H9N2 Avian Influenza A virus: Impact of serial passaging by aerosol exposure on pathogenicity in chickens.

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Abstract

The H9N2 low pathogenic avian influenza virus (LPAIV) is considered a prime candidate for evolving into a highly pathogenic AIV (HPAIV) following circulation in domestic poultry, due to its widespread endemism in Eurasia and the donation of internal genes to H5 and H7 HPAIV. In this study, we investigated if a LP H9N2 virus could acquire increased pathogenicity following nineteen serial passages in week-old chickens. We show that the H9N2 virus remained low pathogenic in chickens after nineteen serial passages, evidenced by a lack of mortality in chickens despite efficient viral replication in chicken organs. An overregulation of cytokine response to infection, typical of highly pathogenic viruses, was not observed in tissue organs analyzed by quantitative PCR. The serial passaging of the H9N2 virus led to reduced virulence in chicken embryos and reduction in Hemagglutinin heat stability after passaging. The positive selection of Leucine at amino acid position 226 (L226) after 19 passages suggests the H9N2 can gain mammalian adaptation markers following circulation in domestic poultry. In addition, we carried out four experiments to determine the effectiveness of aerosol exposure of H6N1, H10N7, H10N8 and H13N6 LPAIV's in causing infection, immune dysregulation and mortality in chickens compared to intranasal and oral inoculation routes. From our study, we observed mortality of chickens exposed to H6N1 and H10N8 viruses via aerosols. Aerosol exposure also resulted in more efficient replication in the respiratory tracts of chickens than intranasal or oral inoculated chickens. In addition, overexpression of pro-inflammatory (IL-6, IL-1ß) and antiviral (INF- γ) cytokines was observed in chickens exposed to aerosols compared to intranasal and oral inoculation. Our results show that the aerosol route of exposure is efficient at causing infection in chickens and should be factored into control and prevention strategies against AIV.

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Table of Contents

ABSTRACT II
ACKNOWLEDGEMENT III
LIST OF ABBREVIATIONS VI
LIST OF TABLES VIII
LIST OF FIGURES IX
1. INTRODUCTION 1
1.2 INFLUENZA VIRUS CLASSIFICATION 2
1.3 GENOME STRUCTURE AND COMPOSITION 3
1.4 VIRUS LIFECYCLE 6
1.5 VIRUS ISOLATION AND LABORATORY DIAGNOSIS 7
1.6 HOST RANGE AND TRANSMISSION 8
1.7 PATHOBIOLOGY IN POULTRY 12
1.8 DETERMINANTS OF HOST RANGE AND PATHOGENESIS OF AIV 13
1.8.1 Hemagglutinin 13
1.8.2 Neuraminidase 21
1.8.3 Polymerase complex and other viral proteins 23
1.8.4 Immune response 25
1.9 RATIONALE OF STUDY 27
1.10 HYPOTHESIS 29
1.11 OBJECTIVES 29
CHAPTER 2 30
2 INTRODUCTION 31
2.1 MATERIALS AND METHODS 35
2.1.1 Chickens and embryonated chicken eggs (ECEs) 35
2.1.2 Virus 35
2.1.3 Virus titration and Hemagglutinin test (HA test) 36
2.1.4 Thermostability assay 36
2.1.5 Chicken experiments 37
2.1.6 Sample Collection 37
2.1.7 RNA Extraction and Real time reverse transcriptase-PCR (RRT-PCR) 40
2.1.8 cDNA synthesis and Quantification of cytokine gene expression 41
2.1.9 Sequencing and sequence analysis 42
2.1.10 Statistical Analysis. 43
2.2 RESULTS 44
2.2.1 Positive selection of Q226L mutation observed following passaging in chickens 44
2.2.2 H9N2 AIV exhibits low pathogenicity in chickens following nineteen serial passages. 49
2.2.3 H9N2 AIV's have a tropism for the respiratory tract. 49
2.2.4 P0 and P19 AIV's replicate efficiently in systemic tissues 52

2.2.5 *Early induction of proinflammatory and antiviral cytokines in lungs of infected chickens.* 52
2.2.6 *Induction of proinflammatory and antiviral cytokines in spleen tissues.* ... 58
2.2.7 *Virulence in chicken embryos* ... 58
2.2.8 *Reduction in HA thermostability* ... 63
2.3 DISCUSSION ... 66

CHAPTER 3 ... **71**
3 INTRODUCTION ... 72
3.1 MATERIALS AND METHODS ... 76
3.1.1 *Virus* ... 76
3.1.2 *Chicken experiments.* ... 76
3.1.3 *Sample collection* ... 77
3.1.4 *RNA extraction and real time reverse transcriptase-PCR (RRT-PCR)* ... 77
3.1.5 *cDNA synthesis and quantification of cytokine gene expression* ... 78
3.1.6 *Statistical analysis.* ... 79
3.2 RESULTS ... 80
3.2.1 *Aerosol inoculation results in mortality in chickens depending on viral strain* ... 80
3.2.2 *Intranasal and aerosol inoculation cause efficient infection in the upper respiratory tract* 80
3.2.3 *Aerosol route of inoculation is more effective in causing infection in the lower respiratory tract of chickens* ... 84
3.2.4 *Virus in cecum tonsil, cecum pouch tissues.* ... 87
3.2.5 *Aerosol inoculation results in increased expression of cytokines in trachea and lungs of chickens.* 87
3.3 DISCUSSION ... 96

CHAPTER 4 – GENERAL DISCUSSION AND CONCLUSION ... **99**
4.1 DISCUSSION ... 100
4.2 CONCLUSION AND FUTURE WORK ... 104

CONTRIBUTION OF COLLABORATORS ... **123**

List of abbreviations

AIV	Avian Influenza A virus
AGID	Agar gel immunodiffusion
CFIA	Canadian food inspection agency
DPI	Days post infection
EID_{50}	50% Embryo infectious dose
ELD_{50}	50% Embryo lethal dose
ELISA	Enzyme Linked Immunosorbent Assay
ECE	Embryonated chicken eggs
Gal	Galactose
HA	Hemagglutinin
HI	Hemagglutinin inhibition test
HPAIV	High pathogenic avian influenza A virus
IFN	Interferon
IL	Interleukin
IVPI	Intravenous pathogenicity index
LBM	Live bird market
LPAIV	Low pathogenic avian influenza A virus
MDCK	Madin-Darby Canine Kidney epithelial cells
M1	Matrix 1
M2	Matrix 2
M42	Matrix 42

mRNA	Messenger RNA
NA	Neuraminidase
NEP	Nuclear export protein
NP	Nucleoprotein
NS1	Non-structural protein 1
NS2	Non-structural protein 2
NS3	Non-structural protein 3
RT-PCR	Real time reverse transcriptase polymérase chain reaction
P0	Wild type H9N2 virus
P8	Passage 8
P19	Passage 19
PA	Acidic polymerase
PB1	Basic polymerase 1
PB2	Basic polymerase 2
RBS	Receptor binding site
RNP	Ribonucleoprotein
SA	Sialic Acid
SPF	Specific pathogen free
TLR	Toll like receptor
vRNP	Viral ribonucleoprotein
vRNA	Viral RNA

List of Tables

TABLE 1: Nucleotide and aminoacid substitutions in gene segments of the H9N2 virus 48

TABLE 2: Tissue tropism of H6N1 and H10N8 viruses in dead chickens 81

List of Figures

FIGURE 1: The structure of influenza a virus. 4

FIGURE 2: Diagram of an aerosol chamber 38

FIGURE 3: Amino acid sequence alignment of the HA1 and HA2 subunits 45-46

FIGURE 4: H9N2 virus infection in oropharyngeal swabs, tracheal and lung tissues 50

FIGURE 5: H9N2 virus infection in cecum tonsil, kidney and spleen tissues 53

FIGURE 6: Cytokine mRNA expression of IL-6 IL-1ß and INF-γ in Lung tissues 55

FIGURE 7: Cytokine mRNA expression of IL-6, IL-1ß and INF-γ in spleen tissues 59

FIGURE 8: Pathogenicity assessment of H9N2 viruses in 10-day-old ECE 61

FIGURE 9: Thermal inactivation kinetics of heat treatment on HA activity 64

FIGURE 10: H6N1, H10N7 and H10N8 virus infection in oropharyngeal swabs 82

FIGURE 11: H6N1, H10N7 and H10N8 virus infection in trachea and lung tissue 85

FIGURE 12: H6N1, H10N7 and H10N8 virus infecion in cecum tonsil and pouch tissues 88

FIGURE 13: H6N1 virus cytokine mRNA expression in tracheal and lung tissues 90

FIGURE 14: H10N8 cytokine mRNA expression in tracheal and lung tissues 94

1. Introduction

Chickens have long been reared by human communities as a source of meat and eggs for food. Due to its importance as an agricultural commodity, poultry rearing increased in size and production standards, from open range rearing of chickens in the late 1800's to large scale commercial poultry rearing farms generating billions in revenue from broiler and egg sale. The economic gain brought by the poultry industry, however, comes with its disadvantages. The overcrowding of poultry barns and cages results in a favourable environment for the spread of the avian influenza A virus (AIV)[1]. Outbreaks of AIV in poultry cause significant economic loss for the poultry industry, affecting both producers and consumers. Economic losses are usually associated with increased costs of prevention and eradication strategies as well as trade restrictions to prevent spread of virus infection[2,3].

AIV is introduced in its Low pathogenic (LP) form causing mild to no disease in poultry, but after circulation in chickens over a period of time, can evolve into a highly pathogenic (HP) form exhibiting increased mortality in avian and mammalian species[3]. The circulation of AIV seems more likely to occur via aerosol transmission in poultry populations. AIV can be carried in aerosols generated from chickens in crowded conditions and dispersed across farm houses[4]. Feathers of poultry birds, waste matter, fodder, and dust are common sources of aerosol generation[5]. AIV has been detected in the air around poultry and livestock houses which is worrisome considering the low dose requirement of the virus in aerosols for causing infection in susceptible hosts[6,7]. HP outbreaks in poultry are documented to spill over to humans either by contact or aerosol transmission[3]. To date, only H5 and H7 viruses have been able to generate high pathogenic viruses from low pathogenic progenitors for yet to be determined reasons[3].

However, LP viruses of the H6, H9 and H10 subtypes are documented to cause infection in humans in the last few decades following possible transmission from poultry[8–10]. Of importance is the H9N2 virus which is widely circulated in domestic poultry and capable of being spread through airborne transmission, crossing species barrier, and donating its internal genes to HP H5 and H7 viruses[9,11–14]. It is important to study the evolution and transmission of AIV in poultry to gain further understanding of the AIV in view of preventing or controlling pandemic outbreaks. This thesis research focuses on the evolution of an H9N2 LP AIV following nineteen passages in chickens via aerosol inoculation and the importance of aerosol inoculation in infection of low pathogenic avian influenza viruses in chickens.

1.2 Influenza virus classification

Influenza viruses are negative sense, single stranded, segmented, enveloped viruses of the Orthomyxoviridae family. Six genera are classified in this family of viruses namely, three influenza viruses A, B, and C, Isavirus, Thogotovirus, and Quaranjavirus. Influenza B and C viruses infect mainly mammalian species while Influenza A viruses infect both avian and mammalian species. Influenza A viruses are differentiated from the viruses in B and C genera on the basis of the identity of the nucleoprotein (NP) and matrix (Ml) proteins[15].

Influenza A viruses are further classified into subtypes based on the antigenic properties of the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). To date, 18 HA and 11 NA subtypes have been identified. HA (1-16) and NA (1-9) have been identified in birds while H17 and H18 subtypes were isolated in bats[16]. Phylogenetically, the 16 HA subtypes fall into two groups with the first represented by H1, H2, H5, H6, H8, H9, H11, H12, H13 and H16, while the second group by H3, H4, H7, H10, H14 and H15 subtypes. The 9 NA subtypes are also separated into two groups with group 1 represented by N1, N4, N5 and N8, and the second by

N2, N3, N6, N7 and N9 subtypes[17].

The Influenza A virus will be the focus of this thesis. The current nomenclature system for influenza A viruses includes the host of origin, geographic location of first isolation, strain number and year of isolation. The HA and NA subtype of influenza A viruses is specified in parentheses, e.g., A/Chicken/Henan/01/98 (H9N2). In poultry, the AIV is further classified based on the severity of infection into low pathogenic AIV (LPAIV) and highly pathogenic AIV (HPAIV). Classification as HPAIV is based on the ability to cause at least 75% mortality in a minimum of eight 4-8 week old chickens within ten days of being inoculated by the intravenous route and also on the possession of polybasic cleavage sites in the HA protein associated with HPAI viruses[18]. Only H5 and H7 viral subtypes have been designated as HPAI viruses to date following isolation during natural outbreaks in poultry[3].

1.3 Genome structure and composition

The virus consists of eight segments of single stranded RNA ranging from 890 – 2341 nucleotides in size and has a total length of about 13,600 nucleotides encoding more than 13 viral proteins (Figure 1). Influenza A viruses are 80 to 120 nm in diameter and are pleomorphic in nature although laboratory strains are usually spherical while clinical respiratory tract isolates are filamentous forms about 20 µm in length[19,20]. The AIV envelope is composed of a lipid bilayer with the Hemagglutinin (HA) and Neuraminidase (NA) surface glycoproteins, which are essential in viral attachment and exit from host cells[21]. The membrane ion channel protein (M2) resides on the outside of the lipid layer while the matrix protein (M1) resides beneath the lipid layer forming a matrix layer bridge between the viral envelope and the ribonucleoprotein (RNP) core. The ribonucleoprotein core consists of eight negative sense, single stranded viral RNAs

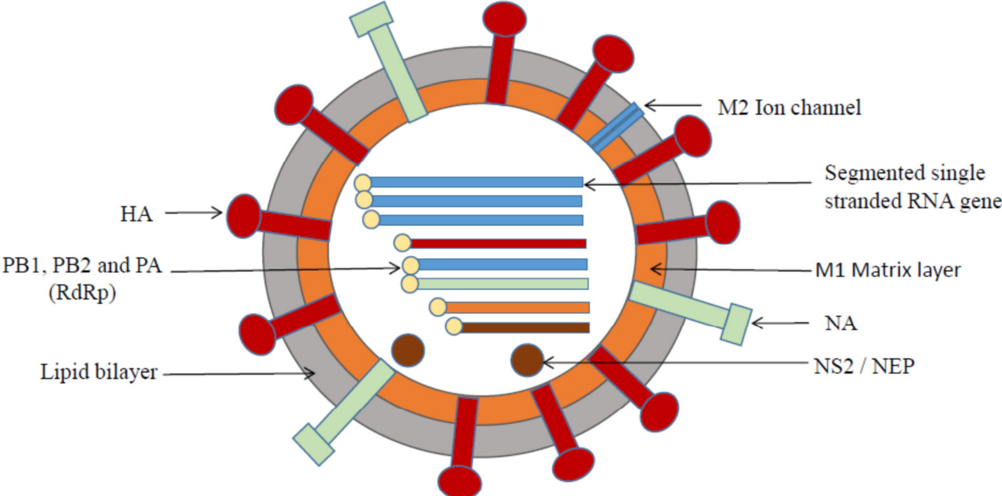

M2 Ion channel
Segmented single
stranded RNA gene
HA
PB1, PB2 and PA
(RdRp)
M1 Matrix layer
NA
Lipid bilayer
NS2 / NEP

Figure 1: The structure of influenza A virus. The virus is composed of eight negative sense single stranded RNA segments ranging from 890 – 2341 nucleotides arranged from the largest to smallest (PB2, PB1, PA, HA, NP, NA, M and NS genes) encapsidated by nucleoprotein. RNA dependent RNA polymerase RdRp (PB2, PB1, and PA) binds to coding regions on the ends of each segment. The viral envelope is composed of hemagglutinin (HA), neuraminidase (NA) and matrix proteins (M1 and M2) which are essential in viral attachment.

(vRNA) and a RNA-dependent RNA polymerase (RdRp) comprising polymerase basic proteins 2 (PB2), polymerase, basic protein 1 (PB1) and acidic polymerase protein (PA) which are all encapsidated by a nucleoprotein (NP) protein scaffold[22].

RdRp binds to highly conserved terminal sequences of 13 and 12 nucleotides at the 5' and 3' ends respectively of each vRNA segment. Terminal sequences contain the necessary signals for the transcription and replication of the viral RNA genome segments. The PB1 gene encodes additional proteins PB1-N40 and PB1-F2 by using alternative translation initiation sites while the PA gene encodes PA-X by a ribosomal frameshift in avian viruses[23]. The Matrix (M) and non-structural (NS) genes of the AI virus also encode ion channel proteins (M1, M2 and M42) and non-structural or nuclear export proteins (NS1, NS2/NEP and NS3) through alternative mRNA splicing following transcription[24,25].

1.4 Virus lifecycle

The avian influenza A virus begins its viral life cycle with the attachment of its HA surface glycoprotein to sialic acid (SA)-galactose (gal) receptors[26]. SA molecules (N-acetylneuraminic acid, NeuAc, and N-glycolylneuraminic acid, NeuGc) are linked to galactose in α-2,3 and α-2,6 SA-gal linkages on the surface of host epithelial cells. Avian AI viruses tend to bind to α-2,3 SA-gal linkages while mammalian viruses favour α-2,6 SA-gal linkages. However, the restriction is not absolute as AI viruses have varying degrees of sensitivity to either linkage and sometimes may bind efficiently to both. Sialic acid distribution in host tissue organs contribute to host specificity, transmission, and pathogenesis of AIV[27,28].

The HA protein must be cleaved into HA1 and HA2 subunits for fusion between host and viral membranes to occur, and subsequently endocytosis into the host cell. Following endocytosis, the M2 protein opens and permits the entry of H^+ ions into the virion thereby

creating a low pH environment in the endosome. Acidifying of the virus interior results in the release of viral ribonucleoprotein (vRNP) and accessory proteins into the cytoplasm[29]. Amantadine drugs can be used in targeting of the M2 ion channel thereby preventing infection by blocking the release of viral proteins[30]. The vRNP are transported to the nucleus via nuclear localization signals mediated by M2 and NP proteins[31,32]. In the nucleus, negative sense, and single stranded vRNAs are transcribed to positive sense messenger RNA (mRNA) utilizing RdRp (PB2, PB1, and PA). The AI virus makes use of the host cellular machinery to initiate mRNA synthesis for viral protein synthesis by stealing a 5' capped primer from host mRNA by the PB2 protein in a process known as cap snatching[33].

The positive sense mRNA either migrates to the cytoplasm to begin viral protein translation (in the case of HA, NA and M2 surface glycoproteins) or remains in the nucleus to serve as a template for negative sense viral RNA synthesis. vRNPs and M1 are packaged into virions and transported to the budding site from the nucleus[34]. Packaging of vRNPs is an inefficient process due to incomplete vRNA segments in the virion or multiples of single segment in one virion. Greater than 90% of viral particles are suggested to be non-infectious due to errors in packaging[35]. At the budding site, the virion buds off the cellular membrane acquiring its surface glycoprotein coated viral membrane. The NA protein plays a major role in exit from the host cell by enzymatic removal of SA-gal on the cell surface[36]. The AI virus depends on constant transmission from host to host due to its lytic nature.

1.5 Virus isolation and laboratory diagnosis

AIV can be identified by virus isolation which is time consuming but necessary in diagnosing the presence of AIV in individual animals and in generating virus stocks for further laboratory analysis. Samples are collected from suspected or dead birds in RNA stabilizing

reagents to limit RNA degradation. Cloacal and oral swab samples are usually collected along with samples from respiratory, gastrointestinal, and systemic organs. Diagnostic samples are then stored at 4^0C for immediate processing or -80^0C for prolonged storage. Influenza viruses are preferably grown in specific pathogen free (SPF) embryonated chicken eggs (ECE)[18]. Allantoic fluid is recovered from dead eggs and remaining eggs at the end of the experiment for use in serological tests such as the hemagglutinin test (HA test), hemagglutinin inhibition test (HI test), agar gel immunodiffusion (AGID) test[15], or solid phase antigen-capture enzyme linked immunosorbent assays (ELISA)[37].

Isolation of AIV in ECE's has been documented to result in the selection of mutations which alter virus antigenic properties in contrast to cell culture methods utilizing Madin-Darby Canine Kidney epithelial cells (MDCK) which result in fewer mutations[38]. In addition, the requirement of large numbers of SPF ECE's for vaccine production and the time required to collate sufficient ECE's makes cell culture of AIV a more attractive option. Culturing of AIV in cells is highly reproducible and standardized and so vaccine production can be started in a timely fashion which will be beneficial in addressing outbreaks[39,40]. Samples can also be analysed directly following RNA extraction. Molecular techniques such as real-time reverse transcription polymerase chain reaction (RT-PCR) offer rapid results which are comparable to virus isolation techniques in sensitivity and specificity[41,42]. RT-PCR allows the analysis of multiple samples in a 96-well plate using probes to target the matrix or nucleoprotein genes. Genetic analysis can also be carried out using RT-PCR to generate PCR amplicons for further sequencing of AIV isolates[43,44].

1.6 Host range and transmission

LPAIV subtypes circulate in wild bird populations of the orders Anseriformes (i.e., ducks, geese, and swans) and Charadriiformes (gulls and shorebirds) which serve as natural reservoirs

of AIV[45]. AIV is constantly spread around the world via migratory wild birds and as such total eradication of the virus is unlikely. AIV is asymptomatic in wild bird hosts causing localized infection in the intestinal tract. High viral titers are usually found in faeces from wild aquatic birds which are able to shed the virus for weeks[46]. At 15-35^{0}C AIV can persist in faeces for up to seven days. However, at cooler temperatures of 4^{0}C the virus can be detected for 30 days or more. AIV has been shown to be capable of persisting in water for months depending on favourable temperature, pH and humidity conditions and also the environmental resistance of the virus[47–50].

AIV is widely considered to be transmitted from wild birds to domestic poultry via the faecal-oral route of transmission. Domestic poultry may become infected by drinking water retrieved from AIV contaminated lakes and rivers. Poultry may also come in direct contact with migratory wild birds requiring shelter from harsh weather conditions in barns or during hydration at open water sources[51]. For instance, the outbreak of AIV in Minnesota in the 1990's occurred largely due to migratory wild ducks coming in contact with free range turkeys[52,53]. After the switch to indoor rearing of domestic poultry, a decline in the number of outbreaks was observed.

Live bird markets (LBM) also serve as a source of introduction and spread of AIV due to the requirement of live poultry on display[54,55]. In a typical LBM, waterfowl and domesticated poultry are housed in cages in close contact during the display. This encourages cross species transmission of a virus which may be asymptomatic in domestic ducks, geese or quail but not chickens. The threat of interspecies transmission and spread of AIV is further increased due to the shutting down of LBM each day as traders' transport unsold birds back to poultry barns. AIV can be introduced into poultry populations by human error due to carriage of the virus on

clothing, shoes, transport vehicles and contaminated equipment during culling, decontamination and disinfection of barns[56].

