Studies on the cellular and molecular mechanisms of innate host susceptibility and resistance to influenza A viruses in chicken and ducks

by

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For my dearest parents for their motivation,
my lovely daughter and son for their cooperation
and my beloved wife for her love and
support during the course of this work

Abstract

Avian influenza viruses are considered to be key contributors to the emergence of human influenza pandemics. While aquatic birds and ducks are the major reservoir for influenza viruses, they are typically resistant to the effects of viral infection, in contrast to the frequently severe disease observed in chickens. In order to understand whether differences in receptors might contribute to this observation, anatomical distribution of influenza virus receptors (sialic acid SAα2,3-Gal and SA α2,6-Gal) in key organs of both species was studied using lectin histochemistry with linkage specific lectins, and receptor binding assays with swine H1N1 (classical A/sw/Iowa/15/30) and avian H2N3 (A/mallard duck/England/7277/06) influenza viruses. Widespread presence of both SAα 2,6-Gal and SAα2,3-Gal receptors were found in all major organs examined in both chickens and ducks. Interestingly, the predominant receptor type in chicken tracheal epithelium (TE) was SAα2,6-Gal whereas SAα2,3-Gal receptors were most abundant in duck TE. Paradoxically, infection of primary cell cultures (duck and chicken lung cells and embryo fibroblasts) with the swine H1N1, the low pathogenicity avian H2N3, and a highly pathogenic H5N1 (A/turkey/England/50-92/91) virus resulted in more extensive and rapid cell death in duck cells than in chicken cells. Infected duck cells displayed morphological features of apoptosis, increased DNA fragmentation and activation of caspase-3/7. Infected duck cells produced comparable levels of viral RNA but less infectious virus than infected chicken cells. Notably, such rapid cell death was not observed in duck cells infected with a contemporary Eurasian lineage H5N1 virus (A/turkey/Turkey/1/05) which has been shown to be fatal to ducks. Gene expression profiling of infected chicken and duck cells, 24hrs post-infection, with a chicken Affymetrix microarray platform revealed differential transcription of many genes between the two avian species. In particular, the array results suggested a possible role of *BCoR, HSPA-9, STAT-3, AVEN, BCLAF1, IL-18, IFN-α,* and *TNF-α* genes in mediating the contrasting species phenotypic response to influenza infections. In summary, rapid cell death in duck cells, mediated at least in part by apoptosis, results in reduced infective virus production and may well be an important protective host response of resistant ducks. By contrast, longer surviving infected chicken cells produce much higher infective virus load along with high levels of pro-inflammatory cytokines which could account for the susceptibility of chickens to influenza infections.

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Author’s declaration

“I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution

Signature _____

Printed Name ____Suresh Varma Kuchipudi_____”

List of Abbreviations

°C	degree Celsius
µl	microlitre(s)
µM	micromolar
AAF	amnio allantoic fluid
AP	alkaline phosphatase
AI	avian influenza
AIV	avian influenza virus
aRNA	amplified ribonucleic acid
ATP	adenosine triphosphate
AVEN	apoptosis, caspase activation inhibitor
BCoR	Bcl-6 co-repressor
BCLAF1	BCL2-associated transcription factor 1
BNF	buffered neutral formalin
bp	base pair
CCM	cell culture medium
CM	collection medium
cDNA	complementary deoxyribonucleic acid
CEE	chicken embryo extract
Ct	threshold cycle
CTP	Cytidine triphosphate
DAB	3,3´-diaminodbenzidine
DAPI	4', 6-diamino-2- phenylindole, dihydrochloride
DEPC	diethylpyrocarbonate
DM	dissociation medium
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
dNTP	a premixed solution containing sodium salts of dATP, dCTP, dGTP and dTTP
DTT	Dithiothreitol
DNA	deoxyriboneucleic acid
FACS	Fluorescence Activated Cell Sorting
FCS	fetal calf serum
ffu	focus forming units
FITC	fluorescein isothiocyanate
GTP	Guanosine triphosphate
HA	haemagglutinin
Hcl	hydrochloric acid
HRP	horseradish peroxidise
hrs	hours
HPAI	highly pathogenic avian influenza
HSPA9	heat shock 70kDa protein 9

IFN	interferon
IM	infection medium
ING-3	inhibitor of growth family, member 3
IVT	*in-vitro* transcription
kDa	kilodalton
kg	kilogram(s)
L	litre(s)
LPAI	low pathogenic avian influenza
MAA	*Mackia amurensis* agglutinin
mab	monoclonal antibody
MDCK	Madin-Darby canine kidney
mg	milligram(s)
MgCl2	magnesium chloride
min	minutes(s)
ml	millilitre(s)
mM	milli molar
MOI	multiplicity of infection
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NA	neuraminidase
nm	nano meters
NP	nucleoprotein
nt	nucelotides
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pfu	plaque forming units
PI	propidium iodide
RIG-I	retinoic acid inducible gene-I
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
s	seconds
SAα2,3-Gal	Sialic acid linked to galactose by α2,3 linkage
SAα2,6-Gal	Sialic acid linked to galactose by α2,6 linkage
SNA	*Sambucus nigra* agglutinin
STAT-3	signal transducer and activator of transcription 3
Tris	tris (bydroxymethyl) aminomethane
Tris Hcl	tris hydrochloride
TBS	tris buffered saline
UTP	Uridine triphosphate
UV	ultraviolet

Chapter 1

Introduction

1.1 Influenza

Influenza (commonly called '*flu*') is a respiratory disease of man and animals and the name influenza is an Italian version of the Latin word *influentia*, which means influence, as this condition was thought to be caused by the influence of stars in ancient times. History of influenza infections dates back several hundred years and the earliest scientific record was in 1650, though the virus causing this condition was only isolated in 1931 (Potter 2001). Several influenza pandemics in humans have been recorded since 1590AD and the most significant is the so called '*Spanish flu*' pandemic that occurred between 1918-1920 killing around 50 million people globally which is considered as the single most devastating disease outbreak in the human history (Johnson & Mueller 2002).

1.2 Avian influenza

Avian influenza (AI) was first described in 1878 by Perroncito in northern Italy, as a contagious disease of poultry associated with high mortality. The condition termed ''fowl plague'', was initially confused with the acute septicemic form of fowl cholera. It was later shown to be a different condition based on clinical and pathological properties by Rivolta and Delprato who called it *Typhus exudatious gallinarum*. In 1901, Centanni and Savunzzi determined that fowl plague was caused by a filterable virus and in 1955 the classical fowl plague virus was shown to be a type A influenza virus by Schafer (reviewed by Lupiani & Reddy 2009).

1.3 Influenza A viruses

Influenza viruses belong to family *Orthomyxoviridae* (from the Greek *orthos*, meaning "standard, correct," and *myxa*, meaning "mucus"). Influenza viruses were initially isolated from pigs in 1931 (Shope 1931) and later from humans in 1933 (Smith et al. 1933). Orthomyxoviruses belong to any one of the five genera: *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, *Thogotovirus* and *Isavirus*. Classification of influenza viruses into A, B, and C is on the basis of antigenic differences in nucleopcapsid (NP)

and matrix (M) proteins. Orthomyxoviruses have segmented genomes and the number of genome segments differs between genera. While influenza viruses A and B contain 8 segments, influenza C viruses contain only 7 genome segments. The main antigenic determinants of influenza A and B viruses are the haemagglutinin (H or HA) and the neuraminidase (N or NA) trans-membrane glycoproteins (Cheung & Poon 2007).

Influenza A viruses are further classified into subtypes based on the antigenic properties of the external glycoproteins and currently cluster into sixteen antigenically different HA (H1-H16) and 9 different NA (N1-N9) types, with their combination designating the subtype of the virus. Conventional classification of influenza viruses was based on antigenic properties while more recent classification systems are based on phylogenetic analysis of the nucleotide and deduced amino acid sequences of the HA and NA genes (Fouchier et al. 2005). The conventional nomenclature for the influenza virus provides a unique identity for the virus. This identity comprises of the influenza virus type, the host species (other than human origin), the geographical site, serial number, and year of isolation (example influenza A/mallard duck/England/7277/06). For influenza virus type A, the haemagglutinin and neuraminidase subtypes are added in brackets [example influenza A/mallard duck/England/7277/06 (H2N3)].

1.3.1 Properties of Influenza A viruses

1.3.1.1 Structure and organization

Influenza A virions are spherical (100 nm diameter) or filamentous (300 nm length) in shape. The virion core is enclosed by a matrix of M1 layer which is overlaid by a lipid envelope from which project glycoprotein spikes of HA and NA, in a ratio of approximately four to one (Ruigrok, Calder, & Wharton 1989). Electron microscopic studies show that the HA spikes are rod-shaped and the NA spikes are mushroom shaped (Colman, Varghese, & Laver 1983;Varghese, Laver, & Colman 1983). The HA is a homotrimer responsible for the receptor binding and membrane fusion, helping the virus to attach to host cells. The NA is a homotetramer whose function is to destroy host receptors by hydrolyzing sialic acid groups from glycoproteins thus helping to release the

viral progeny from host cells. Viral lipids constitute around 18-37% of the weight of the virus, which are modified cellular lipids derived from plasma membranes of the host cell. Small numbers of matrix (M2) ion channels made of integral membrane tetramer M2 protein, traverse the lipid envelope. Approximately one M2 channel per 10^1 to 10^2 HA molecules are usually found (Zebedee & Lamb 1988). The virion core contains nuclear export protein [NEP; also called non-structural protein 2 (NS2)] and the ribonucleoprotein (RNP) complex. The RNP complex consists of the viral RNA segments coated with nucleoprotein (NP) and the RNA polymerase complex (Figure 1.3-1). The RNA polymerase complex is a RNA-dependent RNA polymerase, which is a heterotrimer composed of two "polymerase basic" (PB1, PB2) and one "polymerase acidic" (PA) subunits (Baudin et al. 1994).

Table 1.3-1 Comparison of major properties of influenza viruses

(Adapted from Cheung & Poon, 2007)

	Influenza virus A	Influenza virus B	Influenza virus C
Number of gene segments	8	8	7
Surface glycoproteins	HA and NA	HA and NA	HEF (Haemagglutinin-Esterase-Fusion
Host range	Wide range of hosts (human, pigs, horses, whales, seals and birds)	Humans and seals	Mainly humans (also found in swine)

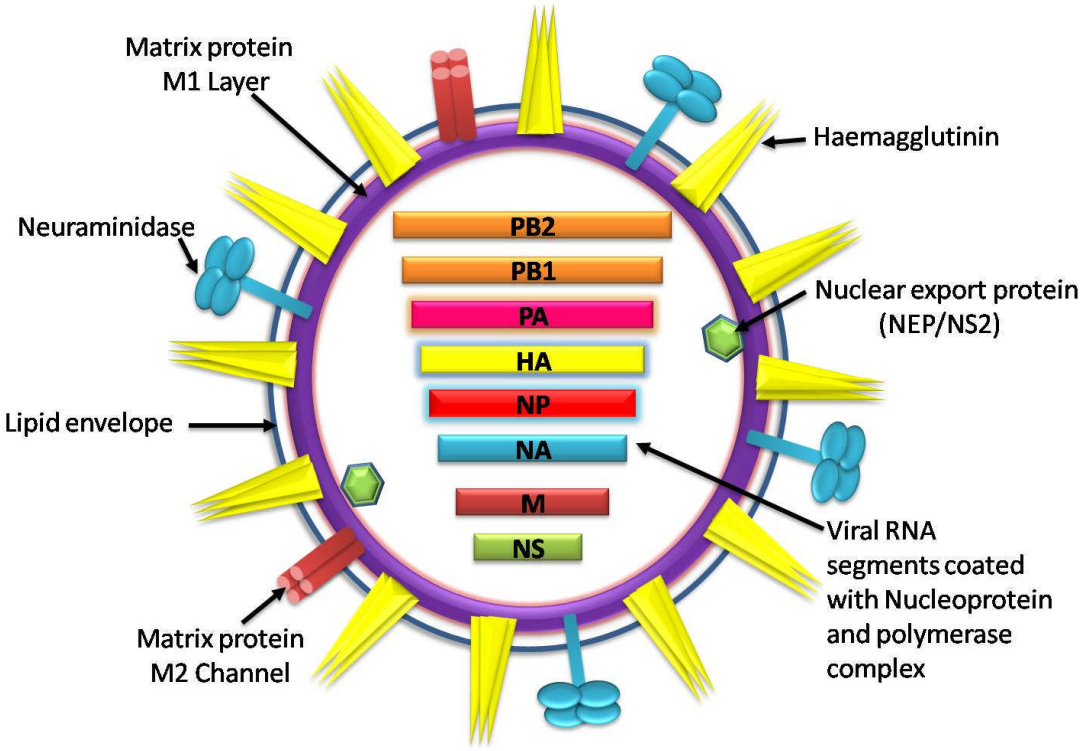


Figure 1.3-1 Schematic illustration of Influenza A virus particle

Influenza virus particles are surrounded by lipid envelope studded with HA and NA spikes and M2 channels. The virion core contains NEP and the RNP complex and is surrounded by a layer of matrix protein M1. The RNP complex consists of the viral RNA segments coated with nucleoprotein (NP) and the RNA polymerase complex.

1.3.1.2 Molecular biology of Influenza A

Influenza A virus genome consists of eight segments of linear negative-sense (complementary to mRNA) single-stranded RNA and the segments are numbered in the order of decreasing length (Palese 1977). Each of these segments at both 3'and 5' ends possesses non-coding regions which form a helical hairpin, bound by the hetero-trimeric RNA polymerase complex which functions as the promoter for viral RNA replication and transcription.

The remaining part of the segment is coated with arginine-rich nucleoprotein (NP) that imparts net positive charge and binds the negatively charged phosphate backbone of the vRNA. The complete genome is 13588 nucleotides long and the 8 gene segments of influenza A viruses are the polymerase genes PA, PB1 and PB2, haemagglutinin (HA), nucleocapsid protein (NP), neuraminidase (NA), matrix (M), and non-structural (NS) genes (Table 1.3-2).

Viral mRNAs from segments 1, and 3 to 6, are monocistronic encoding one protein each which are PB2, PA, HA, NP and NA proteins respectively (Compans, Content, & Duesberg 1972). The viral mRNAs from segment 2 encode the polymerase subunit PB1 and in some strains this segment also codes for the accessory protein PB1-F2 by an alternative open reading frame which is a small, 87-amino acid protein with pro-apoptotic activity (Chen et al. 2001). In contrast, viral mRNAs derived from segments 7 or 8 can undergo alternative splicing, while segment 7 codes for matrix proteins M1 and M2, segment 8 encodes non-structural protein 1 (NS1), an interferon-antagonist protein and NS2 (Kochs, Garcia-Sastre, & Martinez-Sobrido 2007), the nuclear export protein (NEP) (Lamb et al. 1980) .

Table 1.3-2 Influenza A virus gene segments and their proteins

(Adapted from Bouvier & Palese 2008)

Genome Segment	Segment length in nucleotides	Encoded protein	Protein length in amino acids	Protein function
1	2341	PB2	759	Polymerase subunit; mRNA cap recognition
2	2341	PB1	757	Polymerase subunit; RNA elongation, endonuclease activity
		PB1-F2	87	Pro-apoptotic activity
3	2233	PA	716	Polymerase subunit; protease activity
4	1778	HA	550	Surface glycoprotein; major antigen, receptor binding and fusion activities
5	1565	NP	498	RNA binding protein; nuclear import regulation
6	1413	NA	454	Surface glycoprotein; sialidase activity, virus release
7	1027	M1	252	Matrix protein; vRNP interaction, RNA nuclear export regulation, viral budding
		M2	97	Ion channel; virus uncoating and assembly
8	890	NS1	230	Interferon antagonist protein; regulation of host gene expression
		NEP/NS2	121	Nuclear export of RNA

1.3.2 Replication of influenza viruses

Replication of influenza viruses involve various steps from entry in to host cells to the release of newly produced virus particles by budding (Figure 1.3-2). The viral ribonucleoproteins are the minimal functional unit of replication since they are able to transcribe and replicate the viral genome in the nucleus of infected cells.

1.3.2.1 Virus attachment

The initial step in influenza virus infection of a cell is the attachment of virus particle to the target cell surface through the interaction of the viral surface glycoprotein HA, with cell-surface oligosaccharides containing sialic acids (SA) (Gottschalk 1957). Avian influenza viruses show preferential binding to SA receptors linked to galactose by an α2,3 linkage (SAα2,3-Gal), while human and classical swine viruses show preference for receptors with an α2,6 linkage (SAα2,6-Gal) (Matrosovich et al. 2004). Haemagglutinin precursor molecule HA0 should be cleaved to HA1 and HA2 by serine proteases to activate virus infectivity. The HA2 portion mediates fusion of virus envelope and cell membranes, while the HA1 portion contains the receptor binding and antigenic sites. Distribution of activating proteases in the host is one of the determinants of virus tropism and pathogenicity. While the HAs of mammalian and low pathogenic avian influenza viruses are cleaved extracellularly, the HAs of highly pathogenic avian influenza viruses are cleaved intracellularly by ubiquitously occurring proteases. Spread of mammalian and low pathogenic avian influenza viruses is therefore limited to tissues that contain appropriate proteases, notably the respiratory and intestinal tract, whereas the highly pathogenic viruses have the capacity to infect various cell types and cause systemic infections (Steinhauer 1999).

1.3.2.2 Virus entry

Attachment of the influenza virus HA protein to specific receptors generates a receptor-mediated internalization signal, which promotes assembly of clathrin into a coated pit, at the inside face of the plasma membrane. Influenza virus entry in to cells is mainly by

clathrin-dependent receptor-mediated endocytosis, although non-clathrin-dependent endocytic pathways have also been implicated (Sieczkarski & Whittaker 2002)

1.3.2.3 Uncoating of vRNPs and nuclear transport

A drop in endosomal pH to approximately pH5, activates the M2 ion channel, which allows hydrogen ions to enter in to the virus particle. Conformational changes in M1 and acid-catalyzed conformational rearrangements of HA exposing a fusion peptide, cause fusion of the viral envelope with the endosomal membrane. This opens a pore through which the individual viral RNPs are released into the host cell cytoplasm. Influenza virus NP proteins carry a nuclear localization signal promoting the import of vRNPs to host nucleus, whereas M1 proteins inhibit the import.

The M1 protein was found to dissociate from the RNP which enters the nucleus independently by passive diffusion, though the entry of incoming M1 was not a prerequisite for infection (Martin & Helenius 1991). Detachment of vRNPs from M1 proteins is therefore important; viruses with defective M2 ion channels fail to fuse with the endosomal membrane and vRNPs are degraded by lysosomes. Following uncoating, vRNPs are imported to the nucleus through the nuclear pore complexes and this process is promoted by the nuclear localization signal (NLS) carried by the NP proteins (reviewed by Sidorenko & Reichl 2004).

1.3.2.4 Transcription

Inside the host cell nucleus, viral RNA dependent RNA polymerase synthesizes three types of viral RNAs: viral mRNAs of positive polarity (vmRNA), viral genomic RNAs (vRNA) of negative polarity, and complementary RNAs (cRNA) of positive polarity. Influenza viral genome transcription is catalysed by PB1, an RNA dependent RNA polymerase. Until recently the PB1 was also considered to comprise an endonuclease activity which cleaves the CAP structure (m7G or 7-methylguanosine) of the host cell mRNA to provide primers for viral transcription a process referred to as “CAP snatching” (Li, Rao, & Krug 2001). However, more recently the endonuclease activity

has been identified to be associated with the PA subunit rather than PB1 (Dias et al. 2009). Influenza virus vmRNAs contain a cap structure at the 5' end and a poly (A) tail at the 3' end. Splicing of M and NS mRNAs also occurs in the nucleus, which is regulated by NS1 proteins (Krug 1981).

1.3.2.5 Viral genome replication

Influenza virus genome replication is carried out by PB1 and PB2 proteins via the synthesis of full-length vRNA (–) and cRNA (+) strands. The viral RNA dependent RNA polymerase uses the negative-sense vRNA as a template to synthesize complementary RNA (cRNA) intermediates from which the RNA polymerase subsequently transcribes more copies of negative-sense, genomic vRNA.

The poly (A) tail of influenza virus mRNA is encoded as a stretch of five to seven uracil residues in the vRNA, which is transcribed by the viral polymerase into the positive sense strand as a string of adenosines that form the poly (A) tail (Li & Palese 1994; Luo et al. 1991). It was shown that the NP proteins bind to elongating strands of viral genome promoting the initiation of un-primed transcription and block the synthesis of viral mRNAs. The newly produced vRNAs are used for the production of further vmRNAs and cRNAs.

1.3.2.6 Protein production (capsid, non-structural and matrix proteins)

Newly synthesized viral mRNAs are exported from the nucleus into the cytoplasm via nuclear pores and are translated to proteins at ribosomes. Influenza proteins PB1, PB2, PA, NP, NS1, NS2, and M1 are produced in the cellular cytoplasm.

During influenza virus infection host cell protein synthesis is inhibited either by degradation of cellular precursor mRNAs, inhibition of the translation of cellular mRNAs and/or by retarding the transport of cellular mRNAs to the cytoplasm (Park & Katze 1995). While cellular protein synthesis is inhibited, viral proteins are synthesized at a maximum rate by ribosomes. Based on infection studies with recombinant influenza

viruses lacking the NS1 gene (delNS1) or expressing truncated NS1 proteins, influenza virus NS1 protein was suggested to be important for efficient viral protein synthesis (Salvatore et al. 2002).

Newly synthesized polymerase proteins, nucleocapsid, matrix, and non-structural proteins are then transported to the nucleus. These proteins participate in matrix and NS mRNAs splicing, transcription, and genome replication. Some of these proteins will be used for the production of new vRNP complexes (reviewed by Sidorenko & Reichl 2004).

1.3.2.7 Envelope protein production

The envelope proteins HA, NA, and M2 are synthesized, from mRNA of viral origin, on membrane-bound ribosomes. These proteins are then inserted in to the endoplasmic reticulum, where they are glycosylated, folded and trafficked to the Golgi apparatus for post-translational modification. These three envelope proteins have apical sorting signals which direct them to the cell membrane where they are incorporated in to the cell membrane (reviewed by Bouvier & Palese 2008; Sidorenko & Reichl 2004).

1.3.2.8 Encapsidation (packaging)

In the host cell nucleus new vRNP complexes are formed from the newly synthesized PB1, PB2, PA, NP, and NS2 proteins. The transport of vRNPs in to cytoplasm is catalyzed by the M1 proteins which form M1-vRNP complexes.

Nuclear export of vRNPs is directed by NS2 proteins and nuclear export signals (NES) carried by NP proteins and inhibited by the M1 proteins. Therefore, newly synthesized M1-vRNP complexes are unable to penetrate into the nucleus again. Influenza virus particles are not fully infectious unless they contain all the 8 genome segments.

Encapsidation of virus was considered to be a random process (Bancroft & Parslow 2002), until recent evidence makes a strong case for it being a far more selective

process, wherein discrete packaging signals on all vRNA segments ensure incorporation of a full set of 8 segments into the majority of virus particles (Fujii et al.2003).

1.3.2.9 Virus Budding and Release

Influenza virus budding is initiated by an accumulation of M1 matrix protein at the cytoplasmic side of the lipid bilayer. The vRNP-M1 protein complexes interact with the cytoplasmic tails of the cell membrane inserted envelope proteins M2, HA, and NA. This interaction leads to the formation of a bud at the assembly site, which separates from the cellular membrane and a virion is formed.

These newly formed virions remain attached to the cell surface through the HA binding to the host cell surface sialic acids. The sialidase activity of the NA protein cleaves sialic acids from receptors, prevents self-aggregation and facilitates release of virus particles into the extracellular medium (Luo, Chung, & Palese 1993). Sialic acid residues from the virus envelope are also removed by NA, which prevents viral particle aggregation to enhance infectivity (Palese et al. 1974).

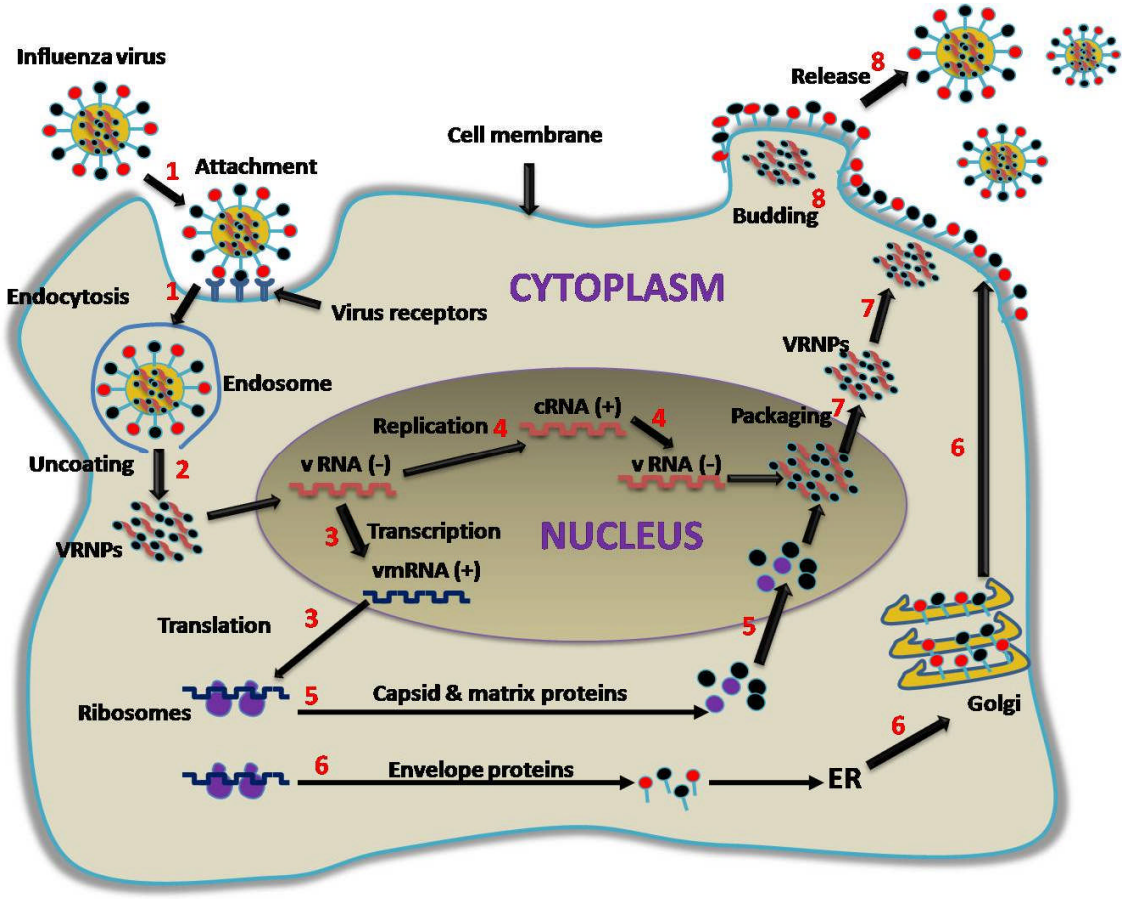


Figure 1.3-2 Replication of influenza viruses

Various stages involved in the replication of Influenza viruses are illustrated

1. Attachment to host receptors and entry by endocytosis,

2. Uncoating of vRNPs,

3. Transcription and translation of vRNPs,

4. Replication of vRNA

5. Capsid, matrix and polymerase protein production,

6. Envelope proteins (HA, NA and M2) production and their transportation through golgi to plasma membrane,

7. Packaging of vRNPs and assembly, and

8. Release of virus particles by budding.

1.3.3 Ecology of Influenza A viruses

1.3.3.1 Host range of influenza viruses

Influenza A viruses have been isolated from humans and also a wide variety of animals, including pigs, horses, minks, seals, whales and birds. Phylogenetic studies show that aquatic birds are the source of the majority of the influenza viruses in other species (Webster et al. 1992). Influenza viruses from aquatic birds have been shown to cross species barriers and cause infections of mammalian hosts (Figure 1.3-3). Avian H1N1 and human H3N2 viruses have been shown to be transmitted to pigs and swine H1N1 viruses to humans. Avian influenza viruses have been shown to cross species barriers and infect horses, seals, whales and mink (Ito & Kawaoka 2000).

1.3.4 Influenza viruses in birds

Aquatic birds are considered to be the major reservoirs of all the known subtypes of influenza A viruses. Influenza viruses established in mammals show a restricted combination of HA and NA subtypes whereas 82 different combinations have been isolated from wild birds. Wild birds frequently become infected with influenza and feral water birds including ducks, geese, terns, shearwaters and gulls are most susceptible to influenza virus infections (reviewed by Van Reeth 2007). Influenza viruses multiply in the cells lining the intestinal tract of waterfowl without causing any clinical signs and are secreted in very high numbers through faecal matter. The virus released into the water is efficiently transmitted to other birds and wild ducks as they migrate through an area and spread the viruses efficiently to other domestic and feral birds. It was hypothesized that all mammalian influenza viruses originate from the avian influenza reservoir. Phylogenetic analysis shows that influenza viruses have evolved into five host-specific lineages which are ancient equine, which has not been isolated in over 15 years; recent equine; gull; swine; and human. The human and classic swine influenza viruses appear to have avian influenza virus as a common ancestor (reviewed by Webster 1998).

1.3.5 Pathogenicity of avian influenza viruses

Influenza A viruses cause mild to very severe infections in domestic poultry and can be divided into two distinct groups based on their ability to cause mortality in chickens. Influenza viruses that cause severe fatal infections are classified as highly pathogenic avian influenza (HPAI). This group is restricted to subtypes H5 and H7 although not all viruses of these subtypes are highly pathogenic. These viruses cause very high mortality often reaching 100%. All other viruses that cause a much milder infection, primarily respiratory disease are classified as low pathogenic avian influenza (LPAI) viruses.

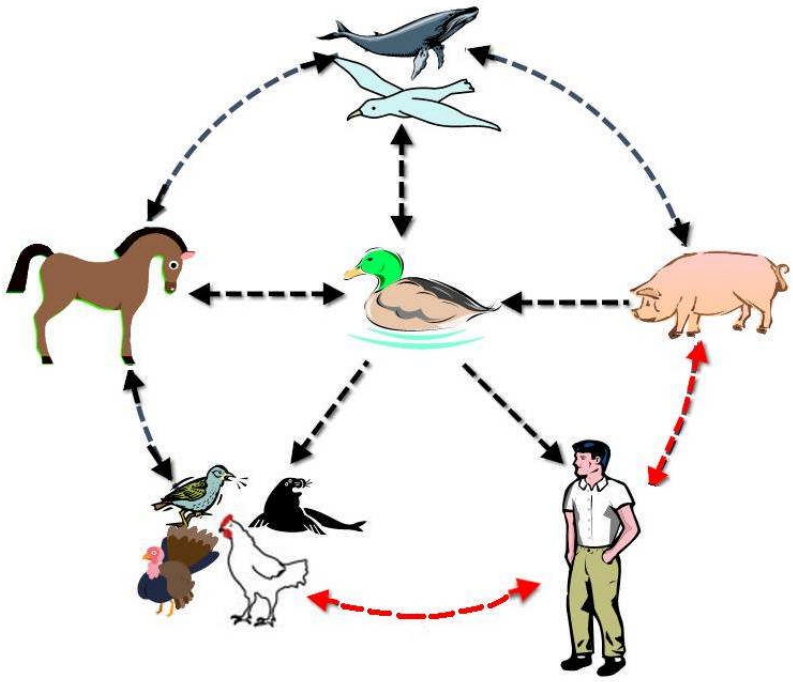


Figure 1.3-3 Reservoirs of influenza A viruses

Wild aquatic birds are hypothesized to be the primary reservoir of all influenza viruses. Five different host groups are constituted based on phylogenetic analysis of the NPs of a large number of different influenza viruses. Transmission of influenza virus has been demonstrated between pigs and humans; humans and chicken (red lines). There are extensive evidences for the transmission of influenza viruses between wild ducks and other species (black lines) (modified from Webster et al.1992).

1.3.5.1 Highly pathogenic avian influenza (HPAI)

Outbreaks of highly pathogenic avian influenza (HPAI) were believed to have occurred in Italy and other European countries prior to its first description by Perroncito in 1878. After the condition was differentiated from fowl cholera by Rivolta and Delprato in 1880, subsequent outbreaks of HPAI have been reported in Italy in 1894 and 1901. These outbreaks later spread with the stock of an itinerary poultry merchant to eastern Austria and Germany and later to Belgium and France. After detecting infected birds in the Brunswick poultry show in 1901, the authorities had closed the show and sent the affected flock to their place of origin leading to further spread of HPAI through Germany. By mid 1901 HPAI became endemic throughout Italy, Central Europe and had been reported in most of Europe, Russia, North America, South America, Middle East, Africa and Asia.

1.3.5.2 Avian Influenza H5N1

Until the mid 1950s, all the HPAI viruses isolated were of the H7 subtype but in 1959 a H5N1 strain from Scotland (Becker & Uys 1967) and a H5N3 strain from South Africa were isolated which produced clinical disease indistinguishable from traditional fowl plague. This lead to the misconception that all the H5 and H7 influenza viruses belong to HPAI. This misconception was later found to be incorrect following the isolation of low pathogenic H5 and H7 viruses from turkeys in Canada (reviewed by Lupiani & Reddy 2009). The first isolation of HPAI H5N1 was from a flock of sick geese in the Guangdong Province of China in 1996 (A/Goose/Guandong/1/96). H5N1 viruses began circulating in Hong Kong in late 1990s ,and later spread beyond Asia into Europe and Africa has raised serious public health concerns following fatal human infections in 1997 (Claas et al. 1998; Subbarao et al. 1998). In May 2001, H5N1 infection of chicken with very high mortality was reported in Hong Kong (Guan et al. 2002). In late 2002, H5N1 infections with high mortality rates were observed in free-flying wild waterfowl in two parks in Hong Kong which suggested that many genotypes of H5N1 were circulating among wild birds in Hong Kong (Ellis et al. 2004). During 2003 and 2004, severe human infections of H5N1 were reported from Vietnam and Thailand along with severe poultry

infections causing up to 100% mortality (Yee, Carpenter, & Cardona 2009). From December 2003 to March 2004, H5N1 infections were reported in three chicken farms and in a number of pet chicken flocks in Japan (Mase et al. 2005). The next wave of H5N1 was reported in 2004 between the months of June and July in China, Indonesia, Thailand, Vietnam and in August from Malaysia. During October 2004, another H5N1 outbreak in tigers at a Thai zoo was reported in which 147 tiers were either died or were euthanized with symptoms of respiratory distress and severe pneumonia (Thanawongnuwech et al. 2005). In October 2005, poultry infections of H5N1 were reported in Turkey and Romania, while Croatia and Hungary reported infection of mute swans. India reported H5N1 outbreaks in 'backyard poultry' in the southern state of Maharashtra in 2006. The United Kingdom reported a single H5N1 case in a wild swan in 2006, and in 2007, a case was reported in a commercial turkey farm in 2007 and sporadic deaths in mute swans were reported in 2008.

1.3.6 Evolution of influenza viruses

Influenza virus genomes exhibit a rapid rate of mutation compared to other DNA viruses due to the low fidelity of RNA-dependent RNA polymerase (replicase) and lack of proof reading mechanism (5'-3' exonuclease activity) (Zambon 1999). Influenza viruses have been shown to evolve by two different mechanisms antigenic drift and antigenic shift.

Antigenic drift is the process in which mutation in few genes leads to an accumulation of amino acid sequence changes that alter the antigenic sites. These antigenic changes will mean that the host immune system is no longer able to recognize the virus for what it is. The virus is then able to multiply without any hindrance from the host immune system. Within an influenza virus, different genes evolve differently because of different selective pressures and evolutionary constraints. Genes coding for surface proteins like HA and NA are subjected to strong selection pressure by neutralizing antibodies of host immune systems. Hence virus surface antigens, the HA and the NA can potentially mutate minutely each time the virus replicates (Figure 1.3-4). Genes coding for internal proteins like the NP are not subjected to strong host immune selection pressure as the surface antigens are, but are thought to undergo significant host-specific adaptive

evolution (Webster et al. 1982). Antigenic shift is the other mechanism, which results when influenza viruses from two different host species infect a single host simultaneously. This co-infection is believed to result in exchange of genome segments between the viruses, a process called reassortment, and the development of a variant virus containing genes from both parent viruses (Figure 1.3-4). Antigenic shift unlike antigenic drift results in dramatic changes in the virus antigenicity, often resulting in the development of a new strain which can be significantly different antigenically from both the parent viruses (reviewed by Zambon 1999). Antigenic 'drift' resulting from the accumulation of amino acid substitutions, in HA helps the virus to escape pre-existing antibodies leading to seasonal annual influenza epidemics in humans. Antigenic 'shift' resulting from a more radical change in the HA and/or NA proteins of influenza A viruses could potentially lead to new influenza pandemics (Donatelli et al. 2003).

1.3.6.1 Interspecies transmission

Despite the presence of all HA and NA subtypes in aquatic birds, very few subtypes have crossed the species barrier and established stable lineages in mammals. Since 1918 three HA (H1–H3) and two NA (N1–2) subtypes have circulated in humans. In horses, only H7N7 and H3N8 are found, and only H1, H3, N1, and N2 subtypes are recovered from pigs in the natural world though they were susceptible to many avian subtypes in experimental settings. The precise molecular, biological or ecological factors determining the ability of the few influenza virus subtypes that can and do cross species barriers and spread among a range of hosts remain largely unresolved (reviewed by de Jong & Hien 2006).

Owing to their inefficient replication, it was believed that avian influenza viruses may need to establish infection in a mammalian intermediate host in order to be able to infect humans (Zhou et al. 1999). Pigs have been proposed to be acting as 'mixing vessels' for the reassortment of avian and human influenza viruses (Scholtissek et al. 1985) as they can be infected by both avian and human influenza viruses (Ito et al. 1998). However, human infections of highly pathogenic H5N1 avian influenza in Hong Kong demonstrated that avian viruses can directly infect humans without the need of an

intermediate host like pigs (Claas et al. 1998; Subbarao et al. 1998). The H5N1 strain has now established in poultry in Asia and Africa and continues to infect humans causing acute respiratory disease syndrome and death. Human infections of H5N1 have a very high reported case-fatality rate ranging from 33% in Hong Kong in 1997 to 61% more recently (reviewed by Chan et al. 2009).

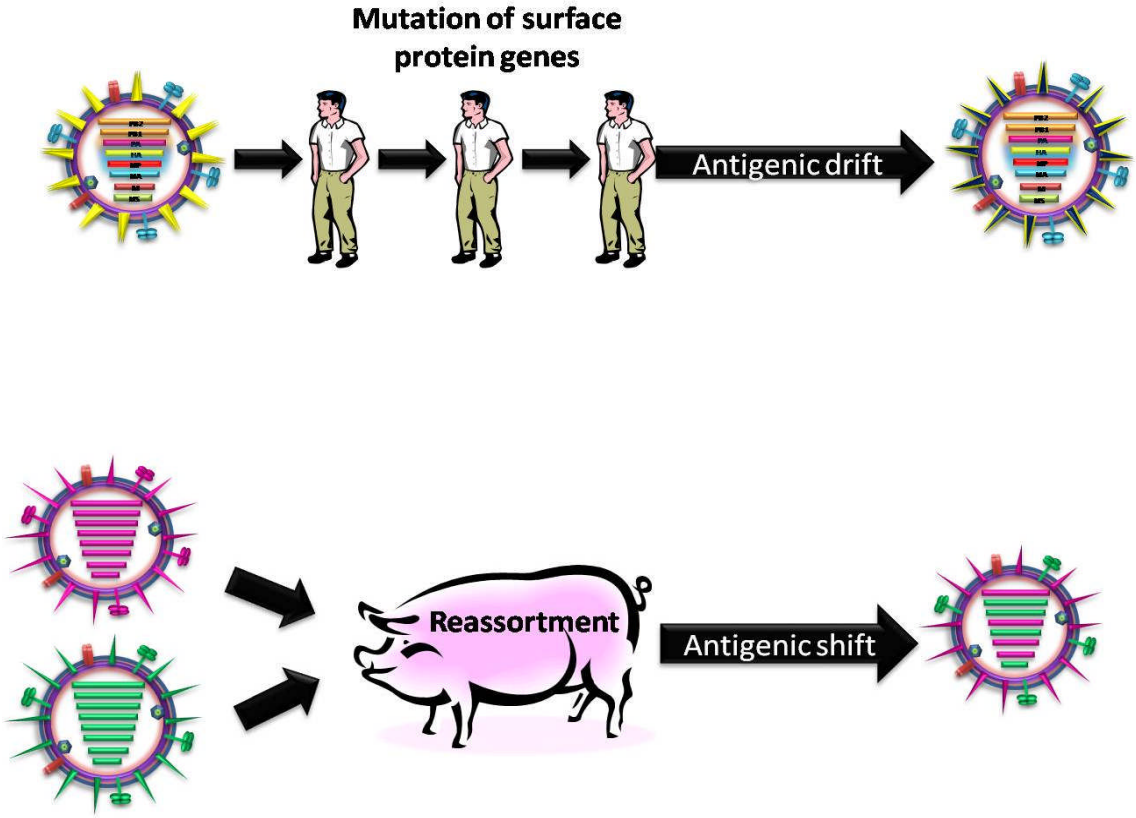


Figure 1.3-4 Mechanisms of influenza virus evolution

Influenza viruses are subjected to host immune selection pressure by circulating pre-existing antibodies causing mutations in surface proteins HA and NA genes leading to amino acid sequence changes in these antigens (antigenic drift). Co-infection of a host by two different influenza viruses leads to exchange of genetic material (reassortment) between the viruses leading to development of a new strain (antigenic shift).

1.3.6.2 Avian influenza and human pandemics

Influenza pandemics in humans can be potentially caused by influenza A virus with a novel genotype for which there is no pre-existing immunity in the human population and be able to spread efficiently between humans. There are records of several pandemics in humans, with the most significant pandemics of the 20th century recorded in 1918, 1957, and 1968, killing millions of people worldwide. In all these three pandemics, the viruses have been shown to have originated from avian influenza viruses (reviewed by de Jong & Hien 2006).

Ten influenza pandemics defined by clinical and epidemiological records have occurred in the last 300 years, with an average of one pandemic in every 33 years. Based on virological and seroprevalence studies the virus types that caused these pandemics were, H1N1 (1977), H3N2 (1968), H2N2 (1957), H1N1 (1918), possibly H3N8 (1900) and H2N2 (1889) (reviewed by Yuen & Wong 2005).

More recently a new influenza pandemic emerged in April 2009 in Mexico which was caused by a swine-originated influenza A (H1N1) virus (S-OIV) containing unique combination of gene segments that have not been previously identified among human or swine influenza A viruses. This novel influenza virus contains a mixture of avian, porcine and human influenza virus gene segments. This virus has spread to many countries and is able to transmit from man to man but has no greater virulence than ‘seasonal flu’ in humans (reviewed by Petrosillo et al. 2009).

1.4 Host resistance to influenza infection

Migratory ducks and other waterfowl serve as the reservoir for most influenza A viruses, including the haemagglutinin (HA) and neuraminidase (NA) subtypes that caused previous human pandemics (Sharp et al. 1997). Despite infection with a wide variety of influenza viruses, ducks often show very little or no clinical signs (Hulse-Post et al. 2005; Kida, Yanagawa, & Matsuoka 1980; Perkins & Swayne 2002). Studies on the effect of influenza infection on migrating mallard ducks by experimental infections

found that LPAI did not produce any clinical disease and the infected ducks have been found to develop transient immunity for LPAI infection (Latorre-Margalef et al. 2009). Most of the HPAI H5N1 strains caused mild or no clinical signs in ducks, whereas they produce a very severe disease in chickens and quails often causing up to 100% mortality within 2-3 days (Jeong et al. 2009; Saito et al. 2009). Experimental inoculation of commercial layer ducks with classical HPAI H5N1 influenza viruses that cause very high pathogenicity for chickens did not produce any clinical signs (Wood, Parsons, & Alexander 1995).

Chickens and ducks thus show a very contrasting outcome following infection with various influenza viruses. The rapid onset of fatal disease in chickens and no evidence of clinical symptoms in ducks suggest the potential role of innate immunity as a resistance mechanism in ducks. However the recent evidences suggest that the resistance of ducks to HPAI infections is not absolute. Asian lineage of H5N1 virus has caused large numbers of deaths in both poultry and wild-bird populations. Some isolates of this virus have also been reported to cause disease and death in commercial ducks, which has not been seen with other HPAI viruses. Experimental infection of Pekin ducks (*Anas platyrhynchos*) with a Eurasian lineage of HPAI H5N1 strain (A/turkey/Turkey/1/2005) caused fatal infection (Londt et al. 2008). These studies suggested that the contemporary Eurasian lineage H5N1 viruses could cross the barrier of innate resistance of ducks. The resistance to H5N1 strains also varies between different species of ducks.

A challenge study involving five species of North American ducks including mallard (*Anas platyrhynchos*), northern pintail (*Anas acuta*), blue-winged teal (*Anas crecca*), redhead (*Aythya americana*), and wood duck (*Aix sponsa*) with a HPAI H5N1 strain found that out of the five species tested, only wood ducks (*Aix sponsa*) developed a severe clinical disease and died (Brown et al. 2006).

