in China from 2011 to 2014. PLoS One 13(7): e0199260.

Summary

Low pathogenic avian influenza virus (LPAIV) H9N2 is one of the three avian influenza viruses (AIVs) primarily threatening poultry industry and human health, together with H5 and H7 subtype AIVs. The H9N2 AIV has become endemic in poultry in many countries all over the world. H9N2 AIVs also serve as gene donors for other subtype influenza viruses that can infect humans. In several Asian countries, such a South Korea, China, Pakistan and Iran, vaccination against H9N2 AIV was permitted to reduce economic losses in the poultry industry. However, surveillance programs initiated after the introduction of vaccination identified the persistence of H9N2 AIV in poultry (especially in chicken in South Korea and China). The aim of the research described in this PhD thesis was to quantify the transmission of LPAIV H9N2 in vaccinated chicken and to identify the characteristics and evolutionary path of the H9N2 AIV currently circulating in vaccinated poultry in China.

In **Chapter 2** the transmission parameters of H9N2 AIV in vaccinated chicken were determined. Therefore, the natural infection and transmission process was mimicked in transmission experiments in chicken vaccinated with the inactivated H9N2 vaccine and challenged with the same H9N2 strain. Using a stochastic SIR model, the estimated reproduction ratio (R) of the H9N2 strain in non-vaccinated and vaccinated chicken was above one. The inactivated H9N2 vaccine was able to provide individual protection by reducing the virus shedding in high-antisera individuals, but could not stop the transmission of the H9N2 AIV in chickens vaccinated with a homologous inactivated virus vaccine.

Under the observed condition that the transmission continues in the vaccinated poultry population, **Chapter 3** aimed to analyze surveillance data from local markets in southern China from 2013 till 2018 to determine how the H9N2 AIV in a partially vaccinated bird population changed over time. This surveillance consisted of 13981 samples from chickens, ducks, geese and pigeons collected at a number of local markets located in nine provinces. This survey revealed that the isolation rate of H9N2 AIV remained high in local markets, although vaccination was initiated in poultry in 1998. The H9N2 subtype was found in approximately 78% (1211 /1549) of avian influenza-positive samples. Next, the whole genome of 126 H9N2 isolates was determined and used for phylogenetic and antigenic analyses to be able to follow the evolution of H9N2 from 2013 to 2018. The phylogenetic tree showed that current strains are rooted from the BJ94 lineage and evolved into two new subgroups (subgroup II and III) with a large genetic distance from the vaccine strain (F98), which belongs to subgroup I. Based on the antigenic distances comparing to F98 strain in HI assay, isolated strains in the two new subgroups clustered into two new antigenic clusters, respectively. The estimated mutation rate of subgroup III viruses was 6.23×10^{-3} substitutions/site/year, which was 1.5-fold faster than that of the average H9N2 HA open reading frame (3.95×10^{-3} substitutions/site/year). New antigenic properties of subgroup III viruses were identified at eleven amino acid positions in the HA protein sequence, which are examples of antigenic drift and potentially change the immunogenic properties of the H9N2 AIVs.

In **Chapter 4** the continuous transmission of the H9N2 strain (the same strain as used in Chapter 2) was simulated via serial *in vivo* passaging in different hosts, with and without homologous vaccination, and in cell lines. The HA genes from the progeny viruses were sequenced with the Illumina MiSeq platform using Next Generation Sequencing (NGS) to determine evolutionary parameters, e.g. genetic diversity, general substitution frequency, genetic distance, and dN/dS ratios. In the chickens' respiratory system, the average substitution

frequency was approximately 2.38×10^{-3} s/n/g (substitutions/nucleotide/infection generation), whereas *in vitro* it was 6.77×10^{-4} s/n/g. Besides, the genetic distances of progeny viruses to parent virus were significantly lower *in vivo* than those *in vitro.* Furthermore, I identified a total of six non-synonymous mutations, which were potential mutations related to antigenic changes due to their location in the encoded HA protein. The methods and observations of the molecular evolution of the H9N2 AIV under continuous selective pressure provide references for evolutionary research on other low pathogenetic RNA viruses.