Transmission of AIV from wild birds is usually transient and subtypes are frequently stamped out of flocks due to restricted replication in host cells or failure to transmit to a new host[57]. Of the abundance of subtypes found in wild bird species, only few AI viruses transmitted from wild birds replicate efficiently in poultry. Of note are viruses of the H5, H6, H7, H9, and H10 subtypes which sometimes crosses the species barrier infecting mammals[8–10]. Adaptation may occur because of the high mutation rate of the viral polymerase due to a lack of a proofreading mechanism (antigenic drift), or reassortment with virus of varying subtype following coinfection of a host due to AIV segmented nature (antigenic shift). AIV can thus evade host immune responses and adapt to new species due to the wide variation of subtypes reproduced[58,59].

Domestic waterfowl such as duck and geese species, act as bridge hosts in the transmission of AIV to domestic poultry[60]. Quail species have been shown to be more susceptible to infection by AIV isolated from wild birds than chicken species. Following serial lung passaging in Quail species, a LPAIV was shown to have increased replication fitness and transmission in chickens[61]. Adaptation in chickens was enhanced by the deletion of amino acids in the NA stalk region following passaging in quail which was maintained in subsequent chicken passages[61]. AIV is introduced into domestic poultry populations in its LP form and is shed by infected birds in saliva, nasal secretions, and faeces. Susceptible birds become infected following contact with the virus shed by infected birds or on fomites, and airborne routes. Numerous reports suggest that AIV is more likely to be spread by respiratory routes in chickens. This is likely due to the

observation of more efficient viral shedding in oropharyngeal swabs compared to cloacal shedding in infected chickens[28,62].

In poultry barns, AIV can circulate in aerosols generated from feathers, dander, feed, fecal material, and bedding[63,64]. Aerosols are minute in size, less than 5 µm in diameter, and as a result can stay suspended in the environment for longer periods of time compared to large droplets[7]. The outbreak of H7N3 HPAI in poultry farms in BC, Canada in 2004 is suggested to have occurred due to wind mediated dispersal of AIV[65]. Wind patterns correlated with the spread of the virus from infected populations to susceptible ones. In addition, AIV was also isolated in the air outside of the barns in minute quantities[6,66].

Circulation of LP viruses in poultry populations may go undetected due to the lack of visible clinical signs in infected birds. Outbreaks of HPAIV are considered to arise from LP progenitors circulating for days, weeks or sometimes months in domestic poultry before acquiring a unique set of genomic segments for increased virulence[3]. H5N3 HPAIV was first detected in shorebirds in South Africa 1961 killing over a thousand ferns within a few weeks which was unusual considering AIV is usually asymptomatic in these hosts[67]. HPAIV H5N1 was first detected in southern China on a goose farm causing mortality in 40% of the birds in 1996[68]. The following year, reassortant viruses with the internal genes of H9N2 and H6N1 viruses were detected in domestic bird species, causing outbreaks in Hong Kong poultry farms[69,70]. After depopulation of affected farms, the HPAI H5 virus continued to circulate in Asia before spreading to other European and African countries.

The increase in the incidence of HP outbreaks are speculated to be due to multiple factors such as increased poultry population, commercial rearing of poultry, and climate change affecting migratory routes [56,71]. Chickens and pigs are known to possess both avian and

mammalian receptor types in multiple organs although at varying degrees of expression and are considered intermediate hosts in the transmission of AIV to humans and other mammalian species[72]. Infection of mammalian species such as dogs and tigers has occurred following ingestion of infected dead birds[73,74]. Poultry farm workers and visitors of live bird markets have been infected with the AIV following contact with infected birds or most probably via respiratory droplets with mortality occurring in about half of the cases[71]. However, the lack of efficient transmission by respiratory droplets limits human to human transmission of AIV thus preventing a pandemic outbreak.

1.7 Pathobiology in poultry

Infection in domestic poultry is characterized by two extremes of virulence, low pathogenic AIV (LPAIV) and high pathogenic AIV (HPAIV). AIV is usually introduced to domestic poultry in its low pathogenic form from wild birds. In chickens, infection with LP viruses lead to low to moderate mortality rates and morbidity characterized by decreases in egg production, depression, decreased feed and water consumption, weight loss, ruffled feathers, sneezing, rales (crackling noises made by lungs of chickens with a respiratory disease during inhalation), and diarrhea. In field infections, mortality is observed in chickens infected by LP viruses albeit in few numbers and depending on the viral isolate. However experimentally, inoculation of specific pathogen free (SPF) chickens with LP isolates usually results in a lack of morbidity and mortality[75]. Mortality observed in field infections could occur as a result of immunocompromised hosts or co-infection with bacteria which exacerbate infections by secreting proteases which support activation of the HA through proteolytic cleavage[76,77].

HP viruses are known to arise from low pathogenic progenitors after circulating for months or years in poultry. Wild birds are not considered reservoirs of HP virus, although in the last five

years, HPAI H5N2 has been isolated from wild waterfowl species causing mortality in large numbers. AI viruses that exhibit an intravenous pathogenicity index (IVPI) in 6-week-old chickens greater than 1.2 or kill at least 75% of 4- to 8-week-old chickens within 10 days is defined as a HP strain. HP infection in chickens is characterized by systemic spread and increased mortality in chickens. Birds display outward signs of disease such as depression, edema of the comb and wattles, respiratory congestion, and hemorrhage of the conjunctiva and bursa. Birds may sometimes die within a few days without showing signs of disease. HP designation applies to infections in chickens and severity of infections in other species does not necessarily result in clinical signs or mortality[58].

1.8 Determinants of host range and pathogenesis of AIV

1.8.1 Hemagglutinin

The HA is the most abundant surface glycoprotein on the AIV envelope (approximately 300 spikes) along with the NA tetrameric protein (approx. 40 spikes) which forms clusters on the envelope. The HA protein is a type 1 membrane protein embedded in the bi-lipid layer of the viral envelope. It is expressed as a trimer with SA-gal receptor binding sites (RBS) on each monomer[78].

1.8.1.1 Receptor binding specificity

The RBS consists of a pocket of conserved amino acids bordered by the 190-helix (residue 189-199), the 130 loop (residue 133-138) and 220 loop (residue 220-229)[78]. Structurally, mammalian viruses which bind to α2,6 SA-gal linkages present in tracheal epithelial cells of humans have wider RBS (~1-1.5 Å) when compared to avian viruses which bind predominantly to α2,3 SA-gal linkages found in respiratory and intestinal tracts of birds[78]. The restriction in binding specificity is not absolute as the HA in a number of AIV isolates are capable of binding

to dual receptors[79,80]. The restriction and distribution of available galactose receptors for binding plays a major role in limiting interspecies transmission of AIV and subsequent human to human transmission via respiratory droplets[81,82]. In chickens and pigs, both α2,3 and α2,6 SA-gal linkages have been detected in many organs although at varying levels of expression implying that these species could serve as intermediate hosts in the transmission of AIV to humans[72,83].

Mutations in the HA protein of AIV are required to confer the switch from avian to human receptor binding depending on the viral subtype. The acquisition of aspartic acid at positions 190 and 225 (HA numbering throughout thesis is based on H3 HA[84]) in the HA of H1 viruses is sufficient for receptor binding and transmission in humans as opposed to glutamic acid and glycine[85,86]. The H1N1 South Carolina pandemic virus of 1918 possessed aspartic acid at both positions and was able to transmit efficiently via respiratory droplets in ferrets[85]. H2 and H3 human viruses on the other hand can switch to human receptor binding via Q226L and G228S mutations[87].

Majority of H5 AIV isolates preferentially bind to α2,3 SA-gal receptors expressed in avian species and so can infect and replicate in poultry species efficiently. Humans possess α2,6 SA-gal receptors on epithelial cells of the respiratory tract restricting infection to viruses with human receptor specificity. Additionally, histological studies show that mucins present in the upper respiratory tract of humans inhibit AIV which bind to α2,3 SA-gal linkages thus restricting human to human transmission of avian viruses by respiratory routes[88]. The possession of α2,3 SA-gal receptors in alveolar cells of the lung explains cases of human infections with HP H5 viruses, with aerosols suggested to enable viral penetration of the lower respiratory tract[89].

Amino acid mutations at positions 190 and 225 of the HA protein which confer human receptor binding in H1 viruses do not produce similar effect in H5 viruses[90]. Multiple amino acid

mutations are suggested to facilitate the switch in receptor binding specificity of H5 AIV. In the HA protein of H5 viruses, combinations of N158D, T160A, N186K, E190D, K193R, Q226L, S227N, and G228S mutations are suggested to increase binding to α2,6 SA-gal receptors or decrease α2,3 SA-gal binding[91,92]. The acquisition of human receptor specificity in the HA's of H7 AIV's can also be facilitated by mutations at residues 226 and 228 like H2, H3 and H5 AI viruses. H7N9 viruses are also known to encode valine at position 186 which is thought to contribute to the change in receptor binding specificity[80,93].

Unlike H5 viruses, natural H7 isolates tend to display dual receptor binding properties and as such enables efficient transmission in animal models[94,95]. Isolates from the 2013 H7N9 outbreak in China possessed human leucine residue at position 226 and avian glycine residue at position 228 which enabled binding to both human and avian receptors[80]. H1N1 viruses which possess both human and avian type residues at position 190 and 225 respectively are considered to bind to dual receptors and in some cases cause increased virulence[96–98]. The H9N2 AIV is known to contribute internal genes to both H5 and H7 HPAIV. Infections of humans have also been documented by the H9N2 virus which is not surprising due to the fact that majority of Asian H9 viruses isolated possess leucine at position 226[99].

1.8.1.2 Pathogenicity

In addition to determining the host range of AIV, the HA protein plays a major role in determining tissue tropism and pathogenicity in susceptible hosts. For infection to occur, the HA0 precursor must be cleaved into HA1 and HA2 subunits thereby revealing a fusion peptide (20-25 residues) for membrane fusion[100]. Cleavage of the HA0 precursor occurs at the C-terminus of HA1 and the N-terminus of HA2 and the presence of single or multiple basic residues in the cleavage site may potentially influence the pathogenicity of AIV in chickens.

LPAI viruses are characterized by the possession of a monobasic cleavage sites such as R-S-S-R or R-S-R-R and are cleaved extracellularly by trypsin like proteases such as tryptase Clara, TMPRSS2 and HAT in avian and human lung epithelium and also factor Xa-like proteases in the intestinal tract of birds[101]. Infection is usually mild and localized to the respiratory and intestinal tracts due to the restriction of proteases[102]. Mortality may be observed in LPAIV infection in poultry due to co-infection with bacterial species capable of secreting proteases that cleave the HA protein[103].

Highly pathogenic viruses of the H5 and H7 subtypes possess multiple basic amino acids in the HA cleavage site (R-X-K/R-R or K–K/R-K/T-R) which enables intracellular cleavage of the HA precursor by ubiquitous proteases such as furin and proprotein convertase 6 (PC6)[104,105]. The polybasic cleavage site is considered a prime virulence determinant in poultry species and can be acquired by LPAIV via various means, most commonly by mutations in the HA gene due to polymerase slippage resulting in the increase in the number of basic residues in the cleavage site[106,107].

Increased pathogenicity has been observed in outbreaks of H7N3 AIV in chickens in Chile following homologous recombination. Sequences coding for viral proteins of the NP gene were inserted into the HA gene resulting in increased mortality in chickens[108,109]. A similar observation was observed in the 2004 outbreak of H7N3 LPAI and HPAI viruses in British Columbia. LP and HP viruses isolated differed by 21 nucleotides in the HA gene resulting from the non-homologous recombination with its M-gene. A seven amino acid insert encoded in the HA gene was sufficient in conferring increased virulence in chickens[110]. Experimentally non homologous recombination between HA and NP of A/Seal/Mass/1/80 has been observed which caused increased mortality in chickens[111]. Increased mortality in chickens has also been observed

following the insertion of a cellular 28s ribosomal RNA sequence into the HA gene of a turkey AIV after passaging in chicken embryo cells[112].

Outbreaks of HPAI H5 and H7 in poultry were usually preceded by LPAI viruses with monobasic cleavage sites. In chickens, reassortant HPAI H5 viruses with monobasic cleavage sites have been shown to cause mild respiratory disease while wild type HPAI H5 counterparts caused increased mortality within a few days. This suggests the acquisition of a stretch of multiple basic amino acids in the HA cleavage site is sufficient to convert a LPAI H5 or H7 virus to high pathogenicity[113,114]. However, experiments using reverse genetic techniques have shown that virulence of HPAI viruses is a multigenic trait involving multiple segments in the AIV[115]. Mutant LP H3N8 AIV bearing polybasic cleavage sites of HPAI H5N2, H5N1, and H7N7 viruses were shown to replicate in tissue culture in the absence of exogenous trypsin unlike the wild type virus.

The viruses did not exhibit increased pathogenicity associated with HPAIV[116]. A LP H5N1 virus was also shown to exhibit in vitro cleavage in the absence of trypsin following the substitution of its monobasic cleavage site with a polybasic motif. The reassortant H5 virus was unable to cause increased mortality in chickens, although compared to the H3N8 study, it could produce more clinical symptoms in chickens. This suggests that H5 LP viruses may possess virulence determinants in other gene segments and as such can mutate into HPAI more readily following the acquisition of polybasic cleavage sites than non H5 viruses[117].

1.8.1.3 HA glycosylation

The HA protein is glycosylated at asparagine residues (N-Glycosylation) during transport through the endoplasmic reticulum / Golgi network. The number of glycosylation sites present on the stem and head of the HA protein vary depending on the strain or subtype of virus. Glycans

found on the stem of the HA protein are usually conserved across AIV strains, however, those found on the globular head exhibit extensive variability[118,119]. Glycosylation of HA molecules has been shown to be essential in proper HA folding, maintaining protein conformation and regulating biological activities. Glycosylation sites are observed to stabilize HA in a metastable form suitable for fusion activation to occur [119].Glycans modulate HA activity by shielding antigenic epitopes from being recognized by antibody and thereby contribute to evasion of the immune system and thus antigenic drift of the virus. Human H3N2 viruses which caused outbreaks in 1974 have been shown to have acquired additional glycosylation sites which interfered with antibody recognition[120].

Glycosylation has also been shown to affect receptor binding specificity and affinity[121,122]. H1 AIV grown in either MDCK cells or chicken embryos showed a variation in receptor specificity due to the gain of a glycosylation site close to the receptor binding site in MDCK cells[123]. In addition, an increase in hemadsorption and cell fusion has been shown to occur following the loss glycosylation sites close to the RBS of the HA protein[119,124]. For successful release of virions from host cells, cleavage of sialic acids by NA is necessary and so optimal HA-NA combination is required. The addition of glycosylation sites close to the HA RBS was shown to increase virus release which was balanced by a deletion in stalk length of the NA protein, thereby reducing virion release from the surface of cells enabling efficient entry and exit from host cells. It was found that HA which possess glycosylated sites at position 158 replicated more efficiently when combined with NA with shortened stalk length and vice versa reducing aggregation of cells[125].

The HA Glycosylation site at residue 158 also influences the transmission of AIV in mammalian species. Loss of the 158-160 glycosylation site by N158D and T160A mutations

conferred ferret transmissible H5 viruses with increased binding to human receptors[91,122,126,127]. The presence or absence of glycosylation sites also impacts the pathogenicity of AIV in different hosts[128]. Introduction of N144 and N177 glycosylation sites resulted in not only a change in affinity for receptors but also increased virulence and pathogenicity of a pandemic H1N1 virus in mice[129]. In addition, the outbreak of H5N2 HPAIV in Pennsylvania came about following the loss of a carbohydrate side chain masking the polybasic cleavage site of its low pathogenic predecessor. The loss of a glycosylation site resulted in viral HA cleavage in the absence of trypsin and contributed to the observed increase in pathogenicity[130].

1.8.1.4 pH of Fusion and thermostability

The pH involved in the HA activation of influenza viruses has a major effect on host range, transmissibility and infectivity in a new host and varies depending on the viral isolate. Following internalization of AIV in endosomes, the HA protein undergoes an irreversible conformational change as a result of the low pH in the endosome which enables fusion of viral and endosomal membranes[120]. Viruses with higher pH of HA activation are more likely to be activated in early endosomes thereby increasing the chance of vRNA detection by the immune system during transport to the nucleus[131].

On the other hand, viruses with low pH of HA activation may be degraded in lysosomes if membrane fusion and release of vRNPs fails to occur. Human and avian influenza viruses differ in pH of HA activation with avian HP viruses being triggered at a higher pH range of 5.6 to 6.0 while LP and human viruses are activated within a pH range of 5.0 to 5.4[132]. The human respiratory airway has a pH of 5.2 to 8.0 and as such, viruses with high pH of HA activation are inactivated before reaching susceptible cells. On the other hand, the intestines and respiratory tracts of wild birds favour the replication of viruses with high pH of HA activation[132].

LPAIV are considered to adapt to a more stable low pH of HA activation following transmission from wild birds to domestic poultry[132]. H7N3 AIV isolated from wild ducks and turkeys a year apart were found to differ in pH of HA activation with the duck isolates having a higher pH of activation compared to turkey isolates[133]. Increased pH of HA activation (range 5.2 to 6.0) is also associated with increased pathogenicity in chickens[128,134]. For efficient replication and transmission in a new host, an optimal range of pH activation is required.

A HPAI A/chicken/Vietnam/C58/04 (H5N1) virus, highly virulent and transmissible in mallards, was shown to become avirulent following increase (6.3) or decrease (5.4) in its pH of HA activation (5.9)[135]. The K58I mutation responsible for the decrease in HA activation pH resulted in increased replication and pathogenesis in mice[136] and productive replication in the upper respiratory tract of ferrets[137]. From these and other studies, it can be inferred that a low pH of HA activation enhances replication and transmission of AIV in mammalian species while high pH of HA activation may contribute to increased pathogenicity in chickens.

Of the low pathogenic viruses in circulation, H9 and H10 subtypes are suggested to have greater acid stability with their activation pH closely similar to human viruses and as such may not require extensive pH adaptation to cross the species barrier unlike LPAIVs with high pH of HA activation[132]. The irreversible conformational change of HA into its fusogenic form induced by low pH can also be achieved by heat treatment at neutral pH[49]. As with pH of HA activation, thermostability of influenza viruses vary across and within subtypes and can be influenced by host factors or geographical locations[138]. Influenza viruses which fuse at low pH are considered to possess higher thermostability than those fusing at high pH[120].

Recent studies show that the adaptation of influenza viruses to new species may require mutations affecting stability of the HA protein. T318I and H110Y mutations are documented to

stabilize the HA protein of H5 viruses following mutations affecting receptor binding specificity. The mutations decreased the pH of HA activation while also increasing the thermostability of the virus leading to increased replication efficiency and transmission[91,139]. Limited studies have been conducted on the thermostability of AIV in the laboratory, however, current work suggest that pH and thermostability of AIV may play a crucial role in replication, transmission and pathogenesis in susceptible hosts[140–142].

1.8.2 Neuraminidase

The Neuraminidase (NA) protein is a sialidase which plays a major role in viral entry and release from host cells. As stated previously, the functional balance between the receptor binding activity of HA and enzymatic cleavage activity of NA surface glycoproteins impacts the replication, transmission and pathogenicity of AIV[143]. The balance between HA and NA genes can be offset by reassortment with other viruses, transmission to new hosts or treatment with inhibitors targeting the NA.

For the efficient release of virions from host cells, NA is required to cleave α2,3 or α2,6 SA-gal linkages on the cell surface[144,145]. A panel of human viruses with N2 proteins were assessed to determine if the HA and NA shared the same substrate specificity. It was found that the NA of the human viruses could bind and cleave the α2,6 SA-gal human receptor preferentially. However the earliest H2N2 isolate, derived from an avian isolate, showed strict preference for α2,3 SA-gal cleavage[145]. Over the decades, following the adaptation of avian viruses to humans, the NA protein gradually drifted to support binding to human receptors thereby increasing viral release from cells. The functional balance between the receptor binding ability of HA and the enzymatic cleavage activity of NA genes is essential for infection to occur[146].

Mutations in the HA gene which affect its affinity for receptor binding has also been observed to reduce the dependence on NA activity allowing adaptation to humans. Mutations of S331R, D339N, N367S, S370L, N400S, and P367S were shown to reduce NA specific activity in reassortant viruses expressing the NA of a H2N2 human virus and the proteins coded by seven genes from an avian H9N2 virus restricting replication in duck intestinal tissues[147]. Unbalanced HA – NA combinations could cause aggregation of viral progeny at the cell surface[145,148]. Human viruses tend to have higher HA affinity for its receptor than avian viruses and so as to maintain an optimal HA-NA combination, a reduction in the enzymatic activity of the NA gene is usually observed thereby allowing efficient exit of progeny from cells[99].

The balance of HA and NA is also suggested to influence the generation of HP viruses. Following serial passage of an avirulent reassortant H5N1 virus, a NA-R293K mutation was observed which was suggested to restore the HA-NA balance by conferring a reduction in NA activity and resistance to antiviral inhibitors ultimately leading to an increase in pathogenicity[149]. The length of NA stalk also influences host range, enzymatic activity and virulence in various host species. The NA stalk separates the active site in the head region from the transmembrane and cytoplasmic domains and its length varies depending on the virus[150]. Deletion in stalk length is considered to occur as a marker of wild bird influenza virus adaptation to domestic poultry[151–154]. Short NA stalk length in influenza viruses has been shown to be inefficient in the release of progeny virus and elution from red blood cells due to limited access to the substrate. A decrease in viral replication in MDCK cells and chicken embryos, as well as a decrease in virulence in mice have been observed in viruses with a shorter stalk length[155–157]. However, naturally occurring avian viruses with shortened length of stalk have been shown to cause increased virulence in chickens and subsequently humans after exposure to poultry[89,151,158].

1.8.3 Polymerase complex and other viral proteins

Although the surface glycoproteins of AIV serve as a major determinant of host range and pathogenicity, the polymerase complex and internal proteins also affect the outcome of infections. The E627K mutation in the PB2 protein is considered as a marker for adaptation of AIV in mammalian species. Avian viruses possess glutamine at residue 627 while lysine is commonly found in human viruses[58]. The E627K mutation was observed to regulate the temperature at which AIV undergoes replication in avian or mammalian species by enhancing replication at 33^0c in mammalian respiratory tract as opposed to 41^0c found in the intestinal tract[159,160].

A HPAI H7N7 avirulent virus chicken isolate from the 2003 Netherlands outbreak in poultry and humans was passaged in mouse lungs to generate mouse-adapted variants. The variant selected after three passages exhibited increased viral replication, tissue tropism, and caused severe disease in mice while also remaining HP in chickens. The E627K mutation in PB2 of this variant was responsible for the increase in pathogenicity, and the mutation was also present in PB2 sequences analyzed from the single fatal human isolate[161]. The E627K mutation has been observed in majority of the H7N9 and H5N1 isolates from human infections. In addition, avian viruses acquire the E627K mutation readily following infection in mammalian hosts. The PB2-D701N mutation has also been observed to increase virus replication by enhancing binding to importin-α1, which translocate viral RNPs into the nucleus for transcription and replication in mammalian cells[32,162].