Ducks have been shown to support genetic reassortment of different influenza viruses in the intestinal tract providing a mechanism of evolution of genetically diverse influenza A viruses (Hinshaw et al. 1980). Several studies have also suggested the potential role of ducks in the evolution of H5N1 HPAI viruses (Hulse-Post et al 2005; Chen et al. 2004;

Guan et al. 2002; Kim et al. 2009b). The precise molecular basis for this contrasting disease outcome following influenza virus infection of ducks and chickens has not yet been identified. Therefore a study of the molecular basis for the contrasting disease outcome in chicken and duck would be most valuable for a better understanding of the innate resistance/ susceptibility mechanisms. Following on from this the study could identify candidate protein or gene targets can be identified for pharmaceutical targeting.

1.5 Hypothesis

There are innate molecular differences between chickens and ducks that would account at least in part for the contrasting phenotypic outcomes of susceptibility and resistance to influenza infection in the two respective host species.

1.5.1 Aims and objectives

To identify the molecular factors or pathways that mediate innate host resistance to influenza A infection in chicken and ducks with the following objectives.

1. To study influenza virus receptor distribution in chicken and ducks

To understand whether differences in receptors might contribute to the contrasting outcomes of infection, influenza virus receptor distribution in organs of ducks and chickens. The study was to involve lectin histochemistry and virus binding assays.

2. To establish *in-vitro* models for the study of influenza virus-host interactions

To understand the potential mechanisms underlying susceptibility/ resistance to influenza virus infection, virus-host interactions will be studied using suitable *in-vitro* models. To this end *in-vitro* models comprising of organ and cell cultures from chicken and ducks will be explored for their suitability for the study of influenza virus-host interactions.

3. To study the cellular responses following influenza infection

To study the host cellular responses following influenza virus infection that might contribute to innate susceptibility /resistance in chickens and ducks.

4. To study the molecular mechanisms underlying influenza resistance

To study the differential gene expression between chicken and duck cells following influenza infection in order to identify molecular mechanisms contributing to susceptibility /resistance. Gene expression analysis of primary cells following influenza virus infection is to be carried out to analyse the transcriptome using Affymetrix GeneChip® probe arrays.

Chapter 2

Materials and methods

2.1 Introduction

The general materials and methods used are described in this chapter and the more specialized materials and protocols are described in the appropriate chapters following. Most of the general materials and methods described were derived from the standard operating procedures that can be found in any common laboratory manuals (for example Maniatis, Fritsch, & Sambrook 1989).

2.2 Experimental animals

Animals used during this study comprised of chicken and ducks. Commercial layer chickens of 35-40 week old were obtained from Glenrath Farms Ltd, East Lothian, UK and 4 week old broiler chickens were obtained from PD Hook Hatcheries, Bampton, Oxfordshire, UK. Pekin ducks, 3 and 6 weeks old, were obtained from Cherry Valley Farms, Rothwell, Lincolnshire, UK. The animals were euthanized by intravenous (i/v) injection of Euthasol (pentobarbital sodium and phenytoin sodium solution).

2.3 Tissue collection

Euthanized birds were dissected with sterile scalpel and scissors. Lungs were collected aseptically in sterile containers with a collection medium (CM) containing Dulbecco's modified Eagle's medium (DMEM) with Glutamax (Invitrogen) supplemented with 100 units/ml penicillin, 100µg/ml streptomycin (Invitrogen) and 2.5µg/ml amphotericin B (Invitrogen) for primary cell cultures.

Organ parts of approximately 2-3cm of tracheae, kidneys, lungs, small intestines, large intestines, hearts, brains and skeletal muscles were dissected, washed in PBS and fixed in 10% buffered neutral formalin (BNF) for histochemistry.

2.4 Tissue sections

2.4.1 Embedding tissues

Tissues were dehydrated by immersion in an ascending series of aqueous alcohol solutions (70%, 80%, 90% and 100% ethanol) using a histokinette (TP 1020 Leica, Germany). Following dehydration, the tissue was cleared using Histoclear (National Diagnostics, Atlanta, USA) as this fluid is miscible with both paraffin wax and alcohol, thereby allowing molten paraffin wax to penetrate the tissue. The schedule used for processing the tissue samples in the histokinette is outlined in Table 2.4-1.

Table 2.4-1 Schedule for tissue processing in histokinette

Chemical	Duration
70% ethanol	60 minutes
80% ethanol	60 minutes
90% ethanol	60 minutes
100% ethanol	4 x 30 minutes (2 hours in total)
Histoclear	3 x 60 minutes (3 hours in total)
Wax	60 minutes
Wax	90 minutes

2.4.2 Sectioning of tissues

After processing, the tissues were embedded in paraffin wax blocks, and orientated as desired using heated forceps. Following embedding, the tissues were sectioned using a rotary microtome (RM 2255 Leica, Germany) with the specimen feed set at 5µm. After cutting, the transverse tissue sections were stretched flat onto Polysine adhesion glass microscopic slides (Thermo Scientific), then placed on a hotplate and baked overnight at 45°C, prior to staining.

2.5 Virus production and titration

2.5.1 Viruses

2.5.1.1 Low pathogenic viruses

A low pathogenic avian influenza H2N3 strain (A/mallard duck/England/7277/06; hereafter referred to as avian H2N3), and a low pathogenic swine influenza H1N1 strain (classical A/sw/Iowa/15/30; hereafter referred to as swine H1N1) were used in this study. Both of these viruses were propagated in 7-8 day old embryonated chicken eggs, by allantoic inoculation and the virus was harvested at 48hrs post-inoculation. It was then stored at -70°C in readiness for further use.

2.5.1.2 Highly pathogenic avian influenza viruses (HPAI)

Two highly pathogenic avian influenza H5N1 strain viruses were used in the study. A H5N1 strain (A/turkey/England/50-92/91; hereafter referred to as HPAI50-92), did not produce any clinical disease in Pekin ducks but produced rapid high mortality in chickens (Wood, Parsons, & Alexander 1995).

Another very high pathogenic H5N1 strain (A/turkey/Turkey/1/05 hereafter referred to as HPAI ty-Ty) caused severe clinical signs in ducks (Londt et al. 2008) and caused severe clinical signs in chickens. Both strains were grown by allantoic inoculation of 10 day old embryonated chicken eggs.

2.5.2 Virus cultivation in chicken embryos

Developing chicken embryos have been earlier employed for cultivation of influenza viruses (Burnet & Lush 1938) and have been shown to be useful for the propagation of influenza viruses (Veeraraghavan & Sreevalsan 1961). Avian influenza viruses used in

the study were grown by allantoic inoculation of 10 day old embryonated chicken eggs. Embryonated chicken eggs were obtained from Cobb Farms and were incubated in an "*Octagon20 eco*" egg incubator (Brinsea, UK).

The embryos were candled at regular intervals using an Egg-Lume Candling Lamp (Brinsea, UK) in dark to check the viability of the embryos. On the 7 – 8th day of incubation the embryos were candled and the air sac was outlined with a pencil. After wiping the egg surface with 70% ethanol, a small hole was made into the shell in the middle of the broad end with a drill carefully without damaging the shell membranes. With the help of a hypodermic syringe and needle, 0.1ml of the virus was carefully injected in to the allantoic cavity through the air sac (Figure 2.5-1).

After virus inoculation, the hole on the shell was sealed with molten paraffin wax, date of inoculation was labelled on the shell and the eggs were transferred back to the egg incubator. Eggs were candled after 24 hrs of inoculation and any death within 24 hrs was considered non-specific and the embryos were discarded. After 72 hrs, embryos were chilled at 4°C for 15 min before harvesting the virus. With the help of a scissors and forceps the egg shell at the air sac was opened and the blood free amnio-allantoic fluid (AAF) was aspirated with a syringe and needle. Harvested virus was aliquoted and stored at -80°C in readiness for further use. The virus was titrated in MDCK cells by an immunocytochemical method.

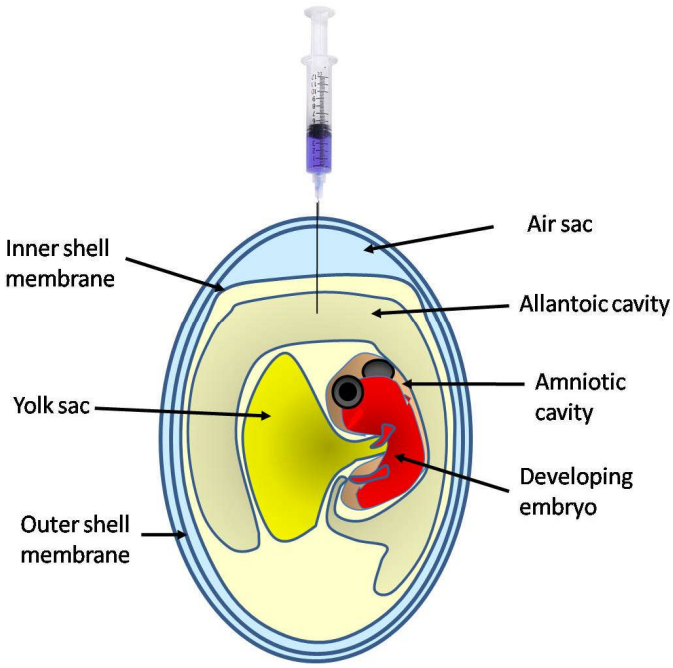


Figure 2.5-1 inoculation of virus in to 10 day old chicken embryo

A healthy 10 day old embryo is represented schematically; 0.1 ml of virus was inoculated through the air sac in to the allantoic cavity as indicated with a syringe

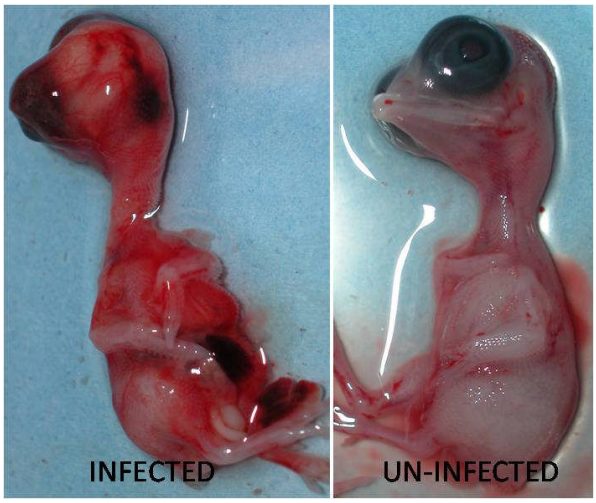


Figure 2.5-2 Chicken embryos inoculated with influenza viruses

Embryopathy in a 10 day old chicken embryo inoculated with low pathogenic avian influenza virus strain H2N3 showing occipital haemorrhages

2.5.3 Virus titration

2.5.3.1 Infection of MDCK cells

Madin-Darby Canine Kidney (MDCK) cells were seeded into 24-well cell culture plates (Costar) at a seeding density of 10^5 cells/well in DMEM with 10%FCS, 100 units/ ml penicillin and 100µg/ml streptomycin. The cells grow to 100% confluence within 24 hrs. Serial 10 fold dilutions from 10^{-1} to 10^{-10} of the virus in a 24 well plate were made with a serum free infection medium (IM) containing 2% Ultroser G (Pal Biosepra), 500ng/ml TPCK Trypsin (Sigma) and antibiotics in Ham's F12 (Invitrogen). Then the medium from MDCK cells was removed and the cells were rinsed twice with PBS. Five hundred micro litres of the diluted virus was added to each well and duplicate wells were infected for each dilution of the virus.

Mock infections were performed in two wells with PBS. After two hours incubation at 37°C with 5% CO_2, the cells were rinsed three times with PBS. Then 1ml of fresh infection medium was added to all the wells. After further 4hrs incubation, the cells were fixed with acetone: methanol (1:1) for 10 min before immunocytochemical staining as described in chapter 4 following.

2.5.3.2 Immunocytochemical staining

Influenza viral protein expression was studied by immuno-staining of fixed cells with a primary mouse monoclonal antibody to influenza nucleoprotein (NP) (AA5H, Abcam) followed by secondary detection by means of a VECTASTAIN ABC-AP Kit with BCIP/NBT substrate Kit (Vector labs) following the manufacturer's instruction. Cells were pre-soaked in TBS for five minutes.

The cells were blocked with 250µl of diluted horse serum (HS) (3drops in 10 ml TBS) per well for 30 min at RT. The cells were rinsed for 5 min in TBS then incubated with

1:1000 primary antibody (mouse monoclonal to influenza A nucleoprotein) for 1 hr at RT. After washing for five minutes in TBS, the cells were incubated with biotinylated anti-mouse IgG for 30 min at RT. While the cells were incubating VECTA STAIN ABC AP reagent was prepared by mixing two drops of reagents A&B each in 10 ml TBS and allowed to stand for 30 min before use. After a 5 min wash in TBS, cells were then incubated with VECTA STAIN ABC-AP reagent for 30 min at RT. Cells were washed and incubated with alkaline phosphatase substrate for 20 min at RT. Substrate solution was prepared by adding two drops each of reagents 1,2,&3 to 5 ml of 100mM Tris Hcl buffer (pH9.5). The cells were rinsed in water and then examined under a microscope (Leica).

2.5.3.3 Calculation of focus forming units (ffu)

For calculation of focus forming units (ffu) per microlitre of the virus, the dilution of the virus that produced less than 20% positive cell staining was used. Number of positive cells per each low power field (10x) was counted. Counting was repeated in five random fields and the average number of positive cells per field (A) was calculated. The average number of MDCK cells per low power field was counted to be approximately 3000 using Image Pro MC software. This number might change depending on the type of microscope used. Using average number of positive cells, percent positive cells was calculated.

Percent positive cells = 3000 x A/100 =B,

Approximate number of MDCK cells in a 24 well plate is 240000

Number of positive cells in 24 well plate = 240000 x B/100 =C

Amount of un-diluted virus used in the well =D (microlitres)

The number of infective virus particles per microlitre = C/D

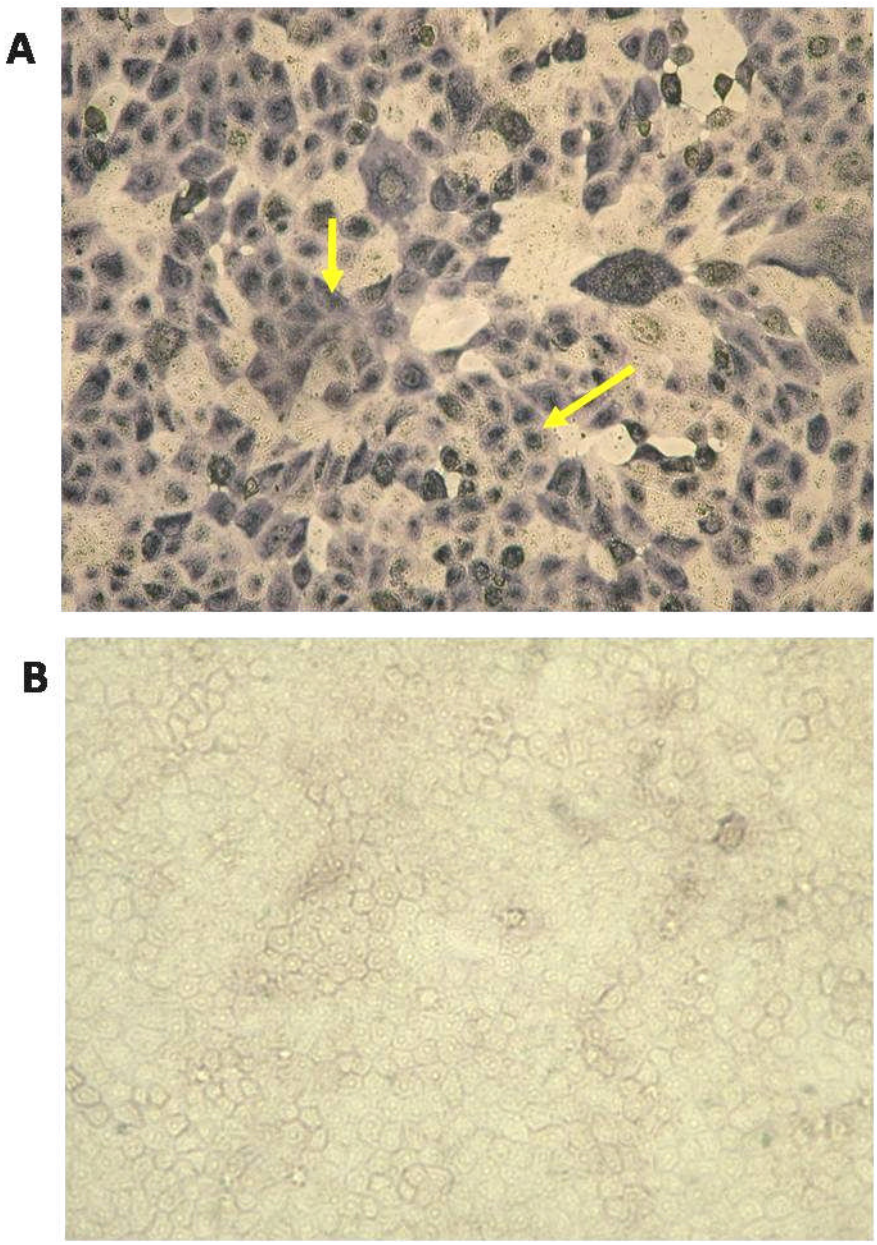


Figure 2.5-3 Immunocytochemical staining of MDCK cells

Influenza virus infected MDCK cells stained with a monoclonal antibody to virus nucleoprotein:

A) Infected cells expressing viral proteins stained dark blue (pointed with arrows)

B) Un-infected control cells did not show any staining.

2.6 Production of influenza viral RNA by *in-vitro* transcription

2.6.1 Extraction of viral RNA

Extraction of viral RNA from influenza viruses was performed by using *High Pure* viral RNA kit (Roche) following the manufacturer's instructions. Supernatants were collected from influenza virus infected MDCK cells and 200µl of supernatant was used for RNA extraction. To a 1.5 ml micro-centrifuge tube, 200µl of supernatant and 400µl of carrier RNA supplemented binding buffer was added and the contents were mixed well with micropipette.

The mixture was incubated at 25°C for 10min and transferred to a *High filter tube* and collection tube assembly. The filter tube assembly was then centrifuged for 15 s at 8,000 x g in a table top centrifuge. Then the filter tube was removed from the collection tube, and the collection tube containing the flow through liquid was discarded. The filter tube was reinserted in to a fresh collection tube and 500µl of inhibitor removal buffer was added to the upper reservoir of the filter tube assembly.

After centrifuging the filter tube assembly for 1 min at 8,000x g, collection tube containing the flow through liquid was discarded and the filter assembly was inserted in to a new collection tube. Then 450µl of wash buffer was added to the filter tube assembly, centrifuged for 1 min at 8,000x g and collection tube containing the flow through liquid was discarded. Washing was repeated with a new collection tube, after initial centrifugation for 1 min at 8,000x g, the filter tube assembly was further centrifuged for 10s at highest speed (approx. 13,000x g) to remove any residual wash buffer. The filter tube assembly was then inserted to a clean 1.5ml RNAse free micro centrifuge tube and viral RNA was eluted by adding 50µl of elution buffer to the upper reservoir of the filter tube assembly and centrifuging for 1 min at 8,000x g. Eluted RNA samples were stored at -80°C until further use.

2.6.2 Quantification of RNA

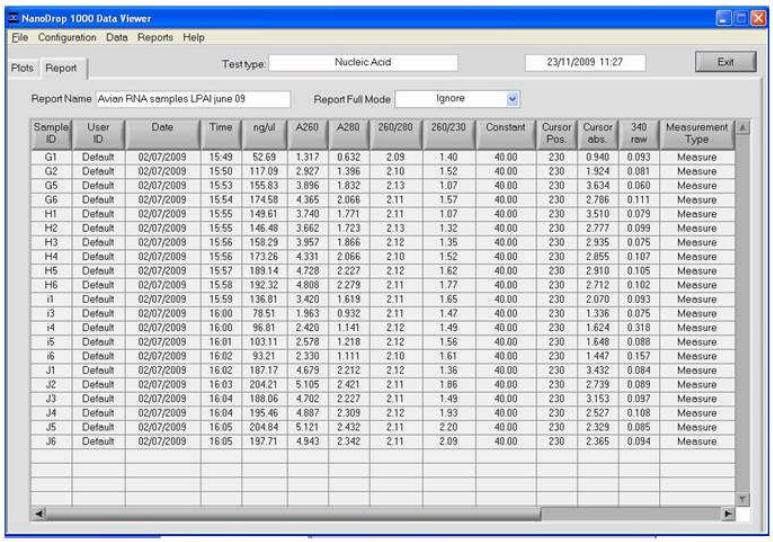
Concentration of extracted RNA samples was determined using UV absorption with a NanoDrop1000 spectrophotometer (Thermo Scientific) and the data was analyzed with NanoDrop1000 3.7.1 software. This instrument measures the absorbance at 260 and 280 nm.

The concentration of nucleic acid is determined using the Beer-Lambert law, which predicts a linear change in absorbance with concentration. For determining the concentration of RNA in solution, OD at 260 nm is used, where the OD value of 1.0 at A260 is equivalent to about 40 µg/ml of RNA. RNA has its absorption maximum at 260 nm and the ratio of the absorbance at 260 and 280 nm is used to assess the RNA purity of an RNA preparation and the ratio of 1.8-2.1 for A260/A280 is considered ideal.

Measurement of RNA concentration using the Nanodrop was performed as per the manufacturer's instruction which is described here briefly. With the sampling arm of the instrument open, the lower measurement pedestal was cleaned with RNase free water. A blank measurement was made by pipetting 1 µl of RNAse free water on to the measurement pedestal. Then 1µl of RNA sample is loaded on to the lower measurement pedestal and the sampling arm was closed.

Absorbance was recorded at 230 and 260nm using the software and the RNA concentrations were expressed in nanograms per microlitre (Figure 2.6-1).

A



Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw	Measurement Type
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G2	Default	02/07/2009	15:50	117.09	2.927	1.396	2.10	1.52	40.00	230	1.924	0.081	Measure
G5	Default	02/07/2009	15:53	155.83	3.896	1.832	2.13	1.07	40.00	230	3.634	0.060	Measure
G6	Default	02/07/2009	15:54	174.58	4.365	2.066	2.11	1.57	40.00	230	2.786	0.111	Measure
H1	Default	02/07/2009	15:55	149.61	3.740	1.771	2.11	1.07	40.00	230	3.510	0.079	Measure
H2	Default	02/07/2009	15:55	146.48	3.662	1.723	2.13	1.32	40.00	230	2.777	0.099	Measure
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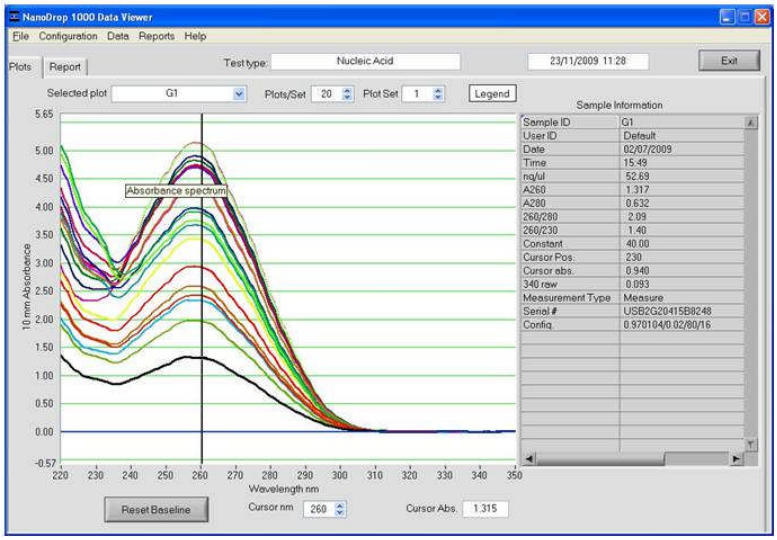
H4	Default	02/07/2009	15:56	173.26	4.331	2.066	2.10	1.52	40.00	230	2.855	0.107	Measure
H5	Default	02/07/2009	15:57	189.14	4.728	2.227	2.12	1.62	40.00	230	2.910	0.105	Measure
H6	Default	02/07/2009	15:58	192.32	4.808	2.279	2.11	1.77	40.00	230	2.712	0.102	Measure
i1	Default	02/07/2009	15:59	136.81	3.420	1.619	2.11	1.65	40.00	230	2.070	0.093	Measure
i3	Default	02/07/2009	16:00	78.51	1.963	0.932	2.11	1.47	40.00	230	1.336	0.075	Measure
i4	Default	02/07/2009	16:00	96.81	2.420	1.141	2.12	1.49	40.00	230	1.624	0.318	Measure
i5	Default	02/07/2009	16:01	103.11	2.578	1.218	2.12	1.56	40.00	230	1.648	0.088	Measure
i6	Default	02/07/2009	16:02	93.21	2.330	1.111	2.10	1.61	40.00	230	1.447	0.157	Measure
J1	Default	02/07/2009	16:02	187.17	4.679	2.212	2.12	1.36	40.00	230	3.432	0.084	Measure
J2	Default	02/07/2009	16:03	204.21	5.105	2.421	2.11	1.86	40.00	230	2.739	0.089	Measure
J3	Default	02/07/2009	16:04	188.06	4.702	2.227	2.11	1.49	40.00	230	3.153	0.097	Measure
J4	Default	02/07/2009	16:04	195.46	4.887	2.309	2.12	1.93	40.00	230	2.527	0.108	Measure
J5	Default	02/07/2009	16:05	204.84	5.121	2.432	2.11	2.20	40.00	230	2.329	0.085	Measure
J6	Default	02/07/2009	16:05	197.71	4.943	2.342	2.11	2.09	40.00	230	2.365	0.094	Measure

B



Sample Information	
Sample ID	G1
User ID	Default
Date	02/07/2009
Time	15:49
ng/ul	52.69
A260	1.317
A280	0.632
260/280	2.09
260/230	1.40
Constant	40.00
Cursor Pos.	230
Cursor abs.	0.940
340 raw	0.093
Measurement Type	Measure
Serial #	USB2G20415B8248
Config.	0.970104/0.02/60/16

Figure 2.6-1 Measurement of RNA concentration

Measuring RNA concentration with NanoDrop1000:

A) Report view with the summary of all samples data showing concentration, 260/280 absorbance ratio

B) Plots view of histograms plotted against wavelength and absorbance

2.6.3 Reverse transcription

The first strand cDNA from the viral RNA was synthesized using the Superscript III First-strand synthesis system (Invitrogen). To a PCR tube 1µl of each of Random hexamers, 10mM dNTP mix and 5µg of the RNA sample were added and the volume was made up to 10µl with nuclease free or diethyl-pyrocarbonate (DEPC) treated water. The tube was incubated at 65ºC for 5 min, and then placed on ice for 1 min. Next 10µl of cDNA synthesis mix was added to each RNA/primer mix. A cDNA Synthesis Mix was prepared by adding each component in the indicated order by 2µl of 10x RT buffer, 4µl of 25mM MgCl2, 2µl of 0.1 M DTT, 1µl of Ribonuclease Inhibitor, RNase Out and 1µl of the Superscript III RT enzyme. The tubes were then centrifuged briefly. Then incubated for 10 min at 25ºC, followed by 50 min at 50ºC and the reaction is terminated at 85ºC for 5 min and the tubes were chilled on ice.

2.6.4 Matrix (M) gene polymerase chain reaction (PCR)

Amplification of part of the influenza virus matrix protein gene was performed using primers designed to a highly conserved sequence among influenza viruses following the method described previously (Spackman et al. 2002).

2.6.4.1 PCR conditions

For performing the M gene specific PCR reaction, Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen) was used. The forward primer (5 '–3') AGA TGA GTC TTC TAA CCG AGG TCG and the reverse primer TGC AAA AAC ATC TTC AAG TCT CTG were supplied by Eurofins MWG Operon (London, UK). To a DNase/RNase-free microcentrifuge tube the following components are added to prepare a master mix of common components for multiple reactions to minimize reagent loss.

Component	Volume	Final Concentration
10X High Fidelity PCR Buffer	5µl	1X
10 mM dNTP mixture	1µl	0.2 mM each
50 mM $MgSO_4$	2µl	2 mM
Forward primer (10 µM)	1µl	0.2 µM
Reverse primer (10 µM)	1µl	0.2 µM
Template DNA	5µl	
PlatinumR *Taq* High Fidelity	0.2µl	1.0 unit
Autoclaved distilled water to	36µl	not applicable

The tube was capped, tapped gently and briefly centrifuged to collect the contents. The tube was then placed in a thermal cycler (Mastercycler® gradient, Eppendorf) and the following program was used for the reaction:

Initial denaturation: 94ºC for 1 min followed by 35 cycles of: denaturation at 94ºC for 30 s; annealing at 52ºC for 30 s; extension at 68º C for 90 s. After 35 cycles the reaction was held at 72ºC for 5 min followed by cooling to 4ºC.

2.6.5 Agarose gel electrophoresis

The size of the DNA sample was determined using 0.8% agarose gels made with agarose (Invitrogen) in TAE buffer (40mM Tris acetate, 1mM EDTA). The agarose was melted in a microwave oven, and was cooled to around 55ºC and 75µg/ml ethidium bromide (Fisher Scientific, UK) was added. The gel mix was then poured in to a gel casting tray (Biorad) with appropriate gel comb inserted. When the gel had set, the comb was

carefully removed and DNA samples were loaded. The DNA samples for loading the gel were prepared by adding 9µl of the DNA sample and 1µl of the 10x loading buffer [0.25% bromophenol blue (sigma), 0.25% xylene cyanol FF (USB Corp, Ohio) and 30% glycerol (Fisher Scientific) in water]. A 1kb DNA ladder (Invitrogen), which is a standard DNA marker with a mixture of DNA segments of known size was also loaded in one of the wells. The gel was then placed in the electrophoresis tank (Biorad) containing TAE buffer with the samples near to the cathode. The electrodes were connected to the power pack and the gel was run for 60-90 min with 70 volts. The gels were then examined under a UV trans-illuminator (ImageQuant 300, GE Healthcare, Buckinghamshire, UK). The size of the sample DNA was estimated by comparing with the standard DNA marker to confirm that the PCR reaction yielded the desired product.

2.6.6 Cloning of PCR product into plasmid vector

For cloning the amplified influenza virus M gene cDNA, TOPO-TA (Invitrogen) cloning system, a specialized cloning kit designed for PCR products cloning system was used. The non-template terminal transferase activity of *Taq* polymerase leaves a single deoxyadenosine residue overhang on PCR products. The plasmid vector (pCR®4-TOPO®) is supplied linearized with a single deoxythymidine residue overhang, allowing any PCR product with deoxyadenosine residue overhang to be ligated in to the vector. The cloning reaction was performed following the supplier's protocol. Four microlitres of PCR product, 1µl of TOPO-TA vector, 1µl of salt solution (containing 200 mM NaCl, 10 mM $MgCl_2$) and 5µl water were added to a microcentrifuge tube. The contents were mixed gently and incubated for 5 min at RT.

2.6.7 Transformation of bacteria with plasmid

One vial of One Shot TOP10 competent *E.coli* cells (Invitrogen) were added to 2µl of the TOPO cloning reaction. The contents were mixed gently and incubated on ice for 30 min. The cells were then subjected to heat-shock for 30s at 42ºC without shaking and

transferred immediately on to ice. Two hundred and fifty microlitres of SOC medium (0.5g of tryptone, 5g of yeast extract and 20mM glucose in 1lt of sterile distilled water) was added to the tube at room temperature and the tube was incubated at 37ºC for 1 hr in an orbital shaker at 200rpm. Following incubation, 10-50µl from each transformation was plated on to a pre-warmed Luria-Bertani (LB) agar supplemented either with 50-100 µg/ml ampicillin or 50 µg/ml kanamycin and incubated overnight at 37ºC.

2.6.8 Isolation of plasmid DNA

Plasmid DNA isolation was carried out by a modified version of the alkaline lysis technique described previously (Birnboim & Doly 1979).

2.6.8.1 Large scale plasmid preparations

For large scale plasmid preparations a Maxiprep kit (Qiagen) was used. Single colony from LB agar was inoculated in to 5ml of LB broth and incubated at 37ºC overnight in an orbital shaker. Two millilitres of the overnight culture was inoculated in to 400ml of LB broth supplemented with 50-100 µg/ml ampicillin or 50 µg/ml kanamycin in a conical flask. The conical flask was incubated at 37ºC in a shaking incubator overnight. The cells were harvested the following day which were in mid-log phase of the growth. The culture was centrifuged at 6000 x g for 15 min at 4ºC. The cell pellet was re-suspended in 10ml of buffer P1.

The cells were lysed by adding 10ml buffer P2 and the tube was mixed vigorously by inverting. After incubating for 5min at room RT, 10ml of pre-chilled buffer P3 was added, mixed thoroughly and centrifuged at 10,000x g for 40 min at 4ºC. While centrifuging, Qiagen maxiprep columns were equilibrated with 10 ml buffer QBT. The supernatant from the centrifugation step was carefully poured into the column through a 0.2µm cell strainer (Qiagen) to remove any particulate matter. Columns were washed

twice with 30ml buffer QC and the flow-through was discarded. Plasmid DNA was eluted with 15ml of buffer QF.

2.6.9 Determination of DNA concentration

The concentration of DNA from large scale plasmid preparations was determined using a spectrophotometer (DU-600 spectrophotometer, Beckman) with optical density (OD) measurements at 260 and 280nm against a distilled-water blank. Plasmid DNA was diluted 1:200 in distilled water (Millipore) to a total volume of 800µl, and transferred to quartz, optical cuvette (LCM, LA). An OD260 measurement of 1.0 was taken to indicate a double stranded plasmid DNA concentration of 10µg/µl. The OD ratio of 260/280nm was used to estimate the purity of the DNA preparation, with a ratio approaching 1.8 is considered ideal (Hirschman & Felsenfeld 1966).

2.6.10 Sequencing of the plasmid

2.6.10.1 Cycle sequencing reaction

Sequencing of the cloned influenza matrix gene was performed based on the chain termination method (Sanger, Nicklen, & Coulson 1977) with cycle sequencing method as previously described (Innis et al. 1988) using BigDye® Terminator v3.1Cycle Sequencing Kit (Applied Biosystems). The manufacturer's instructions were followed, briefly cycle sequencing reaction mixture was prepared with 8.0µl of BigDye ready reaction mix with 300ng of DNA template and 1.6 pmol/µl of each sequencing primer and the volume was made up to 20µl with Millipore water.

Sequencing primers to the M13 priming site supplied as a part of the TOPO-TA cloning system (Invitrogen) were used (M13 Forward 5´-GTAAAACGACGGCCAG-3´ and M13 Reverse 5´-CAGGAAACAGCTATGAC-3´). The cycle sequencing was carried out in a thermal cycler (Mastercycler® gradient, Eppendorf) and the following program was used

for the reaction. Initial denaturation: 96ºC for 2 min followed by 25 cycles of: denaturation: 96ºC for 10s; annealing: 50ºC for 10s; extension: 60ºC for 4min. After 25 cycles the reaction was rapidly cooled to 4ºC on ice.

2.6.10.2 Cleaning cycle sequencing reaction products

To remove the dye terminators, dNTPs and other low molecular weight materials from sequencing reactions, Performa CTR™ gel filtration cartridges (Edge Biosystems) were used following the manufacturer's protocol. The gel filtration cartridges were centrifuged for 3 min at 750 x g and were transferred to clean 1.5 ml centrifuge tubes. After adding the cycle sequencing reaction product to the column, the tubes were capped and centrifuged for 3min at 750 x g. Clean sequencing product is collected in the centrifuge tube, while all the unwanted materials were retained in the column.

2.6.10.3 Sequencing

Cleaned cycle sequencing products were re-suspended in 25µl of Hi-Di formamide (Applied Biosystems) and 20µl was added in to each well of a 96 well plate. The plate was then loaded in to ABI PRISM® 3100 genetic Analyzer (Applied Biosystems), a 16 capillary sequencing instrument to acquire the data. Sequence data was analysed using Chromas software version 2.2.3 (Technelysium Pty ltd, USA). Matrix gene sequence alignment and comparison with published sequences was performed using *Contig express* program of the Vector NTI Advance® 10 software (Invitrogen).

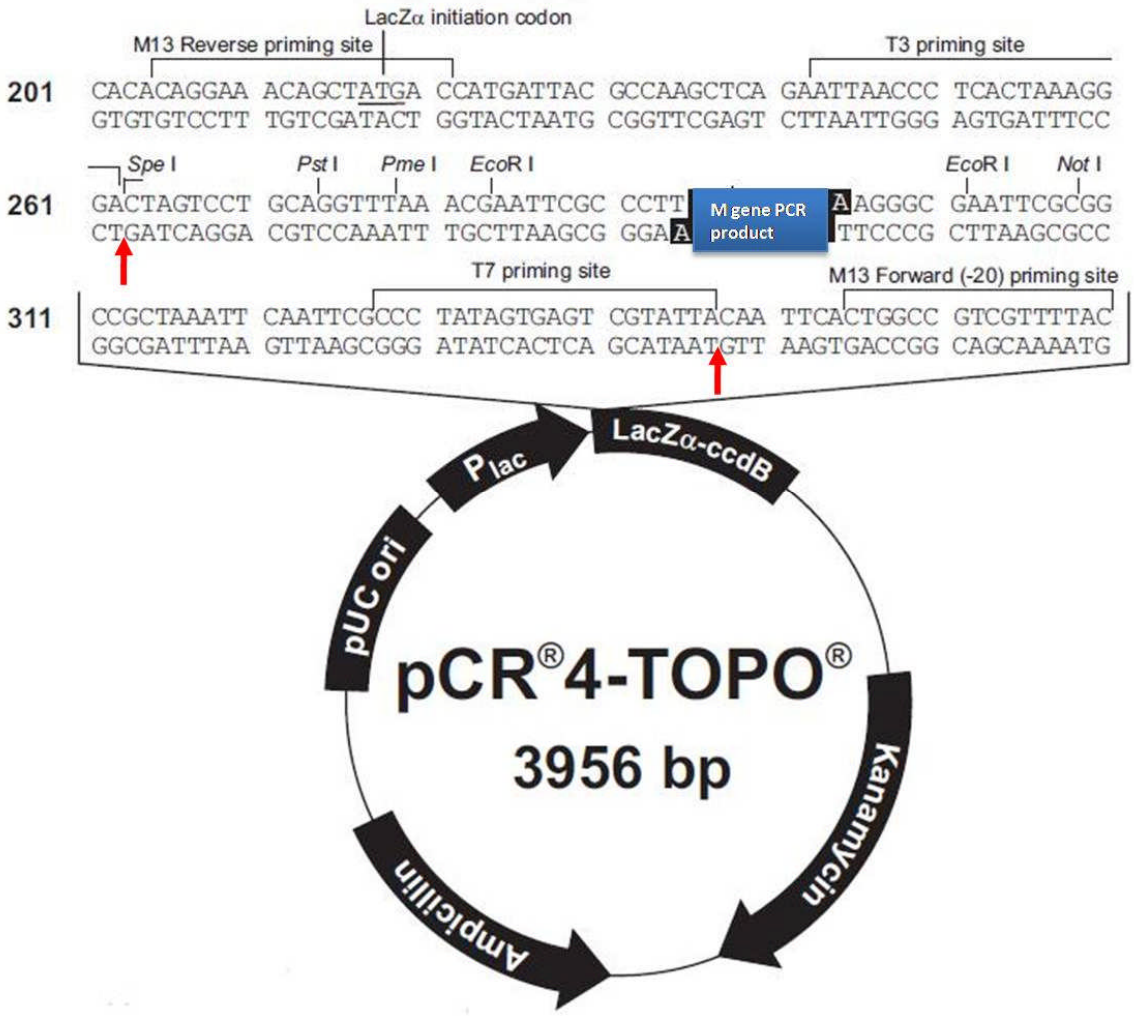


Figure 2.6-2 pCR 4-TOPO vector map showing priming and restriction cleavages sites

Map of the pCR 4-TOPO vector used for cloning and sequencing of a conserved region of influenza matrix gene is shown. After cloning, the vector was linearized with *SpeI*. *In-vitro* transcription starts at T7 priming site.

2.6.10.4 Restriction endonuclease digestion

The plasmid DNA was digested with the restriction enzyme *Spe*I to linearize for *in-vitro* transcription (Figure 2.6-2). Two micrograms of plasmid DNA was mixed with 5-10 units of *Spe*I, 5µl of enzyme buffer and incubated in water bath at 37ºC for 4 hrs.

2.6.10.5 Purification of the linearized plasmid

After digestion with *Spe*I, the reaction was run in an agarose gel as described in section 2.6.5. The agarose gel with appropriate band as determined by the size of the linearized plasmid was sliced with a scalpel blade. The plasmid DNA band in the gel was then purified using QIAquick Gel Extraction Kit® (Qiagen) following the manufacturer's protocol. Briefly the gel slice was weighed and 1–2 volumes of diffusion buffer to 1 volume of gel was added (i.e., 100–200µl for each 100 mg of gel) to a micro centrifuge tube and incubated at 50ºC for 30min.

Incubation was followed by centrifuging the sample at 10,000x g for 1 min. Supernatant was carefully removed with a pipette and was passed through a disposable plastic column to remove any residual agarose. After the volume of the supernatant was determined, three volumes of buffer QG was added to one volume of supernatant and mixed. The colour of the mixture turns to yellow. QIAquick Spin Column was placed in a 2 ml collection tube and the sample was applied to the spin column and centrifuged at 10,000x g for 30–60 s. After discarding the flow-through, the spin column was placed back into the same collection tube and 0.75 ml buffer PE was added and centrifuged for 30–60 s. After discarding the flow-through the spin column was placed back into the same collection tube and centrifuged for an additional 1 min at maximum speed. The spin column was transferred into a clean 1.5 ml micro centrifuge tube and 50µl Buffer EB (10 mM TrisHCl, pH8.5) or water was added to the centre of the column and centrifuged for 1 min to elute the DNA.

2.6.10.6 *In-vitro* transcription

Influenza virus M gene RNA was produced from linearized plasmid by in-vitro transcription using RiboMAX™ Large Scale RNA Production System—T7 (Promega) following manufacturer's instructions. To a 1.5ml micro centrifuge tube the following components are added and mixed gently by pipetting.

T7 Reaction Components	**Volume**
T7 Transcription 5X Buffer	20µl
rNTPs (25mM ATP, CTP, GTP, UTP)	30µl
Linear DNA template (10µg) plus Nuclease-Free Water	40µl
Enzyme Mix (T7)	10µl
Final volume	100µl

The tube was then incubated at 37°C for 2–4 hrs. For removing the DNA template from the *in-vitro* transcribed RNA, the reaction was treated with RNase-Free DNase (Qiagen) following manufacturer's instructions. Briefly, for every 1–2µg RNA, 2µl of 10x DNase buffer (500 mM TrisHCl, pH 8.0; 50 mM MgCl2; 10 mM DTT), 10 units RNase inhibitor, 0.5 Kunitz units of DNase I (RNase-free) was added and the volume was made up to 20µl with water. After incubating for 30 min at 37°C, 2µl of mM EGTA was added and incubated for 5 min at 65°C to inactivate the DNase.

2.6.10.7 Calculation of RNA molecules in the preparation

Concentration of in-vitro transcribed RNA was determined using nanodrop as described in section 2.6.2. Determination of number of RNA molecules per micro litre was calculated as follows. Transcript size = M gene product size is 101 nucleotides + 84 nucleotides of the vector (from T7 priming site to *Spe*I restriction site) =185 (Figure 2.6-2)

Molecular weight of ssRNA = (No of nucleotides x 320.5) + 159 =**A**

= 185 x 320.5 + 159 = 59451.5 (**A**)

Concentration of RNA obtained by nanodrop method = 680.05 ng/µl (**B**)

To convert nanograms to nanomoles = **B**/**A** =680.5/59451.5 = 0.011 nanomoles/µl

Divide by 10^9 to get moles/µl 0.011 /10^9 =1.1 x 10^{-11} moles/µl

Multiplied with Avogadro's number (6x10^{23}) to get molecules / µl

1.1 x 10^{-11} x (6x10^{23}) = 6.6 x 10^{12}molecules / µl

2.7 Extraction of total RNA from cells

Total RNA from cells was extracted using RNeasy Mini - QIAshredder Kit (Qiagen) following the manufacturer's instructions and the protocol is briefly described here. After rinsing the cells with PBS, 350µl of buffer RLT was added to each well to lyse the cells. The lysates were pipetted directly into QIAshredder spin column placed in a 2 ml collection tube, and centrifuged for 2 min at full speed. The homogenized lysate was then transferred to a gDNA eliminator spin column placed in a 2ml collection tube and

centrifuged for 30s at 8000x g. After adding 350µl of 70% ethanol to the flow through the contents were mixed well with pipetting. The contents were then transferred to an RNeasy spin column placed in a 2 ml collection tube. After closing the lid gently, the spin column-collection tube assembly was centrifuged for 15 s at 8000x g (~10,000 rpm). After discarding the flow through, 700µl buffer RW1 was added to the RNeasy spin column and centrifuged for 15 s at 8000x g. After discarding the flow through, 500µl buffer RPE was added to the RNeasy spin column and centrifuged for 15 s at 8000x g. After discarding the flow through, 500µl buffer RPE was added to the RNeasy spin column and centrifuged for 2 min at 8000x g. After discarding the flow through, the spin column was placed in a new collection tube and centrifuged at full speed for 1min. After discarding the collection tube, the spin column was placed in a 1.5ml collection tube. After adding 30-50µl RNase-free water to the centre of the spin column, the spin column was centrifuged at 8000x g for 1min to elute the RNA.