In **Chapter 5** the characteristics and compatibility of novel viruses that may arise by reassortment of human and avian influenza viruses were analyzed. Reassortant viruses were generated by reverse genetics in which genes of a human H1N1 virus (A/WSN/1933) were replaced by genes of an avian H9N2 virus (A/chicken/Jiangsu/A2093/2011). Both the HA and NA (Neuraminidase) genes in combination with one of the genes involved in the viral ribonucleoprotein (RNP) complex (either PB2, PB1, PA or NP) were replaced. Reassortant viruses were able to replicate in canine MDCK and chicken DF1 cells, as well as in mice. In particular, for the reassortant virus with an avian PB1 gene, an enhanced virulence for mice was measured by increased body weight loss after infection. In addition, the avian PB1 gene increased the polymerase activity of the RNP complex in luciferase reporter assays. With the NGS data of reassortant viruses passaged for five generations, a higher substitution rate for the PB1-reassortant virus in mice was estimated. The higher polymerase activity and the increased mutation frequency suggests that the avian PB1 gene drives the evolution and adaptation of novel reassortant viruses to the human host. Therefore, surveillance for infections with H9N2 viruses and the emergence of novel reassortant viruses in humans is important for pandemic preparedness.

In **Chapter 6**, I have integrated the discoveries and conclusions from the earlier chapters and discussed the relationships between weak herd immunity protection and the transmission of H9N2 in host populations. I further elaborated on the evolutionary changes occurring in H9N2 AIV due to: 1) selection by immunogenic pressure from vaccination and 2) reassortment with human strains (for instance, H1N1), and compared my data with previous research. Last but not least, I discussed the advantages and disadvantages of the methods used in this research. The identified molecular changes and evolutionary patterns provide a reference for vaccine development and vaccination strategies in poultry

Acknowledgements

Now is the time to terminate the fantastic journey of my five-year PhD. I am lucky and have been taken great pleasure in meeting so many fabulous people during this journey.

First off, I would like to express my appreciation for the 2016 Sino-Dutch joint PhD program between the Chinese Academy of Agricultural Sciences (CAAS) and Wageningen University & Research (WUR). Being involved in this program was an honour and a challenging opportunity for me to continue in academia. I am very grateful for the fund, and all the facility supports from Prof. Zejun Li's lab group at SHVIR, CAAS. My promotor and co-promotors in WUR and SHVIR have a token of my gratitude. Mart de Jong, Monique van Oers, Nancy Beerens and Zejun Li, you are my supervisors but also my precious friends of life, guiding me with the knowledge from the senior generation:

Mart, how can I express my adoration for your wisdom. Your passion for statistics and modelling explained your patience when you taught me the transmission models' primary mechanism. Our weekly meetings in which we had our "statistical lessons" and efficient discussions guaranteed the process of my PhD and raised me towards the "the shoulders of giants" in Statistical analysis. You were supportive throughout my PhD research with your patience and knowledge in modelling. Moreover, I benefited greatly from the "old" books you lent to me during the "quarantine time". Your appreciation for Modern Art inspired me to appreciate the beauty of life besides the logical reasoning in Sciences. I appreciate your kindness in "carpooling", driving me between Wageningen and Lelystad in the first year of my PhD. I enjoyed chatting with you during the journeys, exchanging our opinions about life.

Monique, I am much obliged to you for your timely feedback and thoughtful suggestions. Your expertise in virology inspired me to consider research in a broader view. I appreciate your effort in the writing of my thesis. The way you guided me to refine the general introduction and discussion taught me how to write logically. All your kind reminding and timely advice promoted the process of finishing my PhD. Your kindness in ordinary life brought me the warmth of friendship. All these have made the tough PhD life enjoyable

Nancy, I am grateful for your supervision of my experiments and the arrangement for the training in WBVR. I enjoyed every moment of working with you in either the period of thesis writing or the experience in HCU at WBVR. Your critical questions on the results led me to clarify the conclusions using logical reasoning.

Zejun, the thesis could not have been done without your original idea and the generous supports from you and your group. I am lucky to have obtained your supervision in both my master's and doctoral research in SHVRI. You have set me an example of being a diligent and insightful explorer in life science.

Claudius, my dear KAKA. I should first thank you for your lectures and your endless effort in the process of this joint PhD program. Your sincere consideration and kindness made me feel at home when I was still a stranger in Wageningen. Secondly, I must thank you for all your challenging questions that pushed me to be myself and live a life without useless worries. Your guides make me not only a Dr. but also a real human enjoying life.

Maria Forlenza, my enthusiastic PhD advisor, I appreciate every professional suggestion from you in my PhD life. All the pleasant conversations we had together have built up competence and confidence for my career after this PhD.

Many thanks to all the colleagues who offered professional help and advice in research: Marc Engelsma, thank you for your time and energy guiding me in phylogenetic analysis and

teaching me the relative technique about BEAST. You Chang, my "teacher" on statistics in R and my office accompany in Radix. I will miss the time we worked together in the office before the outbreak of Covid-19. Qiaoyang Teng, I am grateful for the generous offer of your data and efforts on one chapter in this thesis. Wanwan Yan, Guangsheng Che and Luzhao Li, it was nice working with you on this project. I appreciate your assistance, time and energy in helping me with my experiments in SHVRI. Dawei Yan, Qinfang Liu, Xuesong Li, Jianmei Yang, Chunxiu Yuan, thank you for providing technical help or advice that guaranteed my research's continuation.