The combination of polymerase proteins impacts host specificity and pathogenicity of AIV in avian and mammalian hosts. Attenuation of recombinant viruses has occurred following the mixture of polymerase proteins from different viruses in chickens[163]. Using reverse genetics,

recombinant viruses were created possessing the HA and NA genes of a HPAIV and internal genes of LPAIV derived from either chickens or wild birds. It was observed that the PA and PB1 genes both contributed to the pathogenicity of HPAIVs in chickens by affecting survivability, disease presentation and replication[164]. It was further observed that amino acid substitutions at residues 14 and 38 of the PB1 protein affected pathogenicity by increasing or decreasing polymerase activity[165]. Differences in lethality of highly pathogenic and non-pathogenic H5N1 clones in mallard ducks were observed to be associated with mutations in PB1-Y436H and PA-T515A proteins[166].

In addition to the polymerase complex, the NP protein is considered important in the replication of viral RNA by interacting with PB2, PB1 and PA. Replication is more efficient when the genetic constellation comprises of polymerase complex and NP proteins from the same virus. The NP gene has been shown to be essential and have a major impact on both virulence and replication of AIV in chickens[167]. The Matrix protein is also involved in the outcome of AIV infection due to its function as a regulator of pH through forming a membrane ion channel. M2 protein is thought to stabilize HA proteins with polybasic cleavage sites by maintaining the pH to prevent premature irreversible low pH conformational change. An avirulent H5 AIV isolated from teal, was passaged 12 consecutive times in chicken air sacs to generate a HP virus with a 100% mortality rate[168]. A D44N mutation in the M2 protein was shown to contribute to the increased pathogenicity observed in the chickens in the presence of a polybasic cleavage site.

The NS1 protein acts as an interferon (IFN) antagonist thus enhancing the pathogenicity of AIV in both avian and mammalian species. The NS1 protein plays an important role in enabling the virus evade host immune responses by preventing the development of an antiviral state. The IFN inducing capacity of H7N3 AIV was suggested to play a role in regulating pathogenesis,

virulence and transmission of AIV in chickens. A virus encoding the full length NS1 protein was observed to replicate at higher titers and cause more lesions in chicken organs[169]. In addition, the NS1 protein has been shown to be critical in causing increased pathogenicity in H5N1 HPAI infected chickens. Serine at positon 215 of the NS1 protein enhanced pathogenicity of the reassortant H5 viruses in chickens when compared to viruses bearing proline[168]. A A149V mutation is reported to block induction of interferon protein levels in chicken embryo fibroblasts[170]. These studies show that the generation of highly pathogenic avian influenza viruses is not restricted to the possession of multibasic cleavage sites in the HA gene, but the presence of an optimum gene constellation comprising multiple segments.

1.8.4 Immune response

Following infection by AIV, pro-inflammatory and antiviral cytokines are secreted by the host to initiate an immunological response to control viral replication. Interferon gamma (IFN- γ) is a type II interferon which binds to the IFNGR receptor to induce interferon regulatory factors via the JAK/STAT signalling pathway which also enables activation of type 1 interferons[171]. IFN- γ is known to induce an antiviral state enabling viral clearance[172,173]. Following infection of chickens and ducks with a H7N3 virus, higher viral load was observed in chicken lungs compared to that in ducks. However increased expression of IFN- γ was observed in the lungs of ducks but not chickens suggesting IFN- γ expression contributed to the clearance of virus in ducks[174]. However many studies suggest the expression of IFN- γ may not necessarily lead to the clearance of virus, unless in conjunction with other cytokines[175–177].

Interleukin 6 (IL-6) and interleukin 1β (Il-1β) are early innate response cytokines and are commonly referred to as pro-inflammatory cytokines[178]. These cytokines have been associated with mediating fever, pain, vascular permeability and cellular infiltration[179]. An overregulation

of cytokine responses due to pulmonary infection by HPAI viruses is recognized as a determinant of increased pathogenicity in humans, commonly referred to as a cytokine storm[180]. However induced responses depend on the virus strain as observed in infections with HPAI H5 and H7 viruses which differ in induction of cytokines[181]. Increased expression of IFN- γ, IL-1β and IL-6 has been documented to cause differences in mortality observed in chickens infected by H9N2 viruses of varying pathogenicity[182]. Interleukin 4 (IL-4) is a crucial in the activation of CD4+ helper T cells and humoral activity. H9N2 infected chickens showed a decrease in expression of IL-4 and thus low level of antibodies were detected whereas chickens infected with a H6N2 virus had a robust humoral response compared to controls suggesting viruses differ in the induction of humoral response in the host[183].

In addition to viral differences, the host species also plays a major impact on the outcome of infection. In experiments involving the inoculation of chicken and duck species, it was observed that chickens express more inflammatory cytokines at later time points than ducks which induce expression of pro-inflammatory cytokines earlier on and at lower levels of expression. The differences in cytokine expression correlated with the observed mortality in chickens and the lack of clinical signs in ducks[184–187]. From these experiments, it appears the overregulation of pro-inflammatory cytokines contributes to morbidity and mortality in chickens.

The immune response can also be influenced by the infecting influenza virus. NS1 is documented to act as an IFN antagonist preventing activation of transcription factors by blocking pathogen associated molecular patterns (PAMPs) through retinoid inducible gene 1 (RIG-1)[188]. NS1 has also been implicated in the overregulation of cytokines in H5 infections[189,190]. PA-X is also considered to contribute to immune dysregulation in avian and mammalian hosts. The loss of PA-X expression activated the genes associated with the inflammatory response pathway and

the interferon (IFN), immune cell, and cell death signaling pathways. The exacerbated response by the immune system and viral replication were shown to contribute to the increased pathogenicity of PA-X deficient viruses in birds[191].

1.9 Rationale of study

HPAIVs have been isolated frequently over the past few decades. They are suggested to arise from low pathogenic precursors in poultry following periods of circulation in poultry gaining increased pathogenicity in chickens. Currently H5 and H7 are the only subtypes designated as in HPAIV in poultry, however the H9N2 AIV has gathered wide attention due to its widespread nature, ability to bind human receptors, airborne transmissibility and donation of internal genes to HPAI viruses[99,192,193]. Despite its widespread circulation in poultry, the H9N2 virus has not been designated as HPAIV although it has caused sporadic cases of high mortality in chickens[194].

Researchers have made use of viral passaging in mammalian, chicken embryos, and avian species in studying virus adaptation and pathogenicity. An experiment designed to simulate the evolution of an avian H9N2 AIV in mice revealed the acquisition of PB2-E627K mutation enabling adaptation and virulence in mice after two passages[195]. However, the serial passaging of H9N2 viruses in chicken embryos and chickens has not been observed to result in the generation of HP viruses although minor increase in pathogenicity was observed as passaging progressed in these models[196,197]. Both experiments utilized three serial passages in their respective hosts and additional routes of inoculation and an increase in number of passages were recommended for future experiments.

Repeated passaging of avian influenza viruses has been shown to result in increased replication ability, transmission and virulence in animal and avian host models[198–203]. Using reverse genetic methods, avirulent H9N2 viruses have been shown to gain high pathogenicity

following the introduction of polybasic amino acids into its cleavage site and passaging in chickens[198,201]. It is increasingly important to assess the evolution of the H9N2 virus in chickens and assess its ability to gain high pathogenicity as seen with H5 and H7 LPAIV subtypes to gain more information in preventing and controlling future pandemics. We passaged an avirulent H9N2 virus nineteen times in chickens via aerosol inoculation to assess changes in pathogenicity in chickens mimicking the natural evolution of the virus in poultry.

In addition, we assessed the impact of oral gavage, intranasal and aerosol routes of inoculation on tissue tropism, immune response and virulence in chickens with four low pathogenic viruses. Aerosol inoculation has been shown in mammalian and avian experiments to be more efficient in causing infection and in some cases mortality in AIV infected hosts compared to intranasal or oral routes[204–209]. Limited studies however exist assessing the impact of aerosol exposure in chickens, and such studies suggest aerosol inoculation of AIV results in quicker onset of viral replication and disease presentation compared to oral or intranasal routes[210]. However, the intranasal route is widely used in the experimental inoculation of chickens possibly explaining the lack of mortality seen in SPF chickens following inoculation with virulent field isolates.

Studies comparing the effect of inoculation routes on infectivity and viral shedding in avian species utilizing multiple viruses exist, however the aerosol route is rarely included in such experiments. AIV isolated from quail, duck, and turkeys are suggested to cause severe disease in chickens[211]. As such we selected a H10N8 AIV isolated from quail and a H6N1 AIV isolated from turkey for our experiment. In addition, a H13N6 virus isolated from gulls was chosen because of its lack of replication in domestic poultry. We also selected a chicken isolate, the H10N7 LPAIV, a subtype which has also been documented to cause infections in humans[10]. We

observed the effect of oral, intranasal and aerosol routes of inoculation in the outcome of infection with these viruses in 4-week-old chickens.

1.10 Hypothesis

The consecutive serial passaging of the H9N2 virus via aerosol exposure will cause an increase in pathogenicity following the adaptation of the virus in chickens. Aerosol exposure of the avian influenza virus will result in an increase in clinical presentation of disease in poultry when compared with intranasal or oral routes of exposure.

1.11 Objectives

I. Assess evolution of the H9N2 virus following 19 passages in chickens by comparing tissue tropism, immune responses, thermostability and sequence analysis of the wild type virus (P0), its eighth passage (P8) and its nineteenth passage (P19).

II. Assess the influence of aerosol, intranasal and oral inoculation routes on infection in chickens using low pathogenic virus subtypes H10N8, H10N7, H6N1 and H13N6.

Chapter 2

Impact of serial passaging of a low pathogenic H9N2 avian influenza virus on infectivity, tissue tropism, and virulence in chickens.

2 Introduction

The H9N2 avian influenza virus was first identified in chickens in China in 1994 and has since then become established as the most prevalent subtype in Chinese poultry populations. In addition to its prevalence in China, the virus has been isolated from avian species in many countries in Asia, the Middle East, Africa, and Europe[212,213]. The H9N2 viruses endemic in Eurasia are of three distinct lineages namely, A/chicken/Beijing/1/94-like (BJ/94-like), A/quail/Hong Kong/ G1/97-like (G1-like), and A/duck/Hong Kong/Y439/97 (Y439- like)[214]. The H9N2 AIV was first detected in human populations in 1998, with two cases reported in children in China[215]. Since then the virus has been sporadically isolated from humans causing mortality in some cases[216,217]. H9N2 G1-like viruses have been isolated infrequently from humans and provided the six internal gene segments of the human infecting H5N1 virus isolated in Hong Kong[14]. The BJ/94- like viruses provided internal gene segments to H7N9 and H10N8 viruses which were detected in humans in China[218,219]. The lack of human to human transmission of the virus has so far prevented an outbreak of pandemic proportions.

Chickens are considered intermediate hosts in the transmission of AIV to humans and other mammalian species[72]. The detection of H9N2 viruses in humans and other mammalian species is not surprising as the majority of H9N2 isolates in poultry possess the mammalian adaptation marker, leucine at residue 226 (L226), in their HA proteins[217,220,221]. HA- Q226L and PB2- E627K mutations are said to be positively selected in H9N2 viruses whilst circulating in avian hosts,[140,222] or following adaptation in mammalian hosts[223,224], enhancing replication of the virus in its host. The H9N2 AIV is low pathogenic in chickens, usually causing mild respiratory disease and decreased egg production. The virus is characterised by low mortality rates, however, upon exacerbation by coinfection with bacteria or co-circulation with other viral

subtypes can become highly virulent in chickens[103,225]. The virulent strains isolated from field infections have been observed to be avirulent in specific pathogen free (SPF) chicken when inoculated experimentally, which suggests that immunosuppression or coinfection with other pathogens may account for the mortality observed in domestic chickens[226]. The H9N2 virus's ability to cause widespread organ dissemination without causing clinical signs, along with the intensification of the poultry industry, most likely contribute to its endemism in Eurasia and spread to humans[212,214,227]. HP viruses are known to arise from LP predecessors following circulation in poultry. As such, the ability of the H9N2 AIV to circulate in chickens without exhibiting symptoms of disease, strongly support the virus's consideration as a possible pandemic strain.

The H9N2 virus has been shown to require only a single mutation in the cleavage site of its HA protein to convert it from a LP virus with a R-S-S-R motif into a R-X-R/K-R motif typical of HP virus[194].The acquisition of increased virulence in chickens is determined by multiple segments in the AI virus. However, the presence of multiple amino acids in the cleavage site of the HA protein is a prime determinant[116]. Several studies employ reverse genetic techniques in changing amino acids in the HA proteins of avirulent viruses, in view of recreating the increased viral replication, overregulated immune responses and mortality seen in HPAI infections[113,117]. Following the introduction of polybasic cleavage site in the HA protein of a H9N2 virus, the virus remained low pathogenic whilst a reassortant H9N2 virus bearing the internal genes of a HP H5N1 virus in addition to the polybasic cleavage site was highly pathogenic in chickens[228]; suggesting the H9N2 virus may require adaptations in other gene segments in addition to the HA gene for increased pathogenicity. In another study, a H9N2 avirulent virus with multiple amino acids inserted into its HA cleavage site was passaged ten

times in the air sacs of chicks, resulting in a virus capable of high pathogenicity in chickens when administered intravenously[201]. However, due to the alteration of the virus genetically, these experiments do not necessarily reflect the evolution of the virus in natural poultry infections.

Few studies have followed the evolution of low pathogenic AIV in poultry without alteration of the AIV HA cleavage site. The serial passaging of a wild bird H5N3 avirulent virus twenty four times in the air sac followed by additional five passages in the brain of chicks led to an increase in pathogenicity in chickens[198]. On the other hand, passaging of an avirulent H9N2 virus in chickens three times intratracheally resulted in a LP virus, although an increase in gross lesions was observed as passaging progressed in chicken organs. Additional passages or different routes of inoculation were suggested by the author for future passaging studies[196]. The H9N2 virus poses a pandemic threat due to its donation of internal genes to HPAI viruses, its widespread nature, endemism, and infrequent increase in virulence in poultry; however, the virus is yet to be designated as HPAI. We passaged a LP H9N2 virus in chickens to determine if a highly pathogenic H9N2 virus could be generated following circulation in domestic poultry.

To assess the evolution of the H9N2 virus in chickens under experimental conditions, we passaged a LP H9N2 (A/Chicken/Henan/1/1998) virus nineteen times in approximately week old chickens. The wild type H9N2 virus (P0), its 8th (P8), and 19th passages (P19) were selected for aerosol exposure to 4-week-old chickens. Passaging was done via aerosol inoculation because the selected H9N2 virus had been shown to be more efficient in causing infection in chickens than intranasal or oral routes in a previous experiment[229]. Passaging of the H9N2 virus did not result in the generation of a virus with increased pathogenicity. Chickens inoculated with the H9N2 virus and its consecutive passages displayed a lack of clinical signs and mortality. Sequencing of the virus isolates revealed a LP amino acid motif supporting the lack of mortality

observed. However, the positive selection of the human adaptation marker, leucine at amino acid residue 226, was observed following nineteen passages in chickens. The exposure of the H9N2 virus, its 8th passage and 19th passage to chickens resulted in the moderate induction of proinflammatory cytokines early on after virus infection. In addition, passaging of the H9N2 virus resulted in a decrease in virulence in chicken embryos and a loss in thermostability as passaging progressed.

2.1 Materials and methods.

2.1.1 Chickens and embryonated chicken eggs (ECEs)

All chickens, chicken red blood cells and chicken embryos (ECEs) used in this study were from specific pathogen free (SPF) flock of Single Comb White Leghorn chickens which tested negative for AIV, maintained by the Canadian Food Inspection Agency (CFIA) Ottawa laboratory Fallowfield. Experiments and procedures involving chickens conformed to guidelines established by the Animal Care Committee at the CFIA, Ottawa Laboratory Fallowfield.

2.1.2 Virus

The H9N2 strain of avian influenza virus (A/Chicken/Henan/1/1998) used for this study was a gift from Drs. Earl Brown and Shuai Wang at the University of Ottawa, Ottawa, Ontario. The virus was isolated from diseased chickens in the Henan province of China in 1998[230] and was cultivated in Madin-Darby canine kidney cells. Culture fluids containing the virus were passaged twice in ECEs to prepare a stock. Virus identity was confirmed by real time reverse transcriptase PCR (RRT-PCR) using primers and probes targeting the matrix gene (M) and the hemagglutinin gene (HA)[231,232]. The H9N2 virus was passaged nineteen times via aerosol inoculation in broiler chickens. Briefly, 3 SPF chickens were placed in an aerosol chamber and nebulized with the H9N2 virus for 15 minutes. After three days' lung and tracheal tissue specimens were collected from all three chickens, pooled, and homogenized in PBS (pH 7.2, 0.01 M) at a 1:10 (w:v) ratio and diluted 10-fold before inoculation into another set of three chickens for the next passage. Passage 1 (P1) to passage 19 (P19) were obtained at the end of the passaging experiment. Lung and trachea homogenates of the wild type H9N2 virus (P0), its eighth passage (P8) and nineteenth passage (P19) were selected from the passaging experiment and inoculated into

chicken embryos to generate allantoic fluid stock virus for the inoculation of 4-week-old chickens.

2.1.3 Virus titration and Hemagglutinin test (HA test)

To determine the impact of serially passaged H9N2 virus on pathogenicity in 10-day old chicken embryos, each generated virus passage was inoculated into thirty ECEs. Ten-fold dilutions were made using PBS from 10^0 to 10^{-5} with tissue homogenate from viral passages. Each dilution (0.2 ml) of tissue homogenate was used to inoculate 5 viable eggs by the allantoic route. The eggs were incubated at 37^0C with 80 % relative humidity and allantoic fluid was recovered from all ECEs after 6 days excluding those dead within the first 24 hours after inoculation. Recovered allantoic fluid was tested for AIV by a hemagglutination assay to determine viral propagation. Briefly, 50 µl of allantoic fluid was 2-fold serially diluted in PBS in a 96-well v-bottom Microtiter plate. Chicken red blood cells (50 µl of 0.5% (w/v)) were added to each well. The plates stood for 30 to 45 minutes before recording positive or negative agglutination. The 50 percent embryo infectious dose (EID_{50}) and embryo lethal dose (ELD_{50}) were determined by inoculating ECEs with ten-fold serial dilutions of the sample fluid[233].

2.1.4 Thermostability assay

Allantoic fluid of the three viruses were normalized to 256 HA units using negative allantoic fluid and aliquoted into triplicates for each time point. The viruses were incubated at 50^0c and 54^0c for various time intervals (1, 2, 3, 4, 5, 6, 8, and 16 hours) following previously described protocols[91,141]. At each time point, triplicates of each virus were retrieved and tested for hemagglutinin activity by a hemagglutination assay as above. The time required to produce a 6-$\log_2$ reduction in HA units (from 256 to 4 HA units) was determined using a linear regression

model representing the thermal inactivation kinetics as in Equation (1) using the XLSTAT software for Microsoft Excel: $y = ax + b$ (1)

where y is the virus HA units (log_2), x is the time of incubation (h), a is the slope and b is the y-intercept. The model was considered fit when its coefficient of determination (R^2) value > 0.95 and its F test for the linearity of regression was significant at $P < 0.05$. With the XLSTAT software, the analysis of covariance[234] was used to determine significant differences in the thermal inactivation kinetics or the thermostability between each of the H9N2 viruses. The critical level for significance was set at $P < 0.05$.

2.1.5 Chicken experiments

Eighty chickens of four weeks of age were divided into 4 treatment groups. Each group of 20 chickens were held in a separate self-contained isolator operated under negative pressure and had access to feed and water *ad libitum*. Five chickens were inoculated at a time in an isolator containing a 45 cm (length) × 30 cm (width) × 30 cm (height) chamber constructed of polycarbonate (Figure 2). Allantoic fluids of P0, P8 and P19 H9N2 viruses were diluted to 6.5×10^7 EID_{50}/ml in PBS and were aerosolized into the chamber using a nebulizer (Whisper Jet™, Vital Signs Inc., Totowa, NJ, USA) and an air compressor (Aeromax 3002, Medical Industries America, Inc., Adel, IA, USA) for 15 minutes[229]. Chickens were also exposed to phosphate buffered saline (PBS, pH 7.2) as a control by aerosol inoculation. The exposed chickens were then transferred to their respective isolators for the duration of the experiment.

2.1.6 Sample Collection

To study virus shedding in our H9N2 passaging study, oropharyngeal and cloacal swabs were collected from all chickens in isolators on 1, 2, 4 and 7 days' post inoculation (DPI). Swabs were immediately transferred into 2 ml tubes containing 1 ml RLT buffer (QIAgen) and then

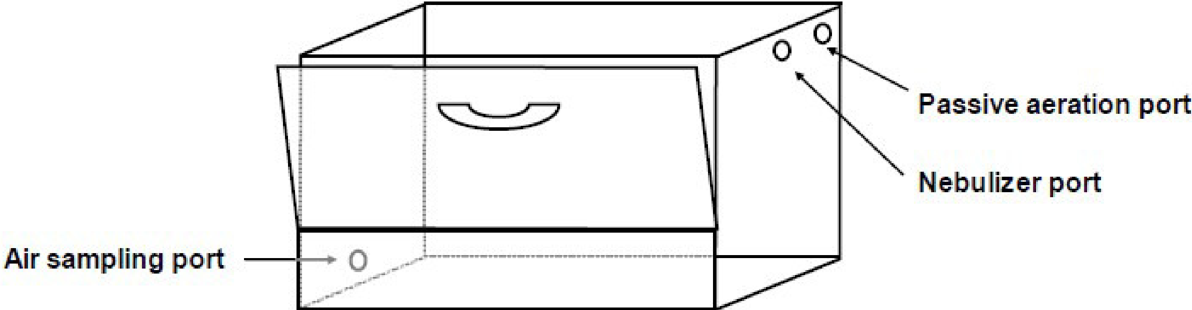

Passive aeration port
Nebulizer port
Air sampling port

Figure 2: Diagram of the aerosol chamber that included ports for a nebulizer, a Bio sampler and passive aeration. Chickens were placed in the chamber through the front panel. The flow rate generated by the compressor of the nebulizer was equivalent to that of the Bio sampler, and the port for passive aeration was left open to compensate minor fluctuations in airflow during operation. The diagram was adapted from Guan et al[235].

stored at -80°C. Virus titer in swabs was determined using RRT-PCR targeting the matrix gene and was expressed as EID_{50}/ml. To study virus replication and cytokine gene expression in tissues, 5 chickens from each group were randomly selected for euthanasia after collection of swab samples on 1, 2, 4 and 7 DPI. Trachea, lung, spleen, cecum tonsil, kidney, liver, small intestine and bursa tissue specimens were collected from each chicken in duplicates, each sample weighing approximately 50 mg. The specimens were immediately placed in 2 ml tubes containing 1 ml RNAlater (Invitrogen Canada Inc. Burlington, ON, Canada) and stored at -80^{0}C. Virus titer in tissue samples was determined using RRT-PCR targeting the matrix gene and was expressed as EID_{50}/g[232].

2.1.7 RNA Extraction and Real time reverse transcriptase-PCR (RRT-PCR)

RNA was extracted from tissue samples using the automated sample preparation equipment QIAcube and RNeasy Mini kit (QIAgen, Mississauga, Ontario). Briefly tissue samples were homogenized by bead beating using 2 ml Precellys® beads (Cayman, Ann Arbor, MI, USA) containing 1 ml QIAzol Lysis reagent and a tissue lyser (QIAgen). Chloroform (200 µl) was added to the homogenate and centrifuged at 12000 g for 15 minutes at 4°C. The aqueous phase (500 µl) recovered was transferred to the QIAcube for RNA extraction. For allantoic fluids and swab samples, 500 µl of sample fluid was transferred directly to the QIAcube for RNA extraction. Recovered RNA (10 ug) was treated with RNase-free DNase (Invitrogen).