2.7.1 RNA quality control

Single most important factor affecting the efficiency of RNA amplification and subsequent hybridization is RNA quality. For good results RNA sample should be devoid of impurities such as proteins, DNA, other cellular materials, phenol, ethanol and other salts. An effective measurement of RNA purity is the ratio of absorbance readings at 260 and 280nm. Ideally the ratio of A_{260} to A_{280} should fall in the range of 1.7-2.1.

Another important component of RNA quality is the integrity of RNA sample which is the measure of proportion of full length RNA in the sample. A full length RNA will exhibit a ratio of 28S to 18S rRNA bands approaching 2:1 (Figure 2.7-1). Quality of the RNA sample can be assessed using Bioanalyzer which calculates the RNA integrity number (RIN) which is a metric developed by Agilent that includes information from both the rRNA bands and potential degradation products.

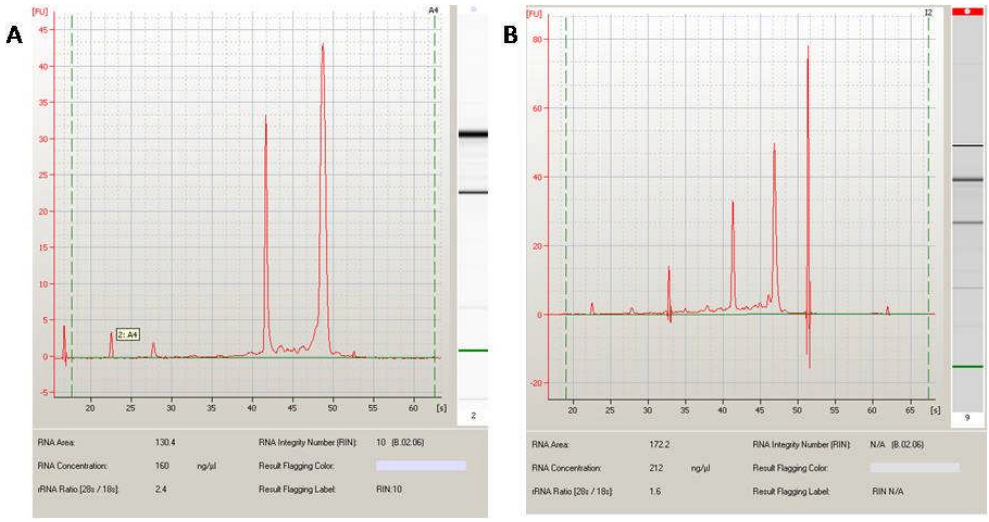


Figure 2.7-1 Analysis of RNA quality using Bioanalyzer

Electropherograms of RNA samples showing good quality RNA with an RIN number of 10.0 (A) with two distinct peaks representing 18S and 28S rRNA and a degraded RNA sample (B) with more than two peaks.

Extracted total RNA samples were analyzed for their suitability for further analysis using Agilent RNA 6000 nano kit (Agilent) following the manufacturer's instructions. Briefly, after equilibrating the reagents at RT for 30 min, the RNA 6000 Nano dye concentrate was vortexed for 10 seconds and spun down for 1 min at full speed. Gel-dye mix was prepared by adding 1µl of RNA 6000 Nano dye concentrate to 65µl of filtered gel. The tube was vortexed for proper mixing of the gel and centrifuged for 10 min at 13000x g at RT.

Prepared gel-dye mix was used within one day. After placing a new RNA Nano chip (Figure 2.7-2) on the chip priming station (Figure 2.7-2) 9µl of the gel-dye mix was pipetted in to the bottom of the well marked ●. Plunger of the syringe was then pressed down until it is held by the clip. After 30 s the plunger was released with the clip releasing mechanism. The plunger should then move back at least to the 0.3 ml mark.

After 5s the plunger is slowly pulled back to 1 ml position. After opening the chip priming station, 9µl of the gel-dye mix was pipette in to each of the wells marked ●. Five micro litres of RNA 6000 Nano marker was pipetted in to well marked ● (with the ladder symbol) and each of the 12 sample wells. One micro litre of the RNA ladder was pipetted into the well marked ● and 1µl of each sample in to each of the sample wells (1-12). The chip was placed horizontally in the adapter of IKA vortex mixer and vortexed for 60s at 2400 rpm. The chip was then inserted in to the Agilent 2100 Bioanalyzer (Agilent) to analyze the samples.

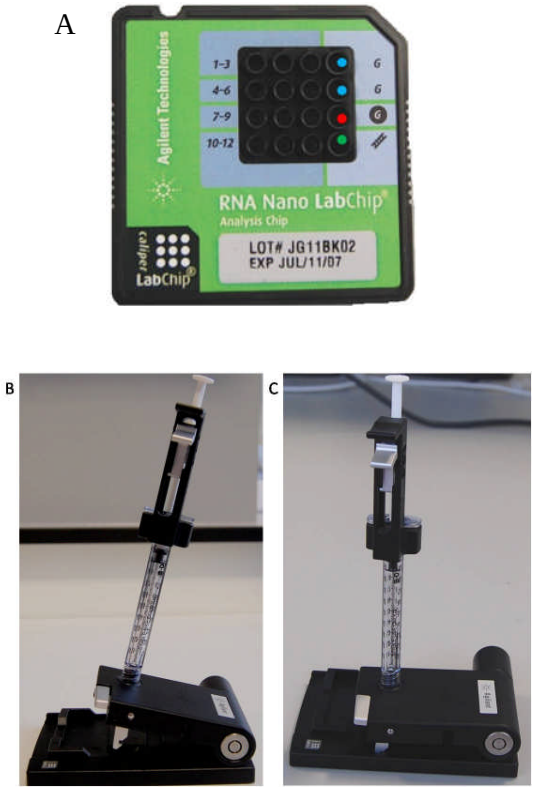


Figure 2.7-2 RNA Nano Lab Chip and chip priming station

Agilent RNA 6000 Nano Lab Chip used for analyzing the quality of total RNA sample is shown. RNA Nano Lab chip (A) and the chip priming station in open (B) and closed (C) positions.

Chapter 3

Influenza virus receptors in chicken and ducks

3.1 Summary

Influenza virus receptor distribution across several tissues of chicken and ducks was studied with lectin-histochemistry followed by confocal microscopy. Wide spread presence of avian influenza receptor SAα2,3-Gal and mammalian influenza receptor SAα2,6-Gal were detected in many tissues of both chickens and ducks. There was a marked difference in the primary receptor type in the trachea of chickens and ducks. In chicken trachea, SAα2,6-Gal was the dominant receptor type, whereas in ducks SAα2,3-Gal receptors were most abundant. This suggests that chickens could be more important as an intermediate host for the generation of influenza viruses with increased ability to bind to SAα2,6-Gal receptors and thus greater potential for infection of humans

3.2 Introduction

Virus receptors are defined as cell-surface molecules used by viruses to attach to target cells and initiate infection. The first step in influenza virus replication is the attachment to host cell receptors and influenza virus infection has been shown to initiate by binding of the virus to sialic acid-containing cell-surface receptors (Gambaryan et al. 1997). A prerequisite for influenza virus infectivity is cleavage of haemagglutinin into HA1 and HA2 and cleavage in the absence of trypsin in tissue culture system has been correlated with virulence (reviewed by Kawaoka & Webster 1988).

A major determinant of the ability of influenza viruses to infect cells is the expression of the appropriate host cell receptor to which viral haemagglutinin can bind. Consequently, a crucial hurdle that influenza A virus needs to overcome when crossing a species barrier is the acquisition of the ability to utilize alternate host cell receptors (Ito 2000).

3.2.1 Receptor tropism of influenza viruses

The study of influenza virus receptors started in 1941, when Hirst found that influenza viruses can agglutinate chicken erythrocytes at 4°C and the agglutinated cells were eluted when the cells were heated to 37°C. The washed cells once eluted from the virus were never agglutinated by the newly added influenza virus (Hirst 1941). This phenomenon introduced the concept that the specific receptor for the influenza virus is present in the erythrocyte membrane which binds to the viral haemagglutinin at 4°C and also indicated that the virus has an enzyme which can destroy the receptor on the host cell surface at 37°C. Later the receptor-destroying enzyme was identified as sialidase (Gottschalk 1957) which is the first evidence suggesting that the cell recognition by influenza viruses is mediated by sialic acid containing sugar chains.

Sialic acids are a family of 9 carbon acid sugars that are abundant at the outer cell membranes and in biological fluids of animal. They occupy terminal positions on oligosaccharide chains of glycoproteins and glycolipids. A variety of sialic acid-containing glycoproteins and glycolipids were thought to support binding of influenza viruses but the exact molecular species serving as receptors for human and avian influenza viruses on their target cells remain undefined (Herrler, Gross, & Brossmer 1995; Suzuki 1994).

Later, resolution of the three-dimensional structure of the haemagglutinin of influenza virus complexed with the receptor analogue sialyllactose by X-ray analysis, N-acetylneuraminic acid residue (Neu5Ac) in the receptor-binding site (RBS) was detected. (Sauter et al. 1992; Weis et al. 1988). This suggested that Neu5Ac is the major component of influenza receptor on cells. Several studies have determined the receptor tropism of a wide range of influenza viruses. Earlier methods were based on generation of specific receptor determinants using sialylytransferases. Human erythrocytes enzymatically modified to contain cell surface sialyloligosaccharides were used to determine the receptor specificity of influenza viruses (Rogers & Paulson 1983). Later

chicken asialoerthrocytes integrated with exogenous gangliosides GM3 (Sia alpha 2-3Gal beta 1-4Glc beta 1-ceramide) and GM2 (GalNAc beta 1-4(Sia alpha 2-3)-Gal beta 1-4Glc beta 1-ceramide) were used (Suzuki, Matsunaga, & Matsumoto 1985).

Ganglioside-coated erythrocyte/virus-binding assay used a bacterial neuraminidase to remove sialic acids from chicken erythrocytes. The determination of influenza virus receptor specificity was based on recovery of virus-specific haemagglutination, fusion and haemolysis in low pH media (Suzuki et al. 1985). Another method used was measuring binding affinity of interaction between influenza virus haemagglutinin with sialosides, sialylglycopolymers and sialylglycoproteins. Assays using monovalent sialosides were based on the ability of these structurally defined monovalent glycosides to block the virus mediated haemagglutination (Kelm et al. 1992;Pritchett et al. 1987) or to inhibit virus binding to peroxidise labelled fetuin in a solid phase assay (Matrosovich et al. 1993). These techniques required large amounts of expensive sialosides because of low binding affinity and could not account for the polyvalency of virus-receptor interactions.

Measurement of binding of polyvalent sialylglycopolymers to influenza virus either by fetuin binding inhibition assay (Gambaryan et al.1997) or by streptavidin- peroxidise (Matrosovich et al. 2000) overcame the drawbacks of using monovalent sialosides. Avian influenza viruses have been shown to preferentially bind to SA receptors that are linked to galactose by a α2,3 linkage (SAα2,3-Gal), while human and classical swine viruses show preference for receptors with a α2,6 linkage (SAα2,6-Gal) (Matrosovich et al. 2004)(Figure 3.2-2).

More recently receptor-binding specificity of avian influenza viruses was characterized by testing their binding to 17 distinct oligosaccharides conjugated with polyacrylamide. This polyacrylamide contained terminal Neu5Aca2-3Gal moiety but have a different composition of more distant (inner) part of the carbohydrate chain. This study indicated that avian influenza viruses are adapted to preferential recognition of sialosaccharides,

with a 1–3 bond between galactose and the penultimate carbohydrate residue (Gambaryan et al. 2005). Among avian influenza viruses, chicken and duck viruses have been shown to further differ in their ability to recognize the structure of the third sugar moiety in SAα2,3-Gal terminated receptors. Influenza viruses from chickens preferentially bound to synthetic sialylglycopolymer containing Neu5Ac-α(2-3)Gal-β(1-4)GlcNAc, whereas viruses from ducks displayed a higher affinity for Neu5Ac-α(2-3)Gal-β(1-3)GalNAc containing polymer(Figure 3.2-1)(Gambaryan et al. 2003). More recent study used binding of fluorescently labeled whole virions to a glycan array of 264 oligosaccharides to determine the receptor specificities influenza viruses (Kumari et al. 2007).

3.2.2 Determination of influenza receptors on host cells

Influenza virus receptors on host cells are glycosylated oligosaccharides that terminate in sialic acid (SA) residues which are bound to glycans through an α2,3 or α2,6 linkage, mediated by sialyltransferases that are expressed in a cell- and species- specific manner (Gagneux et al. 2003).

3.2.2.1 Virus binding assays

Using fluorescent labelled influenza viruses with SAα 2,6Gal and SAα 2,3Gal linkage specificities, it was found that ciliated cells of the human respiratory tract expressed SA α 2,6Gal receptor types whereas mucin-producing cells expressed SAα 2,3Gal receptor type (Couceiro, Paulson, & Baum 1993). Influenza virus receptor distribution pattern in chicken and ducks was studied using extracted total gangliosides from plasma membranes of respiratory and intestinal epithelial cells by virus binding assay. The results demonstrated presence of SAα2-6Gal moieties in chicken epithelial tissues but no presence in duck epithelial tissues (Gambaryan, Webster, & Matrosovich 2002).

3.2.2.2 Lectin binding assays

A lectin isolated from elderberry *Sambucus nigra* bark named Sambucus nigra agglutinin (SNA) was found to be having specific binding affinity to SAα2-6Gal sequence by quantitative precipitation, hapten inhibition, and equilibrium dialysis (Shibuya et al., 1987). Later a lectin from the seeds of the leguminous plant *Maackia amurensis*, named Mackia amurensis agglutinin (MAA) was found to be a potent leukoagglutinin for the mouse lymphoma cell line and a weak haemagglutinin of human erythrocytes. Based on the affinity to purified glycoproteins, MAA was found to be interacting with sialic acid residues linked by an α 2,3 to penultimate galactose (SAα2-3Gal) residues (Wang & Cummings 1988). The two isoforms of MAA, MAAI and MAAII have been shown to differ in their lectin binding specificity. While MAA I show preferential binding to SAα2,3-Galβ(1-4)GlcNAc, MAA II binds specifically to SAα2,3-Galβ(1-3)GalNAc receptor types (reviewed by Nicholls et al 2007).

Linkage specific lectins have been used to determine the distribution of influenza virus specific receptors on host tissues. Study of extracted cell membranes and gangliosides from epithelial tissues of ducks and chickens using lectin binding, found abundant presence of SAα2-3Gal binding in both cell types and SAα2-6Gal Gal binding in chicken cells but not in duck cells (Gambaryan et al 2003). Intact tissue sections of human trachea (Couceiro et al., 1993), chicken embryonic tissues (Feldmann et al. 2000), Japanese quail tissues, chicken trachea, chicken duodenum, pig trachea and duck colon (Wan and Perez, 2006) have been studied for influenza virus receptor distribution pattern by lectin binding followed by fluorescent microscopy. Confocal microscopy on lectin stained tissue sections was used to gain a much better resolution to determine the differences in spatial distribution of influenza receptors (Shinya et al 2006). Fluorescence-activated cell-sorting analysis of lectin stained primary cultured cells was another method used to determine receptor distribution of a culture of normal human bronchial epithelial (NHBE) cells (Kogure et al. 2006).

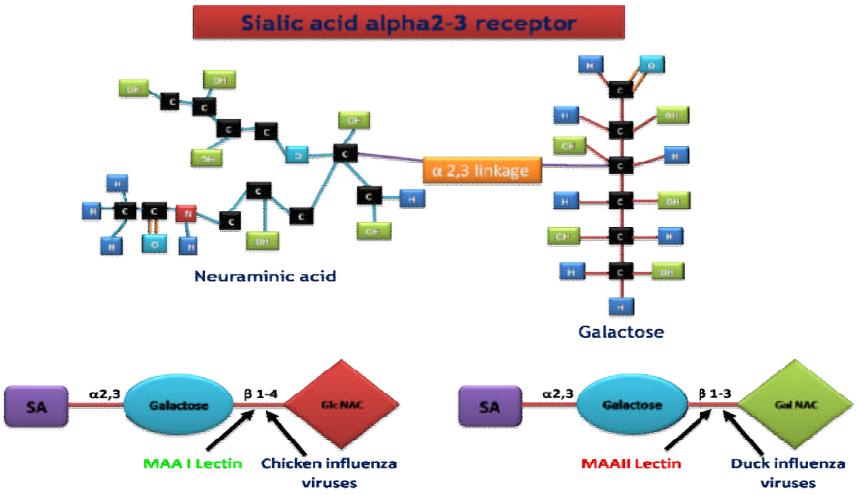


Figure 3.2-1 Overview of receptor predilections of Avian influenza viruses and linkage specific lectins

Mackia amurensis agglutinin I (MAAI) lectin and chicken influenza viruses show preferential binding to SAα2,3 Gal -β(1-4)GlcNAc receptor type, where as MAAII lectin and duck influenza viruses show preferential binding to SAα2,3 Gal -β(1-3)GalNAc receptor type.

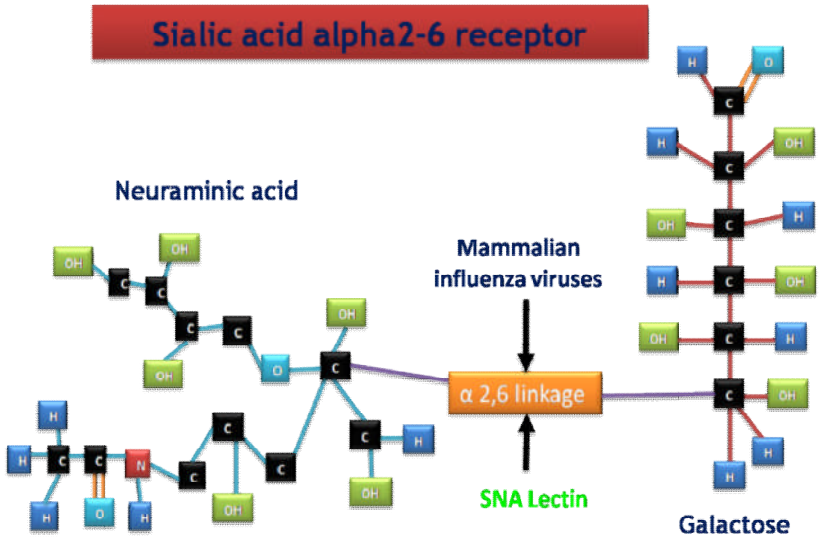


Figure 3.2-2 Overview of receptor predilections of mammalian influenza viruses and linkage specific lectins

Mammalian influenza viruses and *Sambucus nigra* agglutinin (SNA) lectin show preferential binding to SAα2,6 Gal receptor.

3.2.3 Influenza virus receptor distribution in chicken and ducks

Ducks and chickens are the major aquatic and terrestrial hosts for a wide variety of influenza viruses and are valuable natural models of resistance and susceptibility to study influenza. Ducks display resistance to disease by many strains of avian influenza viruses including highly pathogenic strains like H5N1 which cause a very high mortality in chickens. Receptors are important determinants of virus entry and differences in receptor distribution between hosts could therefore account for variation in susceptibility to infection, including the contrasting outcomes following infection with many strains of highly pathogenic avian influenza (HPAI). However, the receptor distribution across intact mucosal surfaces and organ systems was not studied.

More recently, chicken trachea and duodenum have been studied using lectin binding assays followed by conventional immuno-histochemistry and light microscopy (Wan & Perez 2006). Despite this, detailed information on the distribution of influenza virus receptors in these important avian hosts is lacking. To further evaluate the potential role of receptor distribution in ducks and chickens in determining susceptibility to infection, and the potential of these species to support infection of viruses with tropism for SAα2,6-Gal and SAα2-3-Gal (and therefore act as “mixing vessels”) the detailed anatomical distribution of influenza A receptors in key organs of both species has been studied.

3.3 Materials and methods

3.3.1 Animal tissues

Animals for this study comprised four 35-40 wk old, commercial layer chickens (Glenrath Farms Ltd, East Lothian, UK), four 4 wk old broilers (PD Hook Hatcheries, Bampton, Oxfordshire, UK), two 3 wk old and four 6 wk old Pekin ducks (Cherry Valley Farms, Rothwell, Lincolnshire, UK). The animals were euthanized and samples from

trachea, lungs, heart, kidney, brain, skeletal muscle, small and large intestine collected into buffered neutral formalin. Formalin fixed tissue samples were dehydrated and cleared using a histokinette (Leica TP 1020) before being embedded in paraffin wax. After embedding, the tissues were sectioned using a rotary microtome (Leica RM 2255) with a specimen feed of 5µm.

3.3.2 Lectin staining

Lectin histochemistry using linkage specific lectins was performed on normal tissue sections from various organs of chickens and ducks using a method described previously with minor modifications (Shinya et al.2006). Lectins used in the study were: *Sambucus nigra* agglutinin (SNA) specific for SAα2,6-Gal (Figure 3.2-2) (Shibuya et al. 1987), *Maackia amurensis* I (MAA I) and *Maackia amurensis* agglutinins (MAA II) which are specific for SAα2,3-Galβ(1-4)GlcNAc and SAα2,3-Galβ(1-3)GalNAc respectively (Figure 3.2-1) (Konami et al. 1994) (all provided by Vector Laboratories, Burlingame, CA). Sections were pre-soaked in Tris buffered saline (TBS) and blocked using a biotin-streptavidin blocking kit (Vector Laboratories) according to the manufacturer's instructions, and washed 3 times in TBS.

Sections were then outlined with a hydrophobic barrier ImmEdge Pen (Vector Laboratories, Burlingame, CA). Sections were then incubated with fluorescein isothiocyanate (FITC) labelled SNA or FITC labelled MAA I, and biotinylated MAA II lectin, each of these at a concentration of 10µg/ml overnight at 4°C. After three washes with TBS, the sections were incubated with 1:500 dilution of streptavidin-AlexaFluor594 conjugate (Molecular Probes, Inc., Eugene, OR) in TBS for 2 hrs at room temperature (RT). The sections were washed three times with TBS and then mounted with ProLong Gold antifade reagent with 4', 6-diamino-2- phenylindole, dihydrochloride (DAPI; Molecular Probes, Inc., Eugene, OR). Negative controls were performed without the primary reagents.

3.3.2.1 Sialidase treatment of sections

Serial sections of chicken trachea and duck trachea were subjected to lectin staining with and without sialidase treatment to rule out nonspecific binding of the lectins. Tissue sections were treated, prior to lectin staining, with Sialidase A (N-acetylneuraminate glycohydrolase; Prozyme, San Leandro, CA), which cleaves all non-reducing terminal sialic acid residues in the order α(2,6)> α(2,3)> α(2,8)> α(2,9). Deparaffinized sections were outlined with an ImmEdge Pen and incubated with 3ul of Sialidase A in 6ul of 5x reaction buffer (250 m*M* sodium phosphate, pH6.0) and 150µl TBS at RT overnight. The sections were then washed three times with TBS and subjected to lectin staining as described in the section 3.3.2.

3.3.2.2 Confocal microscopy

The sections after lectin staining were imaged using confocal microscope (Leica TCS SP2 AOBS). Appropriate section thickness was adjusted to avoid any fluorescence from the background. Overlay images were captured by sequential scanning between frames for DAPI, FITC and Alexa Fluor with a line average of 4 (Figure 3.3-1).

3.3.2.3 Measurement of mean fluorescent energies

Overlay images captured using a confocal microscope will have both FITC and Alex Fluor channels merged and the resulting image show a combination of green and red colours. As tissues are not homogenous in their composition of glycoproteins, staining is quite different between different parts of the same tissue section. For objective assessment of level of binding of each lectin, we have quantified differences in relative expression of each fluorochrome using LCS Lite software. Images in maximum projection mode were analyzed and the mean energy values were measured for each fluorochrome in a representative area and corrected by subtracting background energy

values (Figure 3.3-2). Corrected mean energy values of FITC and Alexa Fluor were expressed as a ratio. This ratio was used as a measure of the relative expression of SAα2,6-Gal and SAα2,3-Gal receptor types in various tissues.

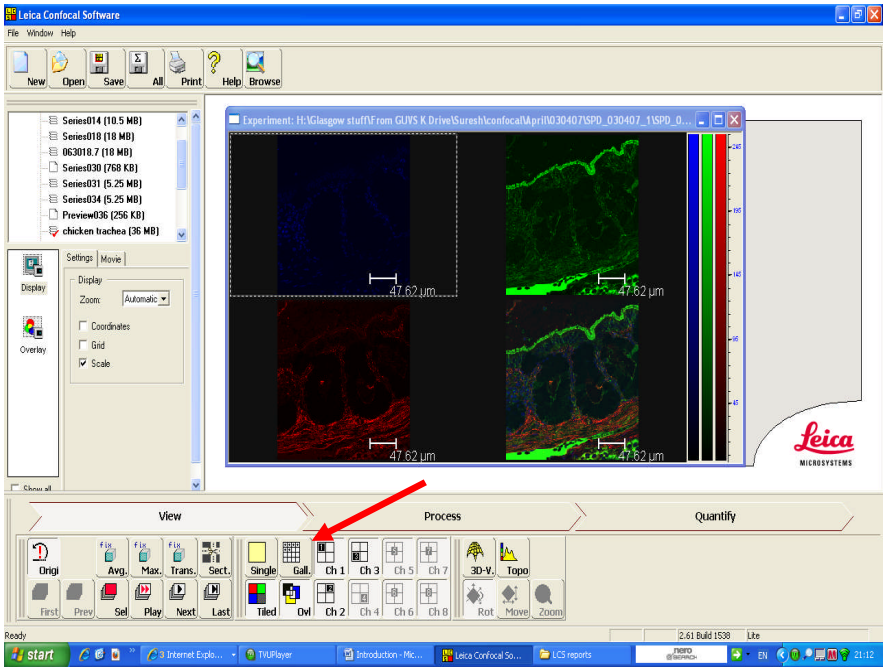


Figure 3.3-1 Overlay image acquisition using a confocal microscope

Sections are scanned for DAPI, FITC and AlexaFluor fluorochromes separately and an overlay of all the 3 channels can be obtained by using the tools under the view tab of the software.

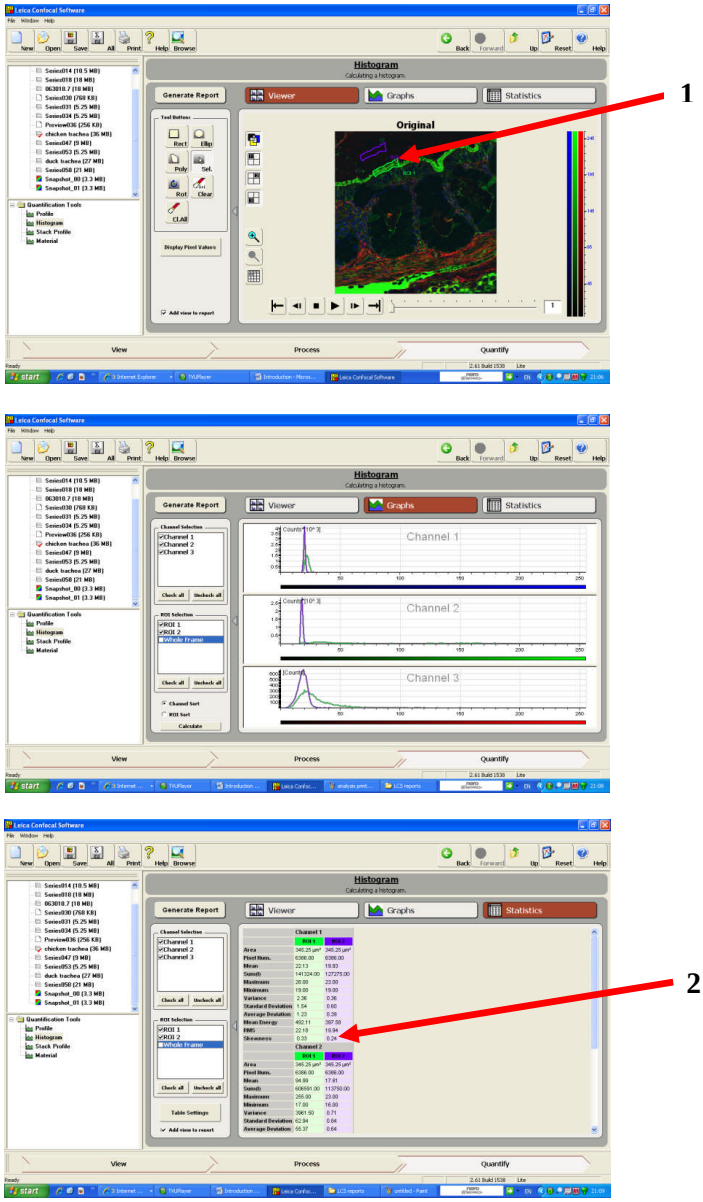


Figure 3.3-2 Calculation of fluorescent mean energy values

Fluorescence of each channel can be measured by outlining a region of interest (ROI1) in an overlay image (1). Another region of same size (ROI2) is also outlined for background correction. Mean energy values (2) for each channel can be obtained in the statistics view.

3.3.3 Virus binding assays

We have performed virus binding assays to study the functional significance of the lectin binding assays using a classical swine and avian influenza virus strains with preferential binding ability to SAα2,6-Gal and SAα2,3-Gal receptor types. Virus binding assays with H1N1 classical swine strain (A/Sw/Iowa/15/30), a subtype closely related to the human 1918 pandemic influenza virus (Tumpey et al. 2004), and a H2N3 low pathogenic avian strain (A/mallard duck/England/7277/06) were performed by a previously reported method, with minor modifications (Couceiro et al. 1993).

Briefly, paraffin embedded 5µm sections of chicken and duck tracheae, small and large intestines were deparaffinised in xylene and rehydrated by alcohol. Sections were incubated with avian or swine influenza virus for two hours at RT. After removing excess fluid, the sections were rinsed with TBST (0.2% Triton x100 in TBS). The sections were then blocked with goat serum diluted in TBST buffer for 30 min at RT. After removing excess fluid the sections were incubated with mouse monoclonal antibody to influenza nucleoprotein (ab20343, Abcam, Cambridge, UK) at 1:1000 dilution in TBST for one hour at RT followed by FITC-labelled goat anti-mouse IgG (ab7064, Abcam, Cambridge, UK) at 1:500 dilution for two hours at RT.

After three further washes with TBST, the sections were mounted with ProLong Gold antifade reagent with DAPI. Negative controls were performed by omitting the initial incubation with virus or primary antibody. The sections were scanned by confocal microscopy (Leica TCS SP2 AOBS) and overlay images with FITC and DAPI channels were obtained as described in section 3.3.2.2.

3.4 Results

3.4.1 Lectin histochemistry

Extensive examination of influenza virus receptor distribution in a range of tissues from chickens and ducks has been performed. No difference in the reported results was observed due to the age or source of animals, and the receptor distribution was consistent between individual animals within each species. Widespread presence of SAα2,6-Gal and SAα2,3-Gal receptors in a range of tissues from each species was observed. The spatial distribution of SAα2,6-Gal and SAα2,3-Gal receptors is different within the organs of the two species.

3.4.1.1 Respiratory system

The relative receptor distribution across the respiratory tract was very different between chicken and duck (Figure 3.4-1). In the upper respiratory tract, ciliated epithelial cells of chicken trachea expressed SAα2,6-Gal receptor predominantly with a low SAα2,3-Gal receptor expression. In contrast, SAα2,3-Gal receptor type was predominant on the ciliated epithelial cells of duck trachea with a low SAα2,6-Gal receptor type expression. Consistent results were observed from tracheae of ages, and sources of broiler and layer chicken (data not shown). Similarly receptor distribution pattern was consistent in trachea of older ducks (data not shown). Intra epithelial mucous glands of chicken and duck both expressed predominantly SAα2,6-Gal receptor type. Receptor distribution pattern of the tracheal sub mucosa was similar between chicken and duck with the expression of both SAα2,3-Gal and SAα2,6-Gal receptor types. In the case of chicken, the amount of SAα2,3-Gal receptor gradually increased further down the airway in bronchi and bronchioles (Figure 3.4-2). Bronchiolar membranes expressed approximately equal amounts of SAα2,6-Gal and SAα2,3-Gal receptor while SAα2,6-Gal receptor was

predominant on alveolar membranes. In the case of duck, the receptor expression pattern in bronchi and bronchioles was very similar to trachea, while SAα2,6-Gal receptor type was more predominant on alveolar membranes.

3.4.1.2 Digestive system

The receptor distribution pattern is very similar between chicken and duck intestines with a predominant expression of the SAα2,3-Gal receptor type across the epithelial lining of villi with no significant expression of SAα2,6-Gal receptor type. Both small (Figure 3.4-3) and large intestines (Figure 3.4-4) of chicken and duck expressed predominantly SAα2,3-Gal receptor across the epithelial lining of the villi, while SAα2,6-Gal receptor expression was found in the goblet cells and sub-mucosa. No difference in influenza receptor distribution as assessed by lectin staining was observed in different parts of the intestines of both chicken and duck.

3.4.1.3 Kidneys

In chicken kidneys, vascular endothelium expressed SAα2,6-Gal receptor type and proximal and distal convoluted tubular cells expressed SAα2,6-Gal and SAα2,3-Gal receptor types respectively with no co-expression. In contrast, in the vascular endothelium, proximal and distal convoluted tubular cells of ducks, co-expression of both SAα2,3-Gal and SAα2,6-Gal receptor types was observed (Figure 3.4-5).

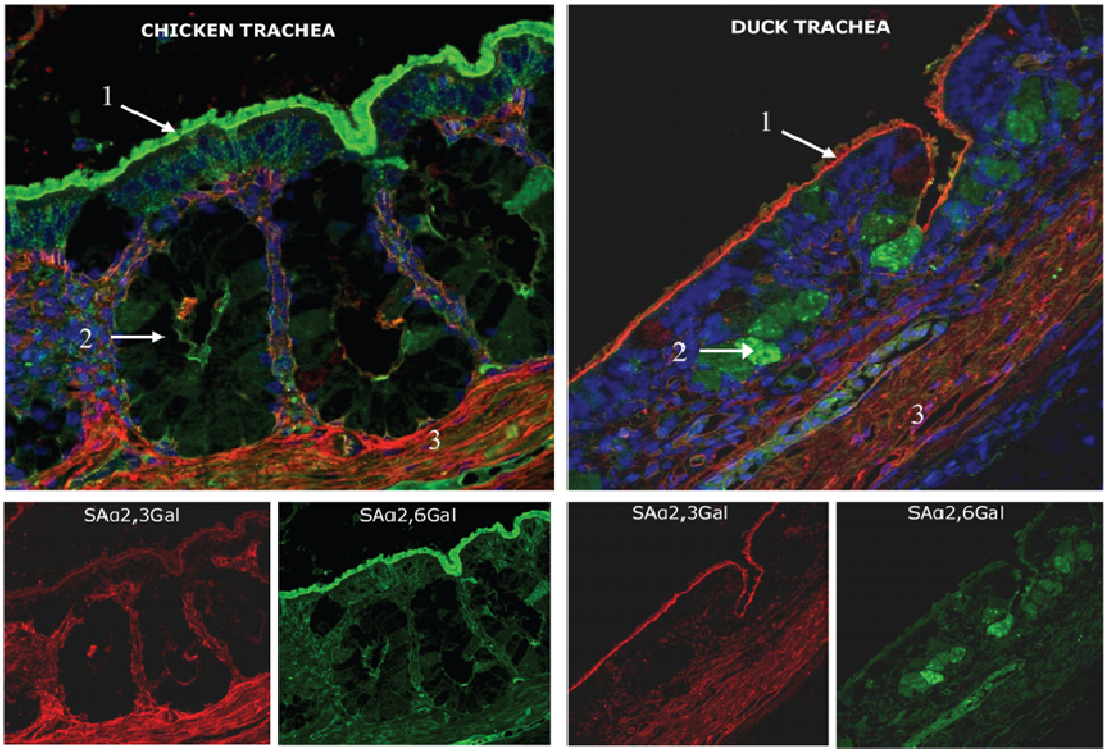


Figure 3.4-1 Influenza receptor distribution pattern in chicken and duck trachea

Composite confocal image shows abundant SAα2,6 Gal receptor (in green) type chicken tracheal epithelium whereas SAα2,3Gal receptor (in red) is abundant in duck tracheal epithelium.

1. Epithelial cells (ciliated),

2. Intra-epithelial mucous gland,

3. Sub-mucosa.

Figures are representative of a number of tissue specimens screened.

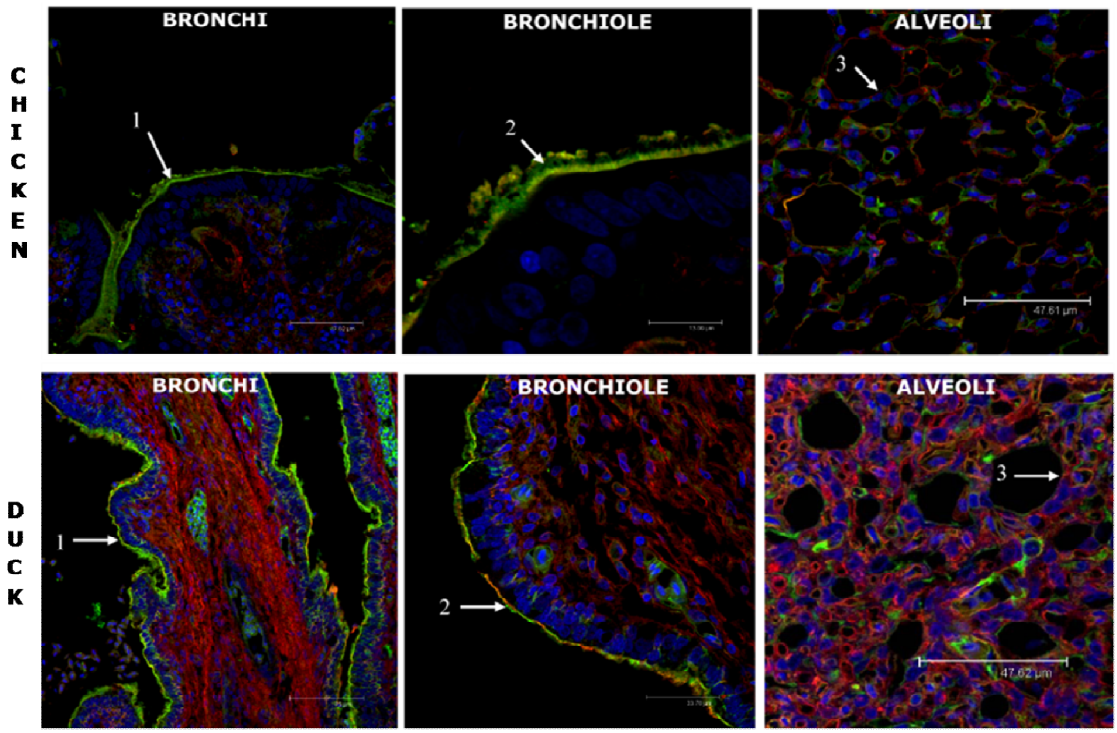


Figure 3.4-2 Influenza receptor distribution pattern in the lower respiratory tract of chicken and duck

Composite confocal image showing approximately equal amounts of SAα2,6-Gal (green) and SAα2,3-Gal (red) receptor types across bronchial and bronchiolar membranes and abundant SAα2,6-Gal receptor type on alveolar membranes in both chicken and ducks.

1. Bronchial epithelium,

2. Bronchiolar epithelium,

3. Alveolar lining

Figures are representative of a number of tissue specimens screened.

3.4.1.4 Other organs

Cardiac muscle, endocardium (Figure 3.4-6), meninges of brain, skeletal muscle fibres (Figure 3.4-7) of both chicken and duck have co-expressed both SAα2,3-Gal and SAα2,6-Gal receptor types with a relatively higher amount of SAα2,6-Gal receptor type in all these organs as measured by the mean fluorescent energy values.

3.4.1.5 Sialidase treatment of sections

Lectin staining on sialidase treated sections has been performed as a control to confirm that the lectins used in the present study did not bind to non sialic acid residues. Sialidase treatment of sections removed all sialic acids as evidenced by lack of lectin binding in the sialidase treated sections. Abundant SNA binding was found in untreated chicken tracheal sections. However, no SNA binding was observed after sialidase treatment. Similarly the abundant MAAII staining pattern in duck tracheal sections was absent in sialidase treated sections (Figure 3.4-8).

3.4.1.6 Measurement of fluorescent mean energies

Relative expression of SAα2,3-Gal and SAα2,6-Gal receptors in tissues of chicken and duck was quantified by measuring the mean energy values of each fluorochrome in overlay images using LCS Lite software and expressed as percentage of the total. The mean energy values for FITC and Alex Fluor which represent binding of SNA and MAAII were used as a measure of SAα2,6-Gal and SAα2,3-Gal receptor type expression in both chicken and duck tissues. Analysis of mean energy values provided a more objective assessment of relative expression of the receptors in all the tissues than was possible observing the overlay images. The ratio between FITC and Alex Fluor determines the predominant colour in the overlay image. When the ratio of FITC to Alex Fluor was more than 5:1 as found in chicken trachea, chicken and duck bronchi, the

overlay image appeared green. When the ratio of FITC to Alex Fluor was more than 1:5 as found in duck trachea and chicken and duck intestines, the overlay image appeared red. When the ratio was less than 1:5 as in chicken and duck endocardium and duck kidney endothelium, overlay images appeared in shades of orange which is a combination of red and green.

Chicken trachea, bronchi and alveoli expressed SAα2,6-Gal receptor type abundantly, while, the bronchioles expressed equal amounts of SAα2,3-Gal and SAα2,6-Gal receptor types (Figure 3.4-9). Chicken cardiac muscle, endocardium, meninges expressed both types of receptors. However, SAα2,6-Gal receptor type was found to be abundant compared to SAα2,3-Gal based on the mean energies values (Figure 3.4-9). In Duck trachea abundant SAα2,3-Gal receptor type expression was observed and SAα2,6-Gal was more abundant in bronchi and bronchioles.

The ratio between SAα2,6-Gal : SAα2,3-Gal in duck trachea was 1:20 where as the ratios in bronchi and bronchioles were 30:1 and 60:1 respectively (Figure 3.4-10). Although expression of both receptor types was noticed in alveoli, endocardium, cardiac muscle and meninges of duck, SAα2,6-Gal receptor type was found to be more abundant in these organs based on the mean energy values.

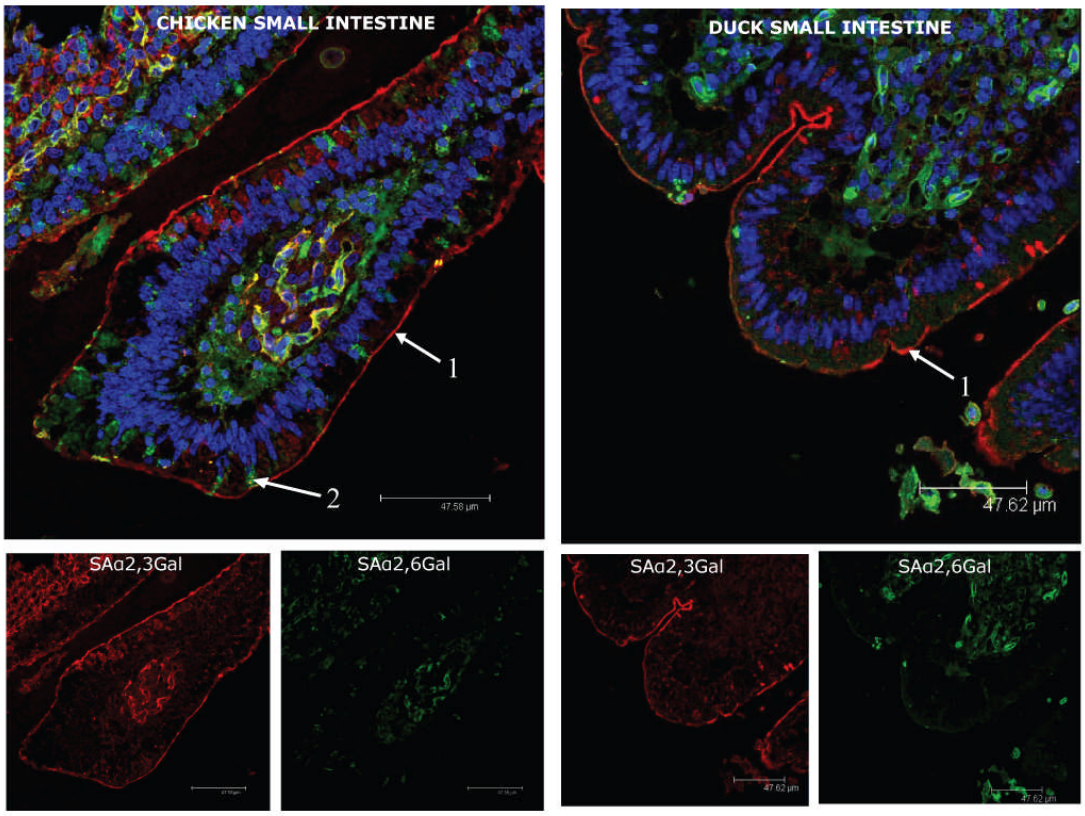


Figure 3.4-3 Influenza receptor distribution in small intestines of chicken and duck

Composite confocal image showing predominant SAα2,3-Gal receptor type expression in chicken and duck small intestines .