My appreciations also go to my colleagues in the QVE group. It is an honour to work in QVE group, experiencing its development in the PhD journey. The pandemic blocked our communication in person, but not online. All the professional discussions we had in the group meetings informed me of broader scientific ideas. Every Wednesday's social time with some of you will be a precious memory of working in a group. I have specific thanks to Kiki; thank you for the inspiring questions guiding me to make my propositions.

Still, I cannot forget the "gezellige" time working in WBVR in 2017. Saskia Oosterbroek, Sandra Venema-Kemper, René Heutink, Sylvia Pritz-Verschuren, Olav de Leeuw, and Jan Priem, thank you for your advice and help with my lab work. I enjoyed your guidance in the lab and the chats in "tea breaks".

Sincere thanks also go to Lisette, Fadma and Danielle; I am impressed that you were always ready to help with enthusiasm. Thank you, Roger, for improving my English in scientific writing. Thank you, Iris, for your lovely favour on the cover design of this thesis. I love it so much because it exactly expressed my ideas and surprised me with your creation. Your Artwork is always inspiring and thoughtful like that. Alan, thank you for painting the portrait of the "chicken family" for my thesis. It perfectly pays tribute to the birds that suffered from influenza in and out of the lab.

All my dearest friends contributing to my welfare and emotional health in my wonderful PhD life: Betty, I feel lucky and happy to be a friend of yours in such a short time in Lelystad. Your friendly invitation and hospitality in Lelystad and Venlo fulfilled the boring life without normal getting-together with friends. Besides, I appreciated that you spared no effort in helping me to make my life easier. Rocio, I love your Mexican tones and dances. What a memorable NEW YEAR eve of 2020 with you and Alan! Wish we can meet again in either China or Mexico City. Elisa, I enjoyed our cooking parties, yoga practices, and chattings with other PhD students exchanging amazing and terrible moments in research life.

With tons of thankfulness to Qiuhong, Fang, Xiaoqian, Fen, Jiaqi, Xiulu, Siyuan and all the other fellows in this joint program, I would like to wish you all the brightest future in academia or career. The time we spent together in Beijing and Wageningen is valuable memory in my life. Sincere gratefulness goes to Mandy (my BUDDY when I was in Wageningen in 2016), Xia Shuwen, Liu Langqing, Wuzhou, Yu Xiaofei, Peng Yebo, Lu Haibo, Akin Fatma, Jibrila Ibrahim, Barber Ann and Panxue, your showing-up in my life in the Netherlands bring me endless happiness and a new idea about real life. Thanks to Suxin, Liu Xingpo, Xu Bangfeng, Lin Weishan, Rong Guangyu, Qu Mengjin, Zhang Pei, Shi Xiaona, Wu Mi, you accompanied me and helped me during the bottleneck of my PhD in Shanghai. All your friendship was the strong mental support for me to be brave and to carry on.

Marlein, how lucky I am to have met you right before the pandemic stormed in the Netherlands in 2020. Covid-19 tore down my last-year PhD life, but you made it rise. You and your family, Henny and Eric, Lotte and Sven, gave me a home when I lived in a foreign country thousands of miles away from my motherland. Your parents' generous offer and selfless help on housing and ordinary life guaranteed my extended stay in the Netherlands. Every piece of memory being with you is the most precious treasures in my life. Your brave accompanies with my life adventures is the most profound confession of love. There is incredible gratitude for you and your family for the past, the current, and in the future.

My mother and father, my lovely sister, and other families, you are always standing by me, ever and forever. You are the root of the tree of my life, giving endless love and courage. Mom and Dad, I am growing up from all the arduous challenges we faced. I dare to be the roof protecting you and give you an enjoyable life for your later years. Hongyang, my dearest sister, thank you for sharing the happiness and pressure with me. With your accompany, I will never feel lonely in any corner of the world.

Last but not least, I am grateful for and grieve sincerely over the animals sacrificed in this project. No matter how careful we are about animal welfare and ethics, I still feel shame to take advantage of the life of other creatures for research. I wish the contributions of this thesis can rescue the animals suffering from influenza in the future. Expect one day in the future, and natural science would reduce the sacrifice of animals.