The RNase-free DNase treated RNA extracted from tissue specimens (5 µl), was used as a template in a 20 µl one step RRT-PCR reaction using Taqman RT PCR mix (Life Technologies), primers targeting the matrix protein, M+25 5′AGATGAGTCTTCTAACCGAGGTCG, M-124 5′TGCAAAAACATCTTCAAGTCTCTG, and M+64 5′FAM-TCAGGCCCCCTCAAAGCCGA-TAMRA probe developed by Spackman

et.al [232]. Sample fluids containing no virus were spiked with known amounts of virus to generate a standard curve for quantification of virus in collected samples. Based on the standard curve, the amount of virus in each sample fluid was calculated by plotting the cycle threshold value (Ct-value) against known virus titers and was expressed as an EID_{50} equivalent (eq-EID_{50}). The quantity of extracted RNA was determined using an ND-1000 spectrophotometer (Nano Drop Technologies, Wilmington, DE). The PCR was performed with a 7500 real-time PCR system (Applied Biosystems, Foster City, CA). Thermal cycling protocol used was reverse transcription at 50^0C for 30 minutes and enzyme activation at 95^0C for 15 minutes, 45 cycles at 94^0C for 5 seconds and at 60^0C for 34 seconds for denaturation and elongation respectively.

2.1.8 cDNA synthesis and Quantification of cytokine gene expression

cDNA was synthesized by reverse transcription using Superscript II (SuperScript^{TM} First-Strand Synthesis System; Invitrogen Life Technologies, Carlsbad, California, USA) and oligo (dT) primer to create the complementary DNA strand from RNase-free DNase treated RNA. Briefly 11 µl of treated RNA was mixed with 1 µl of oligo (dT) primer and 1 µl of 10 mM dNTP to a final volume of 13 µl. The mixture was incubated at 65^0C for 5 minutes. Then, 4 µl of 5× first strand buffer, 1 µl of 0.1M DTT, 1 µl of RNase OUT^{TM} ribonuclease inhibitor (40 units/µl), 1 µl of SuperScript^{TM} RT (200 unit/µl) and 1 µl of RNase free water was added into the tube. The reaction was completed at 42^0C for 50 minutes and was terminated by heating at 70^0C for 15 minutes.

Expression of interferon gamma (IFN-ɣ), interleukin 1 beta (IL-1β), and interleukin 6 (IL-6) were evaluated with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene. RNA extracted from the lung, and spleen tissue specimens were used for synthesis of complementary DNA with Oligo (dT) primer and the Invitrogen kit (SuperScriptTM First-

Strand Synthesis System; Invitrogen Life Technologies, Carlsbad, California, USA) according to the manufacturer's instruction and as previously described[236]. The real time quantitative PCR (qPCR) was performed with the 7500 real-time PCR system (Applied Biosystems, Foster City, CA) using the Power SYBR® Green PCR Master Mix, containing SYBR® Green 1 Dye, AmpliTaq Gold DNA Polymerase, dNTPs, and optimized buffer components for detection of DNA in a reaction volume of 20 µL. Gene-specific primers and 5 µL of a 1:10 dilution of complementary DNA as templates were used for the qPCR. The thermal cycling parameters included pre-incubation at 50^0C for 2 minutes, 95^0C for 10 minutes; 40 cycles at 95^0C for 15 seconds, 60^0C for 1 minute, and dissociation cycle at 95^0C for 15 seconds and 60^0C for 1 minute with a final cycle of 95^0C for 15 seconds.

2.1.9 Sequencing and sequence analysis

Sequencing of the H9N2 virus, and its eighth and nineteenth passages was carried out at the National Centre for Foreign Animal Disease (CFIA Winnipeg laboratory – Arlington). Allantoic fluid generated from the inoculation of chicken embryos with tissue homogenates were used in whole genome sequencing of P0, P8 and P19 viruses. Total RNA was extracted from allantoic fluid using MagMax-96 viral isolation kit (Thermofisher). RNA was amplified using PathAmp™ FluA Reagents (Thermofisher) for the PCR amplification of Influenza A genomic segments for Ion PGM™ library preparation. Amplicons generated were purified and libraries were prepared using the Ion Xpress™ Plus Fragment Library Kit (Thermofisher). The libraries were quantified with the Ion Library Quantitation Kit (Thermofisher) and then amplified using the Ion PGM OT2 200 template kit (Thermofisher). Sequencing was performed with Ion Torrent PGM™ instrument (Life Technologies) and Ion PGM ™Hi-Q™ Sequencing Kit. The nucleotide and deduced amino acid sequences of the H9N2 avian influenza viruses used in the study and the

reference strains in the GenBank database were aligned with the Clustal W method in the MegAlign program (Lasergene 13.0.2 DNASTAR, Madison, WI), and the nucleotide sequences of the eight segments were compared with each other and to those of the avian influenza viruses in GenBank using the BLAST search method.

2.1.10 Statistical Analysis.

Statistical analysis was performed using the students T-Test for differences in viral titer between groups. Statistical analysis was performed using GraphPad Prism statistical software using two-way Anova (Analysis of Variance) on cytokine gene expression in tissue organs. The critical level for significance was set at $P < 0.05$ and data was expressed as mean ± standard deviation, determined from 5 individual birds randomly selected from each group at the designated days.

2.2 Results

2.2.1 Positive selection of Q226L mutation observed following passaging in chickens

The H9N2 viruses were analysed phylogenetically and all eight segments of the three viruses were shown to be derived from those of Dk/HK/Y280/97, a representative virus that derived from Ck/Beij/1/94 lineage. The HA's of the three viruses bore the highest nucleotide homology percentage (P0 99.5%, P8 99.4% and P19 99.2%) to A/Ch/Hubei/01/1999 (H9N2), a chicken virus isolated in the Hubei province in China in 1999. The H9N2 viruses possessed the R-S-S-R monobasic cleavage motif, characteristic of LP viruses, at the HA cleavage site (Figure 3). After 19 passages in 3-day old chickens, non-synonymous mutations were observed in the HA, PBI and NA genes (Table 1).

In the HA gene, 5 non-synonymous mutations were observed. At amino acid residue 226, we detected the presence of viral quasispecies. The wild type virus (P0) contained a mixed population with 54% of variants possessing glutamine at position 226 (Q226) and 46% of variants with leucine (L226) out of a total of 194 reads. Following eight passages in chicks, the resulting viruses (P8) had 87% of variants expressing glutamine and 13% expressing leucine of a total of 177 reads. However, by the 19th passage 100% of reads generated were Leucine at position 226 (Figure 3). Four additional mutations were observed in the HA gene namely, N304D, V318I and HA2-H68Y in both P8 and P19, and HA2-N46D in P19 (Table 1). A N76S mutation occurred in the PB1 gene in both P8 and P19 and a S311R mutation in the NA gene of the P19 virus (Table 1).

```
HA1      11                                                        67
AiH3     ATLCLGHHAVPNGTLVKTITDDQIEVTNATELVQSSSTGKICN---NPHRILDGIDCTLI
P0       DKICIGYQSTNSTETVDTLTENNVPVTHAKELLHTEHNGMLCATNLGHPLILD--TCTIE
P8       DKICIGYQSTNSTETVDTLTENNVPVTHAKELLHTEHNGMLCATNLGHPLILD--TCTIE
P19      DKICIGYQSTNSTETVDTLTENNVPVTHAKELLHTEHNGMLCATNLGHPLILD--TCTIE
Y280     DKICIGYQSTNSTETVDTLTENNVPVTHAKELLHTEHNGMLCATNLGHPLILD--TCTIE
         68                                                       124
AiH3     DALLGDPHCDVFQN-ETWDLFVERSKAFS-NCYPYDVPDYASLRSLVASSGTLEFITEG-
P0       GLIYGNPSCNLLLGGREWSYIVERPSAVNGLCYPGNVENLEELRSLFSSASSYQRIQIFP
P8       GLIYGNPSCNLLLGGREWSYIVERPSAVNGLCYPGNVENLEELRSLFSSASSYQRIQIFP
P19      GLIYGNPSCNLLLGGREWSYIVERPSAVNGLCYPGNVENLEELRSLFSSASSYQRIQIFP
Y280     GLIYGNPSCDLLLGGREWSYIVERPSAVNGLCYPGNVENLEELRSLFKSASSYQRIQIFP
         125                                                      184
AiH3     FTWTGVTQNGGSNACKRGPGSGFFSRLNWLTKSGSTYPVLNVTMPNNDNFDKLYIWGVHH
P0       DTIWNVSYSGTSKACS----DSFYRSMRWLTQKNNAYPIQDAQYTNNRGKNILFMWGINH
P8       DTIWNVSYSGTSKACS----DSFYRSMRWLTQKNNAYPIQDAQYTNNRGKNILFMWGINH
P19      DTIWNVSYSGTSKACS----DSFYRSMRWLTQKNNAYPIQDAQYTNNRGKNILFMWGINH
Y280     DTIWNVSYSGTSKACS----DSFYRSMRWLTQKNNAYPIQDAQYTNNRGKNILFMWGINH
         185                                                      244
AiH3     PSTNQEQTSLYVQASGRVTVSTRRSQQTIIPNIESRPWVRGLSSRISIYWTIVKPGDVLV
P0       PPTDTVQTDLYTRTDTTTSVATEDINRTFKPLIGPRPLVNGXQGRIDYYWSVLKPGQTLR
P8       PPTDTVQTDLYTRTDTTTSVATEDINRTFKPLIGPRPLVNGQQGRIDYYWSVLKPGQTLR
P19      PPTDTVQTDLYTRTDTTTSVATEDINRTFKPLIGPRPLVNGLQGRIDYYWSVLKPGQTLR
Y280     PPTDTTQTNLYTRTDTTTSVATEDINRTFKPLIGPRPLVNGLQGRIDYYWSVLKPGQTLR
         245                                                      303
AiH3     INSNGNLIAPR-GYFKMRTGKSSIMRSDAPIDTCISECITPNGSIPNDKPFQNVNKITYG
P0       VRSNGNLIAPWYGHILSGESHGRILKTDLNSGNCVVQCQTERGGLNTTLPFHNVSKYAFG
P8       VRSNGNLIAPWYGHILSGESHGRILKTDLNSGNCVVQCQTERGGLNTTLPFHNVSKYAFG
P19      VRSNGNLIAPWYGHILSGESHGRILKTDLNSGNCVVQCQTERGGLNTTLPFHNVSKYAFG
Y280     VRSNGNLIAPWYGHILSGESHGRILKTDLNSGNCVVQCQTERGGLNTTLPFHNVSKYAFG
         304                        329
AiH3     ACPKYVKQNTLKLATGMRNVPEKQTR
P0       NCPKYVGVKSLKLAVGLRNVPARSSR
P8       DCPKYVGVKSLKLAIGLRNVPARSSR
P19      DCPKYVGVKSLKLAIGLRNVPARSSR
Y280     NCPKYVGVKSLKLAVGLRNVPARSSR
```

```
HA2     1                                                          60
AiH3    GLFGAIAGFIENGWEGMIDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLNRVIEKTN
P0      GLFGAIAGFIEGGWSGLVAGWYGFQHSNDQGVGMAADRDSTQKAIDKITSKVNNIVDKMN
P8      GLFGAIAGFIEGGWSGLVAGWYGFQHSNDQGVGMAADRDSTQKAIDKITSKVNNIVDKMN
P19     GLFGAIAGFIEGGWSGLVAGWYGFQHSNDQGVGMAADRDSTQKAINKITSKVNNIVDKMN
Y280    GLFGAIAGFIEGGWSGLVAGCNGFQHSNDQGVGIAADRDSTQRAIDKITSKVNNIVDKMN
        61                                                        120
AiH3    EKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLTDSEMNKLFE
P0      KQYEIIDHEFSEVETRLNMINDKIDDQIQDIWAYNAELLVLLENQKTLDEHDANVNNLYN
P8      KQYEIIDYEFSEVETRLNMINDKIDDQIQDIWAYNAELLVLLENQKTLDEHDANVNNLYN
P19     KQYEIIDYEFSEVETRLNMINDKIDDQIQDIWAYNAELLVLLENQKTLDEHDANVNNLYN
Y280    KQYEIIDHEFSEVENRLNMINNKIDDQIQDIWAYNAELLVLLENQKTLDEHDANVNNLYN
        121                                                       180
AiH3    KTRRQLRENAEDMGNGCFKIYHKCDNACIESIRNGTYDHDVYRDEALNNRFQIKGVELKS
P0      KVKRTLGSNAVEDGKGCFELYHKCDDQCMETIRNGTYNRRKYKEESRLERQKIEGVKLES
P8      KVKRTLGSNAVEDGKGCFELYHKCDDQCMETIRNGTYNRRKYKEESRLERQKIEGVKLES
P19     KVKRTLGSNAVEDGKGCFELYHKCDDQCMETIRNGTYNRRKYKEESRLERQKIEGVKLES
Y280    KVKRALGSNAVEDGKGCFELYHKCDDQCMETIRNGTYNRRKYKEESRLERQKIEGVKLES
        181                                     221
AiH3    -GYKDWILWISFAISCFLLCVVLLGFIMWACQRGNIRCNICI
P0      EGTYKILTIYSTVASSLVVAMGFAAFLFWAMSNGSCRCNICI
P8      EGTYKILTIYSTVASSLVVAMGFAAFLFWAMSNGSCRCNICI
P19     EGTYKILTIYSTVASSLVVAMGFAAFLFWAMSNGSCRCNICI
Y280    EGTY--------------------------------------
```

Figure 3: Amino acid sequence alignment of the HA1 and HA2 subunit of H3 (A/Aichi/2/68), wild type H9N2 (P0) (A/Ch/Henan/1/1998), passage 8 (P8), passage 19 (P19) and H9N2 (Y280) (A/duck/HongKong/Y280/97) AIV. Mutations are highlighted in red and the cleavage site highlighted in yellow. Numbering is per the H3 numbering system. Horizontal lines above sequences indicate respective subdomains (Red, for Fusion subdomains, yellow for vestigial esterase, and blue for receptor binding). Multiple sequence alignment was generated in sequence format using sequeence alignment software such as T-coffee and Box shade server.

Table 1: Nucleotide and amino acid substitutions in gene segments of the passaged H9N2 virus

Protein	Nucleotide Alterations			Position			Amino acid Substitutions		
	P0	P8	P19	Nucleotide	Amino acid [a]	[b]	P0	P8	P19
HA	W(A/T)	A	T	734	216	226	Q/L [d]	Q/L [e]	L [f]
	A	G	G	970	295	304	N	D	D
	G	A	A	1012	309	318	V	I	I
	A	A	R(A/G)	1083	332	341	G	G	G
	G	G	A	1183	366	375	D	D	N
	C	T	T	1249	388	397	H	Y	Y
PB1	A	G	G	251	76		N	S	S
NA	W(A/T)	W(A/T)	M(A/C)	922	[c] 311		S	S	R

[a] *HA amino acids were numbered per H9 HA amino acid numbering by (Burke & Smith, 2014)*

[b] *HA amino acids were numbered per H3 HA amino acid numbering by (Nichols, 2008)*

[c] *NA amino acids were numbered per N2 NA amino acid numbering by (Colman, 1993)*

[d] *The percentage of reads for Q-226 (54%) and L-226 (46%) variants out of a total of 194 reads.*

[e] *The percentage of reads for Q-226 (87%) and L-226 (13%) variants out of a total of 172 reads.*

[f] *The percentage of reads for Q-226 (0%) and L-226 (100%) variants out of a total of 151 reads.*

2.2.2 H9N2 AIV exhibits low pathogenicity in chickens following nineteen serial passages.

Chickens inoculated with virus or PBS survived until the end of the experiment or until euthanized for tissue sampling. Corresponding to the low pathogenic HA motif observed in amino acid sequences of the three viruses, a lack of clinical signs and mortality was observed in all chickens over the seven-day duration of the experiment.

2.2.3 H9N2 AIV's have a tropism for the respiratory tract.

To determine if passaging of the H9N2 virus had an influence on tissue tropism, we isolated virus from oropharyngeal and cloacal swabs, as well as tissue specimens. All three viruses replicated efficiently in the oral cavity as virus RNA was detected in oropharyngeal swabs collected from all virus exposed chickens from DPI 1 to 4. Total virus clearance was observed by DPI 7. P19 virus titer was however significantly higher ($P<0.05$) on DPI 1 than P0 and P8 viruses (Figure 4A). In contrast, cloacal swabs recovered from all virus exposed chickens, tested negative for virus on each sampling day (not shown).

The H9N2 viruses showed a tropism for the respiratory organs as the highest viral titers were observed in tracheal and lung tissues. In tracheal tissues the viruses replicated similarly, from approximately 9 $\log_{10}$ EID_{50}/g on DPI 1 to approximately 5 $\log_{10}$ EID_{50}/g by DPI 7. However, on DPI 2 P0 was statistically higher in virus titer than P8 ($p<0.01$) and P19 ($p<0.05$) groups (Figure 4B). In lung tissues, all virus inoculated groups were similar in titer on DPI 1 and 2 (approximately 7$\log_{10}$ EID_{50}/g) before dropping in titer to approximately 3 $\log_{10}$ EID_{50}/g on DPI 7 with statistical difference only detected on DPI 4 where the P0 virus had on average, higher titers than P19 inoculated chickens ($p<0.05$) (Figure 4C).

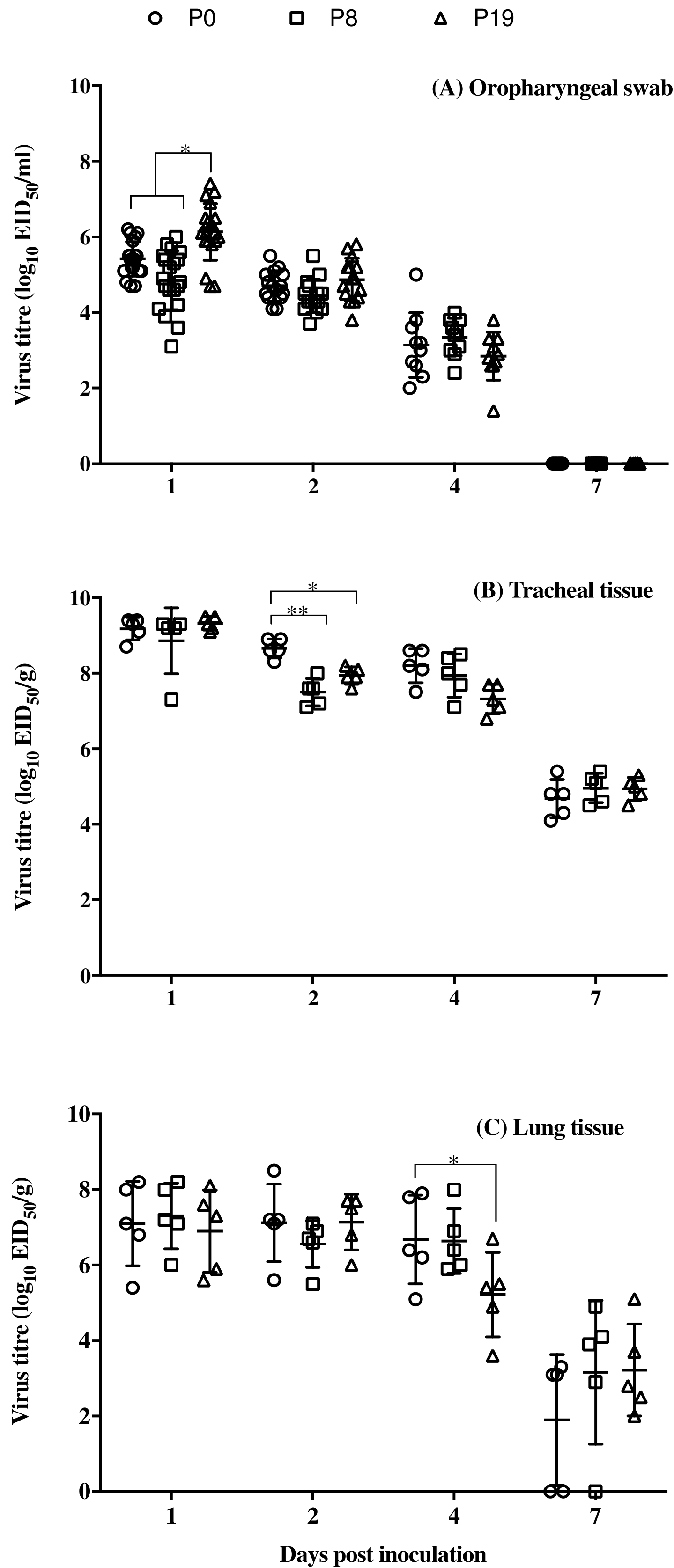

P0
P8
P19
(A) Oropharyngeal swab
Virus titre (log_{10} EID_{50}/ml)
(B) Tracheal tissue
Virus titre (log_{10} EID_{50}/g)
(C) Lung tissue
Virus titre (log_{10} EID_{50}/g)
Days post inoculation

Figure 4: Virus infection in (A) oropharyngeal swabs (B) tracheal tissue and (C) lung tissue from chickens exposed to aerosols generated from 6.5×10^7 EID_{50}/ml of H9N2 virus (P0), passage 8 (P8) and passage 19 (P19). Graphs represent the means and SD of viral titers in swabs (Log10 EID_{50}/ml) and tissue specimen (Log10 EID_{50}/g) in each group of chickens sampled on each day post inoculation. * represents significant differences between groups ($p<0.05$) and ** represents significant differences between groups ($p<0.01$).

2.2.4 P0 and P19 AIV's replicate efficiently in systemic tissues.

We assessed the replication of all three viruses in cecum tonsil, kidney and spleen tissues. A similar trend was observed in the replication of P8 virus in these tissues. In contrast to the significantly higher replication of P0 and P19 viruses on DPI 1 and 2 in cecum tonsil and spleen tissues, P8 inoculated chickens tested negative for virus on these days and was only detected after DPI 4 in these organs (Figure 5A and C). In kidney tissues, P0 and P19 inoculated chickens also had higher and more significant titers on DPI 2 compared to P8. No differences were observed on other days (Figure 5B). The P19 virus was more efficient in replication in systemic tissues. No significant differences were detected in liver, small intestine and bursa tissues assessed and all three groups were detected at low titers (< 4 log_{10} EID_{50}/g) over the seven days (data not shown).

2.2.5 Early induction of proinflammatory and antiviral cytokines in lungs of infected chickens.

The expression of proinflammatory (IL-6 and IL-1β) and antiviral (IFN- γ) cytokine mRNA levels in lung and spleen tissues were quantified by quantitative PCR. IL-6 mRNA expression, in the lungs of chickens inoculated with any of the H9N2 viruses, were significantly upregulated at least 5-fold in lung tissues on DPI 1 ($P<0.01$). In contrast to P0 and P19 viruses which increased in fold change (10-fold) on DPI 2, a decrease was observed in expression of IL-6 in P8 inoculated chickens (4-fold) which was statistically different from the two other groups ($P<0.01$). On DPI 4, the expression of IL-6 mRNA was significantly higher ($P<0.01$) in P0 and P8 inoculated chickens whilst a decline in expression was observed in P19 inoculated chickens. Expression of IL-6 mRNA returned to normal levels on DPI 7 in all virus inoculated chickens (Figure 6A).