1. Epithelial lining of the villus,

2. Goblet cell

Figures are representative of a number of tissue specimens screened

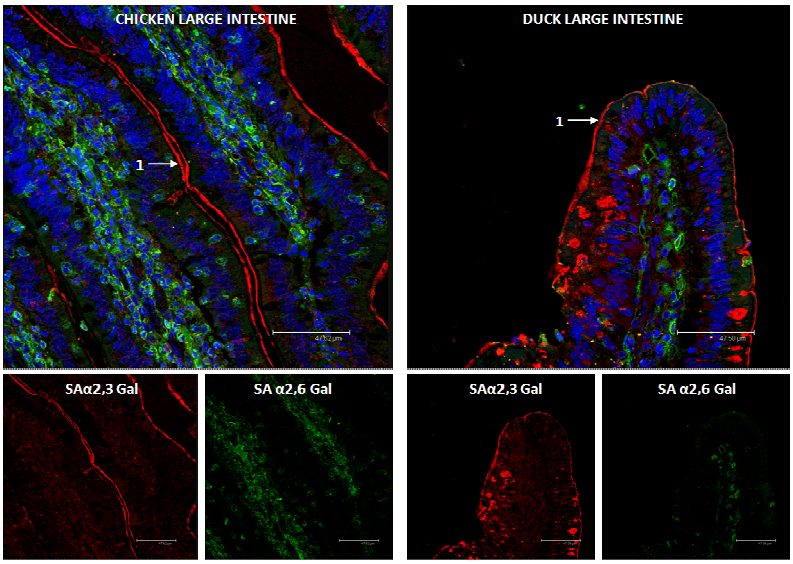


Figure 3.4-4Influenza receptor distribution in large intestines of chicken and duck

Composite confocal image showing predominant SAα2,3-Gal receptor type expression in chicken and duck large intestines . 1. Epithelial lining of the villus**.** Figures are representative of a number of tissue specimens screened.

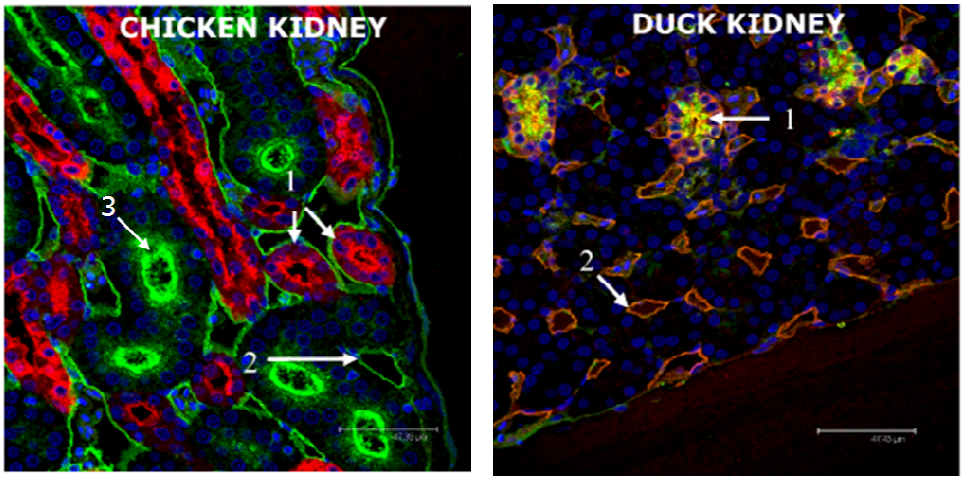


Figure 3.4-5 Influenza receptor distribution in kidneys of chicken and duck

Composite confocal images showing co-expression of both SAα2,3-Gal (red) and SAα2,6-Gal(green) receptor types in vascular endothelium and convoluted tubular cells of duck kidney. Vascular endothelium and proximal convoluted tubular cells of chicken kidneys expressed SAα2,6-Gal while the distal convoluted tubular cells expressed SAα2,3-Gal receptor type. 1. Distal convoluted tubule 2. vascular endothelium 3. Proximal convoluted tubule. Figures are representative of a number of tissue specimens screened.

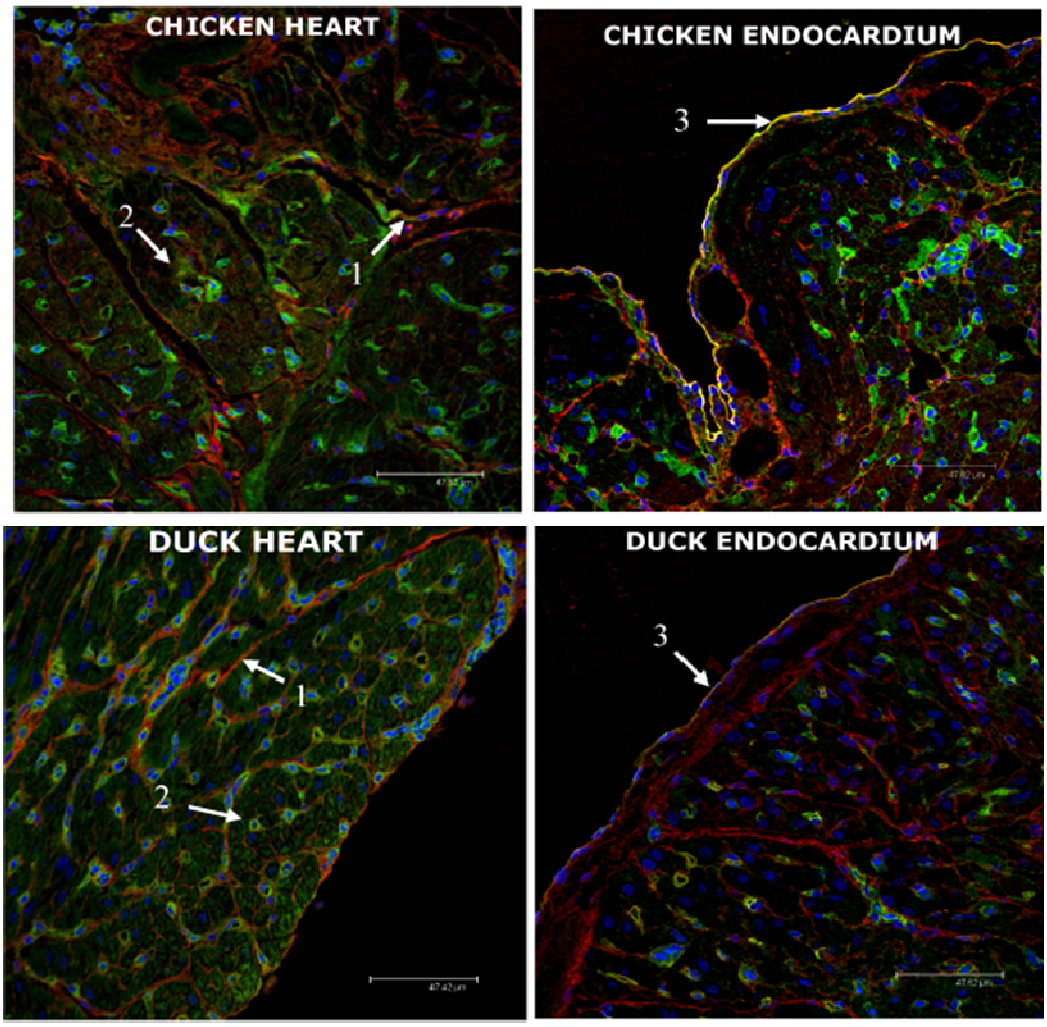


Figure 3.4-6 Influenza receptor distribution pattern in hearts of chicken and duck

Composite confocal image showing expression of both SAα2,6-Gal (green) and SAα2,3-Gal (red) receptor type expression in cardiac muscle and endocardium of chicken and duck . 1. myocardium, 2. connective tissue, 3. Endocardium. Figures are representative of a number of tissue specimens screened.

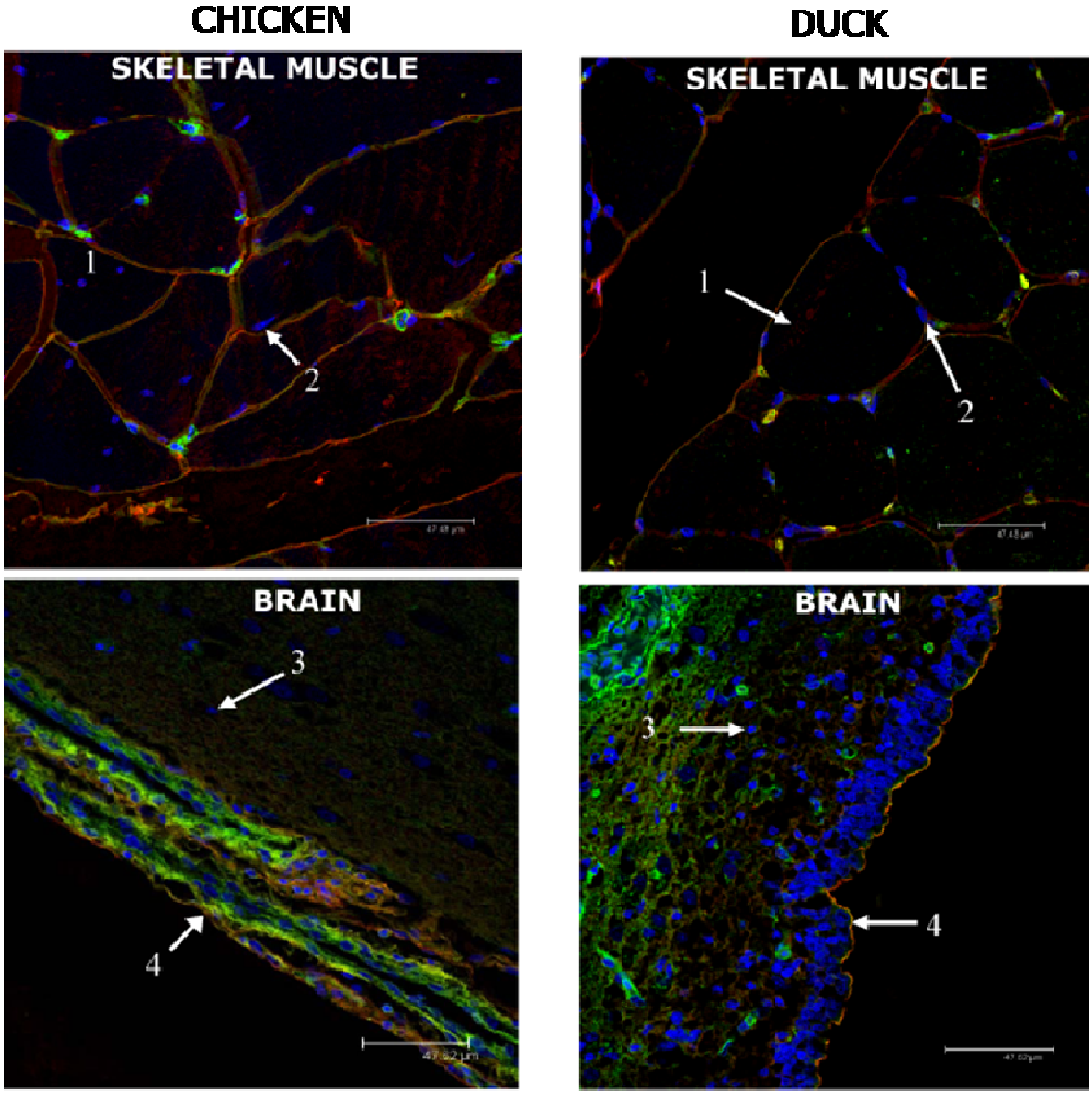


Figure 3.4-7 Influenza receptor distribution in skeletal muscle and brains of chicken and duck

. Composite confocal image showing expression of both SAα2,6-Gal (green) and SAα2,3-Gal (red) receptor type expression in skeletal muscle fibres and meninges of brain of chicken and duck. Figures are representative of a number of tissue specimens screened.

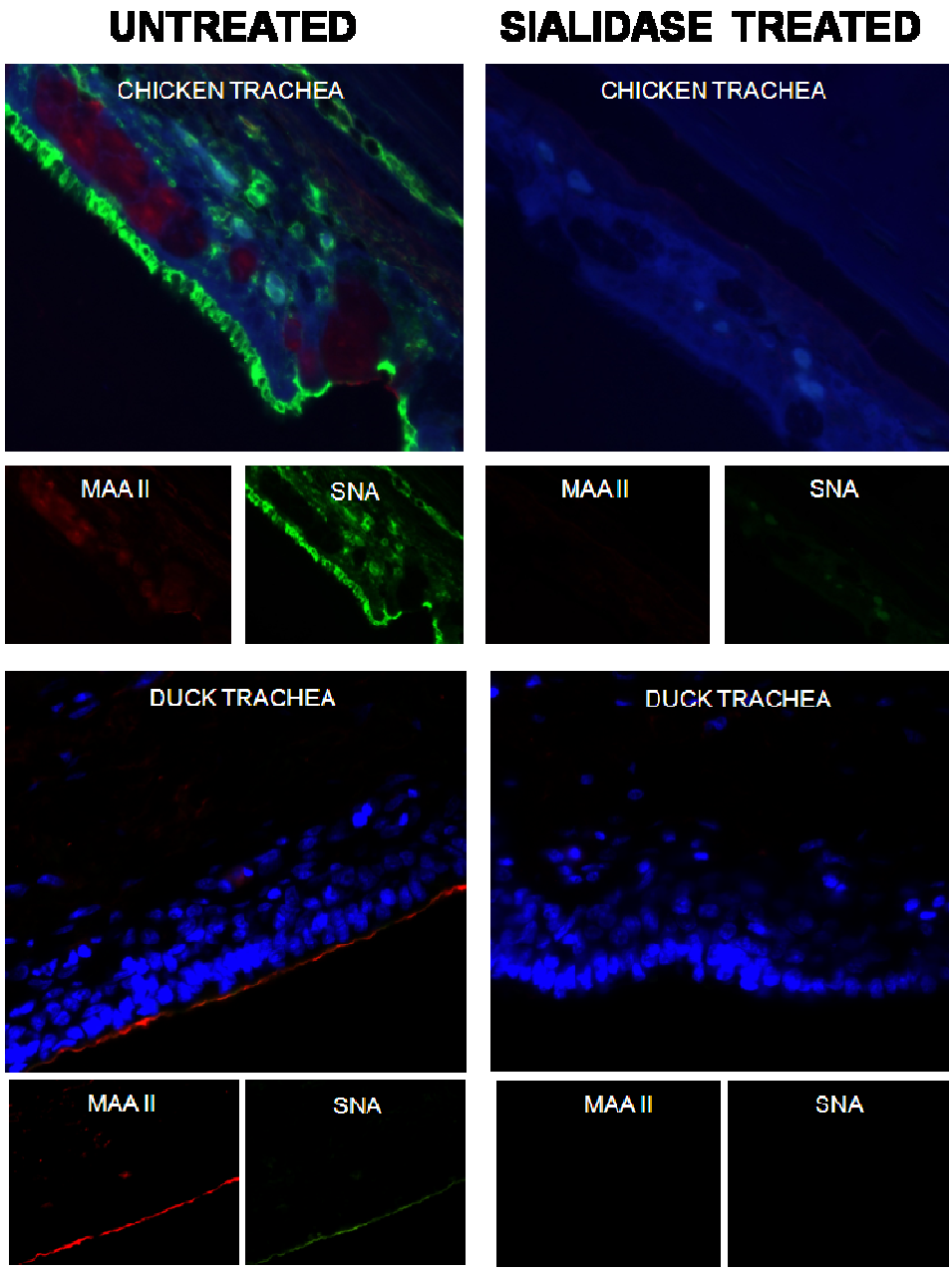


Figure 3.4-8 Effect of sialidase treatment on lectin binding

Abundant expression of SNA binding (green) pattern in chicken trachea and MAAII binding (red) in duck trachea were absent in sialidase treated sections indicating that SNA and MAAII lectins used in this study were specific to SAα2,6-Gal and SAα2,3-Gal respectively and did bound to non-sialic acid residues.

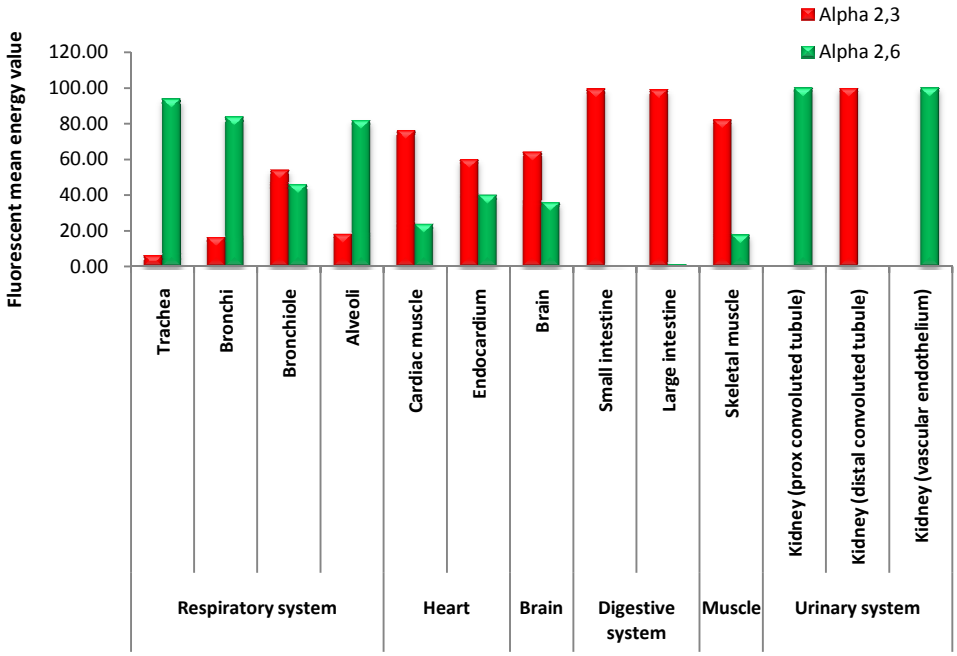


Figure 3.4-9 Distribution of avian and mammalian influenza virus receptors across various chicken tissues

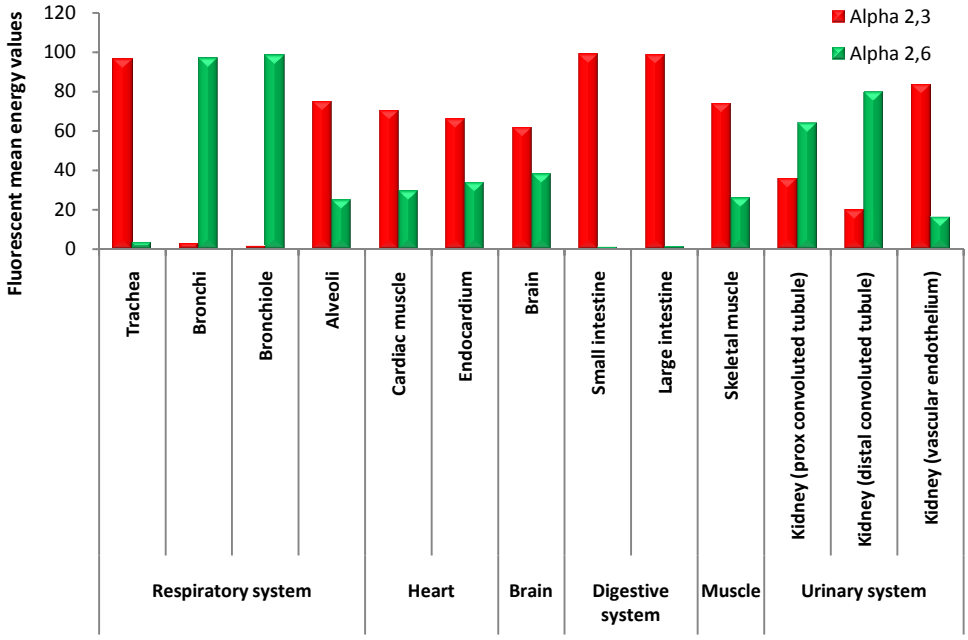


Figure 3.4-10 Distribution of avian and mammalian influenza virus receptors across various duck tissues

Figures 3.4.9 and 3.4.10-Data represents corrected mean energy values of each fluorochrome in overlay images expressed as percentage of the total.

3.4.1.7 SAα2,3-Gal receptor diversity

We further distinguished SAα2,3-Gal receptor subtypes, based on the third sugar residue, in chicken and duck trachea and intestines, with the use of MAA I (SAα2,3-Gal β(1-4)GlcNAc specificity) and MAA II (SAα2,3-Gal β(1-3)GalNAc specificity). In chicken trachea, both SAα2,3-Gal receptor subtypes were detected in the sub-epithelial region (Figure 3.4-11). However, along the chicken tracheal epithelium, SAα2,3-Gal β(1-4)Glc NAc receptor (MAA I lectin) was more dominant than SAα2,3-Gal β(1-3) Gal NAc receptor (MAA II lectin).

In duck trachea, by contrast, minimal MAA I lectin binding was observed along the epithelium; only sub-epithelial mucous glands were MAA I positive. In duck trachea, SAα2,3-Gal β(1-3) Gal NAc receptor (MAA II lectin) was the main subtype detected, with distribution along the epithelial lining and in the mucosa.

In chicken large intestine (Figure 3.4-12), both SAα2,3-Gal β1-4 Gal NAc receptor (MAA I lectin) and SAα2,3-Gal β1-3 Glc NAc receptor (MAA II lectin) expression was observed. In duck large intestine, SAα2,3-Gal β1-3 Glc NAc receptor (MAA II lectin) was the main subtype detected, while the goblet cells were positive for SAα2,3-Gal β(1-4) Glc NAc receptor subtype (MAA I lectin). Similar binding pattern of MAA I and MAA II was also noticed in the small intestines of both avian species (Figure 3.4-13).

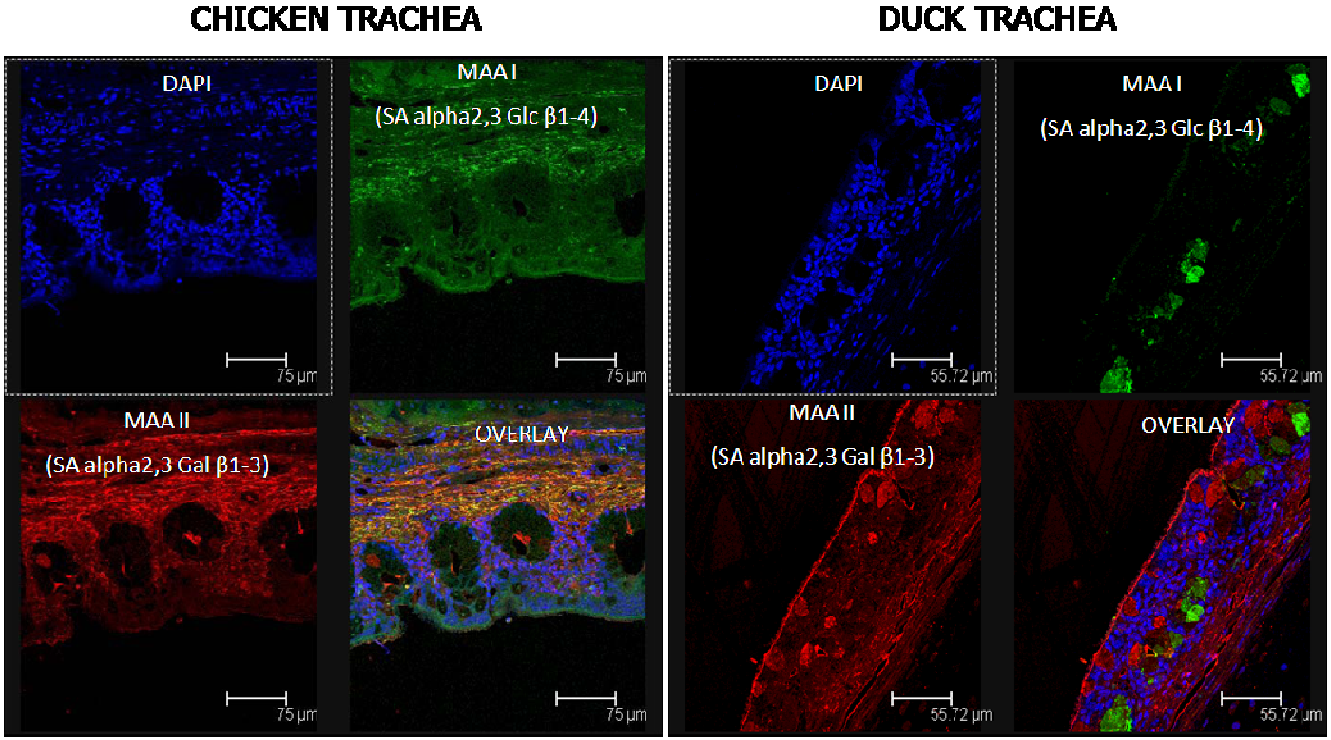


Figure 3.4-11 Diversity of SA α2,3 receptors in chicken and duck tracheae

Chicken tracheal epithelium expressed dominant SAα2,3-Gal β(1-4)Glc NAc receptor (Green, MAA I binding) than SAα2,3-Gal β(1-3) Gal NAc receptor (red, MAA II binding).Whereas, SAα2,3-Gal β(1-3) Gal NAc receptor (MAA II binding) was the predominant receptor type across the duck tracheal epithelial lining and only sub-epithelial mucous glands were MAA I positive. Figures are representative of a number of tissue specimens screened.

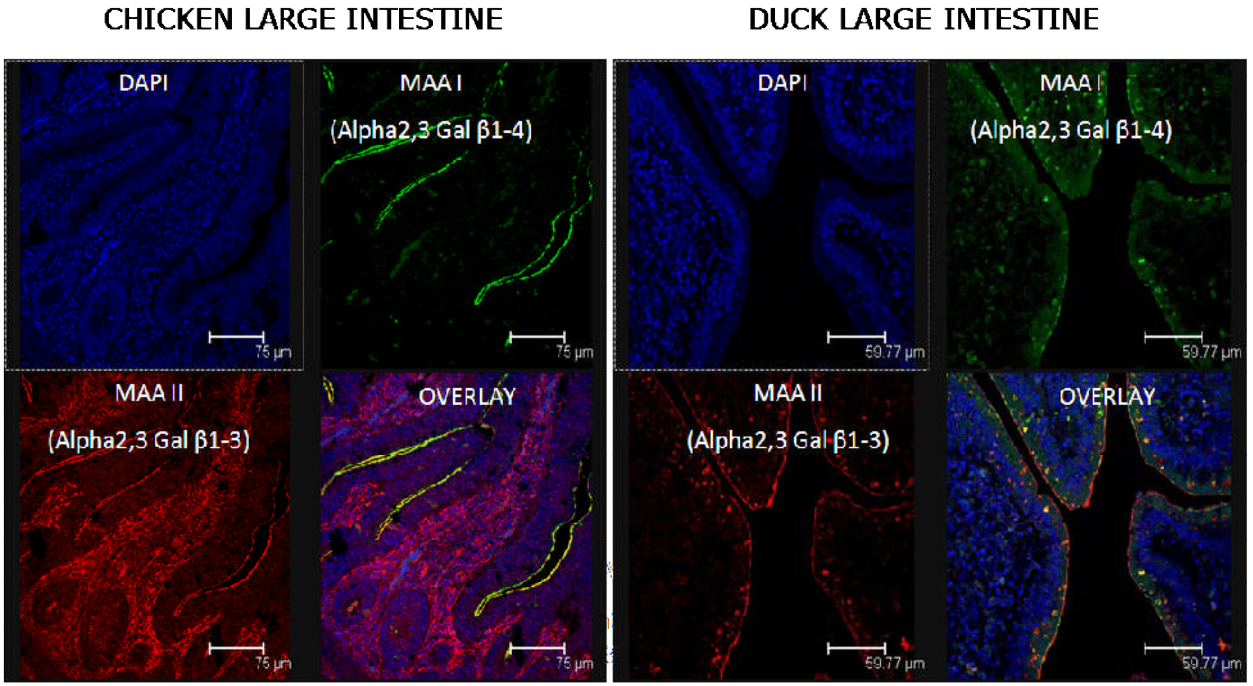


Figure 3.4-12 Diversity of SA α2,3 receptors in chicken and duck large intestines

Large intestine epithelial lining in chicken expressed dominant SAα2,3-Gal β(1-4)Glc NAc receptor (Green ,MAA I binding) than SAα2,3-Gal β(1-3) Gal NAc receptor (red, MAA II binding).Whereas, SAα2,3-Gal β(1-3) Gal NAc receptor (MAA II binding) was the predominant receptor type across the epithelial lining of duck large intestine and only the goblet cells were MAA I positive. Figures are representative of a number of tissue specimens screened.

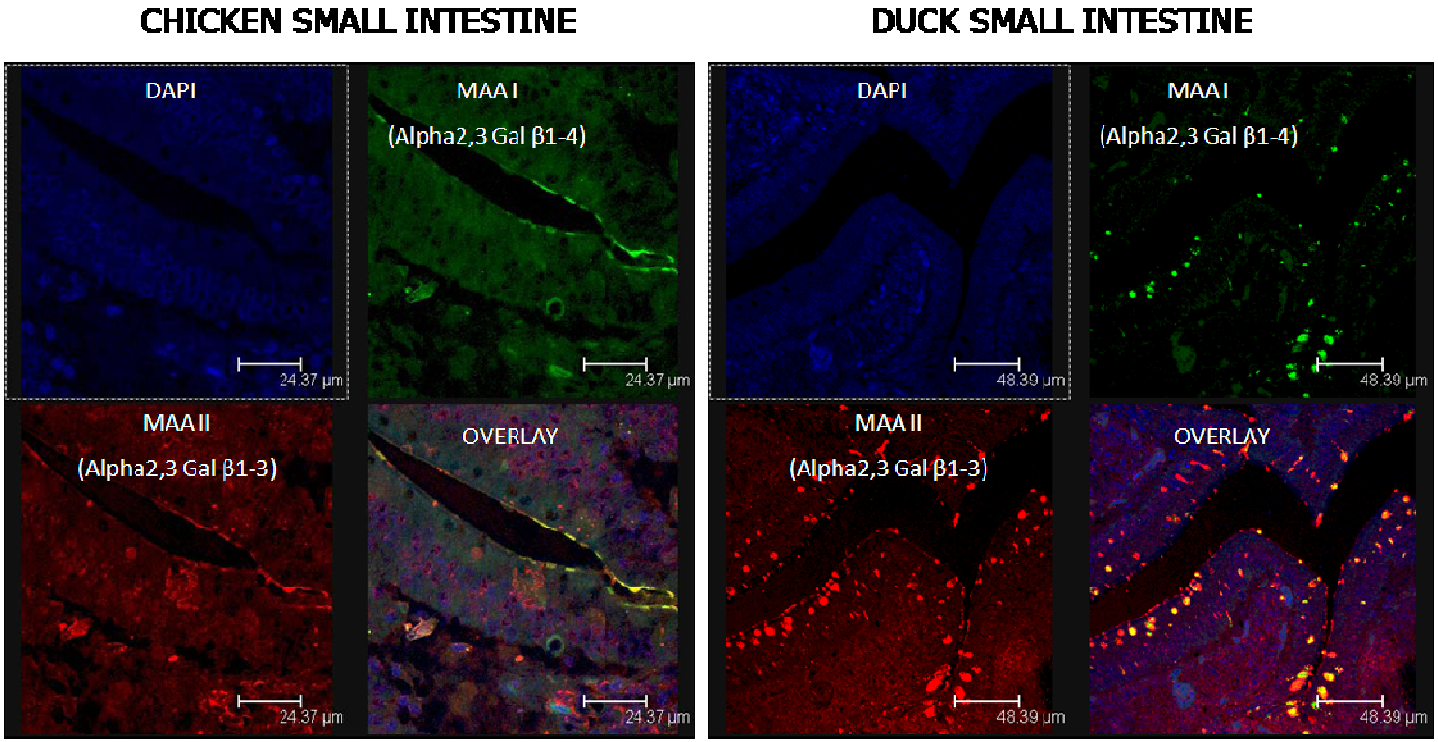


Figure 3.4-13 Diversity of SA α2,3 receptors in chicken and duck small intestines

Small intestine epithelial lining in chicken expressed dominant SAα2,3-Gal β(1-4)Glc NAc receptor (green, MAA I binding) than SAα2,3-Gal β(1-3) Gal NAc receptor (red, MAA II binding).Whereas, SAα2,3-Gal β(1-3) Gal NAc receptor (MAA II binding) was the predominant receptor type across the epithelial lining of duck small intestine and only the goblet cells were MAA I positive. Figures are representative of a number of tissue specimens screened.

3.4.2 Virus binding assay

Virus binding assays were performed on trachea and small intestines sections of chicken and duck. A classical swine H1N1 strain (A/Sw/Iowa/15/30) with SAα2,6-Gal specificity and a H2N3 low pathogenic avian strain (A/mallard duck/England/7277/06) with SAα2,3-Gal specificity were used.

A strong correlation between the results of lectin binding and virus binding assay was observed. The abundant SAα2,6-Gal receptor type found across chicken tracheal epithelium by lectin histochemistry correlated with more preferential binding by the swine H1N1 virus. Similarly the abundant SAα2,3-Gal receptor type found across duck tracheal epithelium correlated with more preferential binding of avian H2N3 virus. Exclusive distribution of SAα2,3-Gal receptor type across the epithelial lining of both chicken and duck intestine by lectin binding assays was found. The results of virus binding assay revealed that the avian H2N3 virus showed extensive binding to intestines of chicken and duck, while no binding of swine H1N1could be detected (Figure 3.4-14).

The virus binding assay described in this thesis is a very useful tool to determine the receptor distribution on host cells and tissues. Although lectin binding assays are easy to perform, it is possible that the levels of sialic acids needed for lectins and influenza viruses to bind could be different. For example, in this study, no binding of swine H2N3 virus was found to chicken and duck intestines, while SNA binding was detected in these tissues. Hence, receptor distribution studies using virus binding assays could be more functionally relevant than lectin binding assays. However, the techniques of virus binding assay need to be further improved for better detection sensitivity, for example, use of labelled viruses.

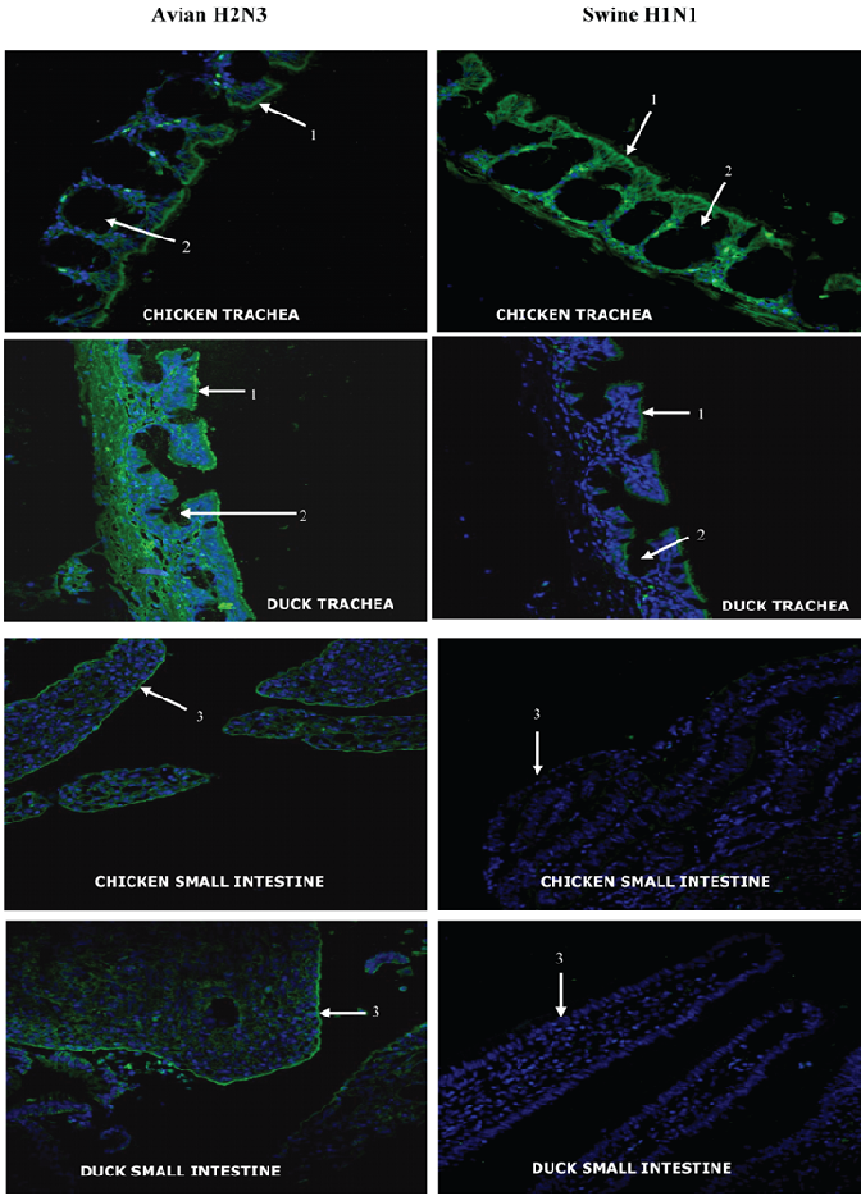


Figure 3.4-14 Virus binding assay

Swine H2N3 showed more preferential binding to chicken tracheal epithelium, while avian H2N3 virus bound more preferentially to duck tracheal epithelium which correlated with the abundant expression of SAα2,6-Gal and SAα2,3-Gal receptor types. Binding of avian H2N3 alone with no binding of swine H1N1 was found to the epithelial lining of chicken and duck intestines.

3.5 Discussion

3.5.1 Lectin histochemistry

Using lectin staining, widespread presence of both SAα2,6-Gal (SNA) and SAα2,3-Gal (MAA II) receptors in a range of tissues from each species was found, suggesting that these organs may be potential targets for both avian and human influenza viruses. The expression of SAα2,6-Gal receptors in duck tissues is in contrast to a previous study using virus binding assays which reported that plasma membranes isolated from duck respiratory and intestinal epithelial cells did not express SAα2,6-Gal-terminated receptors (Gambaryan, Webster, & Matrosovich 2002). The exact reason for this difference is not clear. However, the previous study used mallard ducks rather than Pekin ducks used in this study, further the results were based on virus, rather than lectin binding, on isolated cell membranes rather than intact cells. It is possible that the difference in the methodology and/or choice of duck breed may explain the discrepancy in the two studies results.

3.5.1.1 Respiratory system

The major species difference was observed between chicken and duck in the relative distribution of SAα2,3-Gal and SAα2,6-Gal receptors along the tracheal epithelium. In chicken tracheal epithelium, SAα2,6-Gal (with SNA binding) was the dominant receptor type whereas in ducks the SAα2,3-Gal receptor (with MAA II binding) was more abundant in the ciliated cells of the tracheal epithelium. Based on mean fluorescent energy values, it was found that the ratio of SAα2,6-Gal to SAα2,3-Gal in chicken trachea was approximately 10:1 whereas in duck the ratio was 1:20. The tracheal mucous glands of both chicken and duck predominantly expressed SAα2,6-Gal receptor type. The observed difference in dominant receptor type between chickens and ducks was confined to the upper airway (trachea). In the bronchi and bronchioles the clear presence of both receptor types was found in both species. Chicken alveolar cells expressed both receptor

types. The precise staining pattern was more difficult to determine in duck alveoli due to the extensive presence of vascular tissue which tended to obscure the alveolar staining. However, both receptors could be seen clearly in some areas of the alveoli. The dominant SAα2,6-Gal receptor expression pattern in chicken trachea was in contrast to a previous study (Wan & Perez 2006) which, using lectin binding, found that 85% of the epithelial cells in chicken trachea were positive for SAα2,3-Gal receptors, while only 10% were positive for SAα2,6-Gal receptors. However, it is in agreement with the findings of (Gambaryan, Webster, & Matrosovich 2002), who reported that human influenza viruses with SAα2,6-Gal specificity bound to cell membranes isolated from chicken tracheal cell membranes but not those in duck.

A possible explanation for the discrepancy in the reported receptor distribution in chicken trachea could be the source of the lectin used. Lectins from different suppliers may show different binding specificities; in particular the source of MAA has been shown to significantly affect specificity (Nicholls et al. 2007). Glycan microarray screening of lectins from the supplier used for our study confirms that these lectins bind with a high degree of specificity to the appropriate sialic acid linkages (http://www.functionalglycomics.org). It is also possible that this study was able to detect lower levels of receptor expression due to the methodology used (confocal microscopy with fluorescent detection compared with immunohistochemistry).

The observed predominance of SAα2,6-Gal in chicken trachea was found to be consistent in different ages and breed of chicken. Lectin staining on sialidase treated sections was also performed, which abrogated all staining. This provided confirmation that the lectins used in the present study did not bind to non-sialic acid residues (see section 3.4.1.5). The difference in the predominant receptor type across the tracheal epithelial lining in chicken and ducks could be an important contributing factor to influenza virus entry via the upper respiratory tract. In particular, such differences could impact on the susceptibility of each species to avian H5N1 influenza with its preferential tropism for infection of the respiratory tract rather than the intestines.

The predominant receptor in the human upper airway is SAα2,6-Gal (Shinya et al. 2006;Yao et al. 2008) . The high levels of expression of SAα2,6-Gal receptors in chicken tracheal epithelium implies that this species may be more able to support the evolution of viruses with higher affinity for human type SAα2,6-Gal receptors. This supports the hypothesis that chickens may be the source of emerging H9N2 viruses with a human-virus like receptor specificity (Gambaryan et al. 2002).

3.5.1.2 Digestive system

Chicken and duck intestines predominantly expressed the SAα2,3-Gal receptor type across the epithelial lining of villi supporting the previously reported findings (Ito and Kawaoka, 2000 & Wan and Perez, 2006). The measurement of fluorescent mean energy values along the epithelial lining of the mucosa showed no significant presence of SAα2,6-Gal in the intestines of either chicken or duck. This finding reaffirms the avian digestive tract as a major predilection site for avian influenza virus replication.

3.5.1.3 Kidneys

Although there is widespread distribution of both receptor types in kidneys of both chickens and duck, there are clear differences in their spatial distribution within the organs of the two species. In the vascular endothelium of the kidney, both SAα2,6-Gal and SAα2,3-Gal receptors were found in duck cells, but only the SAα2,6-Gal receptor type was found in the corresponding region in chicken kidney. The significance of this difference is not clear. However, the presence of SAα2,6-Gal receptors in the vascular endothelium in both species indicates that these cells can be potentially infected by mammalian-like influenza viruses. These cells can possibly play a role in the haematogenous spread of the virus. Tubular cells of duck kidney expressed both SAα2,6-Gal and SAα2,3-Gal receptors. However, chicken kidney tubular cells expressed either SAα2,6-Gal or SAα2,3-Gal receptors, but no co-expression was observed.

3.5.1.4 Other organs

The endocardium, meninges and muscle fibres of both species co-expressed both subtypes of receptor. The expression of both host receptors in such tissues in chicken and duck suggests that these avian hosts could possibly serve as “mixing vessels” for virus reassortment following co-infection by human and avian viruses.

3.5.1.5 SAα2,3-Gal receptor diversity

Using MAA I (SAα2,3-Gal β(1-4)GlcNAc specificity) and MAA II (SAα2,3-Gal β(1-3)GalNAc specificity) lectins, SAα2,3-Gal receptor subtypes were further distinguished based on the third sugar residue in chicken and duck trachea and intestines. Dominant expression of SAα2,3-Gal β(1-4)Glc NAc receptor (MAA I lectin) than SAα2,3-Gal β(1-3) Gal NAc receptor (MAA II lectin) across chicken tracheal epithelium was found. In duck trachea, by contrast, SAα2,3-Gal β(1-3) Gal NAc receptor (MAA II lectin) was the main subtype detected, with distribution along the epithelial lining and in the mucosa. In chicken intestines, both SAα2,3-Gal β1-4 Gal NAc receptor (MAA I lectin) and SAα2,3-Gal β1-3 Glc NAc receptor (MAA II lectin) expression was observed. In duck intestines, SAα2,3-Gal β1-3 Glc NAc receptor (MAA II lectin) was the main subtype detected, while the goblet cells were positive for SAα2,3-Gal β(1-4) Glc NAc receptor subtype (MAA I lectin). In humans, MAA I shows more widespread binding throughout the upper and lower respiratory tract compared to MAA II (Nicholls et al. 2007). Avian influenza viruses isolated from chicken and ducks have been shown to preferentially bind to SAα2,3-Gal β(1-4) Glc NAc (recognized by MAA I) and SAα(2,3) Gal β(1-3) Gal NAc (recognized by MAA II) respectively (Gambaryan et al, 2003). This reported virus tropism correlates with the observed receptor distribution in chicken and duck trachea and intestinal tissues. The presence of both SAα2-3-Gal receptor subtypes in chicken suggests that they may be susceptible to infection with avian influenza viruses from chicken and ducks.