在这里要特别感恩父母给我的所有--健康的身体、坚韧的性格和自由的人生。你们坚忍不屈、乐观自信、达观开放、善良勤恳，你们用青春和暮年对抗生活的艰辛却从不想给我施加任何压力；你们用挚诚书写的人生教会了我怎样做人做事，怎样勇敢执着。我将这份终于拿到的博士学历作为送给你们第一份礼物。

还有伴我长大朋友们，马明珠、刘艳雨、崔宏明以及所有生命中不离不弃的朋友，谢谢你们给的爱和支持。时空的距离从来没有拉远我们彼此，生活的浮沉没有抹去我们儿时的天真。不论我们走多远，都在彼此生命中有个安身之所。牛琮，苏平如，柴呈欣，很荣幸在我的人生之旅中与你们相遇，那么多美妙的相聚让我永远对生活报以热忱和感恩。“磊爷”，大为，“凯哥”，我的好“哥们”更是好“哥哥”。在相守的日子里，你们给予的无私帮助和鼓励是我从硕士走到博士的阶石。人生长途，愿诸君安好，有缘常聚。

徐海华，在我拿到人生最后一个学位的时候，还是想谢谢你在我青春叛逆的学生时代所给予的关爱。很感激你曾悉心引导那个迷失的少年；很感激你曾耐心陪伴每一段“黑暗”的时光；每每回忆起促膝长谈的日日夜夜，都会欣然感到仿佛若有光，照进我的心房。于是，愤怒的少年不在，怯懦的自我不在，走来了平平常常、安安稳稳的我，用一生去学习去成长。

石迎，我的最亲爱的姐姐。三生有幸在上兽研成为你的师妹，今生有福我们成了相濡以沫的亲人。感恩你在我读博期间给与的包容、体贴、悉心照顾。在我和家人最艰难的时候，你无私的帮助；在我失眠哭泣的每个日夜，你安静的陪伴；我们一起做过的“可怕的“动物实验，我们一起讨论的科研结果，我们一起吵过的架，我们一起练过的瑜伽。从 2013 到 2020，从我的硕士开始到博士结束，我珍惜和感恩我们的分分秒秒、点点滴滴。从 2021 到很久以后，愿你我开启的新的人生会始终相映相衬，岁月静好。

最后，感恩我生命中的所有遇见。你们是我天空中永远闪烁的明星。

明星，请永远闪耀!
指引我前行
幸福地
坚忍地
从此不必怯懦彷徨

Hongrui Cui 崔宏锐

12 November 2021, Wageningen


Alan

Curriculum Vitae

Hongrui Cui (崔宏锐) was born on 15th October 1990 in Hebei, China. She developed her love of nature in childhood. She has been passionate about biology and life science since middle school. From 2009 to 2013, she studied Animal Medicine at Yanbian University and obtained her bachelor's degree with the thesis titled "Producing a monoclonal antibody (McAb) binds to the E gene of DTMUV". From then on, she started her research study in virology. From 2013 to 2016, she was on her MSc study at Shanghai Veterinary Research Institute, the Chinese Academy of Agricultural Sciences (SHVRI, CAAS). She obtained her MSc degree with the thesis titled "Phylogenetic analysis and pathogenicity of H3 subtype avian influenza viruses isolated in live poultry market of southern China". During these years, she achieved Excellence in Curriculum (2014) and Boehringer-Inglheim Scholarship (2016). She carried on with her enthusiasm on life science and the evolution of viruses for her PhD adventure in the "2016 Sino-Dutch joint PhD program" between the Chinese Academy of Agricultural Sciences (CAAS) and Wageningen University & Research (WUR). She was enrolled through the Quantitative Veterinary Epidemiology Group of Wageningen Institute of Animal Sciences (WIAS) and the Laboratory of Virology of Production Ecology and Resource Conservation (PE&RC). She spent 2.5 years in SHVRI for the primary research of her PhD thesis and minor project on "Evolution and pathogenicity of H6 avian influenza viruses isolated from southern China during 2011 to 2017 in mice and chickens". The PhD project was finished under the supervision of Prof. Mart de Jong, Prof. Monique van Oers, Dr Nancy Beerens, and Prof. Zejun Li. The results of the research are presented in this thesis.

Peer-reviewed publications

Cui, H., De Jong, MCM., et al. Vaccination with inactivated virus against low pathogenic avian influenza subtype H9N2 does not prevent transmission of the virus in chicken. Journal of Virus Eradication, 2021.