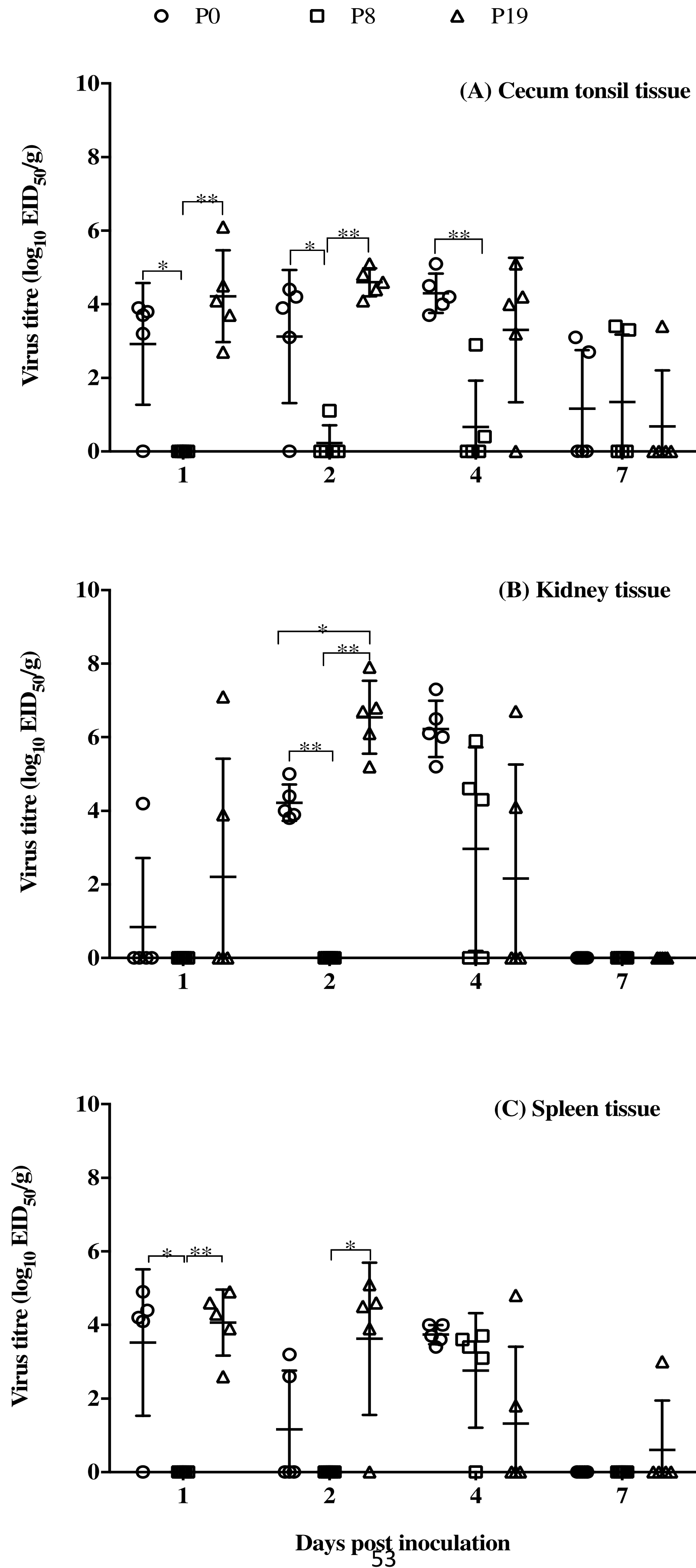

P0
P8
P19
(A) Cecum tonsil tissue
Virus titre (log10 EID50/g)
10
8
6
4
2
0
1
2
4
7
*
**
(B) Kidney tissue
(C) Spleen tissue
Days post inoculation

Figure 5: Virus infection in (A) cecum tonsil tissue (B) kidney tissue and (C) spleen tissue from chickens exposed to aerosols generated from 6.5×10^7 EID_{50}/ml of H9N2 virus (P0), passage 8 (P8) and passage 19 (P19). Graphs represent the means and SD of viral titers in tissue specimen (Log_{10} EID_{50}/g) in each group of chickens sampled on each day post inoculation. * represents significant differences between groups ($p<0.05$) and ** represents significant differences between groups ($p<0.01$).

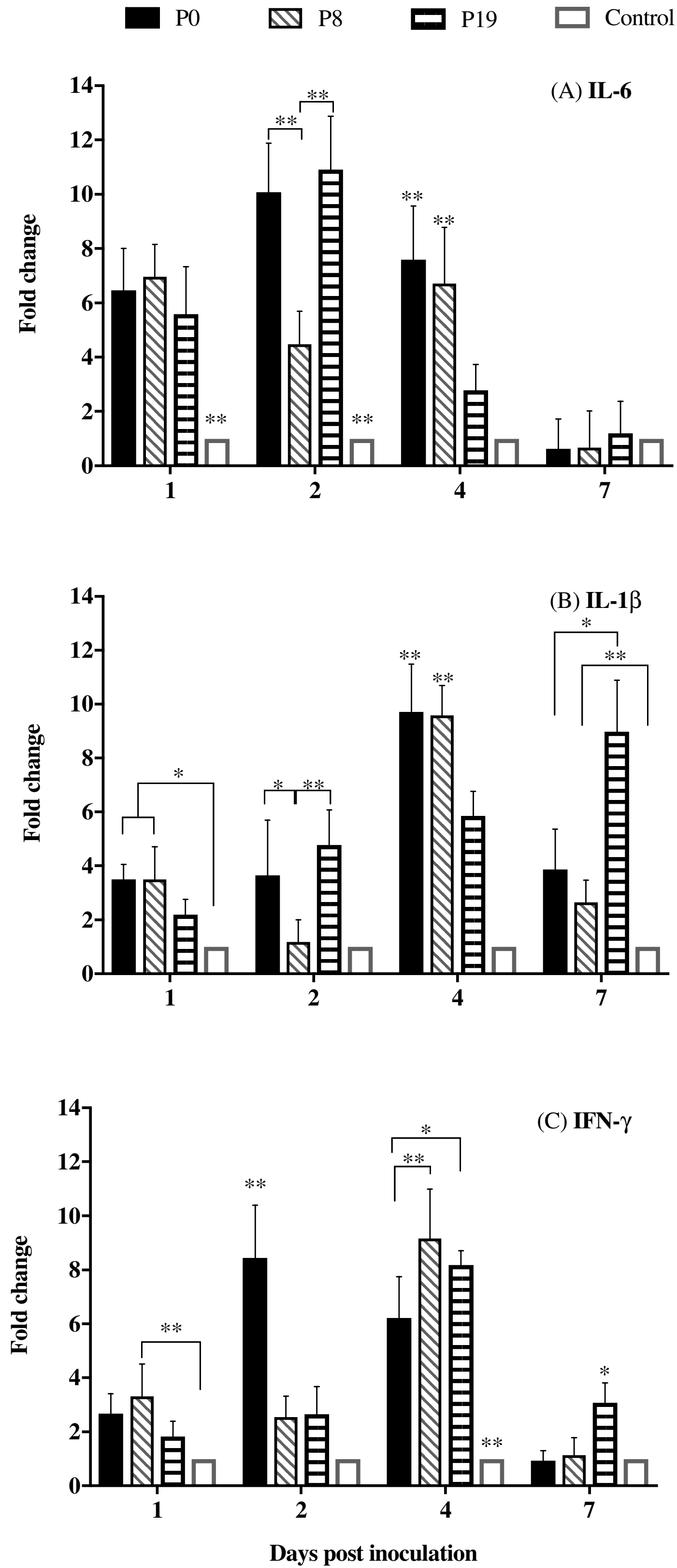

P0
P8
P19
Control
(A) IL-6
(B) IL-1β
(C) IFN-γ
Fold change
Days post inoculation
1
2
4
7

Figure 6: Cytokine mRNA expression of (A) IL-6, (B) IL-1β and (C) IFN- γ in Lung tissues of 5 chickens collected on each day post inoculation from four groups of chickens exposed to aerosols generated from 6.5×10^7 EID_{50}/ml of H9N2 virus (P0), passage 8 (P8) and passage 19 (P19) and PBS as a control. Cytokine mRNA expression was analyzed using real-time PCR. Data are expressed as mean relative expression with standard deviation relative to GAPDH mRNA. Significance of the regulation level compared to controls is analyzed. ** ($p<0.01$) and * ($p<0.05$) represent significant difference between groups.

Expression of IL-1β mRNA was observed to be at lower fold changes than IL-6 mRNA on DPI 1 and 2 although a similar trend was observed. P8 inoculated chickens were also observed to express IL-1β lower than P0 ($P<0.05$) and P19 ($P<0.01$) inoculated chickens on DPI 2. P0 and P8 IL-1β mRNA expression however peaked on DPI 4 and was significantly upregulated compared to P19 mRNA expression ($P<0.01$). The expression of IL-1β mRNA in P19 inoculated chickens increased in fold change each day, eventually peaking on DPI 7 and was significantly upregulated compared to P0 ($P<0.05$) and P8 ($P<0.01$) inoculated chickens (Figure 6B).

IFN- γ mRNA was expressed below 3-fold in lung tissues of all virus inoculated chickens on DPI 1. However, expression in the lungs of P8 inoculated chickens was significantly upregulated compared to control chickens ($P<0.01$). A sharp increase in fold change was observed in P0 inoculated chickens on DPI 2 averaging 8-fold ($P<0.01$) while P8 and P19 inoculated chickens remained at similar levels seen on DPI 1. All three virus inoculated groups were upregulated in mRNA expression on DPI 4. However, the P0 group was significantly lower than P8 ($P<0.01$) and P19 ($P<0.05$) groups. P0 and P8 inoculated chickens returned to control levels on DPI 7 while P19 was statistically upregulated with a 3-fold change observed ($P<0.05$) (Figure 6C).

Overall, all groups expressed IL-6 and IL-1β proinflammatory cytokines early in the lungs during infection. However, higher fold changes were observed in IL-6 expression. Fold changes on all the days assessed were below 10-fold in the lungs. A significant drop in proinflammatory cytokine expression was observed in P8 inoculated chickens compared to P0 and P19 groups on DPI 2, after which mRNA expression peaked on DPI 4. P19 inoculated

chickens in contrast to P0 and P8 groups showed significant upregulation in IL-1β and IFN- γ expression on DPI 7 in lung tissues.

2.2.6 Induction of proinflammatory and antiviral cytokines in spleen tissues.

Lower fold changes (< 5-fold) were observed in the expression of proinflammatory and antiviral cytokines in the spleens of H9N2 inoculated chickens compared to the lungs (Figure 7). Significant upregulation of IL-6 and IL-1β was observed in P8 inoculated chickens on DPI 1 when compared to control chickens ($P<0.05$) (Figure 7A and 7B). Expression of proinflammatory cytokines in H9N2 inoculated chickens were largely like that of controls for the rest of the experiment. Expression of INF- γ followed the same trend with P8 inoculated chickens being significantly upregulated in INF-γ mRNA expression compared to both P0 and P19 on DPI 1 ($P<0.01$) (Figure 7C). P19 inoculated chickens however expressed INF- γ significantly higher than P0 and P19 on DPI 7 ($P<0.05$).

2.2.7 Virulence in chicken embryos

Tissue homogenates recovered from serial passaging of P0, P8 and P19 in less than a week-old chicks were used in the inoculation of 10-day old chicken embryos. Serial passaging of the H9N2 virus in chicks resulted in a decrease in pathogenicity in chicken embryos. The embryo infectious dose (EID_{50}) of the homogenates fell within the range of 4.3 to 7.2 log_{10} EID_{50}/g with an average of 6 log_{10} EID_{50}/g in tissue homogenates for all 19 passages. However, the embryo lethal dose (ELD_{50}) gradually declined as the number of passages increased, more specifically, from 7 log_{10} ELD_{50}/g of P0 in tissues, to the lack of lethality observed in P19 (Figure 8).

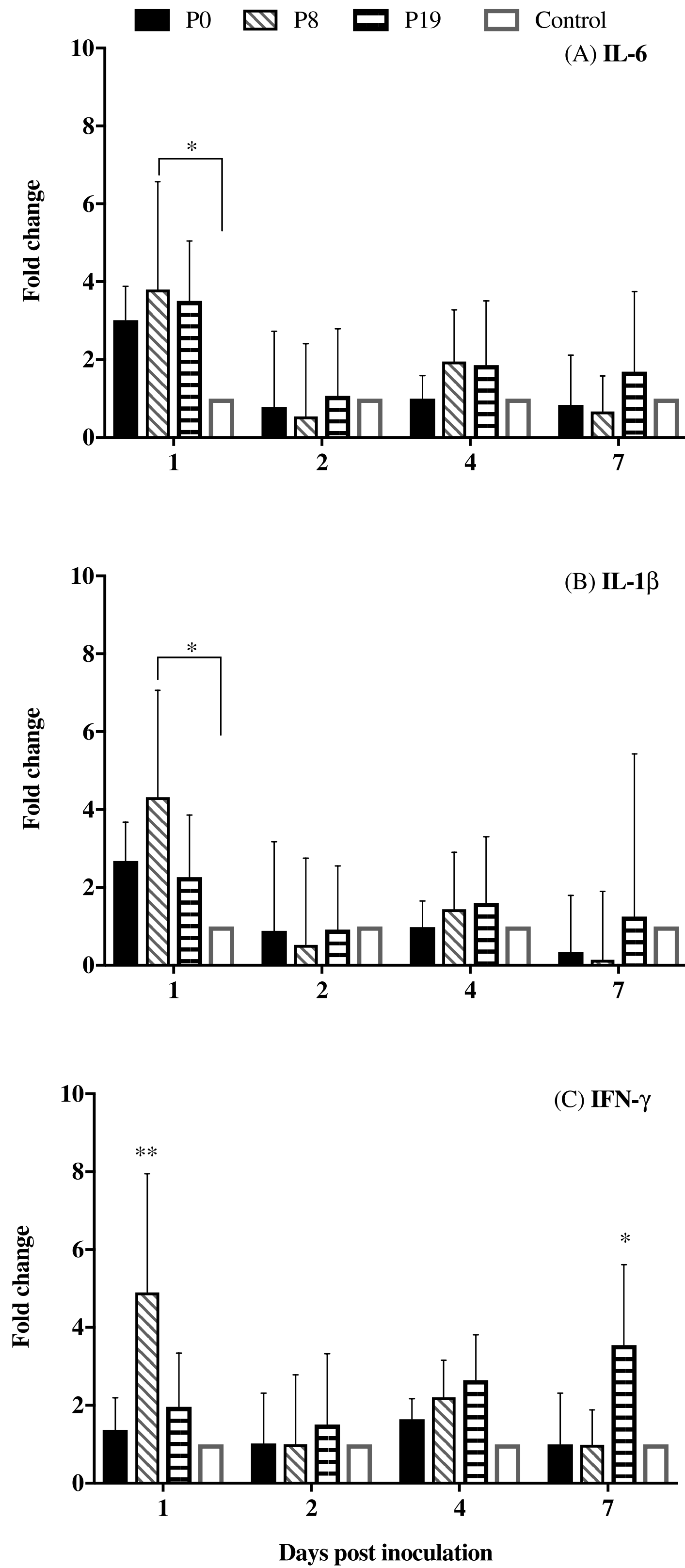

P0
P8
P19
Control
(A) IL-6
(B) IL-1β
(C) IFN-γ
Fold change
10
8
6
4
2
0
1
2
4
7
*
**
Days post inoculation

Figure 7: Cytokine mRNA expression of (A) IL-6, (B) IL-1β and (C) IFN- γ in spleen tissues of 5 chickens collected on each day post inoculation from four groups of chickens exposed to aerosols generated from 6.5×10^7 EID_{50}/ml of H9N2 virus (P0), passage 8 (P8) and passage 19 (P19) and PBS as a control. Cytokine mRNA expression was analyzed using real-time PCR. Data are expressed as mean relative expression with standard deviation relative to GAPDH mRNA. Significance of the regulation level compared to controls is analyzed. ** ($p<0.01$) and * ($p<0.05$) represent significant difference between groups.

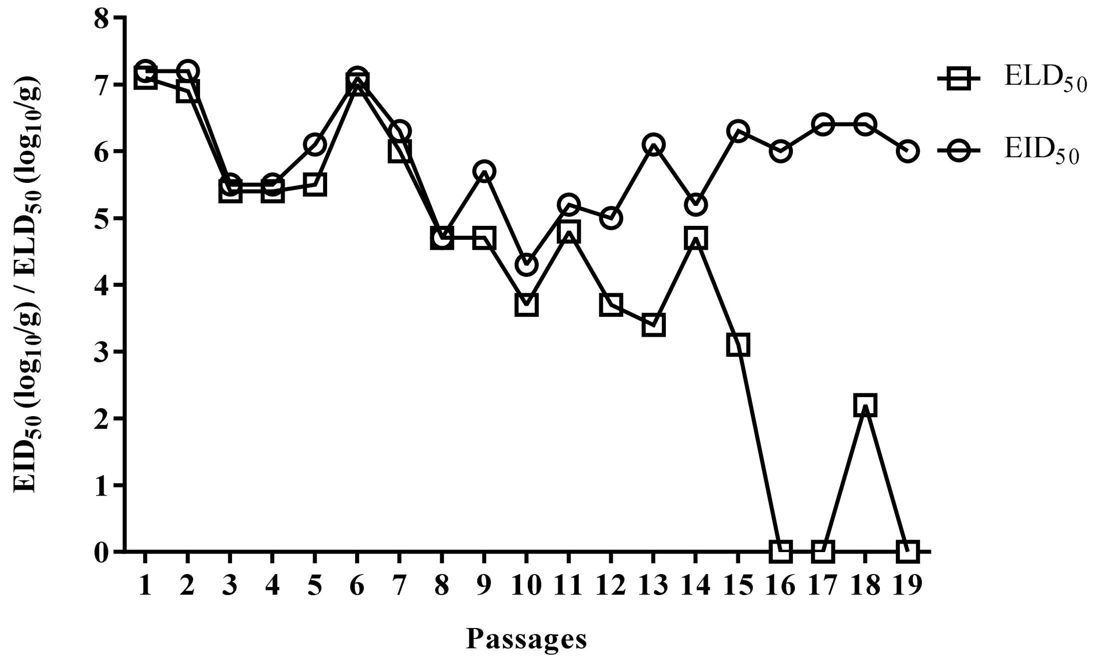
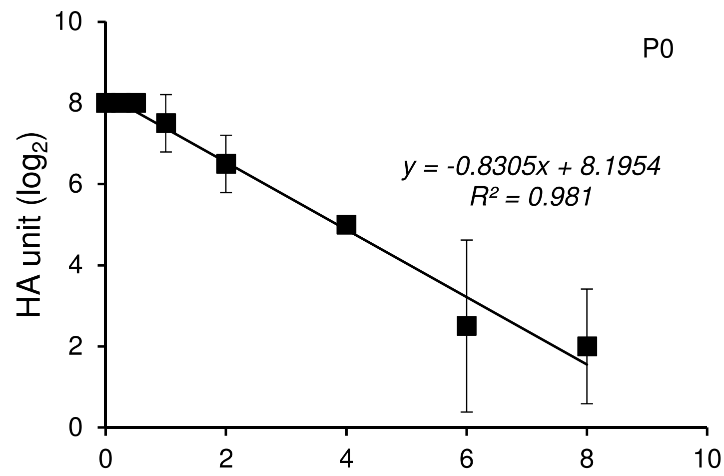

EID50 (log10/g) / ELD50 (log10/g)
0
1
2
3
4
5
6
7
8
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
Passages
ELD50
EID50

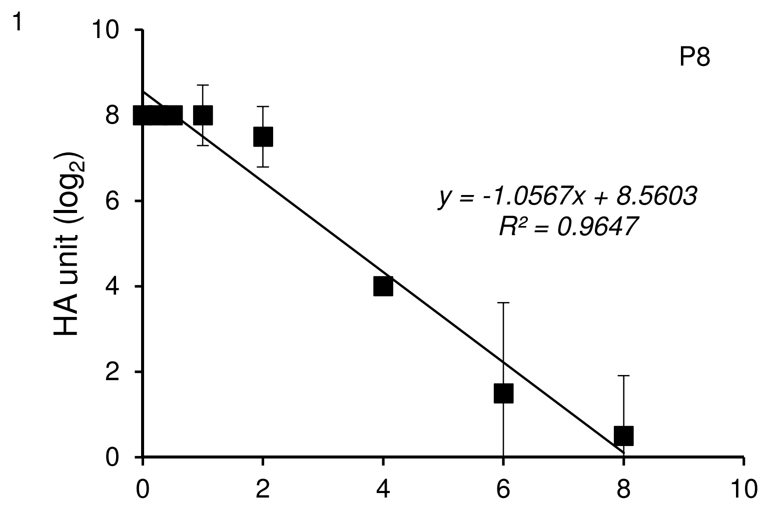
Figure 8: Pathogenicity assessment of Passage 1 to 19 H9N2 chicken virus in 10 day old ECE. Each passage (0.2 ml) was inoculated into chicken embryos for 6 days, after which allantoic fluid collected was tested using the hemagglutination test (HA) to determine viral titer (expressed as $\log_{10}$ EID_{50}/g) and embryo lethal dose (expressed as $\log_{10}$ ELD_{50}/g).

2.2.8 Reduction in HA thermostability

The HA thermal stability of P0, P8 and P19 viruses were assessed by the HA inactivation assay following heat treatment at 50°C and 54°C. At 50°C no change was observed in HA units of all three viruses for up to 6 hours after treatment (data not shown). However, when treated at 54°C a gradual decline in the HA units of each virus was observed from 256 to 4, although at varying times. The time required to reduce HA units of P0, P8 and P19 from 256 to 4 HA units was 7.2, 5.7 and 4.3 hours respectively. P0 and P8 had similar inactivation kinetics, while P19 was significantly (P <0.05) less thermostable than P0 (Figure 9).



1



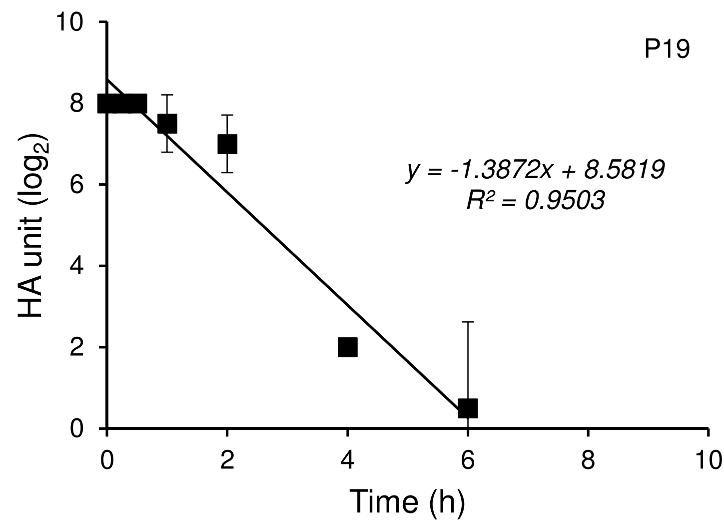


Figure 9: Thermal inactivation kinetics of heat treatment on hemagglutinin activity of H9N2 virus (P0), passage 8 (P8) and passage 19 (P19) at 54°C. The viruses were diluted to 256 HA units with negative allantoic fluid and divided into several aliquots. Aliquots were incubated at 54°C and removed for viral titre determination time points spanning 16 hours via HA test. Values represent the means and S.D. of duplicate experiments.