3.5.2 Virus binding assay

To relate the observed receptor distribution to the ability to bind viruses of avian or mammalian origin, virus binding assays were performed with avian H2N3 and swine H1N1 influenza viruses on tracheal and digestive tract sections. The results showed the predicted preferential binding of the avian virus for SAα2,3-Gal receptor and the swine virus for SAα2,6-Gal receptors. The main SAα2,3-Gal receptor type in duck tracheal epithelium showed greater affinity for the avian H2N3 virus. By contrast, the dominant receptor type of SAα2,6-Gal in chicken trachea showed preferential binding of the swine H1N1 virus. The predominant expression of SAα2,3-Gal receptors type along the small and large intestinal epithelia of chicken and duck showed preferential affinity for the avian H2N3 virus. However, the results showed no significant attachment of the swine virus. Virus-binding specificity was therefore consistent with host receptor type, as determined by lectin staining. The SAα2,6-Gal receptor type expressed by the intestinal goblet cells did not appear to be functionally significant as no virus binding was observed with swine H1N1 virus.

The differences in receptor expression reported in this study suggest that they may be responsible, at least in part, for some of the differences between ducks and chickens in the pattern of disease following influenza infection. While the presence of a virus receptor is clearly not sufficient to confirm that cells or tissue support efficient virus replication or transmission, the widespread replication of influenza virus in multiple organs has been reported in both chickens (Swayne 1997) and ducks (Londt et al. 2008) following infection with highly pathogenic viruses.

Chapter 4

Development of in-vitro models for the study of influenza virus-host interactions

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4.1 Summary

In-vitro models comprising tracheal and intestinal organ cultures, primary lung and embryo cell cultures from chicken and duck were developed. Ability of these models to support influenza virus replication was studied by analyzing viral nucleoprotein expression by immunochemistry and influenza matrix gene expression by quantitative RT-PCR. Although oorgan cultures supported virus infection, there were limitations in the length of cultivation. Primary lung and embryo cell cultures developed from chicken and duck were chosen for further studies on influenza virus host interactions. Using primary cell cultures had several advantages over the organ cultures, including consistent levels of infection, susceptibility to infection by a wide range of influenza viruses and the ability to grow for longer times in culture without any degenerative changes.

4.2 Introduction

In-vitro models have been widely employed in the study of influenza viruses. These models have so far been used either for diagnosis of influenza infections, for propagation of influenza viruses, to study virus pathogenesis or to study host responses to infection.

4.2.1 De-embryonated eggs and embryonic membranes

Recombination of two different influenza viruses by simultaneous infection of a de-embryonated egg was studied showing that a high proportion of the susceptible cells must undergo nearly simultaneous double infection with the two strains to generate recombinant strains (Burnet & Lind 1953). Minced chorio-allantoic membranes from chicken embryos buffered with either ground eggshell (Daniels, Eaton, & Perry 1952) or with sodium pyruvate and glucose (Eaton, Alder, & Perry 1953) were later used rather than whole embryos to study their effect on the growth of influenza viruses.

4.2.2 Tissue and organ cultures

4.2.2.1 Avian organ cultures

Cultures of epidermis of 10- to 12-day chick embryos were shown to support the growth of influenza virus and addition of excess vitamin A was found to increase the daily yield of influenza virus due to conversion of dermal epithelial cells to mucous secreting cells (Huang & Bang 1964). Chick embryo tracheal organ cultures have been used for the propagation of influenza viruses and were found to be as sensitive as rhesus monkey kidney cell cultures to influenza virus infection (Blaskovic, Rhodes, & Labzoffsky 1972). Immunity to influenza viruses was demonstrated using tracheal organ cultures from vaccinated and control chicken and it was found that secretory immune response mediated by immunoglobulin A (IgA) was responsible for the immunity of the organ cultures (Schmidt & Maassab 1974).

Organ cultures comprising of chicken and duck colons have also been used to cultivate influenza viruses from duck and human. While colon cultures of both chicken and duck support the growth of duck influenza viruses, chicken but not duck colon supports growth of human influenza virus (Ohta, Yanagawa, & Kida 1981). Tracheal organ cultures (TOC) from chicken have been recently employed to characterize early immune responses induced by avian influenza viruses (Reemers et al. 2009a).

4.2.2.2 Human and other species organ cultures

Organ cultures of human-embryo nose and tracheal tissue segments have been used for the isolation of viruses causing acute human respiratory infections and the growth of influenza virus was found to be associated with cessation of ciliary activity, commonly within 6 days of inoculation. Although this method was found to be useful for diagnosis, it is not widely used owing to the limited availability of foetal material (Higgins & Ellis 1973). Ferret tracheal organ cultures have been used to measure the therapeutic effect of

anti-influenza drug spiroamantadine (Arroyo & Reed 1977). Hamster tracheal organ cultures were also employed for infection studies with different influenza viruses and the metabolic activity measured using a tetrazolium reduction assay was used to assess the effect of virus infection on the organ cultures (Reeve, Gerendas, & Walzl 1978).

Ependymal organ cultures established from cerebellum of newborn rats were used to study the effect of influenza A virus on the ciliated ependyma and by seven days the ependymal cells were found to be denuded of cilia and microvilli (Kohn, Chinookoswong, & Magill 1981). Organ cultures of human nasal polyps have been used for influenza virus cultivation and it was found that this system supported the growth of human influenza A viruses better than avian strains (Ginzburg et al. 1982). Human adenoid organ cultures comprising of human nasopharyngeal tonsils were used to study the pathogenesis of influenza infections and the local immune response (Edwards et al. 1986).

4.2.3 Cell cultures

Studies relating to isolation and pathogenesis of influenza B viruses have initially employed monkey kidney cell cultures models (Mogabgab et al. 1955) and later, cultures of kidney tissue from several species including man, monkey, cattle embryo, guinea-pig, rabbit, hamster, mouse and chick embryo have been employed to cultivate influenza viruses. These studies have found that serial propagation in monkey kidney cells was readily achieved with mouse adapted virus strains but not with egg-adapted strains (Heath & Tyrrell 1959). Cell culture models from the host of virus isolation were later used for influenza virus propagation. Cell cultures comprising of collagenase-dispersed human and porcine lung cells were used for the propagation of human and swine influenza viruses respectively (Hinz & Syverton 1959).

Several established diploid cell lines have been found to support influenza virus replication and Madin-Darby Canine Kidney (MDCK) cells were found to be one of the most efficient cell systems for the plaque assay of influenza (Gaush & Smith 1968).

Differentiating avian myotubes formed in cultures of muscle cells from 11- to 12-day-old chick embryos were found to support the productive replication of influenza virus (Cox, O'Neill, & Kendal 1977). Effect of influenza virus infection on the muco-ciliary transport of trachea was studied by virus-gland cell interactions by exposing primary cultures of isolated feline tracheal sub mucous gland cells to influenza A (Gentry et al. 1988).

An earlier method described culturing of epithelial cells from adult chicken trachea and reported the usefulness of this system to study the mucin production and synthesis (Douglas et al. 1980). Mucin producing cells cultivated from primary chicken tracheal epithelial cultures derived from 17 day old chicken embryos have been found to be useful to study the mechanisms of early influenza viral infection and cellular host transcriptional responses (Zaffuto, Estevez, & Afonso 2008). This chapter describes the development of suitable *in-vitro* models from chicken and duck to study the differences in host responses following influenza virus infection. *In-vitro* models comprising of organ cultures and primary cells from chicken and duck were developed and their suitability for studying host responses following infection was evaluated.

4.3 Materials and methods

4.3.1 *Ex-vivo* organ cultures

4.3.1.1 Construction of air-interface organ culture

Chicken and duck organ cultures were constructed using a previously described method for canine tracheal tissue cultures with minor modifications (Anderton, Maskell, & Preston 2004). Agarose plugs were made by pipetting 1 ml 1% agarose in DMEM with 100 units/ml penicillin, 100µg/ml streptomycin (Invitrogen) and 2.5µg/ml amphotericin B (Invitrogen) in a 24 well cell culture plate (Costar). After the agarose had set, plugs were removed with a sterile scalpel and placed in a 6-well cell-culture plate (Costar). Semisolid medium made with 0.5% agarose in DMEM medium with penicillin,

streptomycin and 10% FCS was pipetted around the plugs. The lid of the cell-culture dish was replaced and plates were incubated at 37°C in a humidified atmosphere with 5% CO_2 at 37°C in a Hera cell CO_2 incubator (Thermo Scientific).

4.3.1.2 Tracheal organ cultures (TOC)

Tracheae were collected from euthanized birds and were rinsed twice with a collection medium (CM) in 50 ml falcon tubes (Costar) followed by 2 washes with warm PBS to remove antibiotics. Following the washes, the trachea was placed in a sterile petri dish and was cut lengthways into two equal strips with a scissors. Then approximately 5 mm wide strips were made by cutting in between the cartilage rings. Each strip consisted of respiratory mucosa on cartilage, and was placed on the agarose plugs with mucosa side facing upwards (Figure 4.3-1A).

4.3.1.3 Intestinal organ cultures (IOCs)

Small and large intestines from chicken and duck were collected and processed in a similar way to tracheae. Segments of intestines measuring approximately 5cm consisting of intestinal mucosa with the underlying tissue was placed on the agarose plugs with mucosa side facing upwards (Figure 4.3-1B).

4.3.1.4 Infection of organ cultures

Chicken and duck TOCs were infected with low pathogenic avian influenza H2N3 or low pathogenic swine influenza H1N1 viruses in separate culture plates at 2 hrs post-culture. Fifty micro litres of H2N3 or 100 µl of H1N1 virus was instilled with a micropipette on the mucosal surface of the TOC and the plates were incubated 37°C in a Hera cell incubator. Sham infected controls were performed by incubating the sections with PBS. Infected and control organ cultures were collected at 24, 48, 72 and 96 hrs post infection,

rinsed in PBS and fixed in 10% BNF. Fixed tissues were processed and sectioned as described in Chapter 2, earlier.

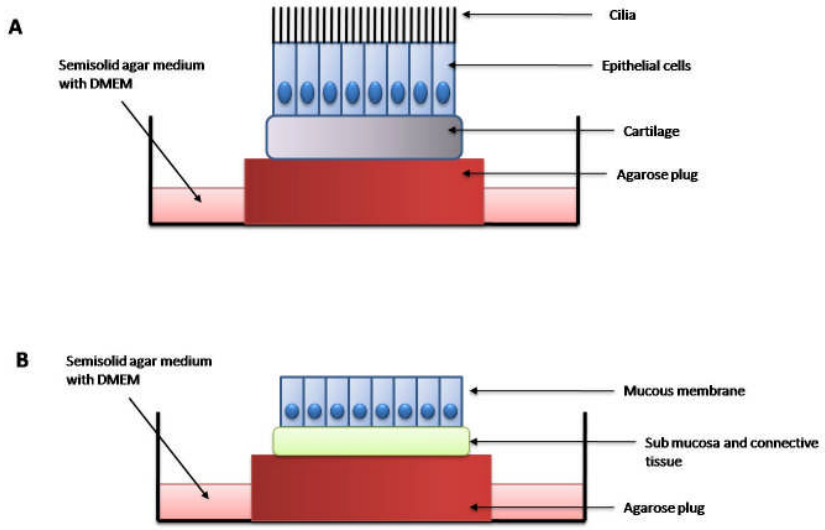


Figure 4.3-1 Schematic representation of the organ culture construction

Construction of avian tracheal organ culture (TOC) (A) and intestinal organ cultures (IOC) (B) is illustrated. Air-interface cultures comprising of small pieces of trachea or intestine placed on the agarose plug with the ciliated surface facing up. Semisolid agarose medium surrounding the agarose plug contained nutrients, creating an air medium interface. (Modified from Anderton, Maskell & Preston 2004).

4.3.1.5 Haematoxylin and Eosin (H&E) Staining

Tissue sections from uninfected control tracheal and intestinal organ cultures harvested at 24, 48, 72 and 96 hrs post-culture were examined for the tissue integrity following haematoxylin and eosin (H&E) staining. Briefly the sections were deparaffinised in Histoclear (National Diagnostics, Atlanta, USA) and rehydrated by alcohol immersion in descending series of aqueous alcohol solutions (100%, 90%, 80% and 70% ethanol). Sections were stained with Lillie Mayer's alum haematoxylin to stain nuclei for 4 minutes before rinsing in running tap water. After differentiating in 0.3% acid alcohol for 30 s, the sections were rinsed in running tap water. The sections were then rinsed first in

Scott's tap water substitute and then in running tap water before staining with eosin for 2 min. The sections were dehydrated by immersion in an ascending series of aqueous alcohol solutions (70%, 80%, 90% and 100% ethanol), cleared using Histoclear and mounted in DPX mountant. (Reagent compositions are provided in Appendix I).

4.3.1.6 Immuno-fluorescent staining of sections

Sections of infected and control TOCs were deparaffinised in xylene and rehydrated by serial dipping in 100% ethanol for 3 min and 95% and 80% ethanol for 1 min each. Sections were rinsed in distilled water; excess fluid was removed and outlined with hydrophobic barrier ImmEdge Pen (Vector Laboratories, Burlingame, CA). Antigen retrieval was performed by proteolytic digestion as described previously (Thomson et al. 2004).

Briefly, the sections were covered with Pronase solution (Dako) and were incubated for 10-20 min at 37°C in a humidified chamber. Sections were rinsed with PBS and allowed to cool at RT for 10 min. After blocking the sections with goat serum (25ul/ml TBS) for 30 min, excess fluid was removed. The sections were then incubated with a primary mouse monoclonal to influenza nucleoprotein (Abcam) (at 1:1000 dilution in TBS) for 2 hrs in a humidified chamber at RT. After removing excess fluid, sections were washed three times with TBS (5 min each wash). Sections were then incubated with a goat polyclonal to mouse IgG-Cy5 (Abcam) (at 1: 500 dilution in TBS) (2µl /ml) in TBS for 1 hr in a humidified chamber in dark at RT. Excess fluid was removed and the sections were washed three times with TBS. Sections were air dried briefly before mounting with ProLong Gold antifade reagent with 4', 6-diamino-2- phenylindole, dihydrochloride (DAPI; Molecular Probes, Inc., Eugene, OR) on a clean glass slide. Sections were allowed to cure overnight at RT in the dark. Images were captured using a confocal microscope (Leica TCS SP2 AOBS) as described in Chapter 2.

4.4 Primary cell cultures

4.4.1 Primary avian airway epithelial cell cultures

Airway epithelial cells from chicken and duck were cultured following a previously described protocol (Davidson et al. 2000) with minor modifications. Four week old broiler chickens and 6 week old Pekin ducks were euthanized and tracheae were collected as described in chapter 2.

The tracheae were cut lengthwise and later 3 cm pieces were made by cutting between the cartilage rings. With the help of a scalpel the mucosal surface was scrapped and the scraped mucosa was transferred to a 50 ml centrifuge tubes (Costar). Later 30ml of dissociation medium (DM) containing equal amounts of DMEM with Glutamax and nutrient mixture Ham's F12 (Invitrogen) supplemented with protease from *Streptomyces griseus* (1.4mg/ml; Sigma), 100 units/ ml penicillin, 100µg/ml streptomycin and 2.5µg/ml amphotericin B was added to the harvested mucosa. The tubes were incubated either at 4°C overnight or at 37°C in a water bath for 2 hrs. After the incubation, tubes were centrifuged at 1200x g for 5 min.

Supernatant was carefully discarded and the cell pellet was reconstituted in small volume of cell culture medium (CCM). The cell culture medium was prepared by mixing equal volumes of DMEM and Ham's F12 supplemented with 2% chicken embryo extract (Biosera), 5% fetal calf serum (FCS), 1% insulin-transferrin-selenium A supplement (Invitrogen), 100 units/ ml penicillin, 100µg/ml streptomycin and 2.5µg/ml amphotericin B was used.

The cells were plated in collagen coated cell culture flasks (Costar) and incubated at 37°C. After 24 hrs of incubation, medium from the cells was removed and fresh CCM without amphotericin B was added to the flasks and these were incubated further at 37°C.

4.4.2 Avian lung cells

4.4.2.1 Extraction of cells from lung tissues

Primary cell cultures were obtained from lungs of 4 wk old broiler chickens and 6 wk old Pekin ducks. The lung samples were washed three times in phosphate buffered saline (PBS, Invitrogen) to remove red blood cells. The lungs were then sliced into fine pieces with a scalpel blade and digested at 4°C overnight in a 50 ml centrifuge tubes (Costar) with dissociation medium (DM) on a rotary shaker. Large undigested tissue pieces after overnight digestion were removed using a cell strainer and the remaining suspension was centrifuged at 1200x g for 5 min. Supernatant was carefully discarded and the cell pellet was reconstituted in small volume of cell culture medium (CCM).

4.4.2.2 Cell counting

The cells were then counted using a Neubauer haemocytometer. For counting the cells 50µl of the cell suspension was mixed with 50 µl of trypan blue, which stains only dead cells which appear blue, whereas live cells exclude the dye and appear colourless. A cover glass was placed on the mounting support of the haemocytometer and 15 µl of the cell suspension carefully pipetted into each side of the haemocytometer for counting. The cells were then plated into collagen coated cell culture flasks (Costar) at a seeding density of 5000 to 6000 cells/cm^2 in CCM. Cell culture flasks were incubated at 37°C. After 24 hrs of incubation, the medium from the flasks was replaced with fresh CCM without amphotericin B.

4.4.2.3 Collagen coating

Cell culture flasks, plates and cover slips were coated with collagen to promote the primary cell adhesion to the surface. Cover slips for cell culture were boiled in 1M hydrochloric acid (Hcl) for 4 hours at 50°C in a water bath. Cover slips were then

washed three times in deionised water and autoclaved. Processed cover slips were aseptically transferred in to each of the wells of a 24 well cell culture plate for collagen coating. Collagen working solution was prepared by diluting stock solution (4mg/ml in 20mM acetic acid) of Type I collagen from rat tail (Sigma) 1 in 4 in 70% ethanol. Tissue culture flasks, plates or cover-slips were coated by adding 0.15 – 0.2 ml working solution per cm^2 and the flask/plates were left overnight. The following day, excess collagen solution was aspirated and flasks/plates were allowed to air dry. Culture flasks and plates were rinsed briefly with PBS before use.

4.4.2.4 Cell passaging

Avian primary lung cells were passaged when they grow to around 90-100% confluence. Culture medium was carefully removed from the flask and the cell monolayer was washed twice with PBS. The monolayer was then trypsinized by adding 0.05% trypsin with EDTA (Invitrogen) and incubating at 37°C for 5 min. The flask was gently tapped to dislodge the cells then the enzyme was inactivated by adding medium containing DMEM with 10% FCS. The contents were then transferred into a 50 ml falcon tube (Costar) and centrifuged at 1200 x g for 5 min. After carefully discarding the supernatant, the cell pellet was re-suspended in fresh CCM. Cells were then returned to fresh culture flasks (1:2 dilution).

4.4.2.5 Cryo- preservation of cells

All the primary cells were stored in liquid nitrogen for long term storage. Cells were trypsinized when they were 80-90% confluent as described in the previous section and re-suspended in a freezing medium (FM) containing DMEM with 50% FCS and 10% dimethyl sulphoxide (DMSO, Sigma). They were then transferred into pre-chilled cryovials (Nunc). The cryovials were placed in isopropanol-filled freezing container “Mr. Frosty” (Nalgene) and frozen at a controlled rate (1°C/min) overnight in a -70°C freezer. The following day the cryovials were transferred to -196°C liquid nitrogen (LN_2) freezer.

When the cryo-preserved cells were required, they were thawed quickly in 37°C water bath. To remove DMSO, the contents were mixed with 10 ml of CCM and centrifuged 1200 x g for 5 min. After discarding the supernatant, the pellet was re-suspended in fresh CCM and transferred to a culture flask.

4.4.3 Avian embryo cells

For extracting embryo fibroblasts nine day chicken, 10.5 day old Pekin duck and 10.5 day old Mallard duck embryos were used. The embryos were candled and healthy embryos were selected. The eggs were placed in a beaker with the pointed end facing down. With the help of a scissors the wide end was cracked open. The embryo was pulled out with a sterile forceps and placed in a petri dish and rinsed with PBS. Head, limbs and internal organs were removed from the embryo, transferred to a fresh petri dish and rinsed with PBS. The embryo was minced with a scissors and forceps and transferred to a 50ml falcon tube with medium containing DMEM, 0.25% trypsin EDTA, 100 units/ ml penicillin and 100µg/ml streptomycin.

Minced embryos were digested in a water bath at 37°C for 2-3 hrs. Large undigested tissue pieces after the digestion were removed using a cell strainer and the remaining suspension was centrifuged at 1200 x g for 5 min. Supernatant was carefully discarded and the cell pellet was reconstituted in a small volume of culture medium (CM) containing DMEM with 10%FCS, 100 units/ ml penicillin and 100µg/ml streptomycin. The cells were then counted as described in section 4.4.2.2 and plated in cell culture flasks (Costar) at a seeding density of 5000 to 6000 cells/cm^2 in CM. The cells were passaged and cryo-preserved as described in section 4.4.2.5.

4.4.4 Growing cells on cover slips

Avian lung cells and embryo cells were grown in 24 well cell culture plates (Costar) containing collagen coated and uncoated cover slips respectively. Approximately 10^5

cells per well in 1ml of appropriate medium were seeded and fixed when the cells were grown to 60-70% confluent after overnight incubation.

4.4.4.1 Cell fixing

Avian lung and embryo cells in cover slips were fixed either with 4% paraformaldehyde (Sigma) or acetone: methanol (1:1). After removing medium from the well, the cells were rinsed twice with PBS. Later 1 ml of 4% paraformaldehyde or 1ml of cold acetone: methanol (1:1) was added to each well and incubated at room temperature for 10 min. After decanting the fluid, cells were rinsed twice in PBS and stored at 4°C after adding 1ml of cold PBS till further processing.

4.4.5 Influenza receptor expression on cultured cells

4.4.5.1 Lectin-cytochemistry

Influenza receptor expression on primary cells was determined by a method previously described with minor modifications (Shinya et al., 2006), using Sambucus nigra Agglutinin (SNA) lectin which is specific to mammalian type sialic acid α2,6-Gal receptor, and Mackia amurensis Agglutinin (MAA II) lectin which is specific to avian type sialic acid α2,3-Gal receptor type. Paraformaldehyde fixed cells on cover slips were presoaked in TBS for 5 minutes.

Endogenous biotin was blocked with the use of a streptavidin blocking kit (Vector laboratories) following manufacturer's instructions. Briefly the cells were incubated with streptavidin solution for 15 min and rinsed briefly with TBS, then incubated for 15 min with the biotin solution. After rinsing with TBS the cells were incubated overnight at 4°C in the dark with fluorescein isothiocyanate (FITC) labelled SNA lectin, and biotinylated MAA II lectin (Vector laboratories), each at a concentration of 10 µg/ml. After three washes with Tris-buffered saline (TBS), the cells were incubated with 1:500 dilution of

streptavidin-AlexaFluor594 conjugate (Molecular Probes, Inc.) in TBS for 2 hrs at room temperature (RT). The cells were washed three times with TBS and then mounted with ProLong Gold antifade reagent with 4', 6-diamino-2- phenylindole, dihydrochloride (DAPI; Molecular Probes Inc.). Negative controls were performed without the primary reagents and the cells were imaged using a fluorescent microscope (Leica).

4.4.6 Staining primary cells for cell markers

Primary lung cells and embryo fibroblast cells from chicken and duck were stained for primary antibodies to pan-cytokeratin (epithelial cell marker), Vimentin (fibroblast marker), and desmin (muscle cell marker) to determine the type of cells in the culture. Cells were fixed with acetone: methanol as described in section 4.4.4.1 and stained using mouse monoclonal antibodies to Vimentin (V9) (Abcam) or pan Cytokeratin [PCK-26] (Abcam) or rabbit polyclonal antibody to desmin (Abcam) followed by visualization with Envision+ system-HRP (DAB) for mouse IgG (Dako) or for rabbit antibodies (Dako) following the manufacturer's instructions.

Cells were washed with TBS and endogenous peroxidise was blocked by incubating the cells with peroxidise for 10 min. After decanting the excess fluid the cells were rinsed gently with TBS and incubated with appropriate dilution of the primary antibody.

Cells were incubated either with 1:300 dilution of pan Cytokeratin antibody or 1:150 of anti desmin antibody or 1:100 of Vimentin antibody for 40 min at RT. Controls were performed with TBS with no primary antibody. After removing the excess fluid the cells were rinsed with TBS and incubated with appropriate labelled polymer (for desmin anti-rabbit IgG and for vimentin and pan cytokeratin anti-mouse anti IgG polymer was used) for 40 min at RT. After removing excess fluid cells were rinsed gently with TBS and incubated with substrate-chromogen solution for 10 min at RT. After rinsing the cells with TBS, cells were counterstained with haematoxylin for 1 min and rinsed with water.

4.4.7 Determination of multiplicity of infection (MOI)

4.4.7.1 Virus infection

Avian lung cells and embryo cells were grown in 24 well cell culture plates (Costar) and when the cells were confluent, medium from the cells was removed and the cells were rinsed twice with PBS. Cells were infected with low pathogenic influenza viruses to achieve multiplicity of infection (MOI) of either 1.0 or 0.1. Volume of the virus for infections was determined based on the titration in MDCK cells as described in Chapter 2. Appropriate volume of avian H2N3 virus or swine H1N1 virus was used per each 24 well to achieve MOI of 1.0 or 0.1.

Two wells of each cell type were infected with avian or swine influenza virus at MOI of 1.0 or 0.1 by adding an appropriate amount of virus in 1ml of infection medium. Control cells were incubated with the infection medium without any of the virus. After 2 hr incubation with virus, medium from the wells was removed and cells were rinsed three times with PBS.

The plates were incubated for a further 4 hrs after adding 1ml of fresh infection medium to all the wells. Cells were then fixed with acetone: methanol as described in section 4.4.4.1.

4.4.7.2 Immunocytochemical staining

Fixed cells were stained for influenza viral protein expression using a primary mouse monoclonal antibody to influenza nucleoprotein (NP) (Abcam) followed by visualization with Envision+ system-HRP (DAB) for mouse IgG (Dako) as described in section 4.4.6.

4.5 Results

4.5.1 Ex vivo organ cultures

4.5.1.1 Tracheal organ cultures (TOCs)

Tracheal organ cultures (TOC) constructed from chicken and duck trachea were cultured for up to 96 hrs. Morphology of the TOCs was near normal at 24 hrs post-culture, with intact mucous membranes and evidence of cilia. By 48 hrs post-culture, the cilia were not clearly visible with minor changes of degeneration in the epithelial surface.

By 72 hrs post-culture, tracheal mucosa was devoid of cilia and degenerative changes characterized by denuded mucosa were evident (Figure 4.5-1). After 96 hours of culture, TOCs exhibited gross areas of tissue degeneration and necrosis with complete loss of tissue architecture.

4.5.1.2 Intestinal organ cultures (IOC)

Organ cultures of small and large intestines constructed from chicken and ducks were cultured for up to 96 hrs. Tissue sections from uninfected control intestinal organ cultures harvested at 24, 48, 72 and 96 hrs post culture were examined for tissue integrity. Small intestine organ cultures showed near normal morphology at 24 hrs post-culture and by 48 hrs tissues exhibited gross areas of degeneration and loss of mucosal integrity (Figure 4.5-2).

At 72 hrs post-culture, small intestinal tissues had completely disintegrated with complete loss of tissue architecture. Similar changes were found in the large intestine organ cultures (Figure 4.5-3).

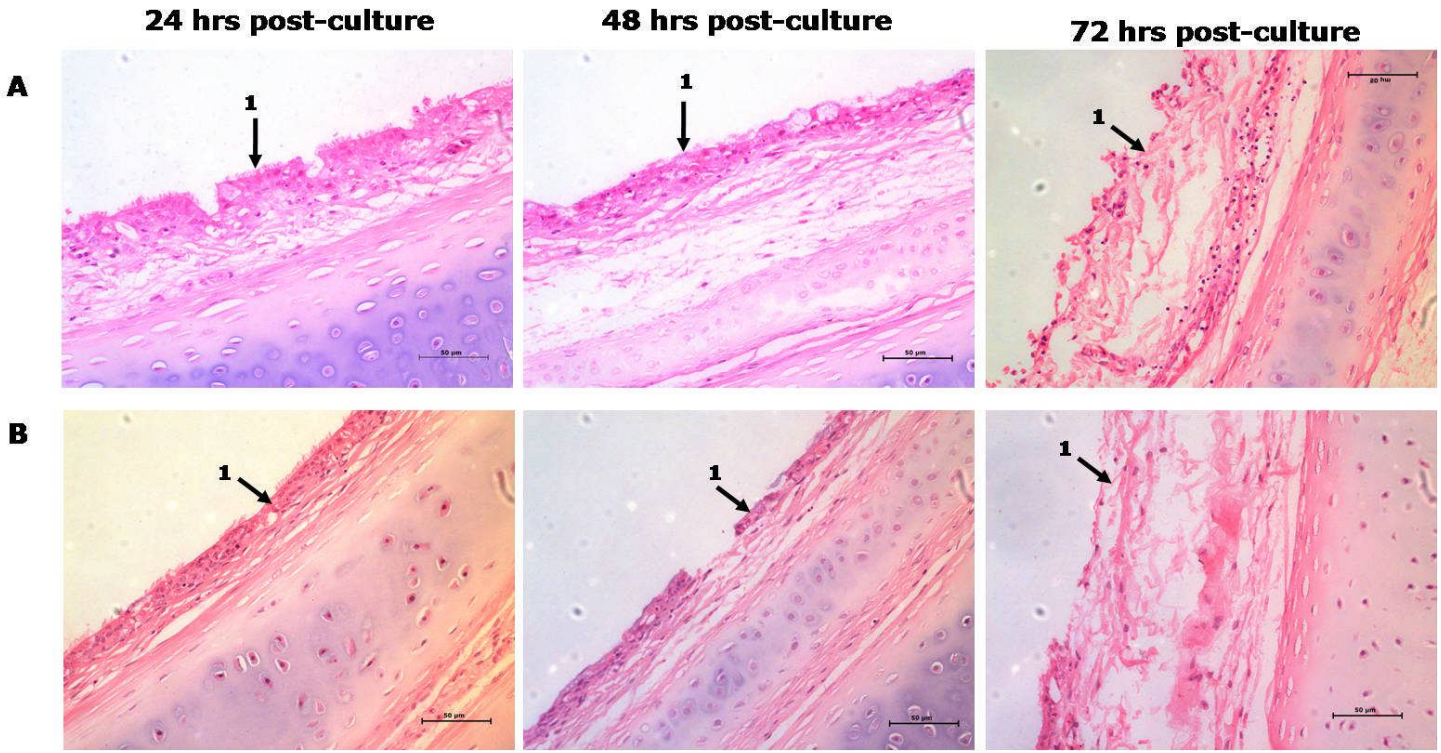


Figure 4.5-1 Histology of Tracheal organ cultures following in-vitro culture

Histological appearance of chicken (A) and duck (B) tracheal organ cultures following *in-vitro* culture is shown. Tissues appeared near normal at 24 hrs post-culture, loss of cilia and few signs of degeneration at 48 hrs and complete loss of cilia and denuded mucosa at 72 hrs post-culture. 1. Tracheal mucous membrane.

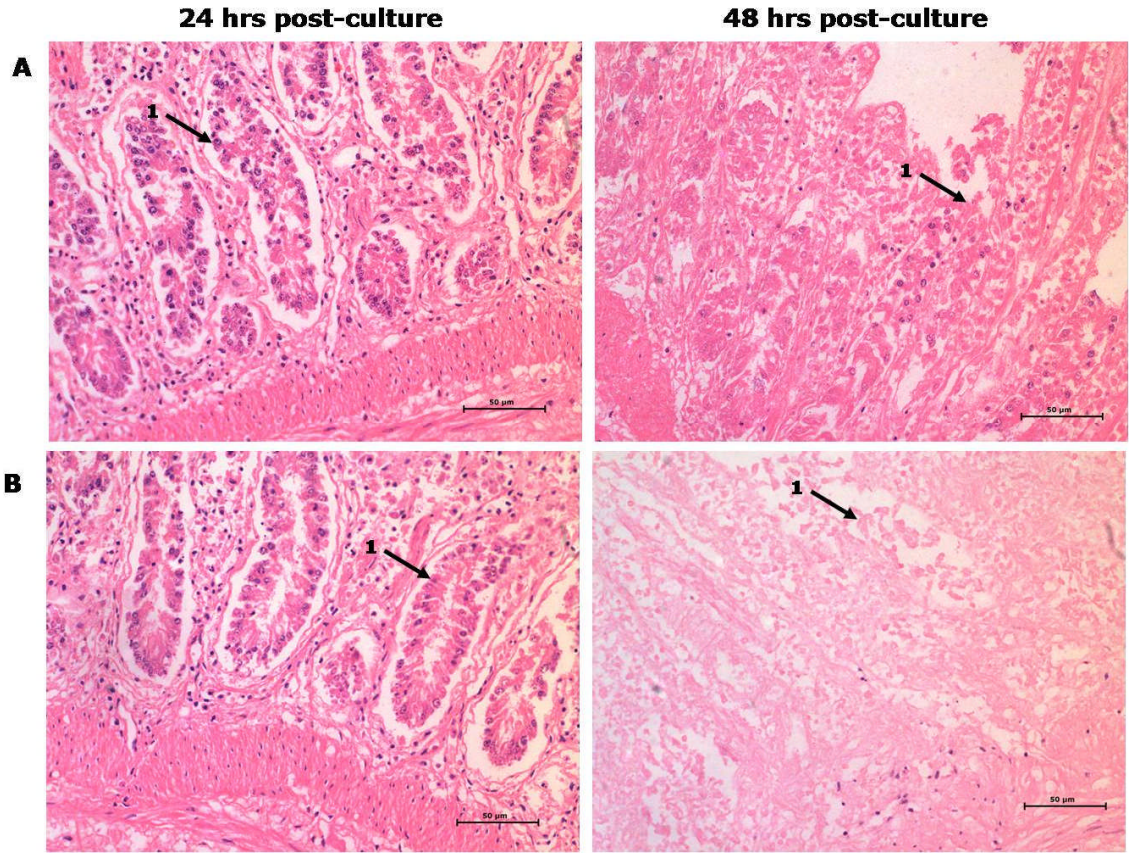


Figure 4.5-2 Histology of small intestine organ cultures following *in-vitro* culture

Histological appearance of chicken (A) and duck (B) small intestine organ cultures following *in-vitro* culture is shown. Tissues appeared near normal at 24 hrs post-culture, at 48 hrs post-culture, gross areas of degeneration and loss of mucosal integrity was found. 1. Intestinal mucous membrane.

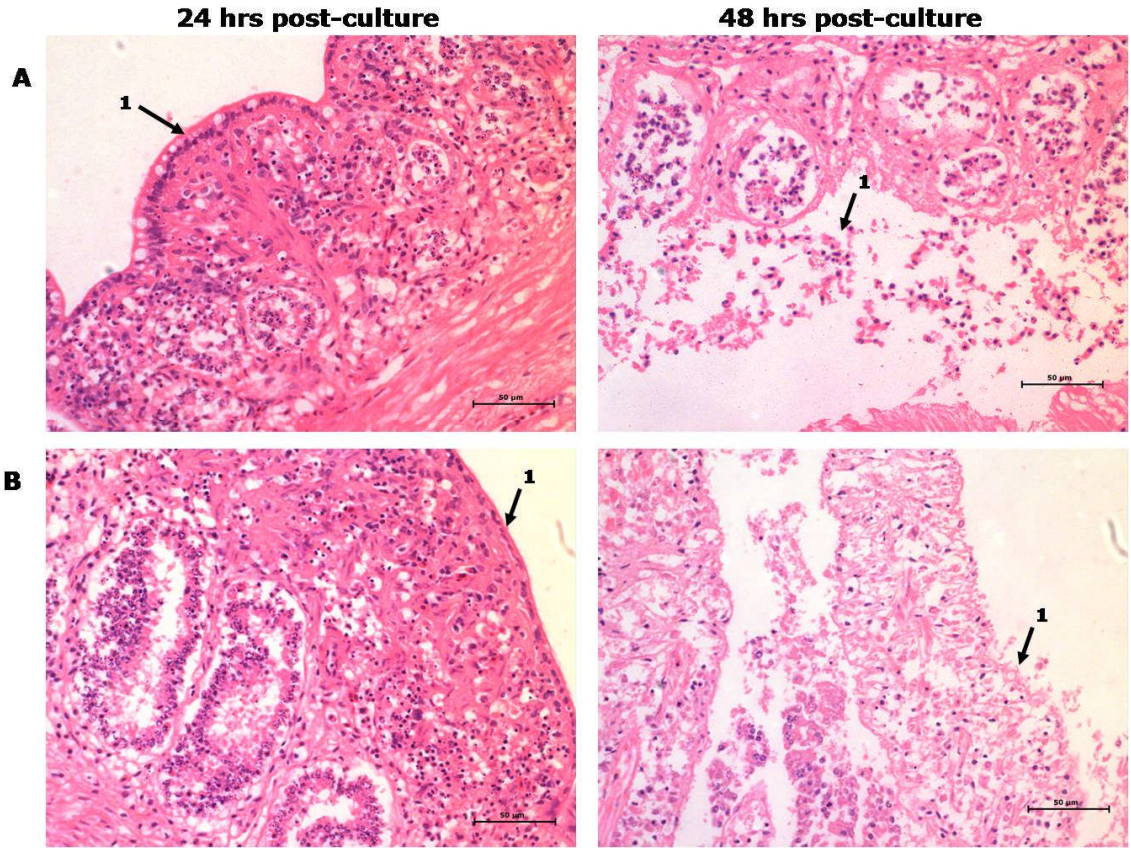


Figure 4.5-3 Histology of large intestine organ cultures following *in-vitro* culture

Histological appearance of chicken (A) and duck (B) large intestine organ cultures following *in-vitro* culture is shown. Tissues appeared near normal at 24 hrs post-culture, at 48 hrs post-culture, gross areas of degeneration and loss of mucosal integrity was found. 1. Intestinal mucous membrane.

4.5.1.3 Infection of organ cultures

Tissue sections of infected and control organ cultures harvested at 24 hrs post-infection were immuno-stained for influenza viral nucleoprotein expression. Discrete areas of influenza nucleoprotein expression were noticed in chicken and duck TOCs infected with avian H2N3 and swine H1N1 viruses at 24 hrs post-infection (Figure 4.5-4).

Viral nucleoprotein expression was observed in the mucosa and the sub-mucosa of the infected TOCs. Both viruses produced a low level of infection in chicken and duck TOCs. Chicken and duck small intestine organ cultures infected with avian H2N3 showed positive nucleoprotein staining at 24 hrs post-infection and no staining was observed with swine H1N1 infection (Figure 4.5-5). A similar staining pattern was also found in chicken and duck large intestine organ cultures (Figure 4.5-6).

Abundant nucleoprotein staining was observed in duck small intestine compared to chicken small intestine while nucleoprotein staining was more abundant in chicken large intestine compared to duck large intestine. However, infection of organ cultures was localized to a few areas following infection with avian H2N3 or swine H1N1 viruses and was not uniform as only a few of the serial sections showed nucleoprotein staining. Some of the infected tissue sections had discontinuous mucous membranes and inconsistent staining pattern making it difficult to compare between species and between virus types.

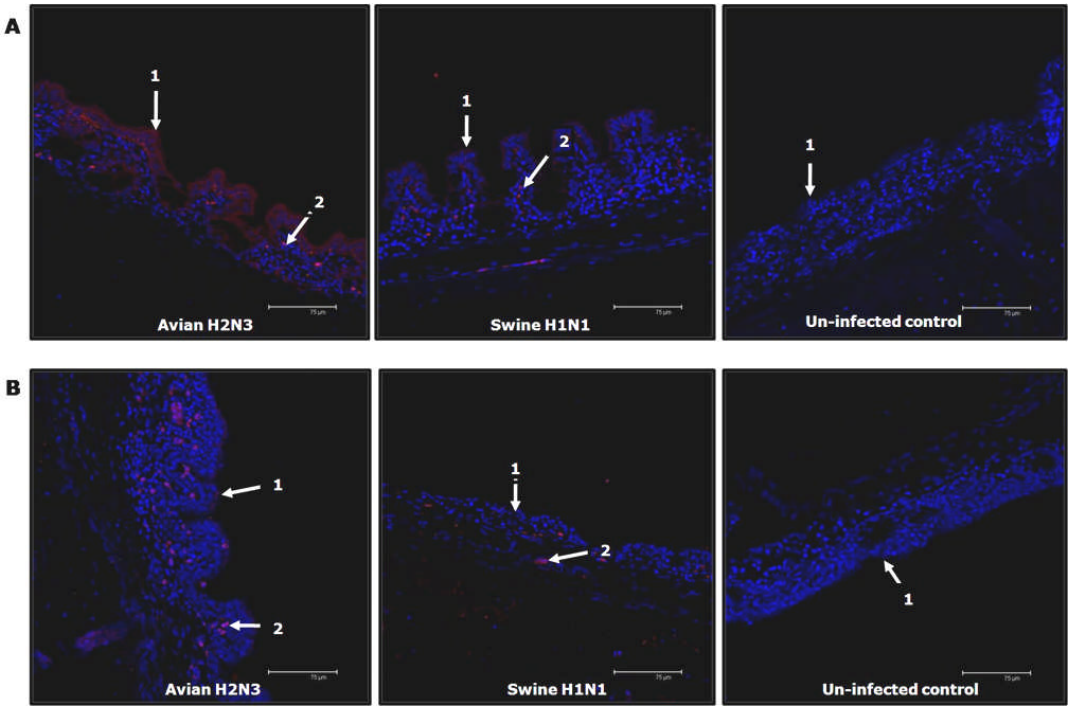


Figure 4.5-4 Immuno-fluorescent staining of *ex-vivo* tracheal organ cultures

Immuno-fluorescent staining of infected and control chicken (A) and duck (B) TOCs 24 hrs post-infection for influenza viral nucleoprotein expression showing positive staining (red colour) with avian H2N3 and swine H1N1 viruses. 1. Mucosal lining of the trachea 2. Cells expressing influenza viral nucleoprotein.

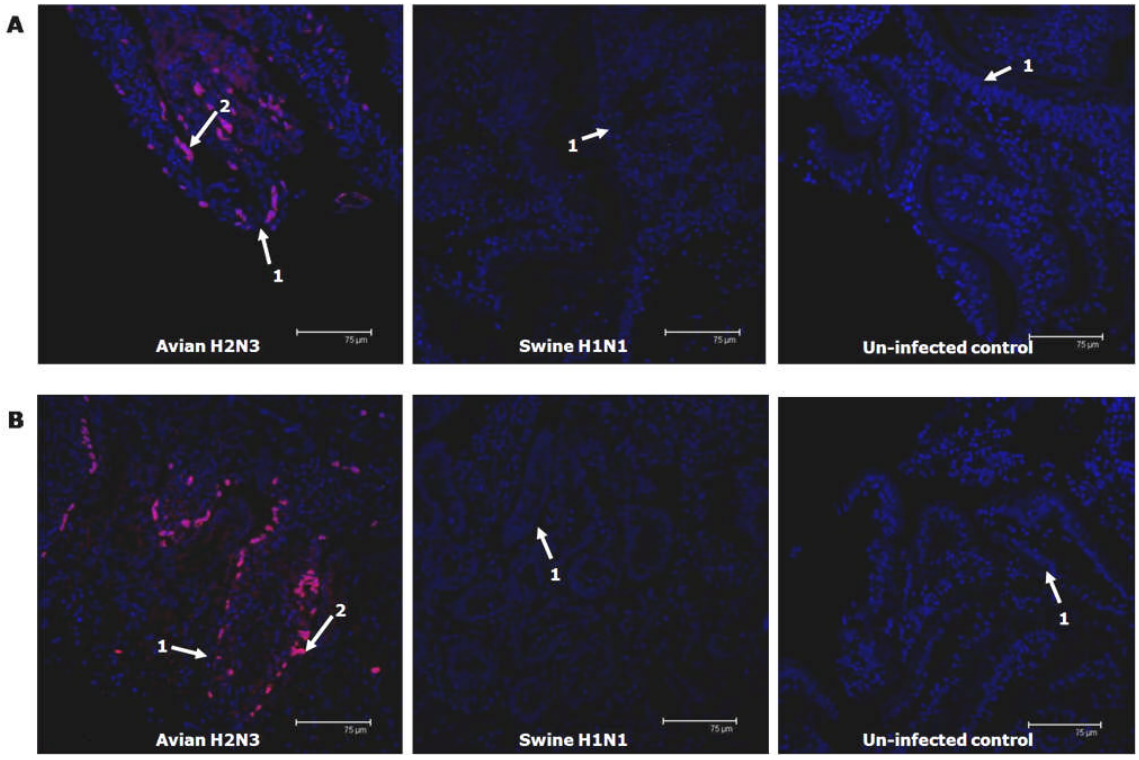


Figure 4.5-5 Immuno-fluorescent staining of *ex-vivo* small intestine organ cultures

Immuno-fluorescent staining of infected and control chicken (A) and duck (B) small-intestine organ cultures 24 hrs post-infection for influenza viral nucleoprotein expression showing positive staining (red colour) with avian H2N3 and swine H1N1 viruses. 1. Intestinal mucosal lining 2. Cells expressing influenza viral nucleoprotein.