Cui, H., Li, Z., et al. Molecular characterization of antigenic drift of avian H9N2 viruses in China. Microbiology Spectrum, 2021. (under review)

Cui, H., Beerens, N., et al. The PB1 gene from H9N2 avian influenza virus showed high compatibility and increased mutation rate after reassorting with a human H1N1 influenza virus. Virology Journal, 2021. (under review)

Cui, H., Lin, W., Zejun Li, et al. Evolution and pathogenicity of H6 avian influenza viruses isolated from Southern China during 2011 to 2017 in mice and chickens. Scientific Reports, 2020, 10(1): 1-12. (shared first authorship)

Yang, J., **Cui, H.**, Teng, Q., et al. Ducks induce rapid and robust antibody responses than chickens at early time after intravenous infection with H9N2 avian influenza virus. Virology Journal, 2019, 16(1): 1-8.

Shi, Y., **Cui H.**, Wang, J., et al. Characterizations of H4 avian influenza viruses isolated from ducks in live poultry markets and farm in Shanghai. Scientific Reports, 2016, 6(1): 1-11.

Cui, H., Shi, Y., Ruan, T., et al. Phylogenetic analysis and pathogenicity of H3 subtype avian influenza viruses isolated from live poultry markets in China. Scientific Reports, 2016, 6(1): 1-11.

Yan, L., Liu, Q., **Cui, H**., et al. Pathogenicity of reassortant H9 influenza viruses with different NA genes in mice and chickens. Veterinary Research, 2016, 47(1): 1-6.

Conference proceedings

Cui, H., Yan, D.W., and Li, Z.J. (2017). Phylogenetic analysis and pathogenicity of H3 subtype avian influenza viruses isolated from live poultry markets in China. in The 7th Wuhan International Symposium on Modern Virology (Wuhan, China).

Cui, H. and De Jong, MCM. (2020). Vaccination effect of inactivated H9N2 vaccine on transmission in chickens. in Society for Veterinary Epidemiology and Preventive Medicine (Westport, Ireland) online version.

Cui, H. and De Jong, MCM. (2021). Evolutionary changes of a low pathogenic avian influenza virus (H9N2) in poultry under the selective pressure from inactivated vaccine. in WIAS Science day 2021 (Wageningen, Netherland) online version.

Training and education

The Basic Package (2.9 ECTs)

WIAS Introduction Day	2017
WGS Scientific Integrity course or alternative scientific integrity course	2020
WGS Ethics in Animal Sciences course or alternative ethics course	2020
Introduction course on Personal Effectiveness	2021

Disciplinary Competences (18.1 ECTs)

Fundamental and Applied Virology(MSc course)	2017
WIAS Course: Statistics for the Life Sciences	2017
Animal Health and Immunology (AHI) Discussion Group	2017
Quantitaive genetics discussion group (QDG)	2020-2021
Literature Survey	2018
External training period (three months) for molecular experiment in HCU of WBR	2018-2020
Management of Infections and Diseases in Animal Populations (MSc level)	2020
Statistics and R-online course from HarvardX	2020
Reviewing Ph.D proposal	2021
Generalized Linear Models	2021

Professional Competences (10.3 ECTs)

Efficient and Effective Academic Development	2016
Academic Writing and Presenting in English	2016
Data Management Planning	2017
The Essentials of Scientific Writing and Presenting	2017
Meta-analysis	2020
Critical Thinking and Argumentation	2020
Reviewing a Scientific Manuscript	2020
Tody data transformation and visualization with R	2020
WAC2021 Organizasion Committee	2020-2021

Societal Relevance (1.5 ECTs)

Societal impact of your research	2020

Presentation Skills (4.0 ECTs)

The 7th Wuhan International Symposium on Modern Virology, Wuhan, China (poster)	2017
FameLab, "A race with Influenza Virus", Wageningen, Netherlands (oral presentation)	2017
Society for Veterinary Epidemiology and Preventive Medicine, Westport, Ireland (poster)	2020
WIAS Science day 2020, Wageningen, Netherlands (poster)	2020
WIAS Science day 2021, Wageningen, Netherlands (oral presentation)	2021

Teaching competences (1.5 ECTs)

Supervising theses (1.5 c MSc minor) in SHVRI, Shanghai, China	2017-2019

Total credits (ECTs)	**38.3**

Colophon

The research described in this thesis was financially supported by the National Key Research and Development Program of China (2016YFD0500204) and the Chinese Academy of Agricultural Sciences of Technology Innovation Project.

Hongrui Cui was sponsored by China Scholarship Council (CSC) fellowship for 18 months (File No. 201903250113).

Financial support from Wageningen University for printing this thesis is gratefully acknowledged.

Cover design by Iris Winkel (iris_winkel@hotmail.com) and Hongrui Cui

Drawing from Alan Heiblum (mulbieh@gmail.com)

Printed by DigiForce | Proefschriftmaken.nl