2.3 Discussion

Genetic analysis revealed that the H9N2 A/Chicken/Henan/1/1998 and its consecutive eight and nineteenth passages possessed low pathogenic R-S-S-R motifs at the HA cleavage site, characteristic of Y280 H9N2 viruses[214,237]. The three H9N2 viruses were asymptomatic in all chickens inoculated via aerosol exposure correlating to the low pathogenic motif observed from genetic analysis. Serial passaging of the H9N2 virus failed to result in the acquisition of multiple amino acids in the cleavage site of the HA gene which has been shown to increase pathogenicity of a H9N2 virus in reverse genetic experiments[238]. Even though the H9N2 virus is currently endemic in China, highly pathogenic H9N2 have rarely been isolated from poultry populations.

Animal models or culture systems have been employed by researchers to demonstrate the acquisition of high pathogenicity by low pathogenic avian influenza viruses[239–242]. For instance, the passaging of low pathogenic H9N2 avian influenza virus in 14 day old embryos has been shown to result in enhanced replication and pathogenicity in embryos and chickens. The virus after 10 passages displayed a wider tissue tropism in chickens while also inducing clinical signs[243]. However, no direct linkage has been made between the passaging of AIV in embryos and the generation of viruses with increased pathogenicity in other hosts. Vaccines for instance, are created by passaging of AIV in embryos, thus decreasing virulence in target hosts[240,244,245]. A Madin-Darby canine kidney (MDCK) cell passaged H5N1 virus showed increased pathogenicity in mice while passaging in ECEs resulted in the adaptation and decreased pathogenicity in mice[199].

In our experiment, we inoculated viruses generated from passages in chickens into 10 day old ECEs to assess pathogenicity in embryos. All viruses replicated to high titers (> 4 logs) in chicken embryos. However, a gradual decrease in virulence was observed as passaging

progressed from the ninth passage up till the nineteenth. This is in contrast to observations made in the passaging of an H5N3 virus in chick's air sacs, in which the passaged virus gained increased virulence in chickens and also chicken embryos[168]. This suggests the virulence of passaged viruses may vary depending on the host in which passaging occurs and the infecting virus strain.

AIV in poultry is considered to be shed predominantly via the respiratory route with limited shedding observed in faecal samples[227,246,247]. Accordingly, in our experiments all chickens inoculated with the H9N2 virus or its passages shed virus orally up until the last day of sampling while in contrast limited cloacal shedding was observed. Whilst no major differences were observed in the replication of the three viruses in the respiratory tracts of chickens, significant differences were found in kidney, spleen, and cecum tonsil tissues between P8 and both P0 and P19 inoculated chickens. Virus was non-existent in these organs on the first and second day post inoculation of the P8 virus in chickens. However, virus was detected in at least four of five chickens in P0 and P19 virus inoculated groups. A study of the systemic distribution of low pathogenic viruses in chickens suggests receptor affinity or replication efficiency may play a role in the observed difference in distribution of H9N2 virus in chicken kidney organs[79,117,248]. In this study, a LP H9N2 virus was not detected in heart and kidney organs of chickens until DPI 4 despite replicating in lung and spleen tissues earlier on[79].

The glutamine (Q) to leucine (L) substitution at amino acid position 226 is well documented to switch the HA receptor binding from α2,3 SA-gal to α2,6 SA-gal which affects the virus host range[249]. Leucine at position 226 increases binding affinity to human type receptors and thus replication in human airway epithelial cells[249]. Viruses possessing avian receptor specificities have been shown to replicate slowly compared to human like counterparts in cell cultures[250]. In

our experiments the serial passaging of the wild type H9N2 P0 virus, which possessed an equal mix of glutamine and leucine variants, led to the acquisition of leucine at position 226 following 19 passages. Interestingly, the P8 virus possessed a majority of glutamine variants (87%) which may explain its restriction in kidney or spleen organs which express both avian and human receptors at varying concentrations[28,83,250–253]. In contrast the nineteenth passage possessed only variants with leucine at 226 and exhibited slightly improved replication in systemic tissues than the wild type H9N2. P19 also acquired D46N mutation in its HA protein which is associated with enhancing binding to avian receptors in guinea pigs enabling dual receptor binding[141].

The expression of proinflammatory and antiviral cytokines may also play a role in the restriction seen in kidney and spleen tissues of P8 inoculated chickens. In spleen tissues P8 inoculated chickens expressed IL-6, IL-1β and IFN- γ mRNA at significantly higher levels on the first day post inoculation compared to other groups. However, on DPI 2 expression of cytokines in P8 inoculated chickens was downregulated before increasing again on DPI 4 in response to viral replication in tissues. Thus, the absence of viral replication in spleen tissues until DPI 4 may be due to the strong cytokine response on DPI 1 however, cytokine mRNA levels obtained via qPCR does not necessarily equate to bioactive protein[254]. A study assessing the mechanism involved in HPAI pathogenesis in ducks and chickens discovered that the timely induction of moderately expressed (<10-fold) proinflammatory or antiviral cytokines contributed to viral clearance in ducks while overexpression (>20-fold) contributed to mortality in chickens[184]. In lung and spleen tissues, the expression of proinflammatory and antiviral cytokines were adequate in response to infection, with fold changes under 15-fold. Chickens also displayed a lack of clinical signs which is enhanced in chickens due to dysregulation of the immune system[183,187,209].

The Q226L mutation, in addition to conferring human type receptor binding specificity, altered the stability of the HA protein of H5 ferret adapted viruses, thereby affecting the pH at which HA is activated and thermostability[91,126]. HA undergoes an irreversible conformational change when exposed to the acidic environment in endosomes or heat treatment at neutral pH[49]. Viruses which fuse at low pH are presumed to possess higher thermal stability than viruses fusing at higher pH[120]. The pH at which this change occurs varies among influenza virus subtypes or host species[255]. In our experiments, we observed the following mutations N304D, V318I, D375N (HA2-D46N), H397Y (HA2-H68Y) which may contribute to changes in thermostability due to their location in fusion domain of the HA gene.

Of note are the V318I and D375N (HA2-D46N) mutations which have been documented to stabilize the HA's of H5 and H9 viruses in mammalian models respectively. A T318I mutation observed in H5 ferret adapted virus reduced the pH threshold for membrane fusion, stabilizing the HA protein following an increase in pH threshold due to the human receptor binding conferring mutations N224k/Q226L[91]. D375N (HA2-D46N) mutation was shown to enhance binding to avian type receptors in a guinea pig model and also increase viral thermostability[141]. Thus, both mutations have been shown to enhance or stabilize the HA's of adapted viruses. In our experiment, we observed a decrease in thermostability as passaging progressed with P19 being less thermostable than P0 and P8. The decrease in thermostability may be related to balancing the stability of the HA protein following the change in receptor binding from avian to human specificity. Human viruses are considered to be more acid stable than avian viruses, however H9 and H10 subtypes have been shown to have acid stability profiles closer to human viruses[132]. This suggests H9N2 viruses may require less evolutionary pressures in crossing the species barrier infecting mammals.

In conclusion, the serial passaging of an H9N2 low pathogenic avian influenza virus 19 times in chicks resulted in low pathogenicity in four-week-old chickens and chicken embryos, adequate immune response of chickens to infection and a loss in thermostability. H9N2 viruses have been shown to acquire mammalian specificity while circulating in avian hosts[140,222]. This is consistent with our data showing the acquisition of (L-226) human receptor binding specificity in avian species following serial passages in chicks.

CHAPTER 3

Effect of Different Routes of Inoculation on Infectivity and Virulence of Low Pathogenic Avian Influenza Viruses in 4-Week-Old Chickens.

3 Introduction

The avian influenza virus has become an increasing threat to poultry and public health over the decades, causing economic losses in the poultry industry and mortality in a wide range of species. LPAIV are reserved in wild bird species from which they are introduced into poultry populations. These viruses mainly replicate in the intestinal tract of wild birds and as such are excreted predominantly in faeces[46]. Wild birds also shed virus via respiratory routes due to replication in respiratory organs although at lower titers. On transmission to poultry populations, low pathogenic viruses cause subclinical infections although infection can be exacerbated by secondary pathogens, immunosuppression of the host or environmental pressures producing mild to moderate disease[226]. It is not uncommon to observe mortality in the field following LPAIV infection. However under experimental conditions, clinical signs or mortality are usually absent in SPF chickens inoculated with LP viruses[226].

AIV is considered to be transmitted by direct contact, large droplets and airborne routes in poultry[57]. Comparative experiments involving the use of avian species however show that infection with AIV is possible via intranasal, intravenous, intratracheal, intracloacal, and intraoviduct routes using wild birds and domestic poultry [256,257]. For instance, mallards were found to be susceptible to infection by either H3N8 or H5N2 LPAI viruses when inoculated via intranasal, intratracheal, intraocular, intracloacal, or intra-ingluvial routes. The mallards were also observed to shed virus similarly through the cloaca or respiratory tract regardless of the route of inoculation[256]. In addition, AIV has been shown to cause infection via intracloacal and intranasal routes in chickens. However, the efficiency of infection was determined by the infecting virus subtype[257]. Using eight H5 subtype viruses, intranasal, intramuscular and contact routes of inoculation were compared in chickens, turkeys, quail, and duck species. Apart from

host and virus associated differences observed, the intranasal route of inoculation was shown to require longer time to cause clinical signs or mortality in chickens compared to the intramuscular route[258]. Another study compared the susceptibility and pathogenesis of chickens and domestic ducks to an H5N1 HPAI virus administered by intranasal and alimentary routes of inoculation. Intranasal inoculation was observed to require approximately 10^{3-4} EID_{50} lower dose of virus than alimentary tract inoculation to produce infection, although both routes were able to produce clinical signs and mortality in chickens[259].

Experimentally the intranasal route of inoculation, which mimics large droplet transmission, is widely used in the inoculation of small animals and birds[205,256,260]. However recent research suggests the intranasal route of inoculation does not adequately reflect the natural exposure observed in the field. A number of studies have been conducted to determine the effect of the route of inoculation of AIV on replication efficiency, tissue tropism, immune responses and virulence in mammalian species[261,262]. Aerosol and intranasal routes of inoculation were compared due to their efficiency in delivering virus into respiratory organs, which serve as the prime target of AIV in both mammalian and avian species. Oral inoculation is less employed in experiments because of the requirement of a larger dose for oral inoculated subjects to replicate at higher titers[259]. Some studies suggest aerosol inoculation is more effective in causing infection, increased replication and exacerbated morbidity via dysregulation of the immune system in mouse models[209]. However others suggest both methods of inoculation are comparable for use in murine studies[205,207].

The importance of aerosols has, however, been less frequently assessed in avian species such as chickens. In the past decade, more research conducted using aerosol routes suggest aerosol

inoculation resulted in higher titers of virus in chicken organs than oral and intranasal routes. Eight HPAI H5N1 viruses were exposed to chickens via aerosol, intranasal, oral and intragastric routes to study their infectivity and tissue tropisms. Birds inoculated via aerosol had higher titres of virus than the other intranasal and oral routes, being thirty and five hundred times higher in sensitivity respectively[263]. The H9N2 AIV has also been shown to be more effective in causing infection in chickens via aerosol exposure, requiring a lower infectious dose when compared to intranasal and oral routes. In addition the virus has also been shown to induce increased antiviral interferon expression following aerosol inoculation compared to intranasal routes in guinea fowl[229,264]. The effectiveness of aerosol exposure in causing AIV infection is associated with the ability of aerosols to penetrate deeper into the lower respiratory tract due to its minute size. Aerosols are less than 5 µm in size and so can stay suspended in the air for extended periods of time compared to large droplets[265]. The small particle size and ability to carry infectious virus is considered to elicit a vigorous host immune response, ultimately exacerbating the outcome of infection[266]. For instance, increased mortality was observed in aerosol inoculated mice when compared to intranasal inoculated mice, due to overexpression of IL-6 in lung tissues[209].

AIV has also been detected outside of poultry and livestock farm houses suggesting aerosols play a major role in the transmission of AIV[66,267,268]. In addition, the spread of AIV in live bird markets is suggested to occur via aerosol exposure due to the housing of birds in stacked cages[269]. Few studies have compared the effect of routes of inoculation of AIV in birds, and even fewer include the aerosol route of inoculation in experiments. To gain further understanding on the effect of aerosol inoculation on the pathogenesis of AIV in chickens, we inoculated chickens with four low pathogenic subtypes, H13N6, H10N8, H10N7, and H6N1 via aerosol, intranasal and oral routes. As such, four individual experiments were carried out in this study. We

compared virulence, tissue tropism and immune responses generated by chickens in response to infection by LP viruses by the different routes. From our study, we observed aerosol inoculation resulted in increased mortality, tissue tropism and cytokine expression in chickens compared to oral and intranasal routes.

3.1 Materials and methods

3.1.1 Virus

The A/Quail /Italy/1117/65 (H10N8), and A/Gull/Maryland/704/77 (H13N6) viruses were from Dr. Earl Brown at the University of Ottawa, Ottawa, Ontario, while the A/Turkey/Mass/3740/65 (H6N2) and 92-20503 Chicken (H10N7) viruses were obtained from Dr. Davor Ojkic at the University of Guelph, Guelph Ontario. All viruses used were passaged twice in chicken embryos (ECE's) to prepare a virus stock. Virus identity was confirmed by whole genome sequencing of allantoic fluid generated form ECE passage.

3.1.2 Chicken experiments.

Four-week old chickens, fifty-four in number, were divided into three treatment groups for experiments using H13N6, H10N8, or H6N1 viruses. Eighteen chickens each were housed in three of the four self-contained isolators operated under negative pressure and had access to feed and water ad libitum with each isolator designated with either aerosol, intranasal or oral routes of inoculation. A fourth isolator housed nine control chickens inoculated with PBS. For our experiment involving the H10N7 virus, only aerosol and intranasal routes of inoculation were attempted due to previous knowledge of a lack of replication in chickens following inoculation by oral routes (unpublished data). Fifty-four chickens were inoculated in total for the H10N7 experiment, eighteen each for aerosol, intranasal and PBS inoculated groups.

H6N1 and H10N7 viruses used in this study had a virus titer of 1.58×10^7 EID_{50}/ml while H10N8 and H13N6 viruses were 4.28×10^6 and 2.81×10^6 EID_{50}/ml respectively. The viruses were aerosolized into the chamber using a nebulizer (Whisper Jet™, Vital Signs Inc., Totowa, NJ, USA) and an air compressor (Aeromax 3002, Medical Industries America, Inc., Adel, IA, USA). Six chickens were exposed to aerosolized virus at a time for fifteen minutes in an isolator

containing a 45cm (length) × 30 cm (width) × 30 cm (height) chamber constructed of polycarbonate for aerosol exposure (Figure 3). Intranasal and oral inoculation was carried out in respective self-contained isolators using a syringe and cannula.

3.1.3 Sample collection

To study virus shedding oropharyngeal and cloacal swabs were collected from all chickens in isolators on 1, 3 and 7 days' post inoculation (DPI). Swabs were immediately transferred into 2 ml tubes containing 1 ml RLT buffer (QIAgen) and then stored at -80^{0}C. Virus titer in swabs was determined using real time RT-PCR targeting the matrix gene and was expressed as EID_{50}/ml. To study virus replication and cytokine gene expression in tissues, 6 chickens from each group were randomly selected for euthanasia after collection of swab samples on 1, 3 and 7 DPI. Trachea, lung, cecum tonsil, cecum pouch and bursa tissue specimens were collected from each chicken in duplicates, each sample weighing approximately 50 mg. The specimens were immediately placed in 2 ml tubes containing 1ml RNAlater (Invitrogen Canada Inc. Burlington, ON, Canada) and stored at -80 ºC. Virus titer in tissue samples was determined using RRT-PCR[232].

3.1.4 RNA extraction and real time reverse transcriptase-PCR (RRT-PCR)

RNA was extracted from samples using the automated sample preparation equipment QIAcube and RNeasy Mini kit (QIAgen, Mississauga, Ontario). Briefly tissue samples were homogenized by bead beating using 2 ml Precellys® beads (Cayman, Ann Arbor, MI, USA) containing 1 ml QIAzol Lysis reagent and a tissue lyser (QIAgen). Chloroform (200 µl) was added to the homogenate and centrifuged at 12000 g for 15 minutes at 4^{0}C. The aqueous phase (500 µl) recovered was transferred to the QIAcube for RNA extraction. For swab samples, 500 ul

of sample fluid was transferred directly to the QIAcube for RNA extraction. RNA (10 ug) was then treated with Rnase-free Dnase (Invitrogen).

Extracted RNA (5 µl) was used as a template in a 20 µl one step RRT-PCR reaction using Taqman RT PCR mix (Life Technologies) and the following primers M+25 5′AGATGAGTCTTCTAACCGAGGTCG, M-124 5′TGCAAAAACATCTTCAAGTCTCTG, and probe M+64 5′FAM- TCAGGCCCCCTCAAAGCCGA-TAMRA developed by Spackman et al. 2002[232]. Sample fluids containing no virus were spiked with known amounts of virus to generate a standard curve for quantification of virus in collected samples. Based on the standard curve, the amount of virus in each sample fluid was calculated by plotting the cycle threshold value (Ct-value) against known virus titers and was expressed as an EID_{50} equivalent (eq-EID_{50}). The quantity of extracted RNA was determined using an ND-1000 spectrophotometer (Nano Drop Technologies, Wilmington, DE). The PCR was performed with a 7500 real-time PCR system (Applied Biosystems, Foster City, CA). Thermal cycling protocol used was reverse transcription at 50°C for 30 minutes, enzyme activation at 95°C for 15 minutes, 45 cycles at 94^0C for 5 seconds and at 60^0C for 34 seconds for denaturation and elongation respectively.

3.1.5 cDNA synthesis and quantification of cytokine gene expression

cDNA was synthesized from Rnase treated RNA by reverse transcriptase reaction using Superscript II (SuperScriptTM First-Strand Synthesis System; Invitrogen Life Technologies, Carlsbad, California, USA) and oligo (dT) primer to create the complementary DNA strand. Briefly 11 µl of treated RNA was mixed with 1ul of oligo (dT) primer and 1 µl of 10 mM dNTP to a final volume of 13 µl. The mixture was incubated at 65°C for 5 minutes. Then, 4 µl of 5X first strand buffer, 1 µl of 0.1M DTT, 1 µl of Rnase OUT^{TM} ribonuclease inhibitor (40 units/µl), 1 µl of SuperScriptTM RT (200 unit/µl) and 1 µl of Rnase free water was added into the tube.

The reaction was completed at 42^0C for 50 min and was terminated by heating at 70^0C for 15 minutes.

Expression of interferon gamma (IFN-γ), interleukin 1 beta (IL-1β), and interleukin 6 (IL-6) and interleukin 4 (IL-4) were evaluated with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene. RNA extracted from the lung, and spleen tissue specimens were used for synthesis of cDNA with Oligo (dT) primer and the Invitrogen kit (SuperScriptTM First-Strand Synthesis System; Invitrogen Life Technologies, Carlsbad, California, USA) according to the manufacturer's instruction and as previously described[236]. The real time quantitative PCR (qPCR) was performed with the 7500 real-time PCR system (Applied Biosystems, Foster City, CA) using the Power SYBR® Green PCR Master Mix, containing SYBR® Green 1 Dye, AmpliTaq Gold DNA Polymerase, dNTPs, and optimized buffer components for the detection of DNA in a reaction volume of 20 µL. Gene-specific primers and 5 µL of a 1:10 dilution of cDNA sample as templates were used for the qPCR. The thermal cycling parameters included pre-incubation at 50^0C for 2 minutes, 95^0C for 10 minutes; 40 cycles at 95^0C for 15s, 60^0C for 1minute, and dissociation cycle at 95^0C for 15 seconds and 60^0C for 1 minute with a final cycle of 95^0C for 15 seconds.

3.1.6 Statistical analysis

Statistical analysis was performed using the students T-Test for differences in viral titer between groups on each day post inoculation. Statistical analysis was performed using GraphPad Prism statistical software using two-way Anova (Analysis of Variance) on cytokine gene expression in tissue organs. The critical level for significance was set at $P < 0.05$ and data was expressed as mean ± standard deviation, determined from 6 individual birds randomly selected from each group at the designated days.

3.2 Results

3.2.1 Aerosol inoculation results in mortality in chickens depending on viral strain

Chickens exposed to the four LPAIV, namely H13N6, H10N8, H10N7 and H6N1, displayed a lack of disease symptoms despite infection. However, inoculation of H6N1 and H10N8 viruses by aerosol route resulted in mortality of one chicken on DPI 5 and two chickens on DPI 6 respectively. In dead H10N8 inoculated chickens, virus was recovered from all organs tested in one chicken at high titers of at least 6.1 log_{10} EID_{50}/g, and from the lung and cecum tonsil of the second chicken at titers of 4.8 and 2.3 log_{10} EID_{50}/g, respectively. Virus was also detected in four of the five organs tested in the dead chicken 5.0 to 6.2 log_{10} EID_{50}/g in H6N1 inoculated group (Table 2).

3.2.2 Intranasal and aerosol inoculation cause efficient infection in the upper respiratory tract

As expected, virus was undetected in all swab samples collected from chickens inoculated with the H13N6 virus via aerosol, intranasal and oral routes of inoculation. Viral RNA was undetected in cloacal swabs collected from chickens inoculated with H6N1, H10N7 and H10N8 viruses by any of the routes assessed (data not shown). Oral inoculation was not attempted in the H10N7 experiment due to previous knowledge of the ineffectiveness of oral inoculation using the H6N1 and H10N7 viruses (Figure 10A and 10C). Viral RNA was detected in oropharyngeal swab samples collected although differences were observed depending on the route of inoculation and the infecting virus. Intranasal and aerosol inoculation proved effective in causing infection in the oral cavity compared to oral inoculation. However, less variation was observed in viral titer of intranasal inoculated chickens than those inoculated by aerosols (Figure 10). Significant differences in titer was only observed on DPI 3 in H6N1 inoculated chickens where oral inoculation resulted in infection of just one chicken compared to all chickens by aerosol

Table 2: Tissue tropism of H6N1 and H10N8 viruses in dead chickens.