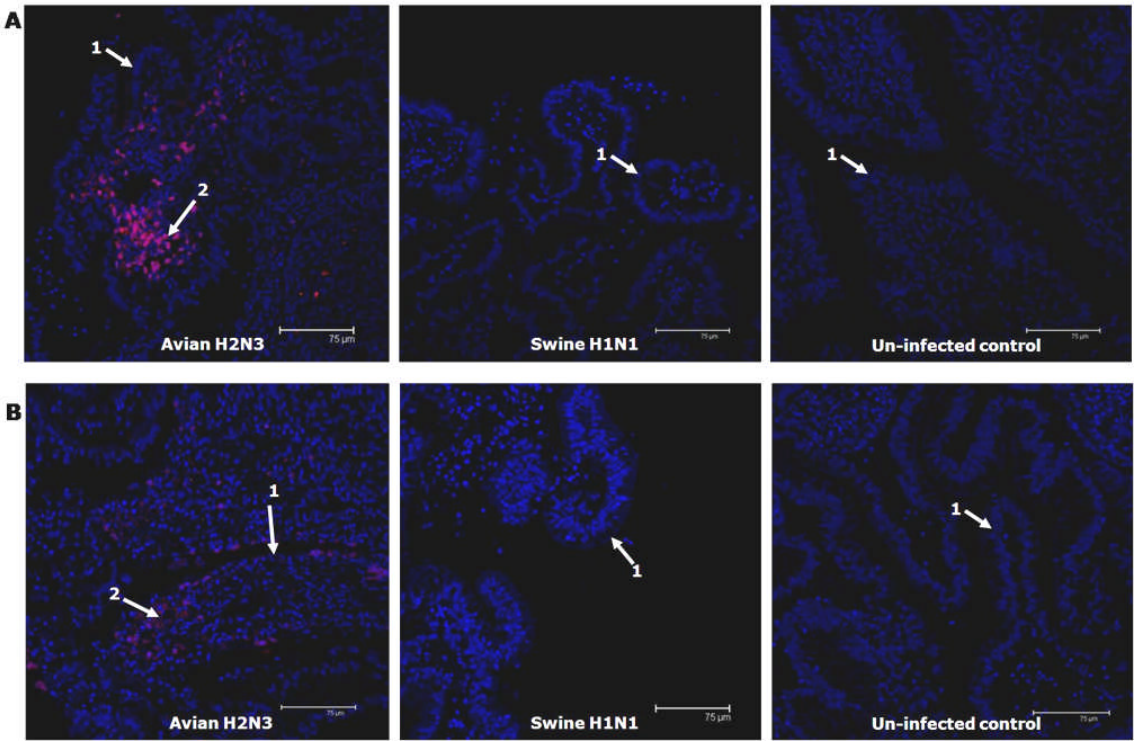


Figure 4.5-6 Immuno-fluorescent staining of *ex-vivo* large-intestine organ cultures

Immuno-fluorescent staining of infected and control chicken (A) and duck (B) large-intestine organ cultures 24 hrs post-infection for influenza viral nucleoprotein expression showing positive staining (red colour) with avian H2N3 and swine H1N1 viruses. 1. Intestinal mucosal lining 2. Cells expressing influenza viral nucleoprotein.

4.5.2 Primary avian cell cultures

Primary lung and embryo cells were successfully extracted and grown in culture. Tracheal epithelial cells extracted from chicken and duck tracheae initially looked healthy at the time of extraction, but the majority of the epithelial cells died after 2-3 days of culture and failed to produce a confluent monolayer.

4.5.3 Influenza receptor expression on avian primary cells

4.5.3.1 Lectin-cytochemistry

Primary lung and embryo cells from chicken and duck were stained with linkage specific lectins to study the expression of influenza specific receptors. Presence of SAα2,6-Gal (SNA binding) and SAα2,3-Gal (MAAII binding) receptors in all the cells was observed (Figure 4.5-7). Co-expression of both SAα2,6-Gal and SAα2,3-Gal receptor types was found in chicken and duck lung and embryo cells. However the relative distribution of SAα2,6-Gal and SAα2,3-Gal receptor types was different between individual cells and the proportions of the two receptor types was also variable.

4.5.4 Staining primary cells for cell markers

Primary lung and embryo cells from chicken and duck were immuno-stained with primary antibodies to cell specific markers of epithelial cells (pan-cytokeratin, PCK-26), fibroblasts (Vimentin-V9) and muscle cells (desmin). All the cells stained exhibited a strong positive staining to Vimentin antibody (Figure 4.5-9). Chicken and duck embryo and lung cells did not show any staining with either pan-cytokeratin or desmin antibodies.

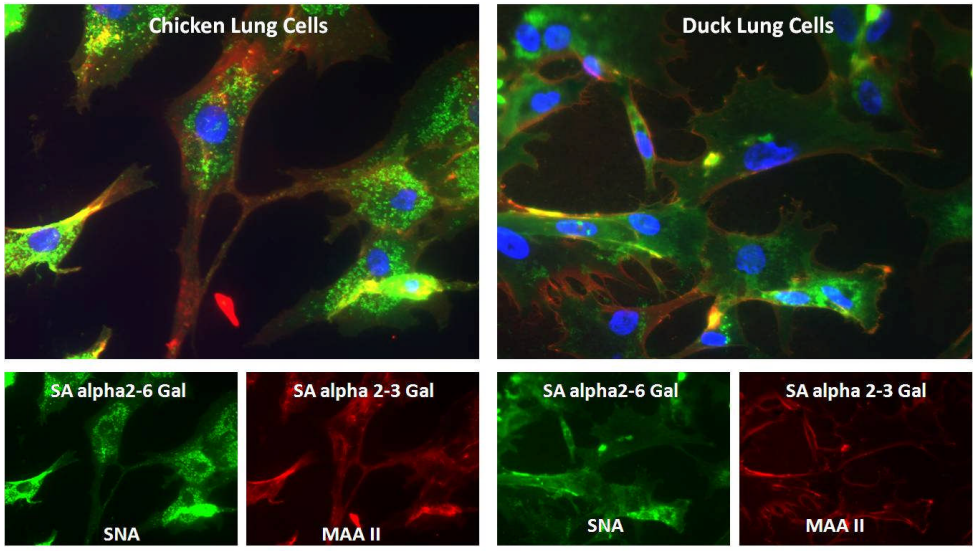


Figure 4.5-7 Influenza virus receptor distribution on avian primary lung cells

Lectin staining of avian primary lung cells revealed that both chicken and duck lung cells expressed mammalian influenza SAα2,6-Gal receptor (green, SNA binding) and avian influenza SAα2,3-Gal receptor (red MAAII binding) receptor types.

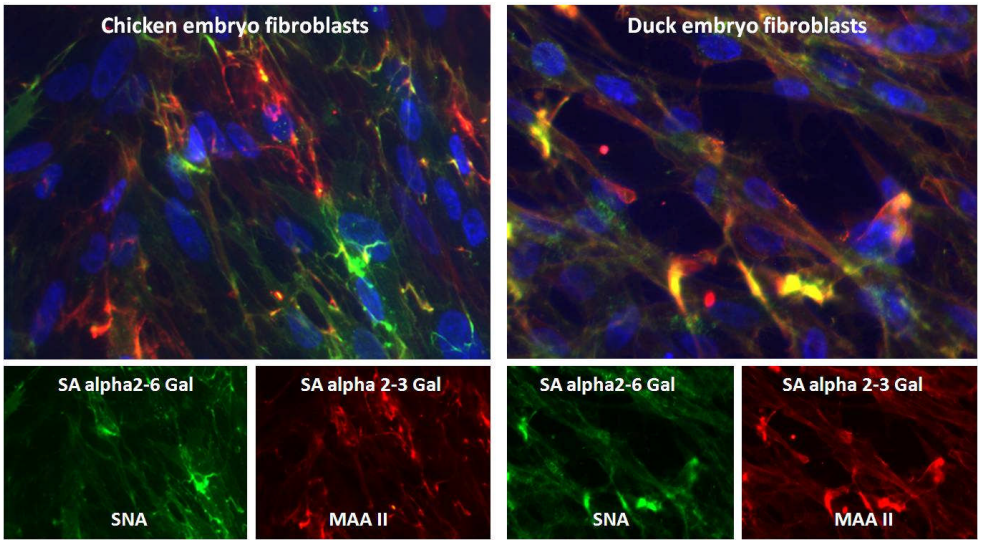


Figure 4.5-8 Influenza virus receptor distribution on avian primary embryo cells

Lectin staining of avian primary cells revealed that both chicken and duck embryo cells expressed SAα2,6-Gal (green, SNA binding) and SAα2,3-Gal (red MAAII binding) receptor types.

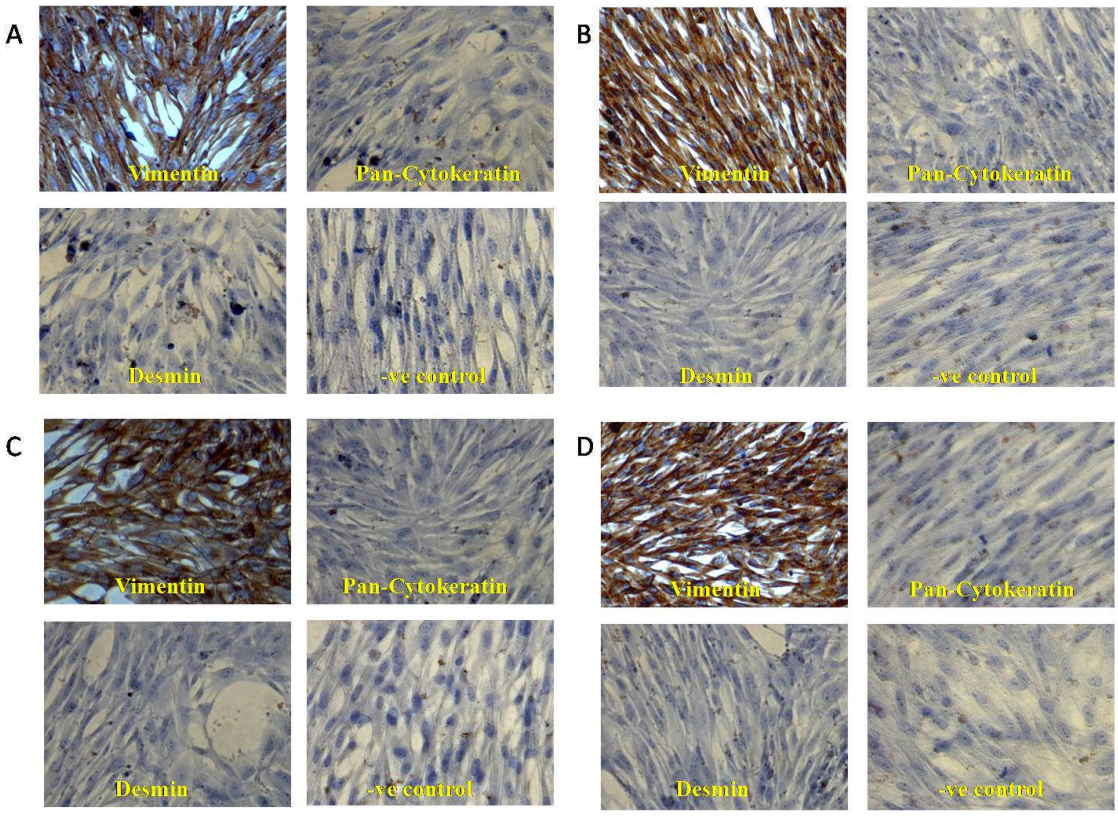


Figure 4.5-9 Immunocytochemical staining of cells for type specific markers

Immunocytochemical staining of avian primary cells with specific markers for fibroblasts (Vimentin), epithelial cells (pancytokeratin) and muscle cells (Desmin) using Dako Envision kit that uses HRP-DAB system. Positive cells appear brown in colour (DAB). Chicken (A) & Duck (C) embryo cells and Chicken (B) & duck (D) lung primary cells were predominantly vimentin positive cells (fibroblasts) and were negative for pancytokeratin and desmin.

4.5.5 Infection of primary cells with Influenza A

4.5.5.1 Avian lung cells

Chicken and duck lung cells were infected with low pathogenic influenza viruses and stained for viral protein expression at 6 hrs post-infection. Based on the viral nucleoprotein expression, both chicken and duck lung cells were found to be susceptible to viral infection. Avian H2N3 and swine H1N1 viruses produced comparable levels of infection in both chicken and duck lung cells (Figure 4.5-10).

Number of infected cells was comparable and no differences were observed in susceptibility to infection between chicken and duck cells. Levels of infection in chicken and duck lung cells produced by avian H2N3 and swine H1N1were as predicted from virus titration in MDCK cells. Non-infected control cells did not show any signs of staining.

4.5.5.2 Avian embryo cells

Chicken, Pekin and Mallard duck embryo cells were infected with low pathogenic influenza viruses and stained for viral protein expression 6 hrs post-infection. Avian H2N3 (Figure 4.5-11) and swine H1N1 (Figure 4.5-12) viruses produced comparable levels of infection in all the three embryo cells. Un-infected control cells did not show any signs of staining.

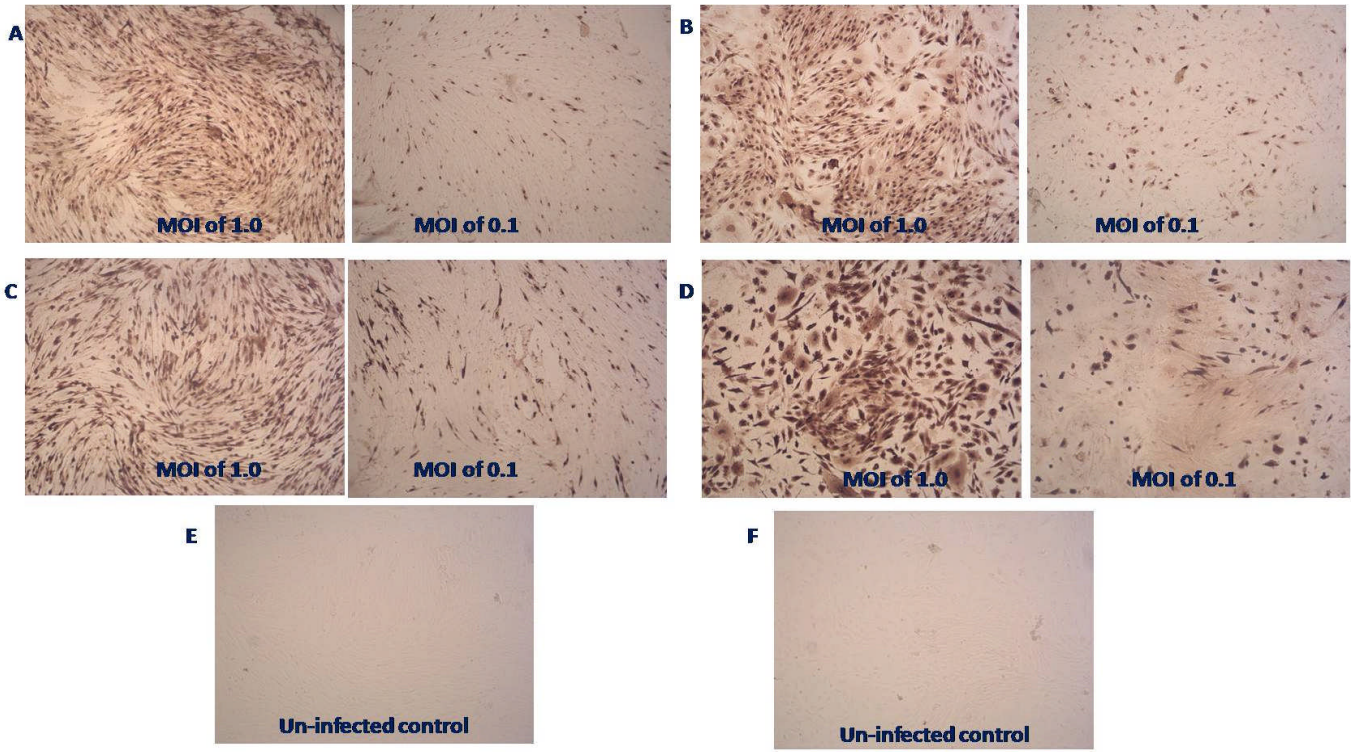


Figure 4.5-10 Determination of MOI of influenza viruses in avian lung cells

Immuno-cytochemical staining of avian lung cells for influenza viral nucleoprotein expression. Chicken (A) and duck (B) lung cells exhibited similar levels of nucleoprotein expression following infection with avian H2N3 virus at MOI of 1.0 and 0.1. A similar pattern was also found in chicken (C) and duck (D) lung cells infected with swine H1N1 virus. Un-infected chicken (E) and duck (F) lung cells did not show any staining.

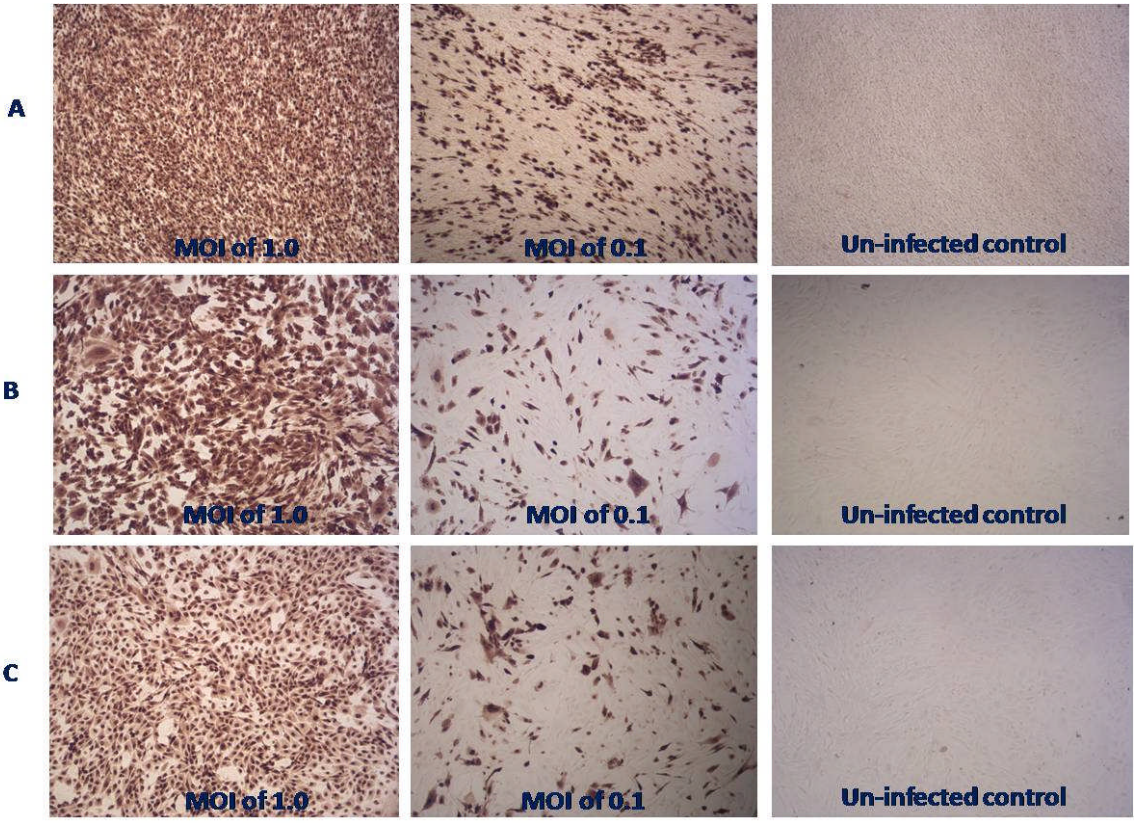


Figure 4.5-11 Determination of MOI of avian H2N3 virus in avian embryo cells

Immuno-cytochemical staining of avian embryo cells for influenza viral nucleoprotein expression. Chicken (A), Pekin duck (B) and Mallard duck (C) embryo cells exhibited similar levels of nucleoprotein expression following infection with avian H2N3 virus at MOI of 1.0 and 0.1. Un-infected chicken (A), Pekin duck (B) and Mallard duck (C) embryo cells did not show any staining.

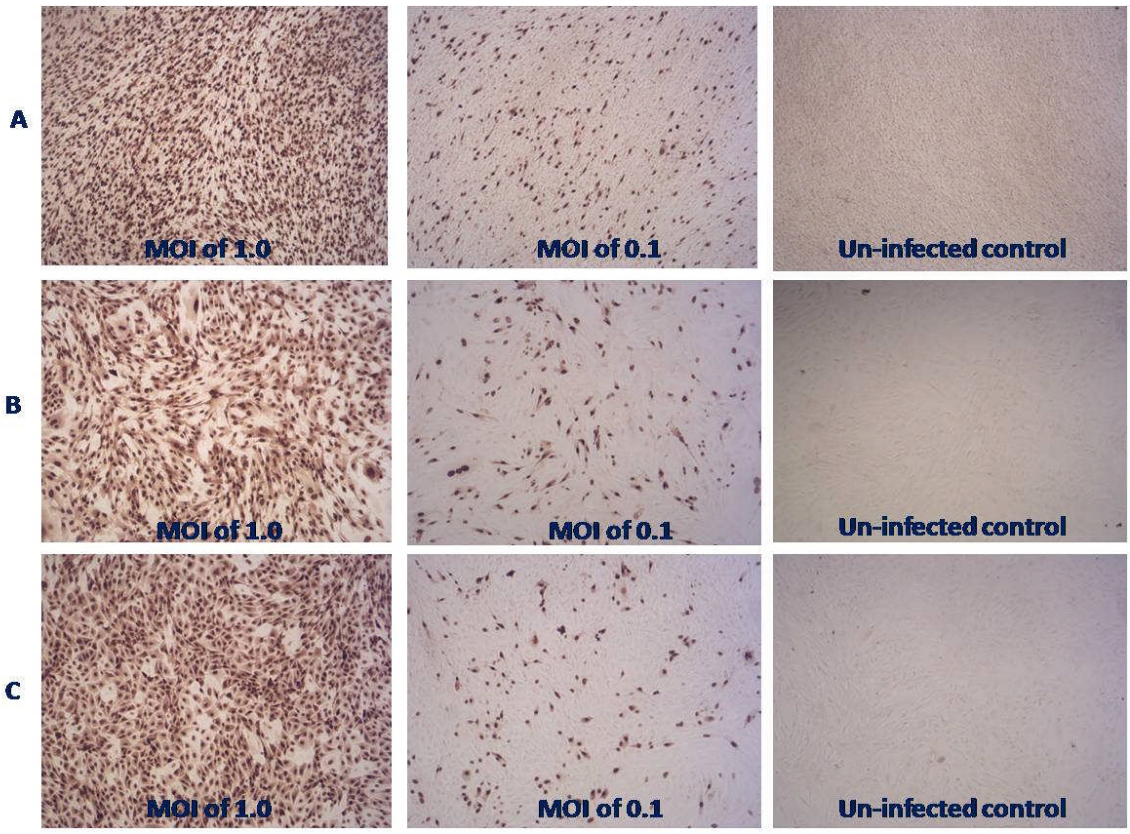


Figure 4.5-12 Determination of MOI of swine H1N1 in avian lung cells

Immuno-cytochemical staining of avian embryo cells for influenza viral nucleoprotein expression. Chicken (A), Pekin duck (B) and Mallard duck (C) embryo cells exhibited similar levels of exhibited similar levels of nucleoprotein expression following infection with swine H1N1 virus at MOI of 1.0 and 0.1. Un-infected chicken (A), Pekin duck (B) and Mallard duck (C) embryo cells did not show any staining.

4.6 Discussion

Tracheal organ cultures (TOC) and IOCs constructed from chicken and duck could be maintained in culture with near normal morphology for up to 48 hrs and 24 hrs respectively. Significant histo-pathological changes were found in TOCs and IOCs cultured beyond these times. These findings suggest that these organ cultures are suitable for study of early responses to influenza viruses. However, these organ cultures are not suitable for prolonged cultivation.

However, a recent study compared host gene expression in TOC and *in-vivo* chicken trachea following influenza infection and found that early host immune responses in TOCs were masked by the wound healing responses in TOCs (Reemers et al. 2009a). Excision of the trachea into rings was found to result in gradual loss of ciliary activity and onset of histopathological changes. A previous study reported cellular and processing damage consisting of tissue shearing, extruded ciliated epithelial cells or the presence of denuded basement membrane in canine tracheal organ cultures (Anderton, Maskell, & Preston 2004).

The critical events occurring during early infection in organ cultures are difficult to study due to the presence of heterogeneous cell population (Zaffuto, Estevez, & Afonso 2008). Chicken and duck tracheae expressed both mammalian type SAα2,6-Gal and avian type SAα2,3-Gal influenza receptors (Chapter 3).

This finding correlated with the susceptibility to infection with avian H2N3 and swine H1N1 influenza viruses as evidenced by detection of viral nucleoprotein by immuno-histochemical staining. Similarly exclusive distribution of avian type SAα2,3-Gal receptor type across the epithelial lining of both chicken and duck intestines correlated with susceptibility to infection with avian H2N3 virus but not with swine H1N1. However, infection of organ cultures was localized to a few areas and immuno-histochemical detection of viral antigen was not consistent between TOCs. This may be

partly due to denudation of infected mucosa during tissue processing, such that cells susceptible to influenza infection were lost.

Earlier studies found that although tracheal organ cultures can be useful to investigate the effect of the virus on the respiratory epithelium (Leeming et al. 2006), their use to study host responses to virus infection are limited (Reemers et al. 2009a). These facts demonstrate the need to optimize culturing conditions of the TOCs in order to improve longevity and consistency of infection. A Recent study has reported an improved air-interface ex-vivo organ culture system for pig trachea, which supported productive influenza infection and remained healthy in culture for up to 7 days (Nunes et al. 2010).

Primary lung and embryo cells from chicken and duck were stained with linkage specific lectins to study the expression of influenza specific receptors. Abundant binding of SNA and MAAII lectins that are specific SAα2,6-Gal and SAα2,3-Gal receptor types was found in all the primary cells tested. Avian influenza viruses have been shown to preferentially bind to SA receptors that are linked to galactose by an α2,3 linkage (SAα2,3-Gal), while human and classical swine viruses show preference for receptors with an α2,6 linkage (SAα2,6-Gal) (Matrosovich et al. 2004). Abundant presence of avian and mammalian influenza receptor types indicate that the primary cell culture models comprising of avian lung and embryo cells can be potentially infected by these viruses.

As predicted chicken and duck lung and embryo cells were found to be susceptible to infection by avian H2N3 and swine H1N1 viruses as evidenced by the nucleoprotein expression. The number of cells positive for virus nucleoprotein in infected lung and embryo cells was comparable and no differences were observed in the staining between species or between viruses. As the cell culture models shows neither differences in the level of virus infection, nor any changes observed in cell response to infection could be considered to be caused by differences in host response. Using cell cultures has the advantages of consistent levels of infection, susceptibility to wide range of influenza

viruses and the ability to grow for longer times in culture without any degenerative changes over the organ cultures. The disadvantage of cell monolayer cultures is that they less closely mimic the natural state compared to organ cultures (Leeming et al. 2006). However, the advantages greatly outweigh these disadvantages. Hence, primary cell cultures comprising of avian lung and embryo cells were chosen for further studies of host responses to influenza virus infections in this project.

Chapter 5

Cellular responses to influenza virus infection

5.1 Summary

Cellular responses in chicken and duck primary lung and embryo cells following infection with a range of influenza viruses were studied. Primary cells from duck exhibited more rapid and abundant cell death compared to chicken cells. Cell metabolic activity following virus infection was measured by an MTT assay. Duck cells consistently showed significantly lower metabolic activity compared to chicken cells following infection with a range of influenza viruses. Influenza matrix gene expression in culture supernatants was analyzed by qRT-PCR. Matrix gene expression in chicken and duck cell culture supernatants was no different between the two species. However, duck cells produced significantly lower infective virus compared to chicken cells. These findings suggested that rapid cell death response of duck cells may interfere with viral assembly rather than the viral gene production. Rapid cell death response of duck cells with a reduced infective virus production observed in-vitro could be a potential in-vivo host defense mechanism in ducks to influenza virus infection.

5.2 Introduction

Influenza viruses after entry must successfully co-opt host cell processes for their replication. Influenza virus infection causes activation of antiviral cellular pathways including interferon response, which are down-modulated by the virus through accessory proteins, such as NS1 and PB1-F2 (Garcia-Sastre 2001). These virus-host interactions play a major role in the pathogenesis and development of clinical signs in infected animals. Influenza viruses from one species may not always effectively multiply in a different host due to species specific restriction. For example many avian influenza viruses can infect mouse cells but cannot replicate because of differences in viral polymerase (Baigent & McCauley 2003).

However, some of the influenza virus strains have evolved to infect a wide range of avian and mammalian hosts. The highly pathogenic avian influenza H5N1 virus is an

example of a zoonotic influenza capable of transmission from animal reservoir species to humans crossing the species barrier (Kuiken et al. 2005).

5.3 Cytopathic effect (CPE) of viruses

Virus infection of cells often leads to certain morphological changes consisting of cell rounding, disorientation, swelling or shrinking, death, detachment from the surface, etc, and such detectable changes in the host cell are referred to as cytopathic effects (CPE) of the virus. Influenza viruses were shown to cause cell death of infected tissue culture cells (Takizawa et al. 1993) and culture of *in-vitro* lymphocytes (Van Campen, Easterday, & Hinshaw 1989). Influenza A virus infection of monocytes and polymorphonuclear leukocytes *in-vitro*, stimulates an oxidative burst leading to subsequent depression of the oxidative metabolic response and bactericidal capacity of these phagocytic cells (Abramson et al. 1982).

Influenza virus infection leads to death of infected cells and is often associated with damage of epithelial cells of the respiratory tract in humans (Sweet & Smith 1980). Destruction of lymphoid tissues and severe systemic lymphoid depletion is usually found in highly pathogenic avian influenza infections of chickens (Van Campen, Easterday, & Hinshaw 1989). Influenza virus infection of cells causes DNA damage leading to cell death or chromosomal aberrations or mutations (Vijaya Lakshmi et al. 1999). Influenza A virus induces apoptotic death in infected epithelial, lymphocyte, and phagocytic cells, and apoptosis plays a major role in the tissue damage during infection (Brydon, Morris, & Sweet 2005;McLean et al. 2008;Schultz-Cherry, Krug, & Hinshaw 1998).

5.3.1 Measurement of cell metabolic activity

Influenza virus infection of MDCK cells causes changes in the cell metabolic activity (Genzel et al. 2004). Metabolic activity of cultured cells can be measured by 3-(4, 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide reduction (MTT) assay (Hanelt,

Gareis, & Kollarczik 1994). This assay measures the reduction of yellow tetrazolium salt MTT by mitochondrial succinate dehydrogenase of metabolically active cells to an insoluble dark purple coloured formazan product. Earlier studies reported use of MTT assay for titrating influenza viruses by infection of MDCK cells followed by the measurement of cell metabolic activity by a colorimetric MTT assay (Levi, Beeor-Tzahar, & Arnon 1995).

5.4 Measurement of virus production from infected cells

Measurement of influenza virus production is essential to understand the differences in virus replication between different hosts and virus types. Influenza virus production in embryos or cell cultures is measured by titrating the viruses using several methods and the earliest methods include haemagglutination test (Hirst 1942) and a modified photoelectric method for influenza haemagglutination (Belyavin et al. 1951). Later, an adsorption-haemagglutination assay was described using monkey kidney cell culture (Vogel & Shelokov 1957).

5.4.1 Plaque assays

Many strains of Influenza A viruses have been shown to form plaques in infected cell cultures which are produced as a consequence of virus replication leading to cell death. However the plaque forming characteristics are genetically determined and differ between strains of influenza viruses (Beare & Keast 1974).

Plaque assay is a widely used quantitative system used to determine the amount of infective virus using cell lines like MDCKs (Appleyard & Maber 1974;Gaush & Smith 1968;Huprikar & Rabinowitz 1980). However plaque assay for virus titration is less sensitive than antibody methods and animal inoculation tests (Moe, Lambert, & Lupton 1981).

5.4.2 Quantification of viral gene expression by qRT-PCR

Real-time polymerase chain reaction (PCR) for the quantification of gene expression and DNA copy number measurements was first documented in 1993 (Higuchi et al. 1993). During PCR, DNA copies are generated in an exponential way and quantification is based on the measurement of the cycle of PCR at which threshold of detection is reached which is termed the threshold cycle (Ct). The TaqMan approach measures an accumulating PCR product in real time by using an internal fluorogenic TaqMan oligonucleotide probe that is cleaved by the endogenous 5 -3 nuclease activity of Taq polymerase (Freeman, Walker, & Vrana 1999).

The oligonucleotide TaqMan probe labeled with two fluorescent dyes specifically binds within the target sequence defined by specific forward and reverse PCR primers. As long as the probe is intact, the emission of the reporter dye (e.g. 6-carboxy-fluorescein, FAM) at the 5' end is quenched by the second fluorescence dye (e.g. 6-carboxy-tetramethyl-rhodamine, TAMRA) at the 3' end. During the extension phase of PCR, the polymerase cleaves the TaqMan probe, resulting in a release of reporter dye. The increasing amount of reporter dye fluorescence emission is measured by a sequence detection system (SDS) with dedicated software.

Quantitative polymerase chain reaction (qPCR) based on the influenza virus Matrix gene, has been described previously, to measure the viral gene expression in cell cultures following infection (Spackman et al. 2002;Ward et al. 2004;Youil et al. 2004). However, qPCR assay measure viral gene production and not the production of fully infective virus particles. Based on the production of influenza matrix gene measured by qRT-PCR assay, MDCK cells were found to produce greater viral gene copies compared to Vero cells, following virus infection (Youil et al. 2004). Such differences in the dynamics of virus replication point to species specific differences between canines and primates to influenza infection.

Study of cellular responses following virus infection could be useful to understand the basis of the relative resistance and susceptibility of ducks and chickens to influenza virus infection. To understand differences in cellular responses between chicken and duck, infections of primary cells with a range of influenza viruses was performed. The effect of influenza virus infection on cell metabolic activity was studied and the pattern of infection was characterized by measurement of virus production.

5.5 Materials and methods

5.5.1 Cell infection

5.5.1.1 Classical swine and LPAI infections

Monolayers of primary lung and embryo cells from chicken and duck were grown in 6 well cell culture plates (Costar) as described in Chapter 2. Cells were rinsed with PBS and infected with appropriate amounts of the virus in serum free infection medium (IM) with LPAI or classical swine influenza viruses at multiplicity of infection (MOI) of 1.0 as described in Chapter 4. Negative controls were performed by sham infection with infection medium without virus. After 2 hr incubation of the virus, the medium was removed and cells were rinsed three times with PBS. After adding 3 ml of fresh infection medium to all the wells, 500 µl of supernatant was collected in micro-centrifuge tubes and was stored at -80°C until further use. Plates were incubated further and 500 µl of supernatant was collected at 24 and 48 hrs post-infection and stored at -80°C. Cells were observed for evidence of cytopathic changes and images were captured at 24 and 48 hrs post-infection by photo microscopy with an inverted phase contrast microscope (Leica).

5.5.1.2 Highly pathogenic virus infections

Chicken and duck lung cells in 6 well cell culture plates were infected with 250 µl of H5N1 50-92 or 25 µl of ty-Ty virus in 2ml of infection medium to achieve a MOI of 1.0.

Cells were observed for cytopathic changes and supernatants were collected as described in section 5.5.1.1 above.

5.5.2 Measurement of cell metabolic activity by MTT assay

Measurement of metabolic activity of primary cells following influenza virus infection was carried out by 3-(4, 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide reduction (MTT) assay. Primary chicken and duck lung cells were seeded in two 96-well cell culture plates (Nunc) at a seeding density of 5000 cells per well. Following 24 hrs incubation at 37ºC, medium from the cells was removed and the cells were rinsed three times with PBS. Cells were infected with avian H2N3 or swine H1N1 viruses at a range of MOI from 0.15 to 5.0 in separate plates, using quadruplicate wells for each MOI. Fifty microlitres of IM containing appropriate amounts of virus was added to the wells. Four wells of each cell type were sham infected with IM without virus.

The plates were incubated for 24 hrs at 37ºC and the metabolic activity of the cells was analyzed at 24 hrs post-infection using CellTiter 96, a non-radioactive cell proliferation assay (Promega) according to the manufacturer's instructions. Briefly 15 µl of the dye solution was added to all the wells and the plates were incubated at 37ºC for 4 hrs. One hundred microlitres of solubilisation solution/stop mix was then added to each well before incubating the plate for 1hr at 37ºC. Following incubation, contents were mixed gently avoiding formation of any bubbles and absorbance was recorded in a plate reader (Labsystems) at 590nm.

5.5.3 Quantification of virus production

5.5.3.1 Measurement of Matrix gene copy number by quantitative RT-PCR

Quantification of influenza virus matrix gene copy number in culture supernatants was performed by a real-time reverse transcription PCR (RT-PCR) assay described

previously (Spackman, et al 2002). This assay targets a highly conserved sequence of the matrix gene in the M1 protein coding region. A one-step RT-PCR assay using influenza virus matrix gene-specific PCR primers and hydrolysis probe was performed using Superscript® III Platinum® One-Step qRT-PCR Kit with reference dye ROX (Invitrogen) using the AB 7500 real time PCR system (Applied Biosystems). Viral RNAs from the culture supernatants collected from the infected cells were extracted using Hipure viral RNA kit (Roche) as described in Chapter 2.

A set of forward primer, (5 '–3') AGA TGA GTC TTC TAA CCG AGG TCG, reverse primer TGC AAA AAC ATC TTC AAG TCT CTG, were provided by Eurofins MWG Operon (London, UK) and a hydrolysis probe, (5 '–3') FAM-TCA GGC CCC CTC AAA GCC GA-BHQ provided by Eurogentec (Hampshire, UK) were used. A range of influenza virus matrix gene RNA standards containing 1 to 1,000,000 copies per microlitre were generated by serial 10 fold dilution of *in-vitro* transcribed viral RNA (Chapter 2). Biological replicates for each species, virus and time point were used for the assay and PCR reactions were performed in duplicate wells. A master mix of common components was prepared in a DNase/RNase-free microcentrifuge tube for multiple reactions to minimize reagent loss.

Component	Volume / reaction (µl)
PCR grade water	3.8
Enzyme Mix	0.4
Reaction Mix	10
RNase OUT	0.2
Probe	0.2
For primer	0.8
Rev primer	0.8
Rox	0.8
Total	17

To a MicroAmp™ Optical 96-well plate (Applied Biosystems), 17µl of master mix was added to required number of wells, followed by 3µl of RNA sample to appropriate wells. Standard RNA samples were added first and the wells were capped using MicroAmp™ Optical 8-Cap Strips (Applied Biosystems).

After adding test and negative control samples all the wells were capped. After inserting the plate in AB7500 system, the following programme was used for PCR reaction and data was recorded in absolute quantification mode. Reverse transcription**:** 45ºC for 15 min and 55ºC for 15 min; denaturation at 95ºC for 2 min, followed by 40 cycles of 95ºC for 15s and 60ºC for 1 min. After 40 cycles, the threshold cycle (Ct) values were analyzed using AB7500 software. A standard curve was generated from the M gene RNA standards and Ct values of the test samples were converted to viral gene copy number values using the standard curve.

5.5.3.2 Virus infectivity assay

Culture supernatants collected from primary avian cells following infection were titrated in MDCK cells to measure the virus infectivity which was expressed as plaque forming units per microlitre of supernatant as described in Chapter 2.

5.5.3.3 Statistical analysis

Data from MTT assay and virus infectivity assay was analyzed by General Linear Model (GLM) analysis of variance (ANOVA) using adjusted (type III) sum of squares followed by pair wise comparison of means using Bonferroni method. Real time RT-PCR data was analyzed with a two sample t test. All analyses were performed using Minitab statistical software version 15.

5.6 Results

5.6.1 Infection of avian lung cells

Following infection of primary chicken and duck lung cells with avian H2N3 or swine H1N1 viruses at multiplicity of infection (MOI) of 1.0, duck cells exhibited rapid cell death compared to chicken cells. Cell death as evidenced by dead, floating cells was found to a greater degree, in duck than in chicken cells by 24 hrs post-infection (Figure 5.6-1) and was more marked by 48 hours post infection (Figure 5.6-2).

Similar rapid cell death was also observed in duck cells compared to chicken cells at 20 hrs following infection with H5N1 50-92 virus. Notably, duck cells showed no evidence of greater cell death at 20 hrs following infection with H5N1 ty-Ty virus. Although few floating dead or dying cells were evident in duck cells, they were also found in infected chicken and un-infected cells (Figure 5.6-3).

5.6.2 Infection of avian embryo cells

5.6.2.1 Cytopathic changes

Duck embryo cells infected with avian H2N3 or swine H1N1viruses at MOI of 0.1 or 1.0 also exhibited more rapid cell death compared to infected chicken embryo cells (Figure 5.6-4). As expected, cell death was also more abundant in duck embryo cells compared to chicken embryo cells at 48 hours post-infection (Figure 5.6-5).

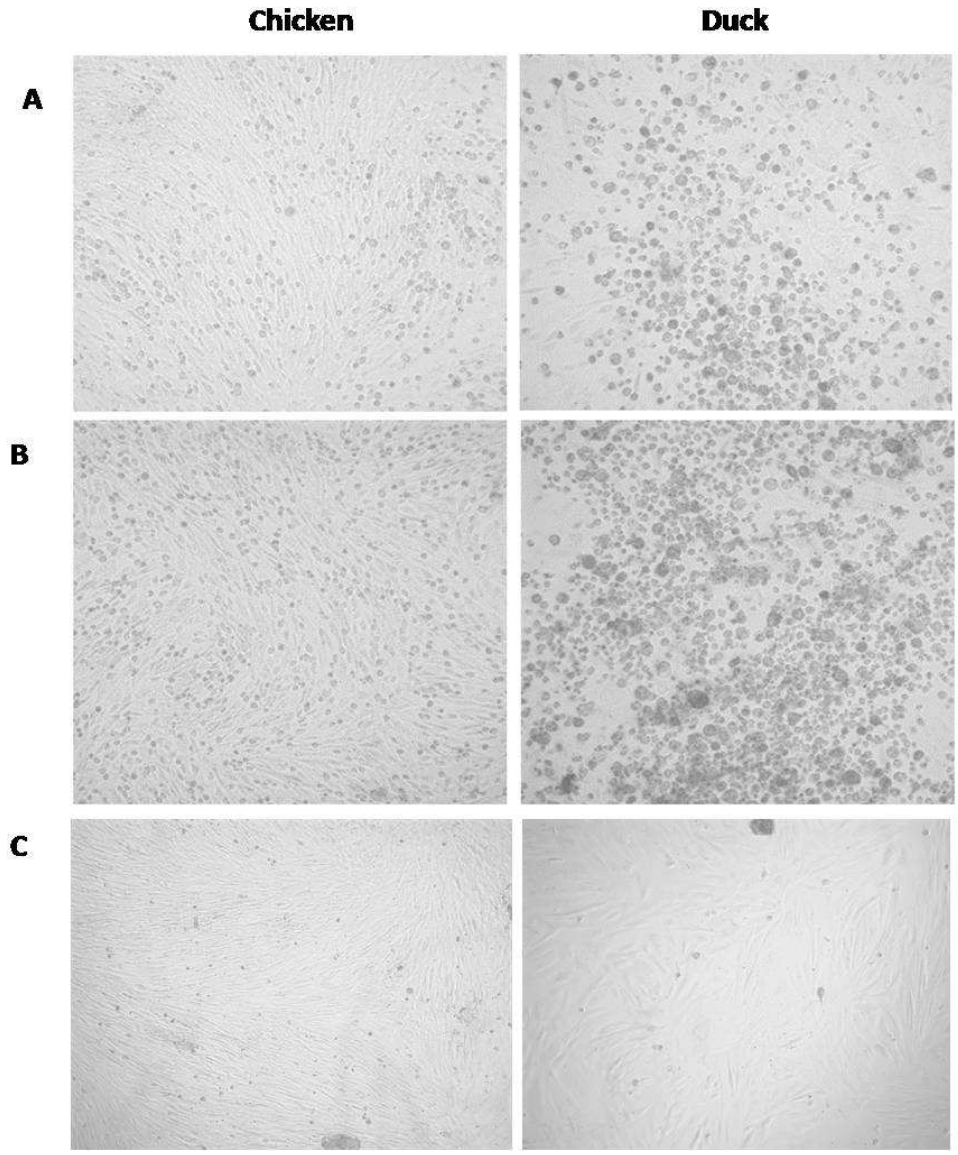


Figure 5.6-1 Cytopathic changes in avian lung cells 24hr post-infection

Photomicrograph showing appearance of chicken and duck lung cells 24 hr post infection with LPAI H2N3 (A) or classical swine H1N1 (B) at an MOI of 1.0. Duck lung cell cultures show large numbers of floating dead and dying cells in comparison with chicken lung cells. Un-infected control chicken and duck cells (C) showed little evidence of such changes.

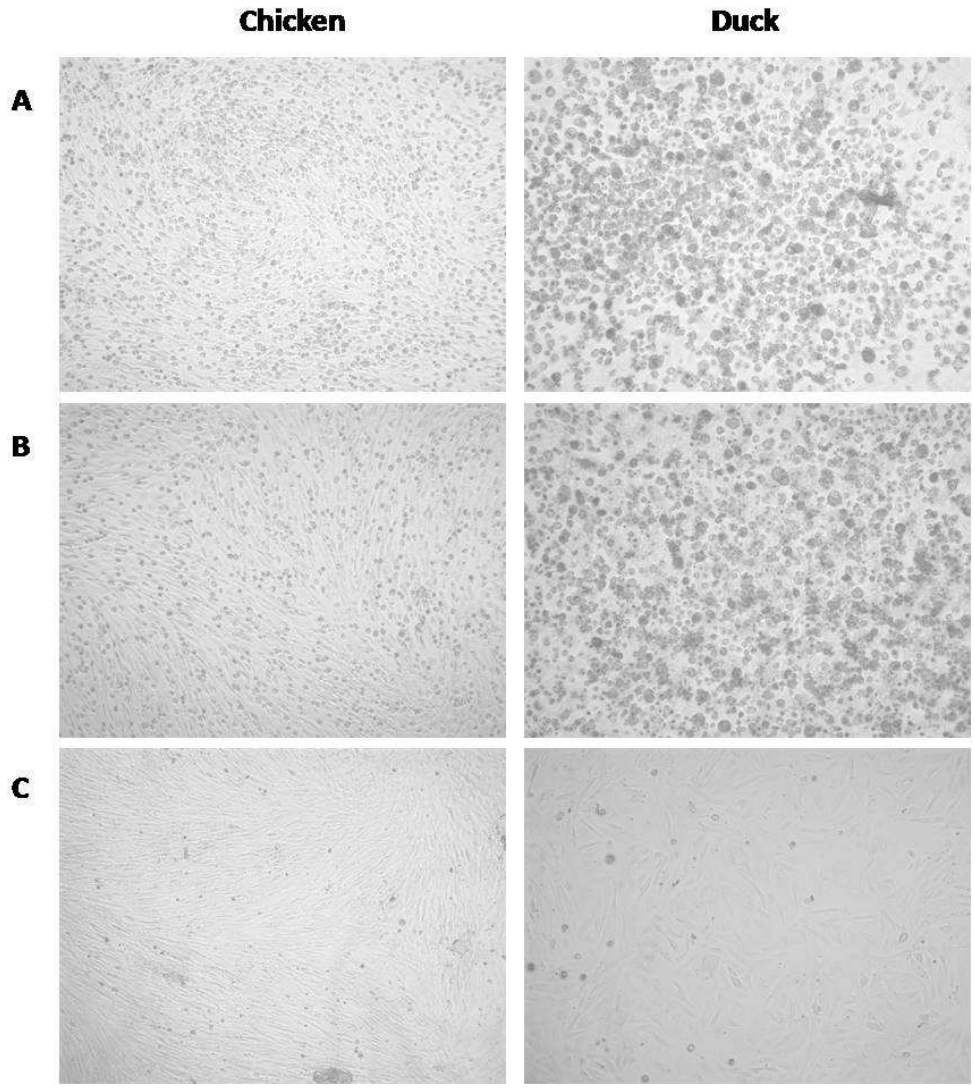


Figure 5.6-2 Cytopathic changes in avian lung cells 48hrs post-infection

Photomicrograph showing appearance of chicken and duck lung cells 24 hr post infection with LPAI H2N3 (A) or classical swine H1N1 (B) at an MOI of 1.0. Duck lung cell cultures show large numbers of floating dead and dying cells in comparison with chicken lung cells. Un-infected control chicken and duck cells (C) showed no evidence of changes.

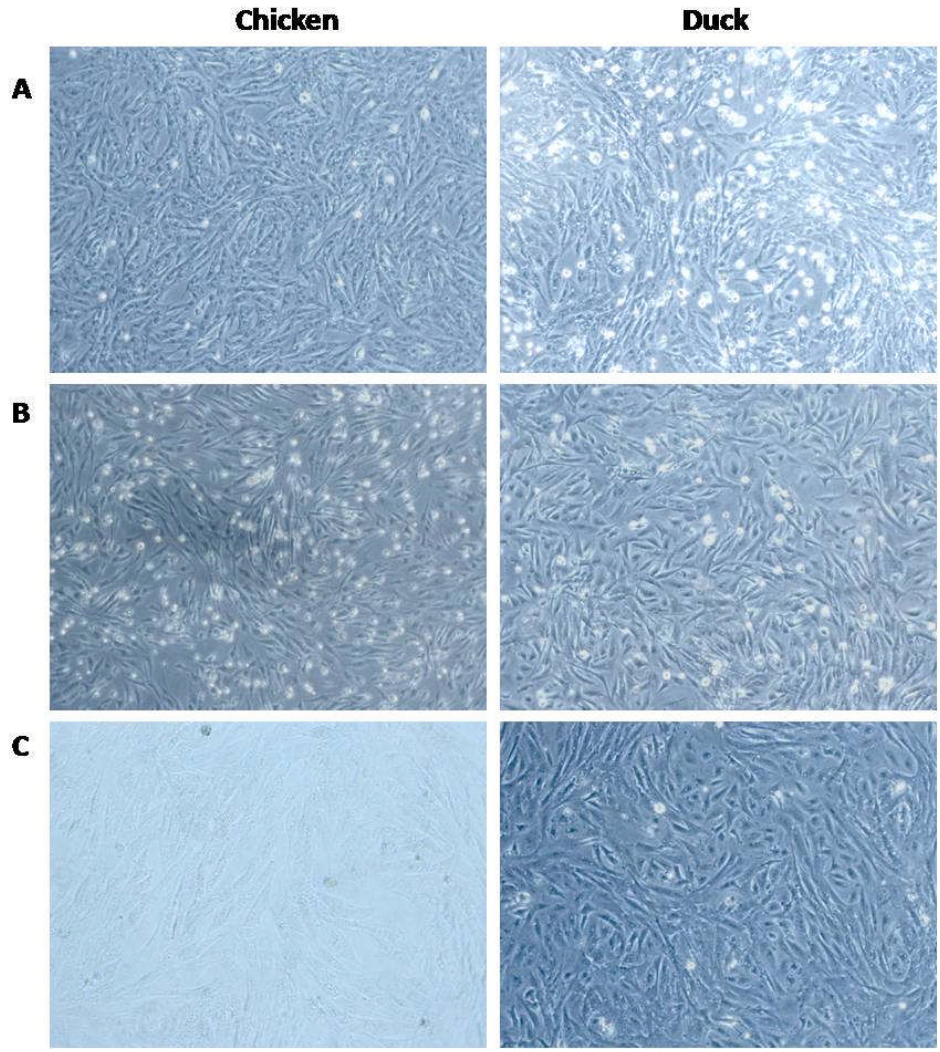


Figure 5.6-3 Cytopathic changes in avian lung cells infected with H5N1 virus

Photomicrograph showing appearance of chicken and duck lung cells 20 hr post infection with highly pathogenic H5N1 influenza strains. Infected duck lung cells infected with 50-92 virus showing more cell death compared to chicken cells (A). The relative rapid death is not evident in ty-Ty virus infected (B) chicken and duck cells, which resembled un-infected control cells (C).

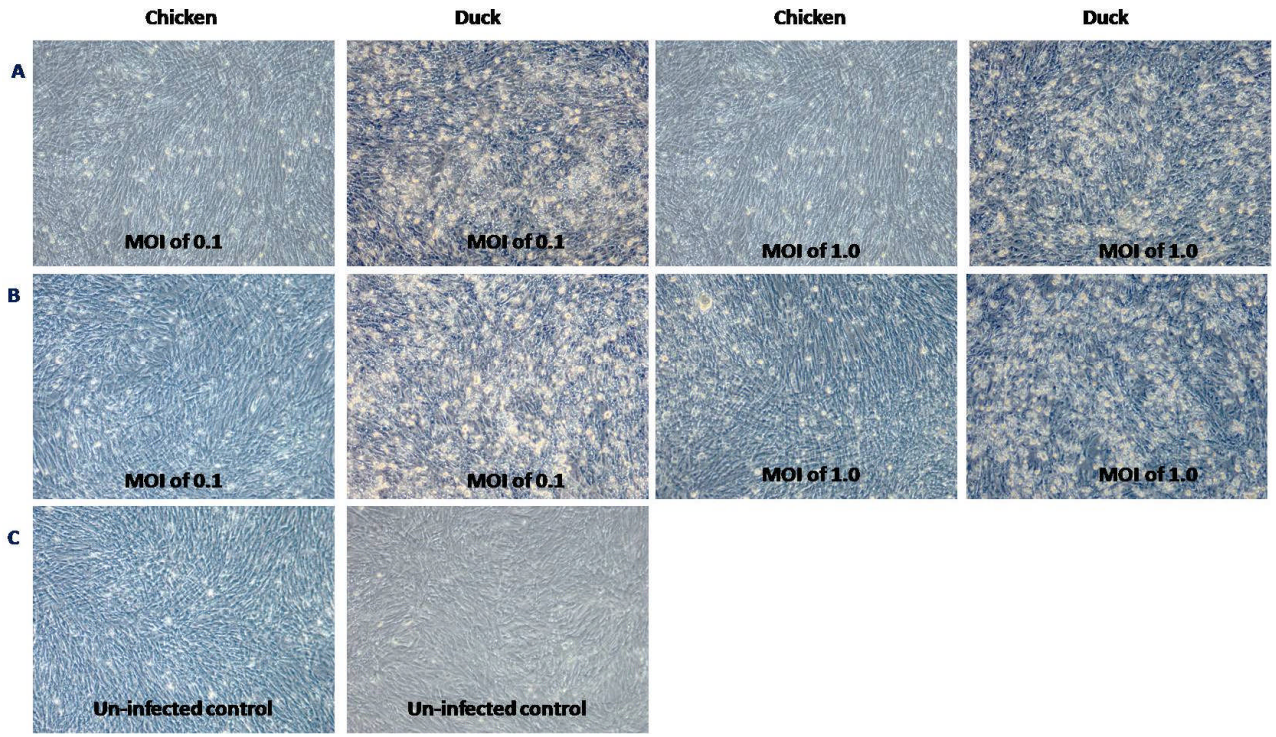


Figure 5.6-4 Cytopathic changes in avian embryo cells 24hr post-infection

Photomicrograph of chicken and duck embryo cells infected with LPAI H2N3 (A) or classical swine H1N1 (B) at a MOI of 0.1 and 1.0 24 hours post-infection. Duck cells exhibited more cell death compared to chicken cells at both MOI of 0.1 and 1.0. Un-infected control chicken and duck cells (C) showed no evidence of changes.

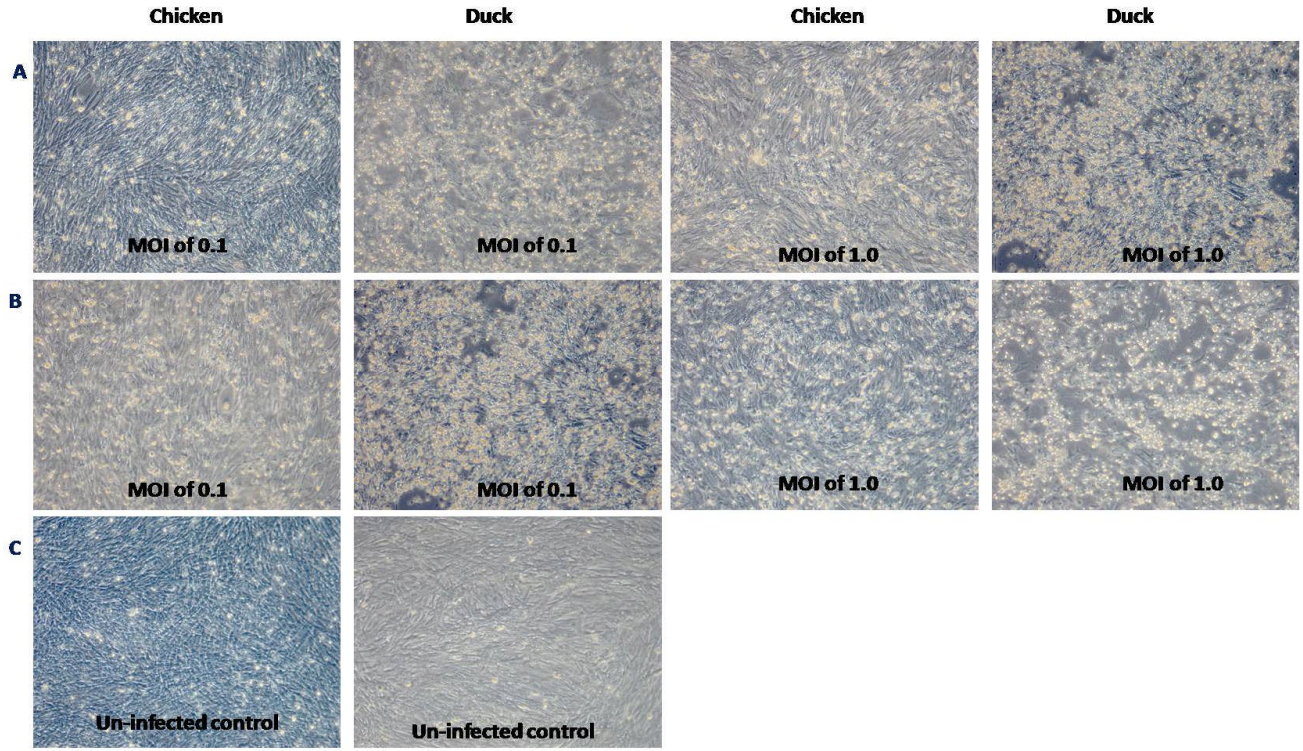


Figure 5.6-5 Cytopathic changes in avian embryo cells 48 hr post-infection

Photomicrograph of chicken and duck embryo cells infected with LPAI H2N3 (A) or classical swine H1N1 (B) at an MOI of 0.1 and 1.0, 48hours post-infection. Duck cells exhibited more cell death compared to chicken cells at both MOI of 0.1 and 1.0. Un-infected control chicken and duck cells (C) showed no evidence of changes.

5.6.3 Measurement of cell metabolic activity by MTT assay

Metabolic activity of chicken and duck lung cells was measured by MTT assay following infection with avian H2N3 and swine H1N1 viruses to quantify the observed difference in cell death between duck and chicken cells. At 24 hours following virus infection duck lung cells consistently showed significantly lower metabolic activity than chicken lung cells following infection with avian H2N3 ($p<0.01$) and swine H1N1 ($p<0.01$) viruses over a range of MOI (Figure 5.6-6). The reduction in cell viability was virus dose dependent, with increasing MOI inducing significant reductions in cell metabolic activity ($p<0.01$).

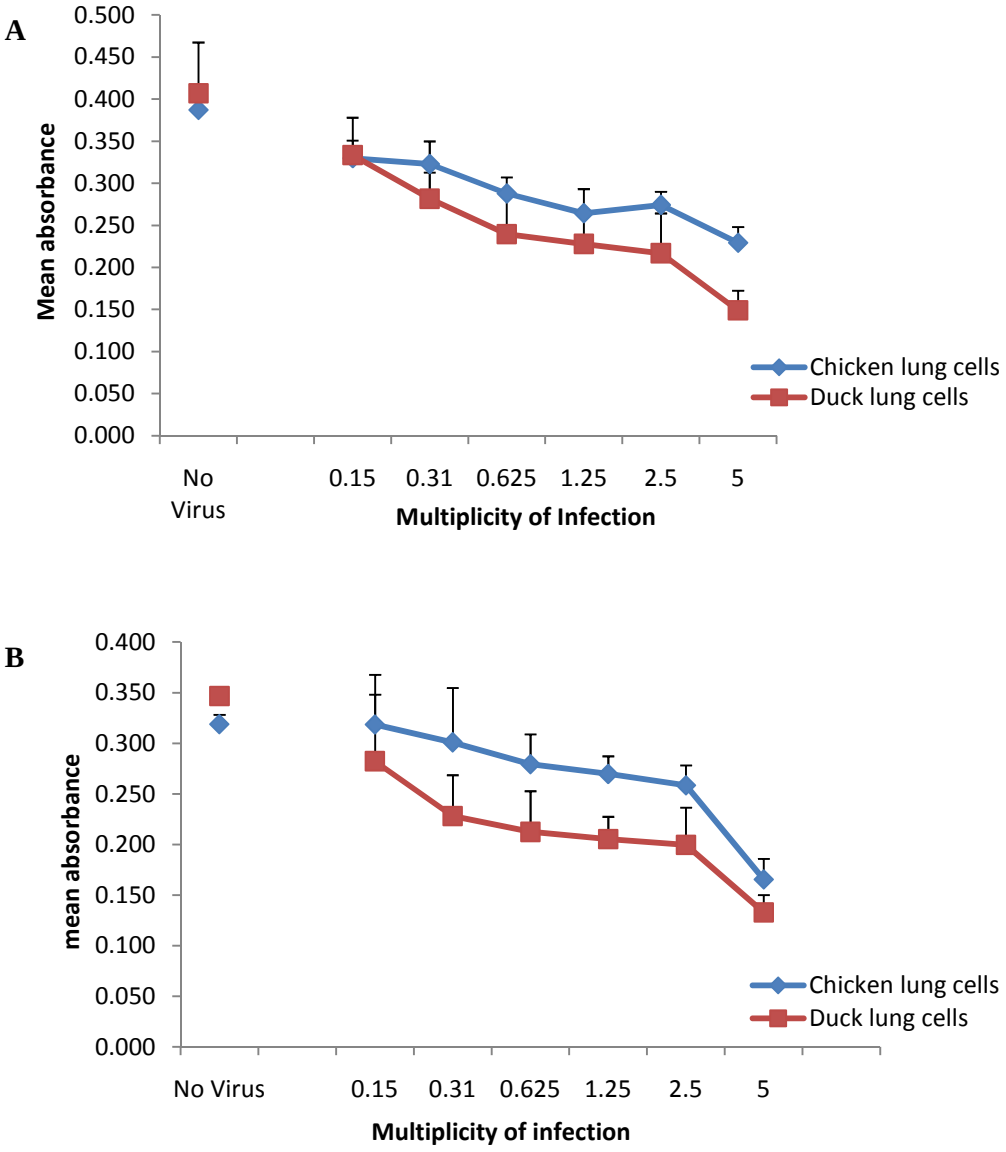


Figure 5.6-6 Measurement of cell metabolic activity following influenza virus infection

Duck lung cells showing significantly higher cell death compared to chicken lung cells following infection with avian H2N3 (A) ($p<0.01$) and swine H1N1 (B) ($p<0.01$). Data points are the mean of quadruplicate wells with error bars showing standard deviation.

5.6.4 Quantification of virus production

5.6.4.1 Measurement of Matrix gene copy number by quantitative RT-PCR

Quantification of influenza virus matrix (M) gene production in culture supernatants from infected chicken and duck cells was performed by one-step qRT-PCR assay. A standard curve was generated with serial dilutions of matrix gene RNA. A linear relationship between average Ct values and log M gene copy number were found with R^2 value of 0.99 and slope of -3.37 (Figure 5.6-7). Using this standard curve, gene copy number of test samples (values on X axis) can be accurately predicted with the Ct values (values on Y axis).

5.6.4.2 Avian lung cells

Measurement of influenza matrix gene production in the culture supernatants of infected chicken and duck lung cells at 8 and 24 hrs following infection with LPAI H2N3 virus revealed that the amount of virus produced in culture supernatants was not significantly different between species at 8 hours ($p>0.05$) or 24 hours ($p>0.05$) post-infection. Matrix gene production in chicken and duck lung cells 20 hrs following infection with HPAI viruses was very similar to LPAI studies such that chicken and duck cells did not show any significant difference following H5N1 50-92 ($p>0.05$) or H5N1ty-Ty ($p>0.05$)virus infections (Figure 5.6-8).

Direct comparison between LPAI and HPAI viruses could not be made as the HPAI qPCR assay was performed at VLA Weybridge (courtesy of Vivien Coward) using a similar method where the calculation of copy number was based on a standard curve generated from whole virus particles rather than *in-vitro* transcribed RNA used in this project.

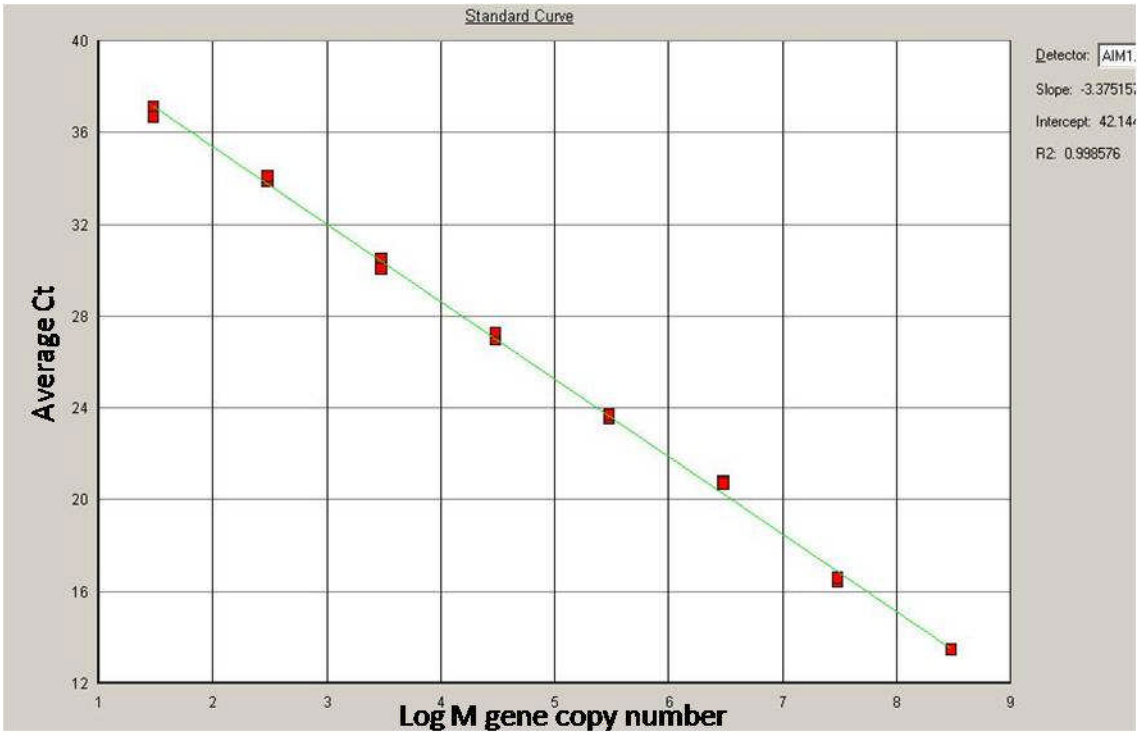


Figure 5.6-7 Standard curve for the calculation of M gene copy number from threshold cycle (Ct) values

Standard curve showing linear relation (R^2=0.998) between matrix gene copy number and Ct value of RT-PCR assay, indicating that m gene copy number (values on X axis) can be accurately predicted using average Ct values (values on Y axis).

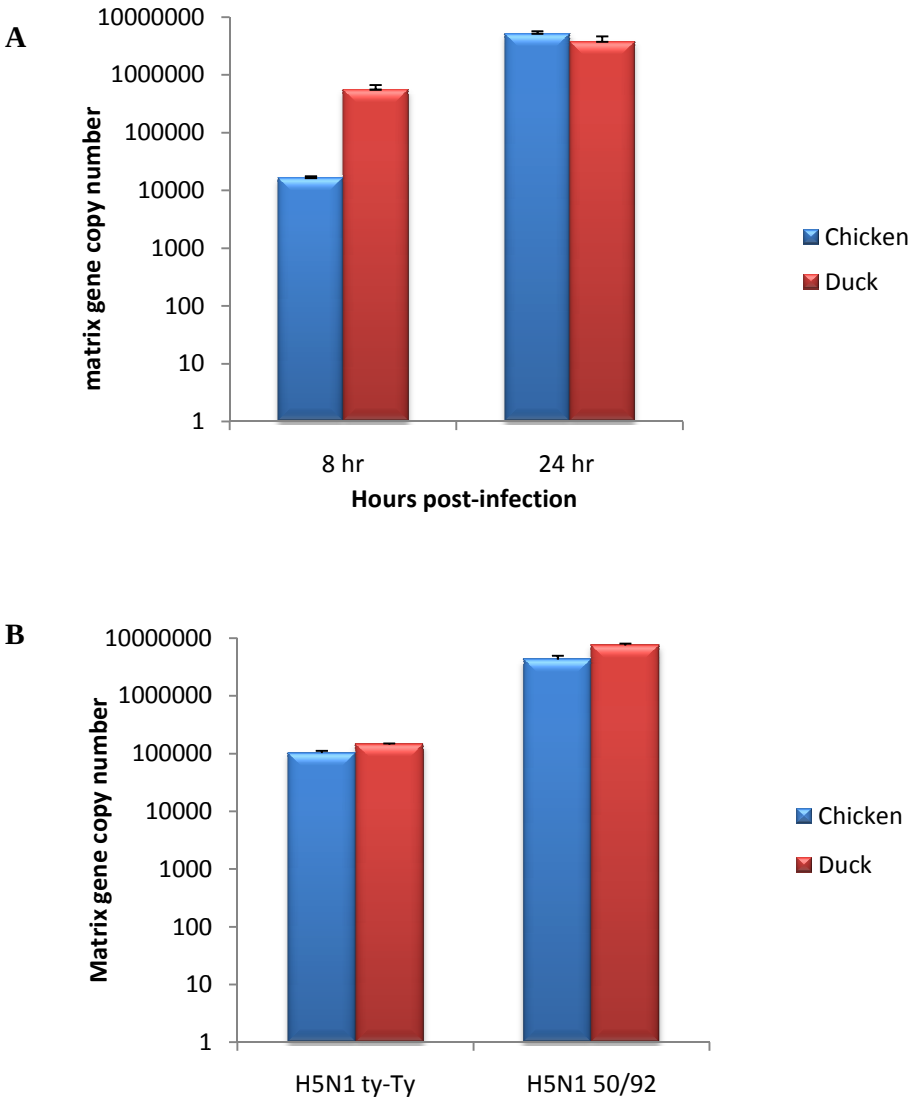


Figure 5.6-8 Measurement of influenza matrix gene copy number by qRT-PCR in avian lung cells

Influenza matrix gene production in culture supernatants of chicken and duck lung cells following infection with LPAI (A) and HPAI (B) is shown. Primary cells from both species did not show any significant difference in matrix gene production at 8 hrs ($p>0.05$) or 24 hrs ($p>0.05$) post-infection following infection with avian H2N3 and 20 hrs following infection with H5N1 50-92 ($p>0.05$) or H5N1ty-Ty ($p>0.05$) virus infections. Data points are the mean of duplicate wells with error bars showing standard deviation.

5.6.4.4 Avian embryo cells

Influenza virus matrix gene production in culture supernatants from infected embryo cells was measured by qRT-PCR assay at 6, 24 and 48 hrs post-infection. Influenza matrix gene RNA production in culture supernatants of infected chicken and duck embryo cells infected with avian H2N3 and swine H1N1 at MOI of 0.1 (Figure 5.6-9) and MOI of 1.0 (Figure 5.6-10). The results were found to be comparable between the species. The results of matrix gene production in embryo cell supernatants were similar to lung cells so that, neither chicken nor duck showed consistently greater amounts of virus production relative to the other species.

Influenza viral RNA production in chicken cells was not significantly different from duck cells at 6 hrs following infection with avian H2N3 at MOI 0.1 ($p>0.05$) or 1.0 ($p>0.05$) or with swine H1N1 at MOI 0.1 ($p>0.05$) or 1.0 ($p>0.05$). At 24 hrs post infection with avian H2N3, chicken cells produced significantly greater amount of viral RNA compared to duck cells at MOI of 0.1 ($p>0.05$) or 1.0 ($p>0.05$). Duck embryo cells produced significantly greater amount of viral RNA compared to chicken cells at 24 hrs post-infection with swine H1N1 at MOI of 1.0 ($p<0.01$), however this difference was not significant at MOI 0.1 ($p>0.05$).

Greater amounts of viral RNA in supernatants from chicken cells was consistent after 48 hrs of infection with MOI 0.1 with avian H2N3 ($P<0.05$), whereas the difference was not significant with MOI of 1.0. Duck embryo cells produced significantly greater amounts of viral RNA compared to chicken cells 48hrs post-infection with swine H1N1 at MOI of 1.0 ($p>0.05$), while this difference was not significant at MOI 0.1 ($p>0.05$).

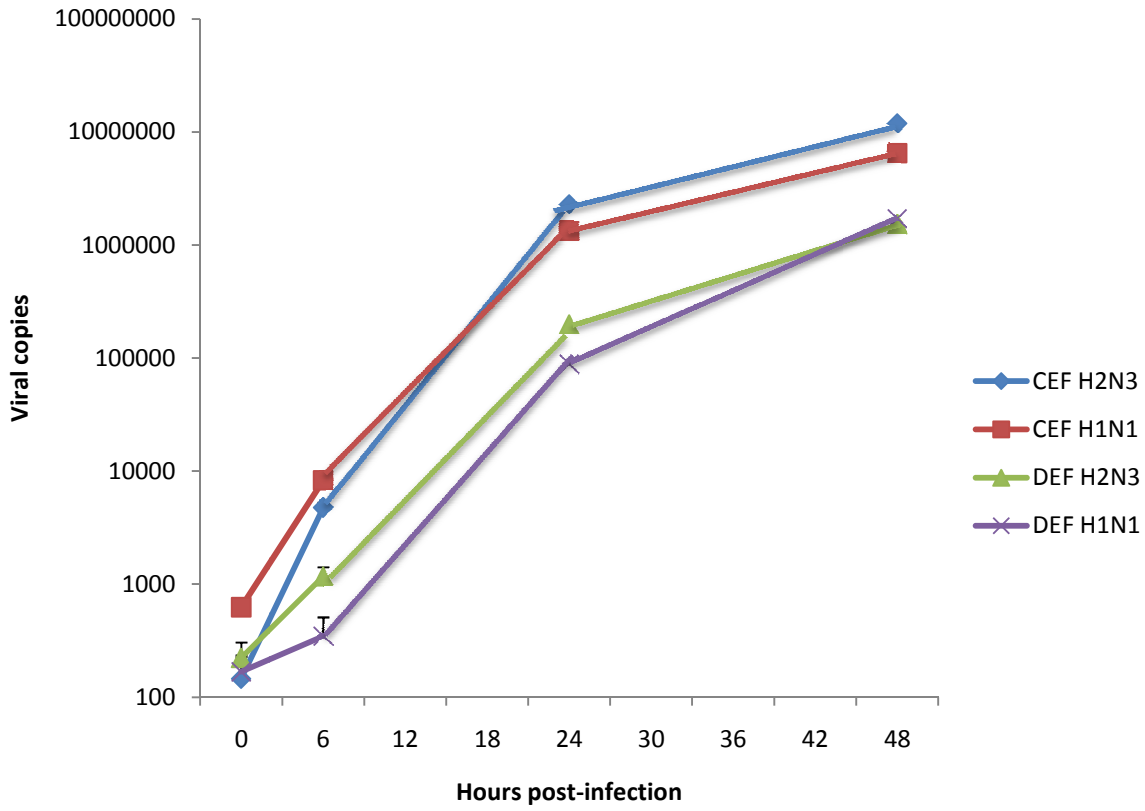


Figure 5.6-9 Quantification of M gene expression in culture supernatants from avian embryo cells following influenza virus infection at MOI 0.1

Influenza matrix gene production in culture supernatants of chicken and duck embryo cells following infection with avian H2N3 and swine H1N1 at MOI of 0.1 is shown. Chicken and duck cells did not show consistently greater amount of virus production. Data points are the mean of duplicate wells with error bars showing standard deviation.

CEF-chicken embryo fibroblasts; DEF- duck embryo fibroblasts.

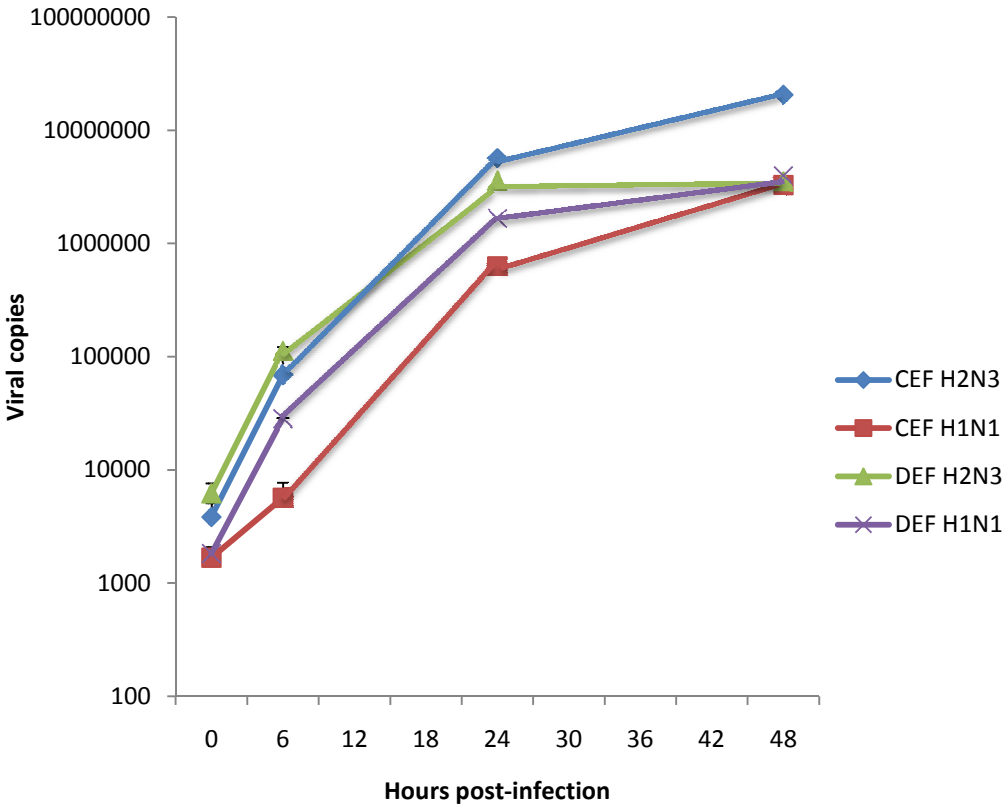


Figure 5.6-10 Quantification of M gene expression in culture supernatants from avian embryo cells following influenza virus infection at MOI 1.0

Influenza matrix gene production in culture supernatants of chicken and duck embryo cells following infection with avian H2N3 and swine H1N1 at MOI of 1.0 is shown. Chicken and duck cells did not show consistently greater amount of virus production. Data points are the mean of duplicate wells with error bars showing standard deviation.

CEF-chicken embryo fibroblasts; DEF- duck embryo fibroblasts.

5.6.4.5 Virus infectivity assay

Supernatants from infected lung cells were titrated in MDCK cells to measure the amount of infective virus production. Greater amount of infective virus was detected consistently in supernatants from infected chicken cells compared to duck cells (Pictures in Appendices III & IV). Chicken lung cells produced approximately 10 times as much infective virus compared to duck lung cells following infection with H2N3 virus at 24 and 48 hours post-infection (Figure 5.6-11).

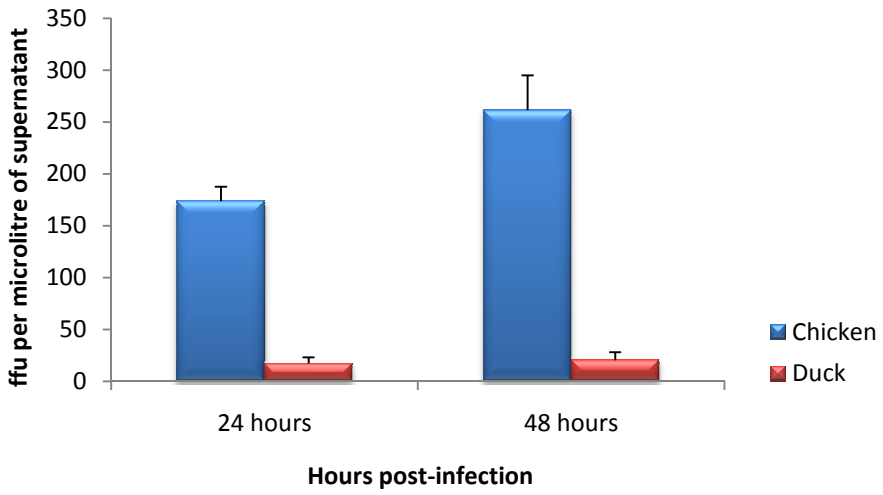


Figure 5.6-11 Measurement of infective virus production following infection with avian H2N3 virus

Measurement of infective virus production in MDCK cells, showed significantly greater ($p<0.01$) levels of infectious virus in supernatants derived from chicken cells compared with duck cells 24 and 48 hours following infection with avian H2N3 virus. Data points are the mean of duplicate wells with error bars showing standard deviation.

Following infection with swine H1N1 virus chicken lung cells produced 3 times and 5 times as much infective virus compared to duck cells at 24 and 48 hours post-infection respectively (Figure 5.6-12).

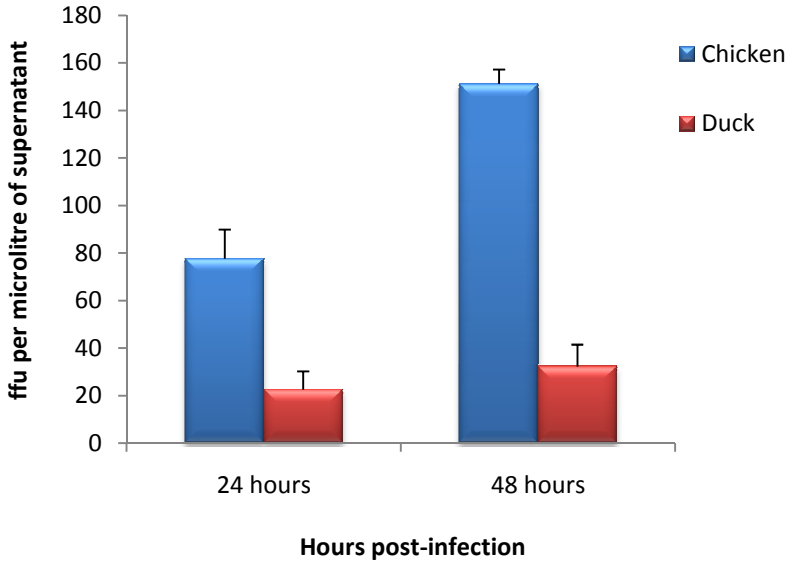


Figure 5.6-12 Measurement of infective virus production following infection with swine H1N1 virus

Measurement of infective virus by titration on MDCK cells, showed significantly greater ($p<0.01$) levels of infectious virus in supernatants derived from chicken cells compared with duck cells 24 and 48 hours following infection with swine H1N1 virus. Data points are the mean of duplicate wells with error bars showing standard deviation.

A similar pattern was also found with HPAI 50-92 infected chicken cells, which produced eight times as much infective virus compared to duck cells at 20 hours post-infection (Figure 5.6-13).

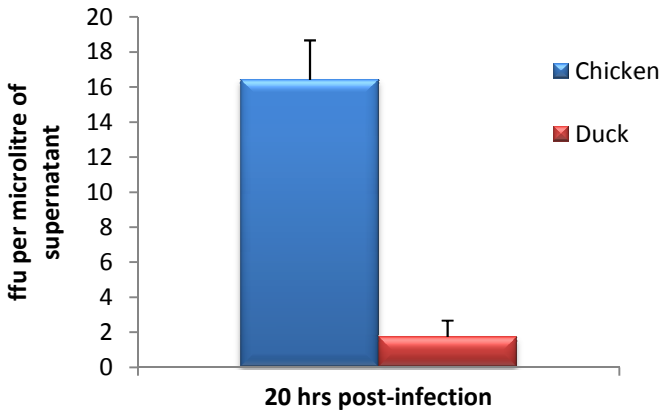


Figure 5.6-13 Measurement of infective virus production following infection with HPAI 50-92 virus

Measurement of infective virus by titration on MDCK cells, showed significantly greater ($p<0.01$) levels of infectious virus in supernatants derived from chicken cells compared with duck cells 20 hours following infection with HPAI 50-92 virus. Data points are the mean of duplicate wells with error bars showing standard deviation.

5.7 Discussion

Differences in cellular responses between chicken and duck lung cells following infection with a range of influenza viruses were studied. Infection studies revealed that cell death was induced more rapidly, and to a greater degree, in primary lung cells derived from Pekin duck cells than in White Leghorn chicken primary lung cells following infection with avian H2N3, swine H1N1 and H5N1 50-92 virus strains. None of these viruses would have been expected to cause disease in Pekin ducks. However it has been shown that, experimental infection of chickens with H5N1 50-92 results in rapid and high levels of mortality (Wood, Parsons, &Alexander 1995). Interestingly duck lung cells did not exhibit rapid cell death compared to chicken cells following infection

with a contemporary Eurasian lineage H5N1 ty-Ty subtype known to cause severe clinical signs in ducks (Londt et al. 2008).

Differences in the levels of influenza virus replication could be dependent on the type of cells and the strain of the virus. For example dynamics of influenza virus replication is different between MDCK and Vero cell lines (Youil et al. 2004). Further, different strains of influenza viruses target different cell types in cultured human airway epithelial cells due to receptor differences (Matrosovich et al. 2004). No differences in influenza virus replication between chicken and duck primary cells used in this study were found based on antibody staining of cells for virus nucleoprotein expression (Chapter 4). This observation affirms similar levels of virus infection in chicken and duck cells. This observation suggests that the observed differential cell death was not a consequence of differences in the levels of virus replication or the number of infected cells.

To confirm that the difference in response to infection was not restricted to lung cells, embryo cells from Pekin duck and White Leghorn chicken were infected with avian H2N3 and swine H1N1. Similar to lung cells there was more rapid cell death in duck embryo cells compared to chicken embryo cells observed in this parallel infection study using embryo cells. Again, there was no underlying difference in the extent of influenza nucleoprotein expression, between species, following infection (Chapter 4). The observed differences in rapid cell death between chicken and duck lung cells were quantified by measuring cell viability using an MTT assay. Duck lung cells consistently showed significantly lower viability than chicken lung cells over a range of multiplicities of infection. As expected, the effect was dose dependent, with increasing MOI inducing greater reductions in cell viability. Measurement of cell metabolic activity following HPAI infections could not be carried out due to the restrictions of working in the containment facilities. The combination of these evidence suggest that rapid cell death could be a potential host resistance mechanism to influenza virus infection in ducks and a loss of such response is associated with a more severe clinical outcome of infection.

Influenza viral M gene production in the infected cell cultures, between 20 – 48 hours following infection with LPAI and HPAI viruses was comparable for chicken and duck cells. Although differences in viral RNA production were observed between chicken and duck cells infected with H2N3 and H1N1 viruses, these were not consistently higher for either species, and it seems unlikely that the relatively small differences seen were biologically significant. Measurement of infective virus by titration in MDCK showed that a significant decrease in the number of infectious virions produced following infection of duck lung cells compared with chicken lung cells. Culture supernatants from duck lung cells infected with avian H2N3, swine H1N1 and H5N1 50-92 consistently showed decreased infectious virus production compared to chicken cells. Cell death characterized by chromosomal DNA fragmentation in HeLa cells following influenza virus infection is associated with low yields of infective virus particles (Takizawa et al.1993).

Influenza virus assembly occurs in infected cells by selective incorporation of vRNAs into virions (Fujii et al. 2003; Odagiri & Tashiro 1997) rather than by random-incorporation (Enami et al. 1991). Similar levels of RNA in supernatants with reduced levels of infective virus detection indicate that virus assembly rather than replication might be inhibited in the dying duck cells. However, viral M gene RNA detected in supernatants by qRT-PCR might be free RNA or virus particle associated or both. Hence, further studies are required to understand the levels of virus particle associated and free RNa in culture supernatants. Death of virus infected cells mediated by apoptosis plays an important role in the virus clearance (Viuff et al. 2002). If the rapid death of duck cells with decreased levels of infective virus production occurs *in-vivo* it could potentially prevent the spread of virus leading to early containment of infection. Reduced levels of infectious virus measured for both species following infection with H5N1 50-92 compared to H2N3 or H1N1 viruses, was most likely due to the use of Vector staining kit, which was less sensitive than DAKO staining kit for detection of the primary nucleoprotein specific antibody. However the relative difference in infective virus

production between chicken and duck cells was consistent with either of the detection systems.

Quantification of infective virus from the supernatants of chicken and duck cells following infection with H5N1 ty-Ty could not be carried out due to restrictions of working in containment facilities. However, it will be interesting to see if the apparently healthy duck cells with no signs of rapid cell death following H5N1 ty-Ty infection produce a more infective virus. Such findings will further strengthen the argument of rapid cell death as a potential host resistance mechanism in ducks.

Cell death can be classified according to its morphological appearance into apoptotic, necrotic and autophagic cell death. Biochemically, cell death can arise with and without the involvement of nucleases or of distinct classes of proteases, such as caspases, calpains, cathepsins and transglutaminases. Cell death can be described based on functional aspects as programmed or accidental, physiological or pathological (Kroemer et al. 2009). Hence, the rapid cell death observed in duck cells merits further investigation to characterize the morphological, biochemical aspects and to explore the underlying triggering mechanisms.