Virus	Chicken group/No.	Virus Titer (Log_{10} EID_{50}/g) [a]					
		Tissue Organ	*Trachea*	*Lung*	*Cecum Tonsil*	*Cecum Pouch*	*Bursa*
H6N1	Aerosol/3.5		9.01E+04	1.61E+06	1.56E+06	5.18E+05	-
H10N8	Aerosol/3.1		-	5.81E+04	1.92E+02	-	-
H10N8	Aerosol/3.3		1.40E+06	5.68E+06	5.24E+07	1.74E+06	4.69E+08

[a] *virus titer in tissue organs of dead chickens infected with aerosolized H6N1 (dead on DPI 5) and H10N8 (deaths on DPI 6) avian influenza viruses.*
[b] *– no virus was isolated*

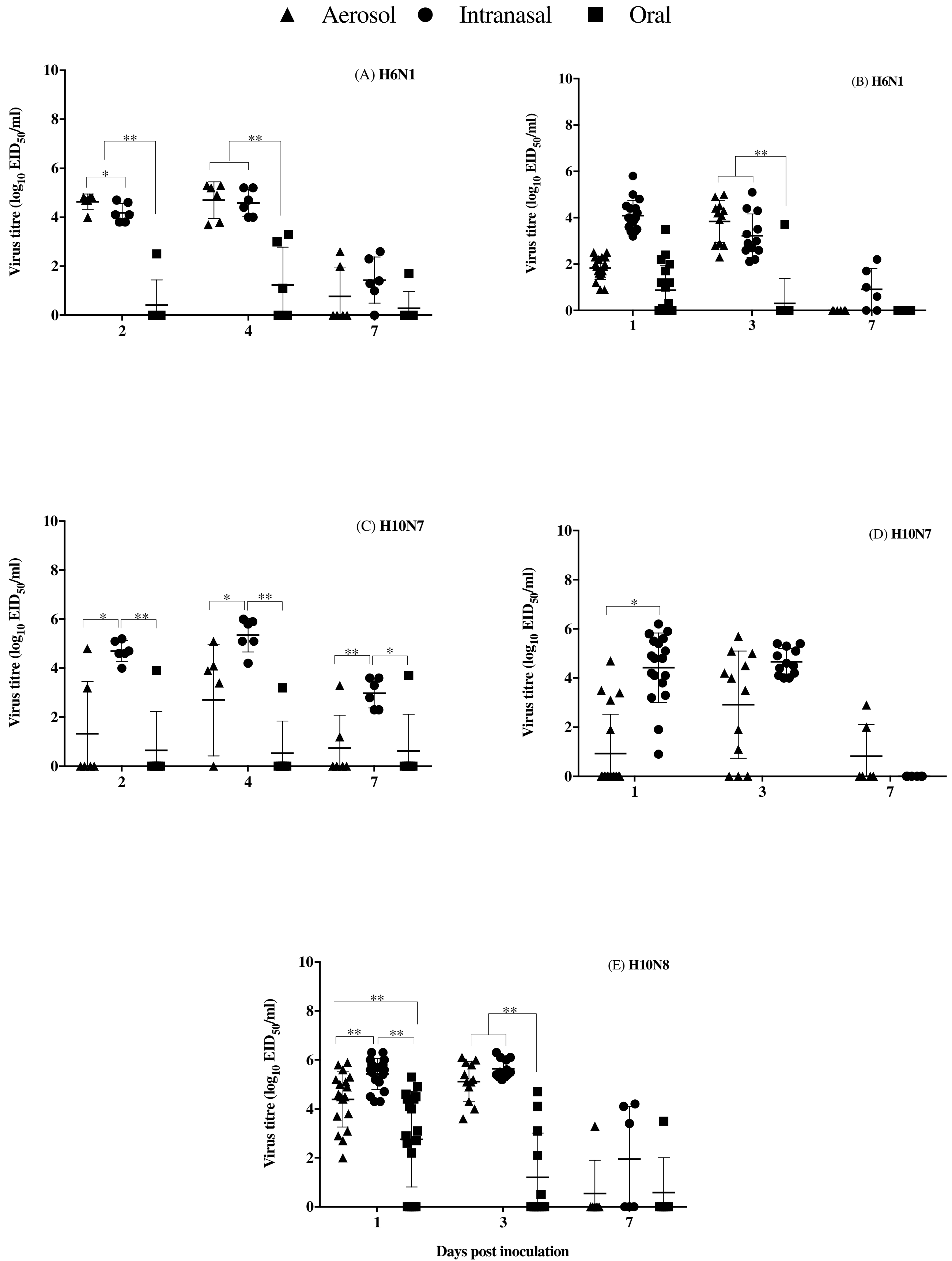

Aerosol
Intranasal
Oral
(A) H6N1
(B) H6N1
(C) H10N7
(D) H10N7
(E) H10N8
Virus titre (log_{10} EID_{50}/ml)
Days post inoculation

Figure 10: Virus infection in oropharyngeal swabs from chickens inoculated with (B) 1.58×10^7 EID_{50}/ml of H6N1 virus, (D) 1.58×10^7 EID_{50}/ml of H10N7 virus, and (E) 4.28×10^6 EID_{50}/ml of H10N8 virus administered by aerosol, intranasal, and oral routes of inoculation. Figure (A) and (C) represent graphs of unpublished data obtained from previous inoculation of H6N1 and H10N7 viruses respectively in chickens via the three routes of inoculation. Graphs represent the means and SD of viral titers in swabs (Log_{10} EID_{50}/ml) in each group of chickens sampled on each day post inoculation. * ($P<0.05$) and ** ($P<0.01$) represent significant differences between groups.

or intranasal inoculation (P<0.01) (Figure 10B). In the H10N7 experiment, significant difference was only observed on DPI 1 between intranasal and aerosol inoculated chickens where the viral titer was significantly higher in intranasal inoculated chickens than that in aerosol inoculated chickens on DPI 1 (P<0.05). In the H10N7 experiment, the intranasal route resulted in infection of all chickens on DPI 1 and 3, whereas virus RNA was detected in 5 of 18 and 9 of 12 chickens inoculated by aerosol on each respective day (Figure 10D). Inoculation of H10N8 virus via the three routes showed more significant differences on DPI 1 and 3. In H10N8 inoculated chickens, aerosol and intranasal groups were significantly higher in titer compared to oral inoculated groups on DPI 1 (P<0.01). The intranasal route was also significantly higher in titer compared to the aerosol route (P<0.01) on DPI 1. The oral route of inoculation proved least effective in causing infection in the oral cavity and lower viral titers were recovered from swab samples (Figure 10E).

3.2.3 Aerosol route of inoculation is more effective in causing infection in the lower respiratory tract of chickens

Aerosol inoculation of the H6N1 and H10N8 viruses proved to be more effective in penetrating the lower respiratory tract. In both experiments aerosol inoculated chickens infected more chickens and had significantly higher titers in tracheal and lung tissues than intranasal and oral inoculated chickens (Figure 11A and 11C). In the H10N7 experiment, no significant differences in titer were observed between aerosol and intranasal inoculation routes in tracheal tissues. In lung tissues, aerosol inoculated chickens were significantly higher in titer compared to intranasal inoculated chickens only on DPI 1(P<0.05) (Figure 11B).

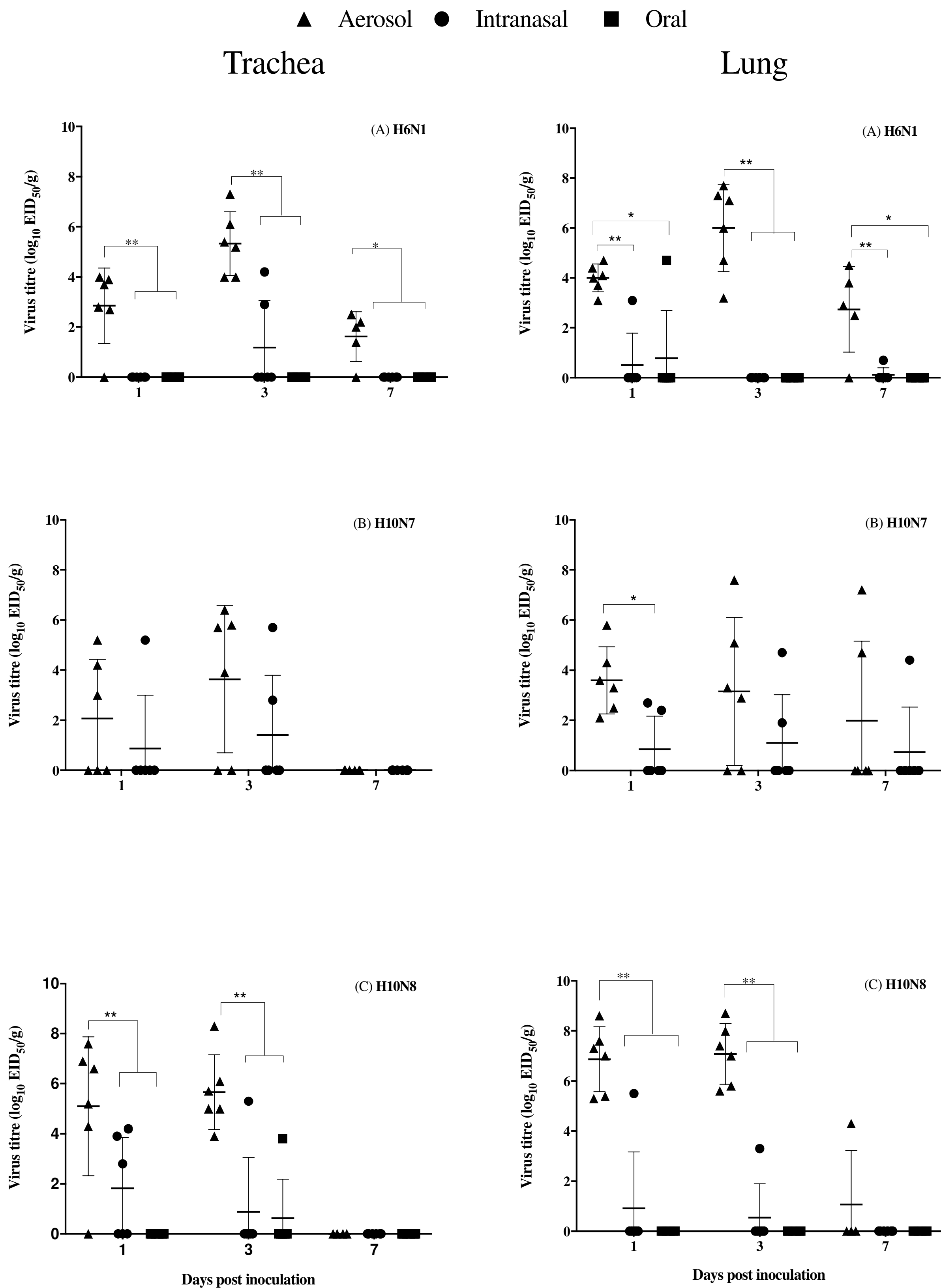

Aerosol
Intranasal
Oral
Trachea
Lung
(A) H6N1
(B) H10N7
(C) H10N8
Virus titre (log_{10} EID_{50}/g)
Days post inoculation

Figure 11: Virus infection in trachea and lung tissues from chickens inoculated with (A) 1.58×10^7 EID_{50}/ml of H6N1 virus, (B) 1.58×10^7 EID_{50}/ml of H10N7 virus, and (C) 4.28×10^6 EID_{50}/ml of H10N8 virus administered by aerosol, intranasal, and oral routes of inoculation. Graphs represent the means and SD of viral titers in tissues (Log_{10} EID_{50}/g) in each group of six chickens sampled on each day post inoculation. * ($P<0.05$) and ** ($P<0.01$) represent significant differences between groups.

3.2.4 Virus in cecum tonsil, cecum pouch tissues.

In the H6N1 experiment, no significant differences were observed in cecum tonsil and cecum pouch tissues assessed during the 7-day experiment. Viral RNA was detected the most in aerosol inoculated chickens. However, the number of positive specimens only exceeded two on DPI 3 in cecum tonsil tissues (Figure 12A). A similar observation was made in the H10N7 experiment where no significant differences were observed and virus was isolated in less than 3 specimens on all days (Figure 12B). Significant differences were however observed in the H10N8 experiment in cecum tonsil and cecum pouch tissue specimens on DPI 3. Virus was detected in all aerosol and intranasal inoculated chickens, which also had significantly higher virus titers compared to oral inoculated chickens (P<0.01) in cecum tonsil tissues. The aerosol inoculated group was significantly higher in virus titer and frequency of infection than the oral inoculated group in cecum pouch tissues (P<0.05) (Figure 12C).

3.2.5 Aerosol inoculation results in increased expression of cytokines in trachea and lungs of chickens.

Following the observed mortality in H6N1 and H10N8 aerosol inoculated chickens, we assessed the immune responses to virus inoculated via oral, intranasal and aerosol routes in lung and tracheal tissues. In tracheal tissues, aerosol inoculated chickens expressed IL-6 mRNA significantly at higher fold changes on DPI 3 and 7 compared to intranasal and oral inoculated chickens (P<0.01) (Figure 13A). Oral inoculated chickens also expressed IL-6 mRNA significantly higher on DP1 3 than control inoculated chickens (P<0.05), and on DPI 7 than intranasal (P<0.05) and control (P<0.01) inoculated chickens.

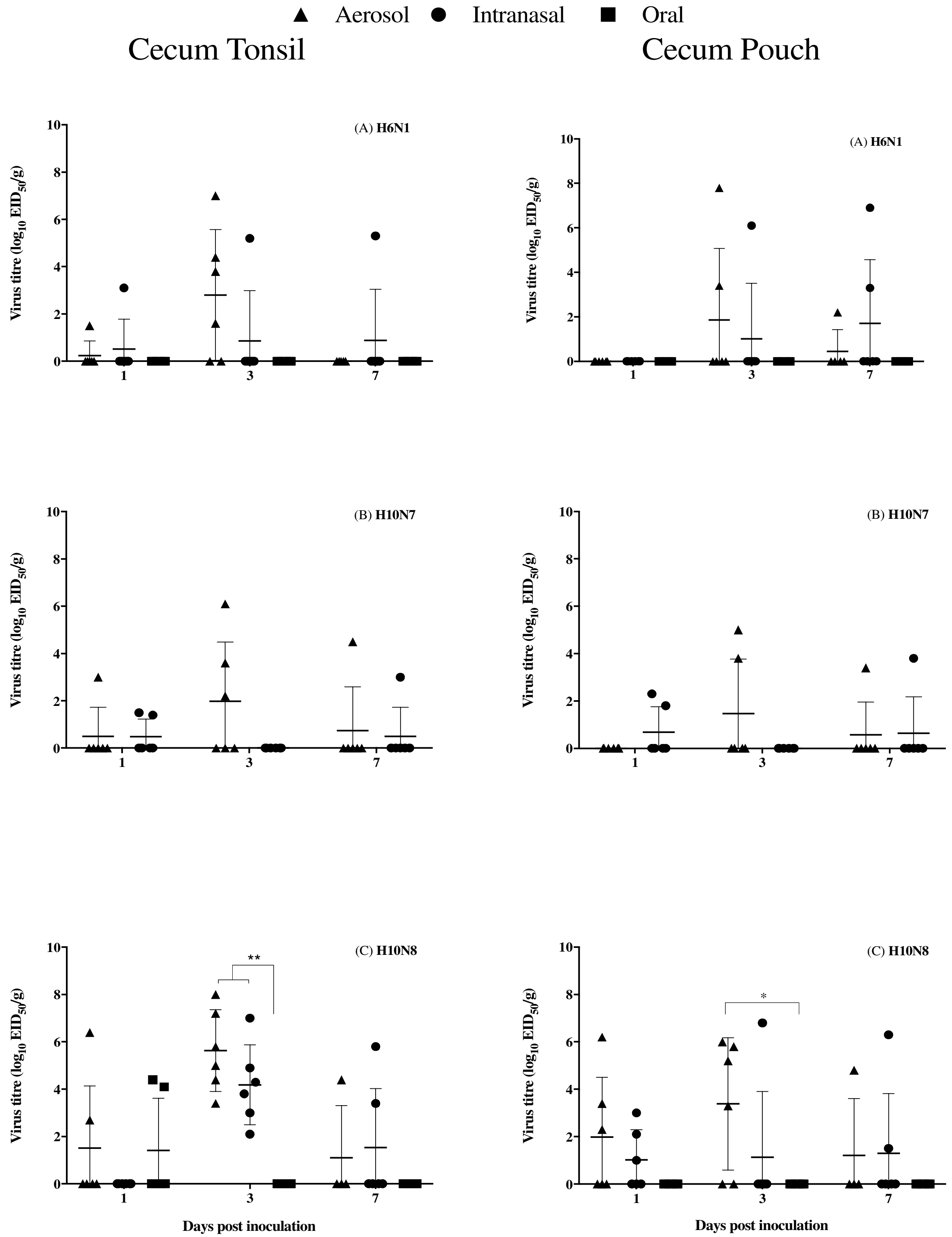
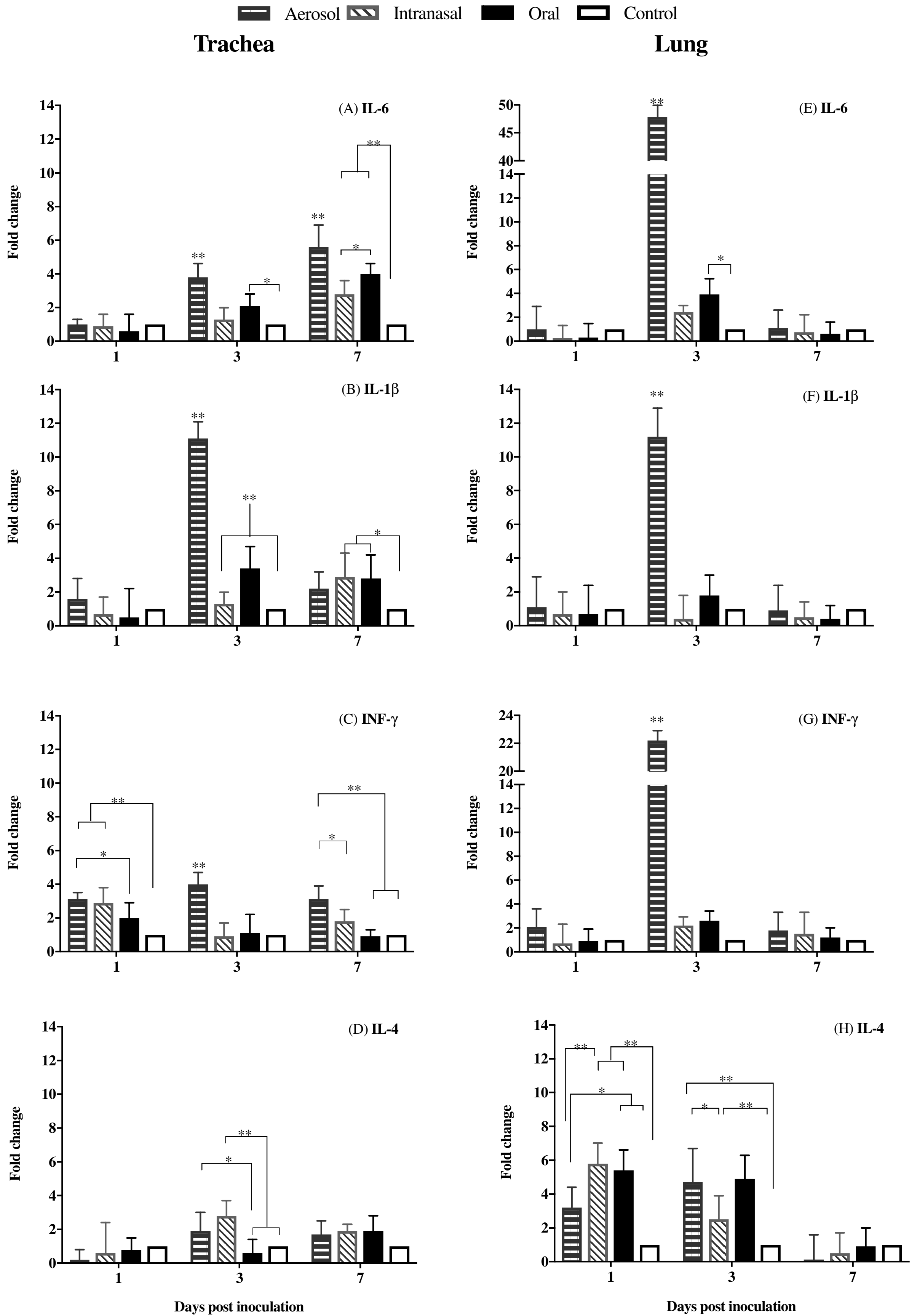

Aerosol
Intranasal
Oral
Cecum Tonsil
Cecum Pouch
(A) H6N1
(B) H10N7
(C) H10N8
Virus titre (log10 EID50/g)
Days post inoculation
**
*

Figure 12: Virus infection in cecum tonsil and cecum pouch tissues from chickens inoculated with (A) 1.58×10^7 EID_{50}/ml of H6N1 virus, (B) 1.58×10^7 EID_{50}/ml of H10N7 virus, and (C) 4.28×10^6 EID_{50}/ml of H10N8 virus by aerosol, intranasal, and oral routes of inoculation. Graphs represent the means and SD of viral titers in tissues (Log_{10} EID_{50}/g) in each group of six chickens sampled on each day post inoculation. * ($P<0.05$) and ** ($P<0.01$) represent significant differences between groups.



Aerosol
Intranasal
Oral
Control
Trachea
Lung
(A) IL-6
(B) IL-1β
(C) INF-γ
(D) IL-4
(E) IL-6
(F) IL-1β
(G) INF-γ
(H) IL-4
Fold change
Days post inoculation

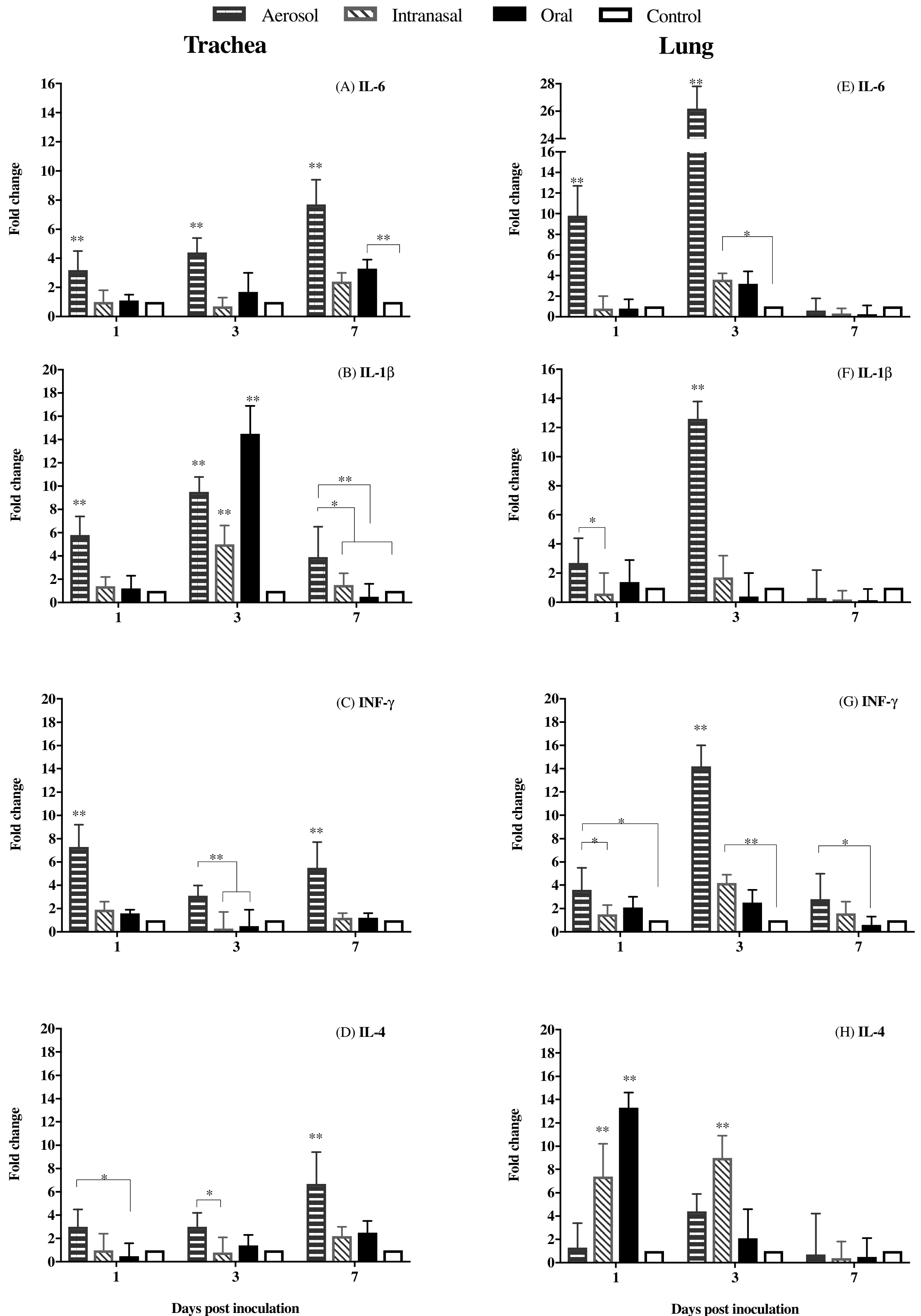
Figure 13: Cytokine mRNA expression of IL-6, IL-1β, IFN- γ and IL-4 in trachea and lung tissues of 6 chickens collected on each day post inoculation from three groups of chickens inoculated with 1.58×10^7 EID_{50}/ml of H6N1 virus administered by aerosol, intranasal, and oral routes of inoculation. Cytokine mRNA expression was analyzed using real-time PCR. Data are expressed as mean relative expression with standard deviation relative to GAPDH mRNA. Significance of the regulation level compared to controls is analyzed. ** ($P<0.01$) and * ($P<0.05$) represents significant difference of the treatment group from that of the control group.

Expression of IL-1β mRNA was comparable to that of IL-6, however aerosol inoculated chickens had significantly higher mRNA expression (at least 10-fold) on DPI 3 compared to intranasal and oral inoculated chickens. Oral inoculated chickens also expressed IL-1β more in tracheal tissues than intranasal and control chickens on this day. On DPI 7, chickens in all three groups expressed IL-1β highly compared to control chickens, but significance was only observed in intranasal and oral groups ($P<0.05$) (Figure 13B). Aerosol inoculation also resulted in the upregulation of IFN- γ mRNA in the trachea of H6N1 inoculated chickens. Significant differences were observed on all three days in IFN- γ expression in aerosol inoculated chickens compared to intranasal and oral groups (Figure 13C). IL-4 mRNA expression was similar in all virus inoculated groups and control chickens. However, significant differences were observed on DPI 3 in the increased expression of aerosol ($P<0.05$) and intranasal ($P<0.01$) inoculated chickens when compared to those inoculated orally (Figure 13D).

In contrast to tracheal cytokine responses where the fold change in expression of mRNA was usually less than 10-fold, aerosol inoculation of the H6N1 virus resulted in significant upregulation of IL-6 by 47-fold (Figure 13E), IL-1β by 11-fold (Figure13F), and IFN- γ by 22-fold (Figure 13G) in the lungs compared to both intranasal and oral inoculation on DPI 3. Intranasal and oral routes of inoculation were significantly upregulated compared to aerosol inoculated chickens in IL-4 expression on DPI 1 ($P<0.01$ and $P<0.05$ respectively) (Figure 13H). However, on DPI 3 aerosol and oral inoculated groups were upregulated and significantly different from the intranasal inoculated group ($P<0.05$ and $P<0.01$ respectively). No difference was observed in IL-4 expression on DPI 7.

Aerosol inoculation of the H10N8 AIV also proved to be more efficient in eliciting expression of IL-6, IL-1β, IFN- γ and IL-4 in tracheal tissues on all days assessed than intranasal

or oral inoculation (Figure 14A, B, C, and D). Aerosol inoculation resulted in significant upregulation of mRNA on all days compared to oral and intranasal routes except on DPI 3 in IL-1β expression, where the oral route had higher fold changes (Figure 14B). Like the expression of cytokines in the lungs of H6N1 infected chickens, significant upregulation of IL-6 by 26-fold (Figure 14E), IL-1β by 12-fold (Figure14F), and IFN- γ by 14-fold (Figure 14G) was observed in the lungs of chickens compared to both intranasal and oral inoculation on DPI 3. Intranasal and oral routes were significantly upregulated in IL-4 mRNA expression on DPI 1 in the lungs of H10N8 inoculated chickens ($P<0.01$). However, significant upregulation was only observed on DPI 3 in the intranasal inoculated group ($P<0.01$), after which expression returned to normal levels (Figure 14H).



Aerosol
Intranasal
Oral
Control
Trachea
Lung
(A) IL-6
(B) IL-1β
(C) INF-γ
(D) IL-4
(E) IL-6
(F) IL-1β
(G) INF-γ
(H) IL-4
Fold change
Days post inoculation

Figure 14: Cytokine mRNA expression of IL-6, IL-1β, IFN- γ and IL-4 in trachea and lung tissues of 6 chickens collected on each day post inoculation from three groups of chickens inoculated with 4.28×10^6 EID_{50}/ml of H10N8 virus administered by aerosol, intranasal, and oral routes of inoculation. Cytokine mRNA expression was analyzed using real-time PCR. Data are expressed as mean relative expression with standard deviation relative to GAPDH mRNA. Significance of the regulation level compared to controls is analyzed. ** ($P<0.01$) and * ($P<0.05$) represents significant difference of the treatment group from that of the control group.

3.3 Discussion

We carried out four individual experiments inoculating chickens with LPAIV isolated from different hosts via aerosol, intranasal and oral routes of inoculation. Few studies have assessed the role of aerosol exposure compared to other routes of inoculation in chicken infections with AIV[229,263]. In our study, the intranasal route of inoculation was more effective in causing infection in the upper respiratory tract evidenced by higher titers in oral swabs, which was likely due to the direct deposition of virus in the nasal cavity. Aerosol exposed chickens tested positive for viral RNA however a wider variation in virus titre was observed in oral swabs compared to the intranasal group. No virus was detected in cloacal swab samples assessed. This adds support to current knowledge which considers AIV to be shed predominantly via the respiratory route in chickens[247].

Interestingly, oral inoculation resulted in detection of viral RNA in majority of oral swabs recovered from chickens however no virus was detected in lung and trachea tissues. Oral inoculation is also thought to require a larger dose to produce similar infection as other routes[259]. Aerosol exposure proved more effective in causing infection in the lower respiratory tract as more chickens tested positive for viral RNA in trachea and lung tissues compared to intranasal inoculation and oral inoculation. This must be due to the ability of aerosols to penetrate deep into the respiratory tract thus causing efficient replication in tracheal and lung tissues[270]. In addition, aerosol inoculated chickens required received virus dose at least 2 logs lower compared to that of intranasal or oral chickens.

The virus strain or subtype had a major effect on the outcome of infection. Inoculation of H13N6 virus resulted in a lack of replication in chickens except in the lungs of three aerosols inoculated chickens on the first day. This is likely due to the minute size of aerosols enabling

deposition of virus in the lungs as no virus was detected on DPI 3. H13 subtypes are considered to replicate efficiently only in wild birds[271]. In contrast the H10N8 quail and H6N1 turkey isolates replicated efficiently in chickens compared to the chicken H10N7 virus. Quail and turkey AIV isolates have been documented to cause efficient replication in chickens and play a role in the adaptation of wild bird viruses to domestic poultry[272].

In addition, mortality was also only observed in H10N8 and H6N1 inoculated chickens when inoculated via the aerosol route. H10N8 and H6N1 viruses have been documented to cross the species barrier infecting mammals and also cause mortality in outbreaks in poultry flocks[273,274]. Intranasal inoculation of birds and small animals is less laborious, especially in an experimental setting using large numbers of study subjects[207]. However, intranasal inoculation may not depict the natural infections occurring in the field effectively. Mortality is rarely observed when inoculating specific pathogen free chickens with LPAI isolates recovered from field infections[275,276]. Aerosol inoculation may cause an increase in morbidity and mortality in infected hosts compared to intranasal or oral routes of inoculation.

The penetration of the lungs with aerosolized virus is suggested to result in a more vigorous immune response which contributes to morbidity and mortality[266]. In mice models, the upregulation of proinflammatory cytokine IL-6 resulted in exacerbated morbidity following infection with a mouse adapted H3N2 AIV[209]. In chickens, excessive expression of proinflammatory and antiviral cytokines has been shown to contribute to mortality in chickens[277,278]. Excessive expression of cytokines is considered to result in a cytokine storm due to a dysregulation of the immune system leading to mortality in humans and poultry[186,279,280]. In both H10N8 and H6N1 experiments, aerosol inoculated chickens showed increased expression of

pro-inflammatory and antiviral cytokines, IL-6, IL-1β, and IFN- γ, compared to oral and intranasal inoculated chickens especially on DPI 3. However, expression of IL-4, which is essential in the differentiation and activation of CD4 helper T cells, was lower in aerosol inoculated chickens compared to both intranasal and oral groups in lung tissues. The reduced expression of IL-4 in aerosol infected chickens may suggest a lack of humoral response to influenza infection which is documented to occur in LPAI infections in chickens[183].

In conclusion, we show that aerosol inoculation of low pathogenic avian influenza viruses resulted in efficient replication in 4-week-old chickens when compared to intranasal or oral routes of inoculation. Aerosol inoculation was more effective in disseminating in tissue organs of infected chickens and in expression of proinflammatory and antiviral cytokines. In addition, mortality was only observed in aerosol inoculated chickens suggesting the route of inoculation may play a role in disease pathogenesis of AIV infections along with the subtype of virus. Our results suggest aerosol transmission should be factored in experimental approaches in understanding of the AI virus in poultry species. Aerosol route of spread should be considered to be an important factor in designing control and prevention strategies against the avian influenza virus.

Chapter 4 – General discussion and conclusion

4.1 Discussion

The avian influenza A virus is considered by researchers to be a possible pandemic pathogen[14]. Over the decades the incidence of outbreaks of AIV in poultry have increased most likely due to the increase in commercialization of poultry to meet food demands of an increasing world population[281]. The crowding of birds in cages or farm houses is said to encourage the transmission of AIV in poultry[1,267]. Aerosols have recently been suggested to play a major role in the transmission of human influenza viruses in hospital patients[282], poultry farm workers and live bird markets[268,283]. However in poultry species the general consensus suggests the faecal oral route accounts for majority of AIV transmission in poultry[227].

This may however be true for AIV infection in wild birds, in which AIV replicates in the intestinal tract enabling the shedding of virus in faeces. In domestic birds, most especially chickens, virus is detected predominantly in nasal secretions or oropharyngeal swab samples and less frequently, or non-existent in faeces or cloacal swab samples[247]. Our experiments with H6N1, H9N2, H10N7 and H10N8 low pathogenic viruses resulted in efficient viral shedding in oropharyngeal swabs of SPF chickens while virus was inexistent in cloacal swabs regardless of the viral subtype. Thus, virus in aerosols may more likely result in AIV transmission in poultry especially in crowded environments.

The shuffling of chickens to create space in crowded cages leads to hyperventilation in chickens generating aerosols in the process. AIV is documented to be capable of persisting in the feathers of AIV infected chickens, in dust and bedding, all sources of aerosol generation[66,284,285]. Human errors can also lead to the creation of aerosols in poultry rearing operations. The cleaning of poultry farm houses and transfer of birds in and out of farm premises have been documented

to encourage spread of AIV via aerosols[286]. Aerosols are documented to have longer settling times as a 5 µm particle can remain suspended in the environment for up to an hour compared to 20 µm particles which possess a settling time of 4 minutes[287]. Aerosol transmission has however been considered ineffective over long distances but evidence from outbreaks of AIV suggest otherwise[66]. In outbreaks of HPAI viruses in Canada, aerosol transmission was suggested to play a role in the spread of AIV to neighbouring farms due to coinciding wind patterns at the time of spread[65]. AIV has also been detected in the air in and around live bird markets[288], livestock houses, and poultry barns[66]. Over short distances aerosol is increasingly effective depending on exposure time. Experimentally aerosol inoculation has been shown to require a lower infectious dose than intranasal or oral routes which mimic large droplet and faecal-oral routes respectively[229,263].

Given that chickens seem to shed AIV mostly via the respiratory tract, it is more likely that virus containing aerosols can be spread via coughing or sneezing[62,289]. Thus, aerosols suspended in the atmosphere in poultry barns would be effective in causing infection in chickens exposed for an extended period especially when increased numbers of infected chickens are present. The lack of adequate ventilation and air purification systems in majority of poultry barns, most likely due to the expensive nature of such systems, may aid the spread of AIV in enclosed barns[290]. The ability of aerosols to penetrate the lower respiratory tract, reaching the preferred target organ of AIV in chickens, may also aid the adaptation of AIV in its host. The H13N6 virus used in our experiment was detected in the lungs of three of six chickens a day after exposure to aerosolized virus. Due to detection by RRT-PCR, we cannot conclude if the virus detected was infectious. The virus however failed to replicate and was not detected on subsequent days. In addition, aerosol exposure may also explain the differences observed in mortality when viral strains

known to cause mortality in the field are avirulent in SPF chickens. The intranasal route is widely employed in experimental inoculations and may not adequately represent natural infections. In our experiments, the aerosol route resulted in mortality of chickens while no mortality was observed in intranasal and oral inoculated groups. This lends support to mammalian studies in which clinical signs or mortality is observed earlier in aerosol inoculated subjects than in intranasal inoculated subjects[204].

The difference in tissue tropism of both H10N8 chickens suggests viral spread or titer in tissue organs may not necessarily correlate with observed mortality. One chicken showed extensive viral replication in all assessed organs while the other replicated in lung and cecum tonsil tissues only. It is likely that the immune response to infection plays a major role in the outcome of AIV infection. Aerosol inoculation of chickens resulted in increased proinflammatory and antiviral cytokine expression when compared to intranasal and oral routes of inoculation. The deposition of virus deep into the lower respiratory tract is considered to elicit a vigorous immune response to infection which may ultimately contribute to mortality[266]. Excessive increases in expression of IL-6, IL-1β and IFN- γ in chickens exposed to aerosolized H10N8 and H6N1 viruses were observed which has been shown to be detrimental in poultry hosts leading to mortality on some occasions[277].

It has been documented that the infiltration of neutrophils into the lungs of AIV infected mice contributed to mortality following aerosol inoculation. IL-6 expression was suggested to play a major role in pathogenesis of AIV infections as deregulation may enable the virus to overwhelm the host while overexpression may lead to pulmonary damage causing mortality[209]. Thus, the observed significant increase in pro-inflammatory cytokines in aerosol inoculated chickens, particularly on DPI 3, suggest the deposition of AIV directly into the lungs elicited a

vigorous response in chickens. In contrast although the H9N2 was aerosolized in chickens, adequate immune responses were observed in response to infection suggesting the virus isolate also determines the extent of cytokine expression. In addition, although the H10N8 experiment was conducted using virus a log lower than in H6N1 and H10N7 experiments, the virus was more efficient in causing infection in chicken organs most especially when compared to the chicken H10N7 isolate. The observed efficient replication and mortality induced by H10N8 and H6N1 viruses may also be related to the virus isolate as viruses from turkeys and quail are considered to be highly pathogenic in chickens [61,211].

The circulation of low pathogenic AIV in poultry populations poses an additional problem due to the generation of HPAI viruses via recombination with other subtypes or mutations in virus proteins[3]. Highly pathogenic avian influenza virus outbreak in poultry pose a major concern not only to the poultry industry but also to human health and wellbeing. In contrast to LPAI infections, HPAI infection in poultry results in increased mortality in poultry sometimes wiping out total flocks of birds. The ease of spread makes the control of HPAI outbreaks more difficult and as such costs are accrued in the quarantine and culling of infected birds, and the disinfection and decontamination of poultry farm houses. In addition, trade restrictions increase economic losses for producers and consumers alike[2,291]. The H9N2 avian influenza virus is widely suggested to be capable of acquiring the ability to cause HP outbreaks. However, the virus has till date remained low pathogenic in chickens despite its donation of internal genes to HP H5 and H7 viruses and occasional spikes in mortality in chickens. It however poses an increased threat to humans and mammalian species due to its ability to bind to human receptors[292].

In our experiment, following the serial passaging of an H9N2 virus in chickens, we generated a virus which remained low pathogenic in chickens but also acquired mutations related to human receptor specificity. This work contributes to current knowledge of H9N2 subtypes which have been documented to acquire mutations conferring mammalian receptor binding following circulation in chickens[61,292]. The widespread nature of H9N2 viruses in poultry is indeed troubling considering the possibility of recombination with other subtypes to generate a virus capable of efficient mammalian transmission[293].

4.2 Conclusion and Future work

In summary, we show the effectiveness of aerosols in causing AIV infection and overexpression of pro inflammatory and antiviral cytokines in chickens compared to intranasal and oral routes of inoculation. We also show that the AIV is predominantly shed by the respiratory tract in chickens supporting the argument for aerosol transmission in domestic poultry. As such aerosol transmission of AIV should be factored into control and prevention strategies, such as good biosecurity practices involving the restriction of people, animals, equipment and materials in controlled areas, monitoring the health status of flocks and air purification systems. Aerosols should also be considered for use experimentally in small animal experiments to adequately mimic natural AIV infections.

In addition, our results suggest improved monitoring of H9N2 AIV outbreaks in poultry is warranted due to the ability of the virus to gain mutations related to species adaptation in poultry. The H9N2 virus however failed to increase in pathogenicity following 19 passages in chicks, in contrast to results observed in the serial passaging of a whistling swan H5N3 AIV eighteen times in the air sacs of chicks which led to mortality in 50% of the birds. The loss in thermostability suggests a corresponding increase in pH of activation which is documented to cause increased

mortality in chickens. As such additional passages may result in increased pathogenicity in chickens, or such adaptive increase in pathogenicity may be restricted to just H5 and H7 viruses. In addition, receptor binding analysis will be carried out to determine the specificity of each virus in binding to avian or human SA-Gal receptors. Also the exposure of the viruses to small animals such as ferrets, will be conducted to assess replication and virulence in mammalian species.

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avian influenza virus infection in vitro differ among chicken cell lines established from different organs. *Vet Immunol Immunopathol*. 2011;144(3-4):187-199. doi:10.1016/j.vetimm.2011.10.002.

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Contribution of Collaborators

Experiments involving the inoculation of chickens with virus and sampling of tissue organs were carried out with Dr. Jiewen Guan, Qigao Fu and Jillian Rohonczy at the CFIA (Fallowfield). Optimization of the conditions for RT-PCR analysis was done previously at the CFIA by Qigao Fu and PCR analysis of tissue samples, oral and cloacal swabs was conducted by myself. PCR analysis of H6N1 and H10N7 oropharyngeal swabs from previous unpublished experiments and cloacal swabs from the H9N2 experiment was performed by Jenn O'Neil. Whole genome sequencing of the viruses used in this study was performed by Dr. Yohannes Berhane and his team at the Canadian Food Inspection Agency, 1015 Arlington Street, Winnipeg Manitoba.

Curriculum Vitae

Akinlolu Jegede

SKILLS

- Respiratory fit tested and trained.
- Standard first aid and CPR level C certified.
- Knowledge of quality assurance ISO-17025 standard principles, general laboratory safety, and principles of disinfection.
- Security clearance under the Human Pathogens and Toxins Act (HPTA).
- Experience working in an ISO 17025 certified level 3 bio safety laboratory.
- Able to learn quickly and work independently or in groups to achieve goals set.
- Excellent written and oral communication.
- Competent at writing research papers for publication and technical reports.
- Over 5 years' experience as a microbiologist in the field of molecular biology and bacteriology.

EXPERIENCE

Laboratory Technologist | Canadian Food Inspection Agency | 2016 December – present

- Experience in working in an ISO accredited level 3 containment laboratory.
- Trained and possess good understanding of ISO/IEC 17025.
- Processing of tissue specimens for culture and isolation of Mycobacterium ssp.
- Filling of data and organizing of records generated from biochemical analysis.
- Ordering of consumables and materials needed for laboratory activities.
- Preparing slides and staining for the Ziehl-Neelsen stain for acid fast bacilli.
- Performing DNA extraction of Mycobacterium ssp.
- Receiving and processing of animal tissue specimen submissions.
- Knowledge of biochemical assays in culture and isolation of Mycobacterium ssp.
- Knowledge of the principles of disinfection and appropriate safety practices in a level 3 laboratory.

Graduate Student | Canadian Food Inspection Agency | 2013 September -2016 December

CFIA-OLF Avian diseases.

- Development and update of experimental protocols.
- Recording and interpreting experimental data in laboratory books.
- Planning of experiments and ordering of supplies required.
- Performing animal experiments using low pathogenic avian influenza viruses.
- Processing of samples using molecular laboratory techniques such as RT-PCR, DNA extraction and sequencing of the influenza virus.

References

- Analysis of data generated using sequence analysis and statistical software's.
- Handling and storage of chemicals.
- Use and maintenance of laboratory equipment.
- Training of students in safe laboratory practice and techniques.

Laboratory assistant | May and Baker PLC. Nigeria (Internship). Feb 2010 – Aug 2010

- Responsible for sampling of raw material samples and finished products for microbiological and chemical testing.
- Prepared and maintained media needed for microbiological testing.
- Documentation of results in the log books for future referencing.
- Dispatch of results to the appropriate departments.

EDUCATION

M.Sc. Microbiology and immunology | 2017 | University of Ottawa.

Department of Biochemistry, Microbiology and Immunology

Faculty of Medicine, University of Ottawa.

Thesis project title: H9N2 Avian Influenza Virus. Impact of Serial Passaging by Aerosol Exposure on Pathogenicity in Chickens.

The Canadian Food Inspection Agency, Ottawa Laboratory Fallowfield, Ottawa, Ontario

Supervisor: Dr. Min Lin and Dr. Jiewen Guan

B.Sc. Microbiology | 2011 | Bells University

Department of biological sciences,

Bells University of Technology, Nigeria.

Thesis project title: Comparative processing and storage studies of hot smoked African catfish raised from three different breeding sources.

Bells University of Technology, Ota. Nigeria.

Supervisor: Dr. C.T Kester