Chapter 6

Role of apoptosis in innate host resistance to influenza

6.1 Summary

Role of apoptosis as a mechanism of rapid cell death in duck primary cells following influenza virus infection was studied. Duck cells exhibited rounding, pyknosis and karyorrhexis which are characteristic morphological hallmarks of apoptosis. Degree of apoptosis in chicken and duck cells was studied by quantifying the percent of hypodiploid cells by Propidium Iodide-flowcytometric assay. Duck cells consistently exhibited significantly greater amounts of hypodiploid cells compared to chicken cells following infection with LPAI, swine influenza and classical H5N1 viruses. No significant difference in the percent of hypodiploid cells between chicken and duck cells was found following infection with a contemporary Eurasian lineage H5N1 virus which causes lethal infection in ducks in-vivo. Significantly higher levels of activated Caspase-3 and-7 were observed in duck cells compared to chicken cells at 18 and 40 hrs following virus infection. These findings suggested the role of apoptosis in the rapid cell death of duck cells following influenza virus infection.

6.2 Apoptosis

The term apoptosis (‘apo’ meaning ‘off’, ‘ptosis’ meaning ‘a falling’) is a Greek word which means “dropping off " or " falling off " of petals from flowers, or leaves from trees (Restifo 2000). Apoptosis is a mechanism of controlled cell deletion, which is complementary but opposite to mitosis in the regulation of animal cell populations. Cell death was first described by Virchow in 1858 and was described by the macroscopic observations like degeneration, mortification and necrosis. Later the terms karyorrhexis and karyolysis were introduced in 1879 which are microscopic observations denoting disintegration and disappearance of the nucleus respectively. The term chromatolysis was introduced by Flemming in 1885 and terms pyknosis (condensation) and chromatin margination were introduced by Arnheim in1890 (Van Cruchten & Van den 2002) all of which described the nuclear changes in dying cells. Later the term apoptosis was introduced to denote the active, inherently programmed phenomenon, that is initiated or

inhibited by a variety of environmental stimuli, both physiological and pathological (Kerr, Wyllie, & Currie 1972).

6.2.1 Cell death by apoptosis

Cells undergoing apoptosis characteristically show certain morphological changes which have been described as hallmark events of apoptosis. The plasma membrane of the cell becomes ruffled and shows blebbing or budding which was also called ‘zeiosis’, referring to the bubbling of yeast in a vat of beer. The cell undergoes remarkable shrinkage resulting from loss of water and ions. One of the changes noticed in the cellular organelles is swelling of cisternae of the endoplasmic reticulum while the nucleus simultaneously undergoes condensation and fragmentation (reviewed by Cohen 1993). Following these changes, the cell is fragmented into compact membrane-enclosed structures, termed 'apoptotic bodies' which contain cytosol, the condensed chromatin, and organelles (Figure 6.2-1). These apoptotic bodies express surface markers which are recognized by macrophages leading to their rapid phagocytosis in the body (Earnshaw 1995).

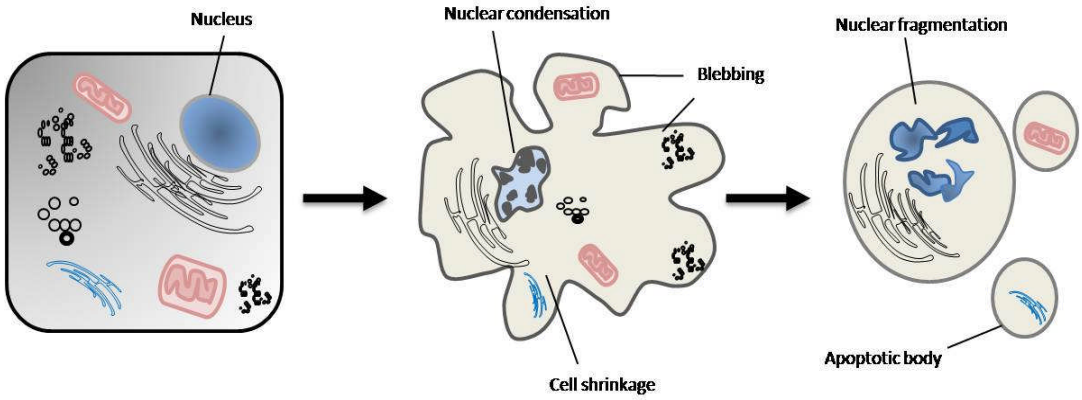


Figure 6.2-1 Hallmark events of apoptosis

Hallmark events of apoptosis showing nuclear condensation, plasma membrane blebbing, nuclear fragmentation and formation of apoptotic bodies.

6.2.2 Apoptosis, necrosis and oncosis

Death of cells resulting from diverse lethal stimuli including severe hypoxia, hyperthermia, viral infection, or exposure to a variety of toxins and respiratory poisons causing rapid loss of cellular homeostasis often referred to as necrosis. Necrosis is marked by cellular swelling, often accompanied by chromatin condensation and eventually leading to cellular and nuclear lysis with subsequent inflammation (Wyllie, Kerr, & Currie 1980). Unlike apoptosis, cells undergoing necrosis exhibit swelling due to accumulation of water and electrolytes, rupture of plasma membrane, and disruption of cellular organelles (Figure 6.2-2). Cytoplasmic budding and formation of apoptotic bodies are typically absent in necrosis and the nuclear chromatin is irregularly clumped. Rupture of plasma membranes occurs and results in leakage of intracellular contents that leads to an inflammatory response (reviewed by Saraste & Pulkki 2000).

The term accidental cell death instead of necrosis was suggested to be more appropriate as necrosis actually refers to changes secondary to cell death, rather than a specific form of cell death. Ischemic cell death is a form of accidental cell death, which results due to failure of the ionic pumps of the plasma membrane. As ischemic cell death is accompanied by swelling, the term oncosis (derived from onkos, meaning swelling) was proposed in 1910 by von Reckling-hausen which means cell death with swelling (Majno & Joris 1995).

However, necrosis may be considered as the end stage of any cell death process and apoptotic cells that are not phagocytosed show several of these necrotic features. The ancient terms pyknosis, karyorhexis and karyolysis are still used to describe apoptosis and oncosis and are not specific for either. The terms pyknosis and karyorhexis are common features of both processes whereas karyolysis seems more specific for oncosis, although apoptotic cells eventually undergo nuclear lysis during phagocytosis (reviewed by Van Cruchten & Van Den 2002).

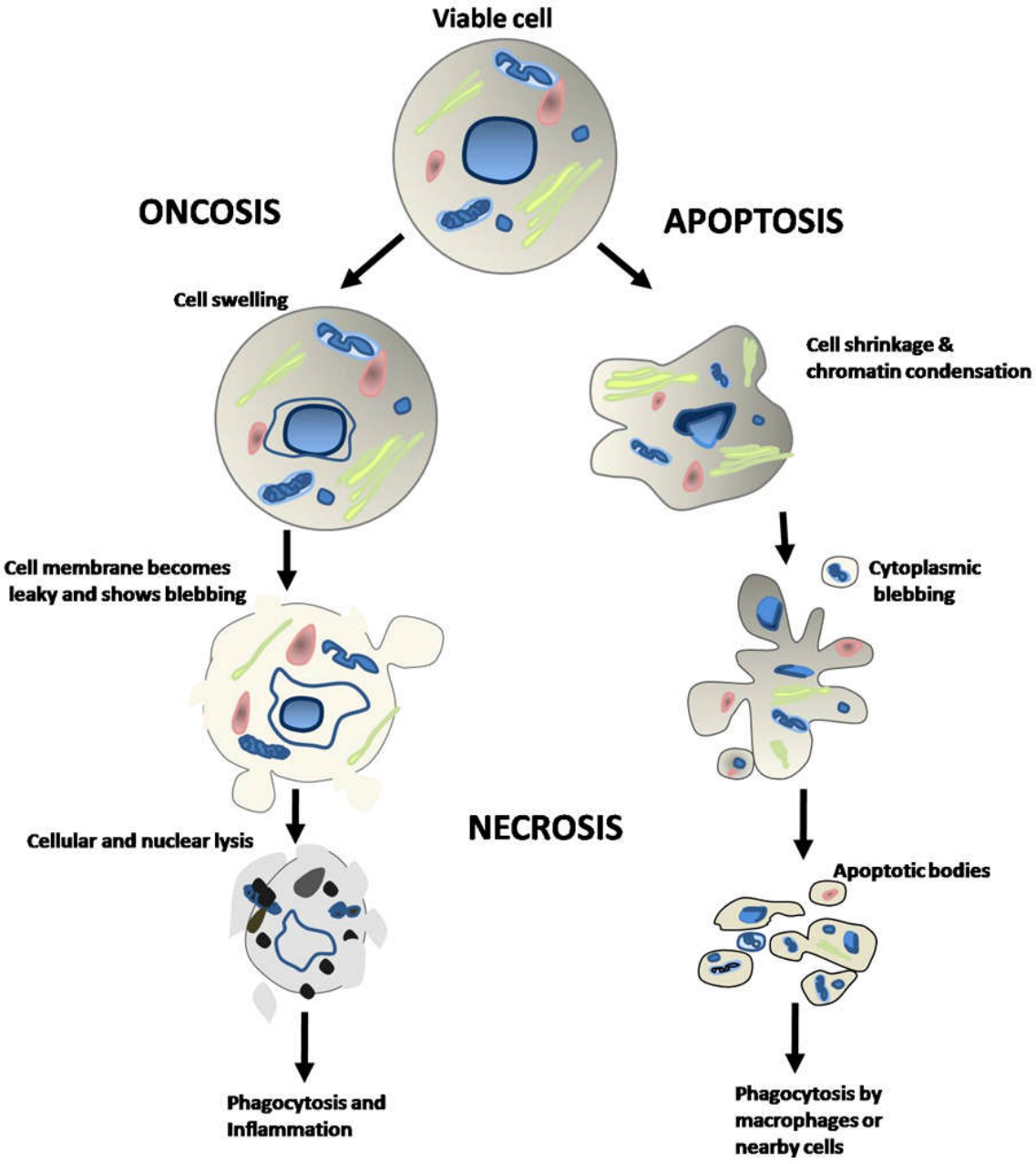


Figure 6.2-2 Pathways of apoptosis and oncosis leading to necrosis

Cell dying by oncosis exhibit swelling, vacuolization, blebbing, increased permeability leading to necrotic changes of coagulation, shrinkage and karyolyis. In contrast apoptotic cells show shrinkage, pyknois, budding, karyorhexis leading to necrotic changes of breaking into apoptotic bodies (Modified from Majno & Joris 1995).

6.2.3 Biochemistry and molecular biology of apoptosis

The process leading to apoptotic death of a cell can be divided into three biochemically and morphologically distinct phases. First is the initiation phase, which involves activation of the central molecular machinery of apoptosis and is triggered by various pro-apoptotic signals. The initiation phase is followed by a committed or effector phase, in which the molecular executioner machinery becomes fully activated. The final phase is called the degradation phase in which the hallmarks of apoptosis including the morphologic changes and DNA fragmentation become evident (reviewed by Saraste & Pulkki 2000).

6.2.3.1 Pathways of Apoptosis

Apoptosis can be genetically encoded or can occur in response to cellular or external stimuli. Apoptosis may be triggered by two distinct pathways namely the intrinsic and extrinsic pathways. The intrinsic cell death pathway also referred to as the mitochondrial cell death pathway involves the initiation of apoptosis as a result of a disturbance of intracellular homeostasis, which is triggered by the release of cytochrome C from mitochondria. The extrinsic pathway involves activation of death receptors (DR) which belong to the tumour necrosis factor receptor (TNFR) superfamily, such as Fas (also known as Apo1 or CD95) (Hengartner 2000). The two pathways with different initiation mechanisms eventually converge to result in cellular morphological and biochemical changes.

The third and least understood pathway is an intrinsic pathway involving the endoplasmic reticulum (ER) which is believed to be a pathologically relevant form of apoptosis occurring in response to cellular stress (Rao et al. 2002;Szegezdi et al. 2006). Apoptosis is characterized by protein cleavage or hydrolysis followed by breakdown of nuclear DNA, and finally recognition of the apoptotic cell by phagocytic cells. The

cleavage of proteins primarily occurs with the activation of a family of cysteine proteases called caspases (**C**ysteine **ASP**artate-Specific Prote**ASE**s) which are synthesized in an inactive form and activated by specific initiation mechanisms (Power, Fanning, & Redmond 2002).

6.2.3.2 Caspases

Caspases are the central executioners of apoptosis and 14 different caspases have been identified to date. However, the major members of the caspase family are Caspases 2, 8, 9, 10 the initiator caspases and Caspases 3, 6, 7 the effector (or executioner) caspases. Initiator caspases activate the effector caspases by cleaving aspartate residue which in turn enzymatically cleaves a set of target proteins. Effector caspases cause cytoskeletal filament aggregation, clumping of ribosomal particles and rearrangement of rough ER to form a series of concentric whorls (Padanilam 2003). Caspases also cleave nuclear lamins which are components of nuclear lamina leading to nuclear shrinking, budding and loss of cellular shape (Buendia, Santa-Maria, & Courvalin 1999).

6.2.3.3 Morphological hallmarks

The onset of apoptosis is characterized by shrinkage of the cell and the nucleus. The nucleus progressively condenses and breaks up (karyorrhexis), which begins with collapse of the chromatin against the nuclear periphery while the nuclear membrane remains intact. During condensation the nucleus may appear as a single dense ball, half moon shape due to condensation to one side or small balls resulting from outward budding of chromatin resembling cluster of grapes. The nuclear pores in the nuclear membrane are redistributed by sliding away from the surface which occurs as a result of dissemination of the nuclear lamina due to proteolysis (Earnshaw 1995). The cell detaches from the surrounding tissue and its outline becomes convoluted and forms extensions, a process referred to as blebbing (Figure 6.2-1). The cytoplasmic processes detach and the plasma membrane seals to form a separate membrane around the detached

cellular material. These are called apoptotic bodies, which contain parts of cellular and nuclear material. Apoptotic bodies contain well preserved closely packed cellular organelles including membranes, mitochondria and fragments of nucleus (reviewed by Saraste and Pulkki 2000).

6.2.3.4 Biochemical hallmark of apoptosis

The biochemical hallmark of apoptosis is DNA fragmentation by endogenous DNases, which cut the inter-nucleosomal regions into double-stranded DNA fragments. Activated caspase 3 migrates to the nucleus and activates a group of nucleases termed caspase-activated DNAases (CAD) which are involved in the breakdown of nuclear DNA into 50–300-kb pieces (Nagata 2000). Biochemical changes in the apoptotic cells also include decreased synthesis and degradation of RNA and proteins (Cohen 1993). Membrane blebbing in apoptosis is driven by the actin–myosin system which is the contractile force that drives bleb formation. During apoptosis, activation of the Rho GTPases, which are intracellular signalling molecules regulating the actin cytoskeleton leads to the activation of effector proteins (Hall 1998). One such effector protein, rho associated kinase ROCK I is activated by cleavage which causes phosphorylation of myosin light-chains, myosin ATPase activity and coupling of actin–myosin filaments to the plasma membrane resulting in the formation of membrane blebs and re-localization of fragmented DNA into blebs (Coleman et al. 2001).

6.2.4 Viruses and apoptosis

Animal virus-induced apoptosis was discovered from the infection studies with an E1B-19K mutant of adenovirus, which was reported to cause a striking phenotype in infected HeLa cells with an accelerated onset of cytopathic effect, and both host cell and viral DNAs were found to be extensively degraded late after infection (Pilder, Logan, & Shenk 1984). Further studies demonstrated the presence of an anti-apoptosis gene in the wild type adenovirus; the mutant virus lacking this gene was found to induce extensive

apoptosis in infected HeLa cells (White et al. 1992). A variety of animal viruses, both DNA viruses and RNA viruses, can induce apoptosis in infected cells (reviewed by (Koyama et al. 1998).

6.2.5 Induction of apoptosis by Influenza A viruses

Influenza A viruses have been shown to induce apoptotic death in infected epithelial, lymphocyte, and phagocytic cells, and apoptosis has been suggested to contribute to tissue damage during infection (Brydon, Morris, &Sweet 2005;(McLean, Ruck, Shirazian, Pooyaei-Mehr, & Zakeri 2008) Schultz-Cherry, krug, & Hinshaw 1998). The precise mechanism of influenza virus-induced apoptosis is unclear and one of the major problems in elucidating the pathways is the large number of cell types being studied, as the apoptotic response can depend on cell type and activation stimulus (Brydon, Morris, & Sweet 2005).

During influenza virus infection protein-kinase R (PKR) is induced by interferon (IFN) and activated by the double stranded RNA (dsRNA). Interferons are known transcriptional inducers of PKR, and type I IFN (IFN-α and IFN-β) are better PKR inducers than IFN-γ (Garcia et al. 2006). Activation of PKR, which is a key regulatory component in many apoptotic pathways, (Gern et al. 2003;Tan & Katze 1999) (Figure 6.2-3) leads to several sequential downstream events. These include activation of nuclear factor (NF)-κB and transcriptional induction of numerous pro-apoptotic genes including those encoding Fas, p53 and Bax. Active PKR, by an unknown mechanism, recruits caspase-8 via the cytoplasmic protein Fas associated death domain (FADD) which results in the initiation of a caspase cascade including the activation of caspase-9 (Gil & Esteban 2000).

Caspase-8 also activates a permeability transition (PT) pore on the mitochondrial membrane resulting in the release of cytochrome c, which in association with apoptosis protein activating factor (Apaf-1) activates caspase-9 family members, thus enhancing

the caspase cascade. Lung tissue injury, following influenza virus infection causes cellular oxidative stress and reactive oxygen species (ROS) generation (Buffinton et al. 1992), while dsRNA dependent-PKR causes induction of nitric oxide synthase (NOS)-2 in human airway epithelium (Uetani et al. 2000). Oxidative stress of infected cells leads to the activation of potential pro-apoptotic and/or pro-inflammatory regulators including the transcriptional regulatory proteins, activating protein (AP)-1, C/EBP, and NF-κB (Choi et al. 1996). Dysfunctional mitochondria release an intermembrane flavoprotein called the apoptosis inducing factor (AIF), which is involved in caspase independent apoptosis by binding to DNA causing chromatin condensation (Reviewed by Brydon, Morris, & Sweet 2005).

6.2.6 Role of apoptosis in antiviral immunity

Apoptosis is a complex, adaptable and flexible mechanism and the immunological consequences of apoptosis vary depending on the circumstances in which it occurs (Restifo 2000). Studies with mutant insect viruses have found that the host apoptotic response interfered with viral replication *in-vitro* and *in-vivo* and hence apoptosis has been proposed to be a potential host antiviral mechanism in insects (Clem & Miller 1993). Based on studies with herpes simplex virus type 1 (HSV-1), animal viruses unlike insect viruses, were shown to multiply in apoptotic cells. It was proposed that animal viruses either multiply rapidly before the onset of apoptosis or inhibit apoptosis in infected cells for their effective multiplication (Koyama et al. 1998).

The precise role of apoptosis in influenza virus infection is not yet completely understood. To determine the role of apoptosis in the rapid cell death observed in resistant duck cells following influenza infection, induction of nuclear fragmentation and caspase activation in infected cells were studied to explore whether apoptosis contributes to the innate resistance of ducks to influenza virus infection.

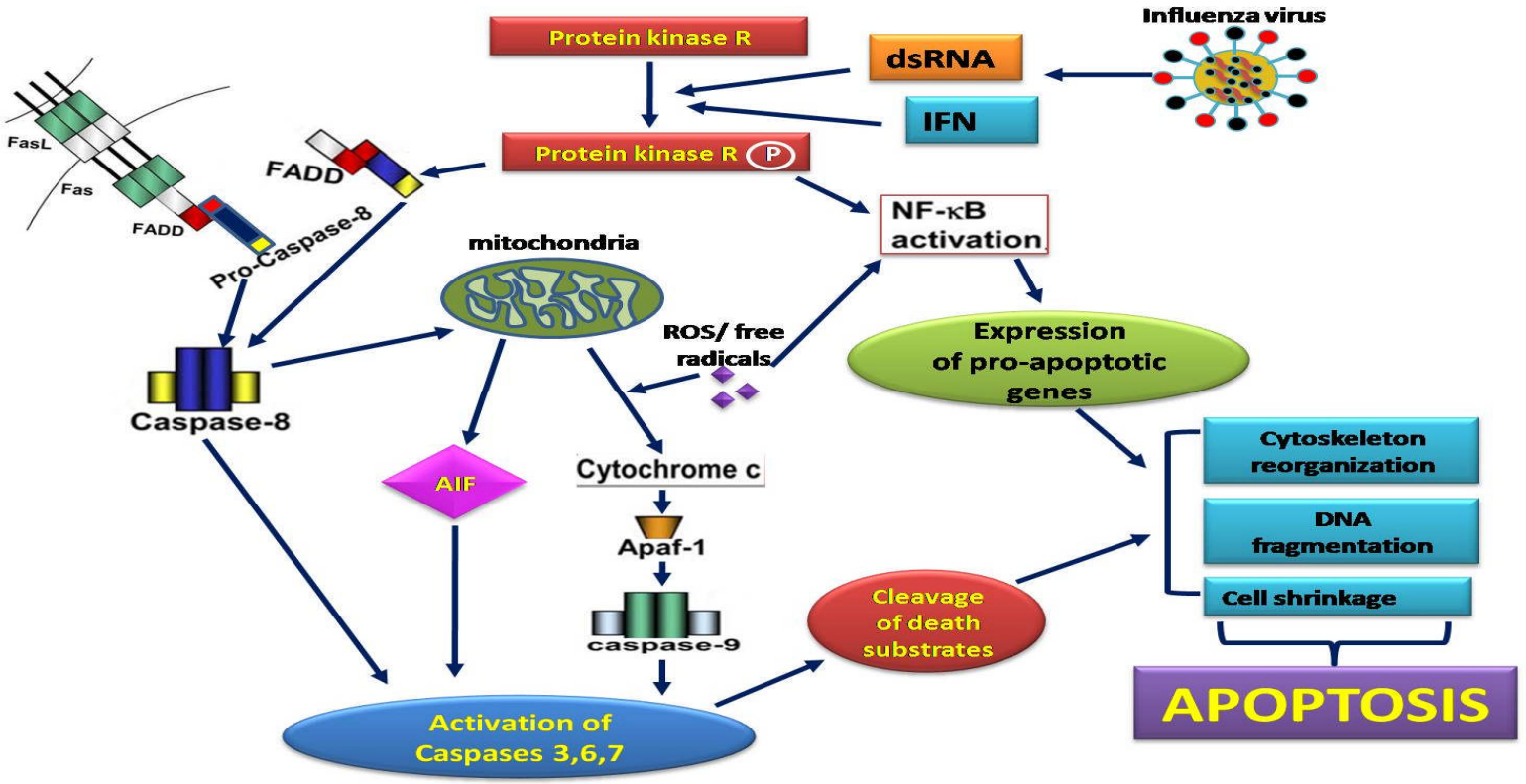


Figure 6.2-3 Signalling pathways of influenza virus induced apoptosis

Molecular pathways involved in influenza virus induced apoptosis are shown. Activation of PKR by dsRNA and IFN triggers a series of downstream pathways including activation of caspase 8 and NFκB leading to apoptosis.Caspase8 either directly or through caspase 9 activation (by activated cytochrome C) triggers the activation of effector caspases 3,6,7 (modified from Brydon, Morris, & Sweet 2005).

6.3 Materials and methods

6.3.1 Morphological characterization of dead cells

Duck lung cells that exhibited rapid death following influenza virus infection were observed for characteristic morphological features to characterize the type of cell death. After examining the cells by phase contrast microscopy (Leica DMIL, Leica, Germany), dead floating cells were collected and stained with 4', 6-diamino-2- phenylindole, dihydrochloride (DAPI; Molecular Probes, Inc., Eugene, OR) and images were captured using a fluorescent microscope (Leica DM 5000B, Leica, Germany).

6.3.2 Measurement of cell death by propidium iodide flow-cytometry

A propidium iodide flow cytometry (PI) assay to measure hypodiploid cells was carried out by a previously described method (Riccardi & Nicoletti 2006). Primary cells (lung and fibroblasts) from chicken and duck were grown in 12 well cell culture plates (Costar) and were infected with LPAI, HPAI and classical swine influenza viruses either at MOI of 0.1 or 1.0 as described in Chapter 4. Propidium iodide is a fluorogenic compound that binds stoichiometrically to nucleic acids so that fluorescence emission is proportional to the content of nucleic acid in cells (Ormerod et al. 1992;Pollack & Ciancio 1990).

6.3.2.1 Cell fixing

Cells were fixed at 24 or 48 hrs post-infection with ethanol for permeabilizing them for propidium iodide staining. Culture supernatants were removed and collected in 15 ml falcon tubes to harvest dead/dying cells detached from the plate. Adherent cells were then washed twice with PBS and trypsinized by adding 0.05% trypsin with EDTA (Invitrogen) and incubated at 37°C for 5 min. Using a micropipette, cells in each well were carefully pipetted several times to break the clumps of cells. The plates were

viewed under inverted phase contrast microscopy (Leica) to ensure that all cells were detached. The trypsinized cells were then removed and added to corresponding well supernatant in 15 ml tubes. Five ml of medium containing DMEM with 10% FCS was added to each tube and centrifuged at 1200 x g for 10 min. After discarding the supernatant, cells were washed with 10ml of cold (4°C) PBS containing 1mg/ml glucose and centrifuged at 1200 x g for 10 min. Supernatants were discarded and washing was repeated a further twice in PBS/glucose using the same procedure. After careful removal of the majority of remaining supernatant, the pellets were resuspended by vigorous vortexing for 10 seconds. While the cells were being vortexed 1 ml of ice-cold 70% ethanol was slowly added to each tube in a drop wise manner. Tubes were capped and allowed to fix for a minimum of 18 hrs at 4°C. Fixed cells were stored for a maximum of two weeks before further processing.

6.3.2.2 Propidium iodide (PI) staining

Propidium iodide staining solution containing 50 µg of propidium iodide, 100 kunitz units of ribonuclease A (Sigma) and 1 mg glucose in 1 ml of PBS was freshly prepared for each assay. Ethanol fixed cells were centrifuged at high speed (~3000rpm) for 5 min as ethanol fixed cells become buoyant and require higher force for sedimentation. After pouring off the supernatant, the cell pellet was re-suspended in residual ethanol. With a micropipette, 1 ml of propidium iodide staining solution was added to the cells and mixed by vortexing. The cell mixture was then transferred to flow cytometer tubes (BD Falcon), capped and incubated at room temperature on a rocking platform for 30 to 90 min in the dark. Stained cells were analyzed in the flow cytometer within 24 hrs.

6.3.2.3 Flow cytometric analysis

Flow cytometry is used to measure certain properties of particles, such as size and internal complexity, using light. In a flow cytometer the particles are moved past a light source and the light that is scattered or emitted by the particles is converted into electrical

signals. The fluidics system of the flow cytometer moves particles in fluid through a flow cell, past a laser beam, and then into a waste tank. As the PI stained cells pass through the focused laser beam, they scatter the laser light and fluoresce. As the cells move rapidly through the flow cell, they scatter or emit fluorescence for a few microseconds, which is collected, filtered, and then converted into an electrical signal by the detectors.

Cells stained with PI were analyzed using BD FACS CantoII (BD Biosciences) flowcytometer and the data was analyzed using BD FACS DIVA software. Twenty thousand cells were analyzed for each sample using the 488nm laser for excitation and red fluorescence (>600 nm) and side scatter were measured. Voltages were adjusted using PI stained uninfected cells and the same settings were used for all the samples (291 volts for forward scatter (FSC) of, 368 volts for side scatter (SSC) and 386 volts for PI).

All the twenty thousand events collected were plotted to show count versus fluorescence in a histogram and FSC versus fluorescence in a scatter plot. Gating was performed to eliminate debris and background in the scatter plot to obtain a histogram showing three distinct peaks representing hypo-diploid (subG1), diploid (G1) and mitotic cells (G2) (Figure 6.3-1) which were expressed as percentages of the total cell population.

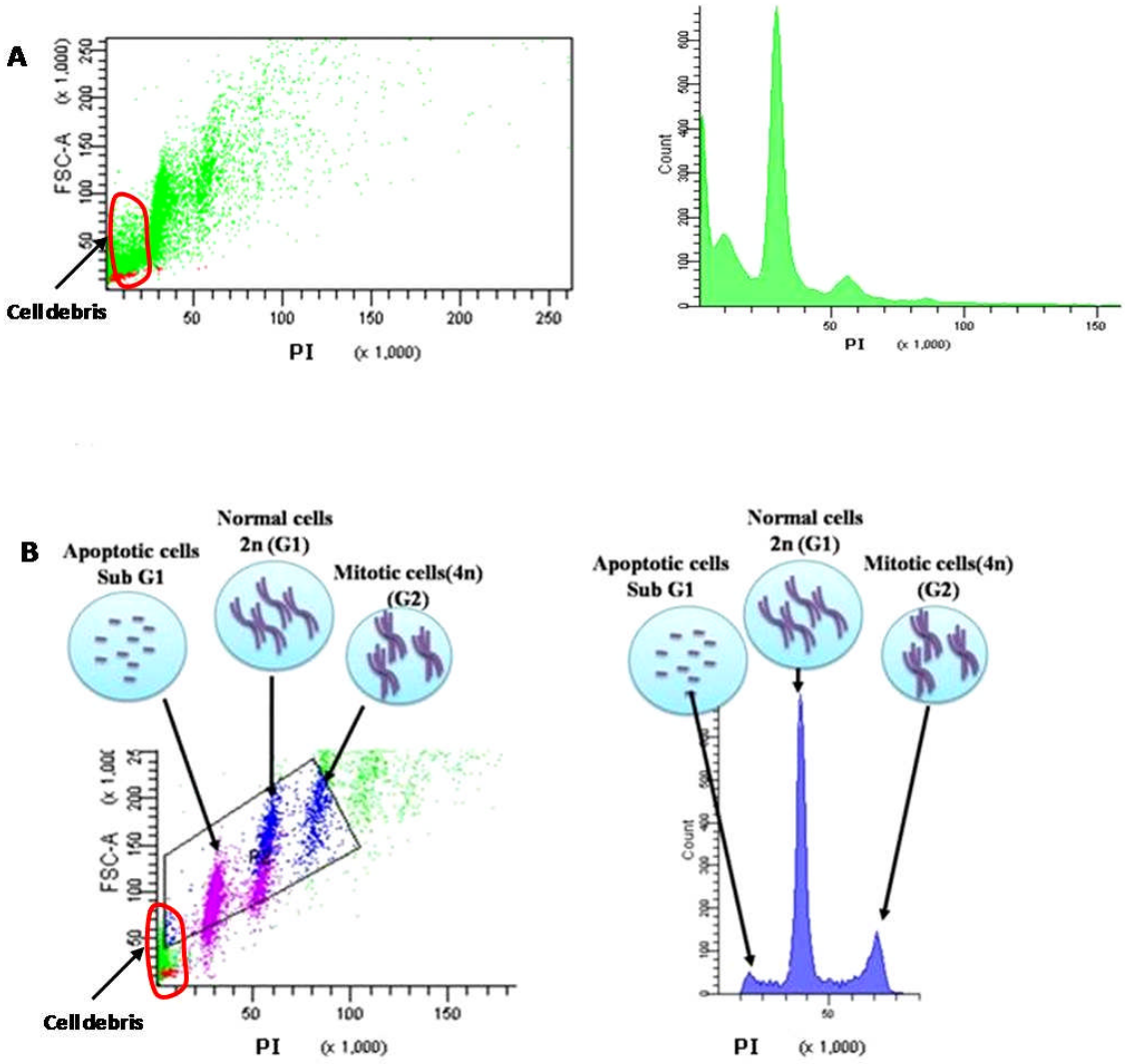


Figure 6.3-1 Cell cycle analysis using propidium iodide flowcytometric assay

Flow cytometric analysis of propidium iodide stained cells showing all the events in scatter plot and histogram before (A) and after (B) gating. After gating (B) cell debris was eliminated and well defined peaks representing hypodiploid (apoptotic), diploid and mitotic cell populations were obtained.

6.3.3 Measurement of caspase 3/7 activation

Levels of activated Caspase 3 and 7 in primary cells following influenza virus infection were quantified using luminescent Caspase-Glo 3/7 assay (Promega), according to manufacturer's instructions. Primary chicken and duck embryo cells were seeded into 96-well cell culture plates (Nunc) and infected with avian H2N3 and swine H1N1 viruses at an MOI of either 0.1 or 1.0 as described in Chapter 5. Negative controls were performed without the virus.

Cells were treated with staurosporine (Sigma; at 4mM final concentration) which induces apoptosis through both caspase-dependent and caspase-independent mechanisms (Belmokhtar, Hillion, & Segal-Bendirdjian 2001) as a positive control. After 24 and 40 hrs of infection, 100µl of Caspase-Glo 3/7 reagent was added to all wells containing cells and reagent blanks with no cells. The contents were gently mixed using a plate shaker at 300–500 rpm for 30s.

After incubating the plates at room temperature for 3 hrs, the contents were carefully mixed and transferred to white-walled 96-well plates. Luminescence of each sample was measured using Orion L micro plate luminometer (Berthold detection systems) and the data was analyzed using Simplicity software version 4.10.

6.3.4 Statistical analysis

Data derived from PI and caspase assays were analyzed by General Linear Model (GLM) analysis of variance (ANOVA) using adjusted (type III) sum of squares followed by pair wise comparison of means using Bonferroni method using Minitab statistical software version 15.

6.4 Results

6.4.1 Morphological characterization of dead cells

Duck lung cells that exhibited rapid death following influenza virus infection were typically were rounded, showing reduced cellular and nuclear volume (pyknosis) (Figure 6.4-1A). Nuclear fragmentation (karyorrhexis) was noticed in phase contrast microscopy as small rounded bodies which was more evident after DAPI staining (Figure 6.4-1B).

6.4.2 Measurement of hypodiploid cells by Propidium iodide (PI) Assay

6.4.2.1 Avian lung cells

Chicken and duck lung cells, 24 and 48 hrs post-infection, were subjected to PI staining followed by flow cytometric analysis to measure the percentage of hypodiploid cells (subG1). Duck lung cells showed significantly greater ($p<0.01$) hypodiploid cells compared to chicken cells consistently following infection with avian H2N3 (Figure 6.4-3A) and swine H1N1 (Figure 6.4-3B) at 24 and 48hrs post-infection. The difference in hypodiploid cells between chicken and duck was more marked following infection at an MOI of 0.1 compared to 1.0. Duck cells showed a significantly greater proportion ($p<0.05$) of hypodiploid cells at 24 hrs following infection with swine H1N1 compared to avian H2N3, this difference between viruses was not significant at 48 hrs post-infection ($p>0.05$). Similarly, significantly higher levels ($p<0.01$) of hypodiploid cells were found in duck lung cells compared to chicken lung cells following infection with H5N1 50-92 (Figure 6.4-2) at 20 hrs post-infection. Interestingly, only low levels of hypodiploid cells were detected after infection of duck lung cells at an MOI of 1.0 or 0.1 (less than 5% compared with 12-17% following infection with H5N1 50-92). Infection of duck cells with H5N1 ty-Ty did not produce significantly higher levels of hypodiploid cells compared to chicken cells ($p>0.05$).

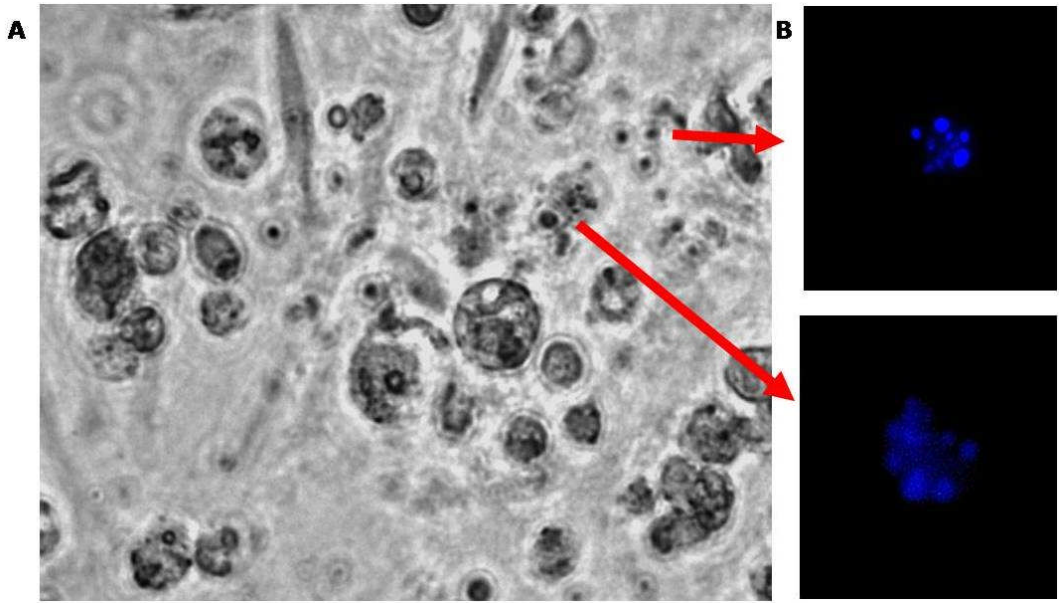


Figure 6.4-1 Morphological appearance of dead/dying duck lung cells

Duck lung cells showing characteristic features of apoptosis i.e rounding, reduction of cellular and nuclear volume (pyknosis) and nuclear fragmentation (karyorrhexis).

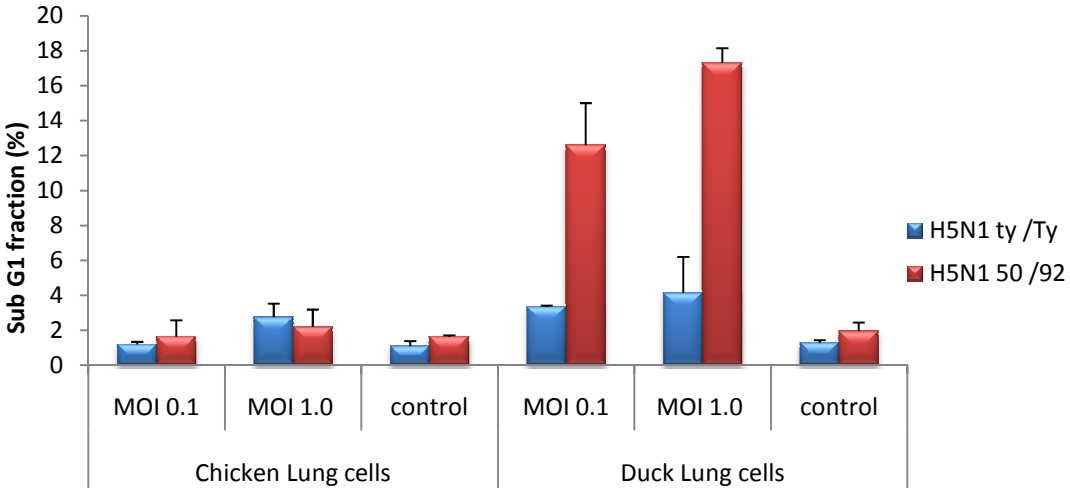


Figure 6.4-2 Quantification of levels of hypodiploid cells in avian lung cells following HPAI infection

Duck lung cells showed significantly higher levels of hypodiploid cells compared to chicken lung cells following infection with H5N1 50-92 ($p<0.01$), whereas the difference is not significant following infection with H5N1 ty-Ty ($p>0.05$). Data points are the mean of duplicate wells with error bars showing standard deviation.

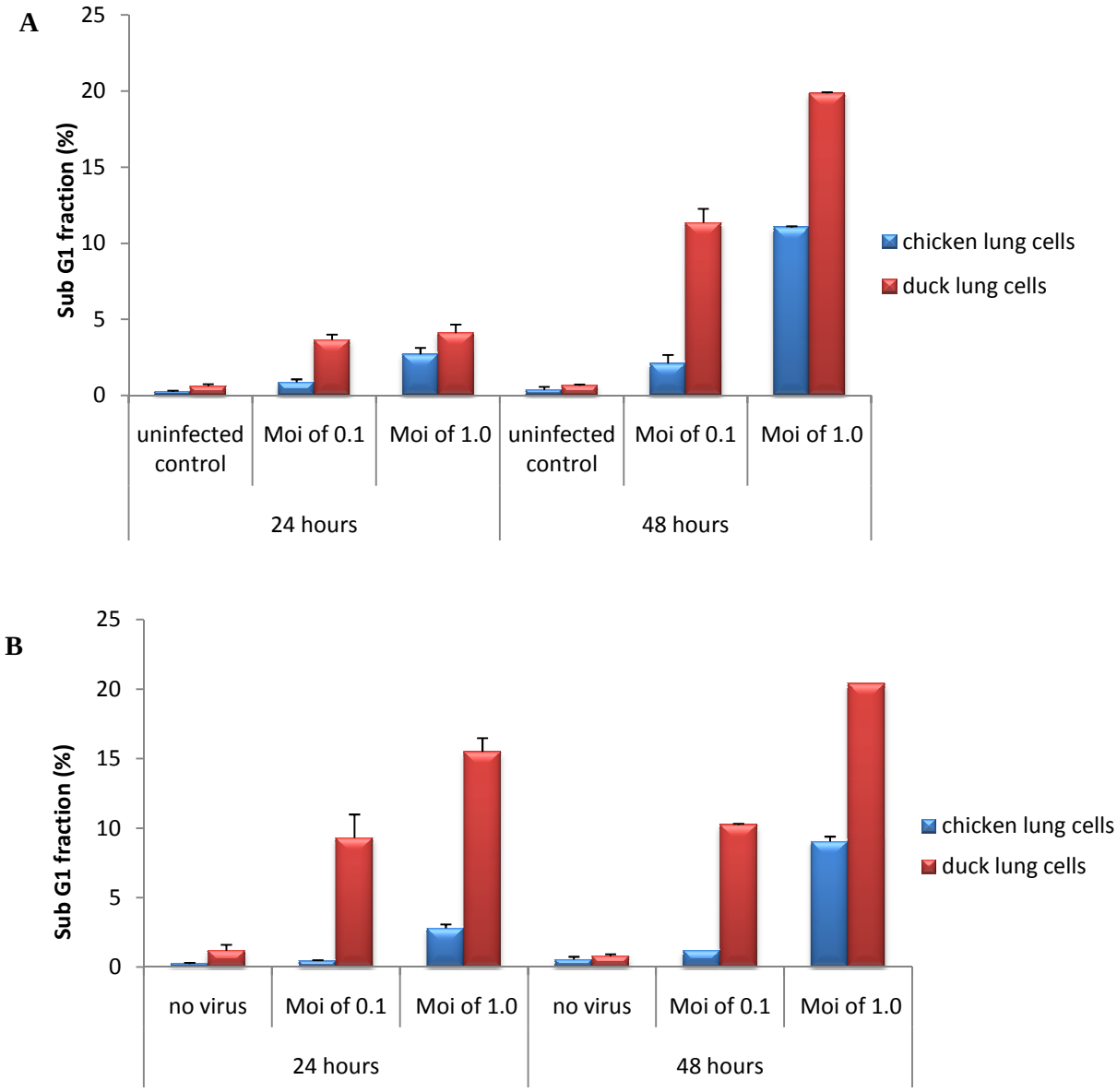


Figure 6.4-3 Quantification of levels of hypodiploid cells in avian lung cells following LPAI and swine influenza infection

Duck lung cells showing significantly higher levels of hypodiploid cells compared to chicken lung cells following infection with avian H2N3 (A) and swine H1N1 (B) ($p=0.01$ for both viruses) at 24 and 48 hrs post-infection. Data points are the mean of duplicate wells with error bars showing standard deviation.

6.4.2.2 Avian embryo cells

Primary cells derived from chicken, Pekin and mallard duck embryos were infected with LPAI and swine H1N1 viruses and the percentage of hypodiploid cells was measured at 24 and 48 hrs post-infection by PI assay. Pekin and mallard duck embryo cells consistently showed a significantly greater ($p = 0.01$ for all time points, both viruses and both doses) proportion of hypodiploid cells compared to chicken cells following infection with avian H2N3 and swine H1N1 at 24 (Figure 6.4-4A) and 48 hrs (Figure 6.4-4B) post-infection.

Mallard duck embryo cells showed a significantly greater proportion of hypodiploid cells at 24 and 48 hrs post-infection with swine H1N1 at an MOI of 1.0 and 0.1 ($p<0.01$) compared to Pekin duck embryo cells. While the level of hypodiploid cells in Pekin and Mallard duck cells was significantly higher compared to chicken cells following infection with avian H2N3 virus ($p<0.01$).

No significant difference ($p>0.05$) between Pekin and Mallard duck embryo cells was observed. The effect of the length of incubation and virus doses were similar to the observations in avian lung cells. Longer incubation and higher doses produced significantly greater proportion of hypodiploid cells at both 24 and 48hrs post-infection.

Swine H1N1 produced significantly higher proportion of hypodiploid cells in Pekin and Mallard duck embryo cells ($p<0.01$) compared to avian H2N3 virus. By contrast the difference caused by the two viruses in chicken embryo cells was not significant ($p>0.05$).

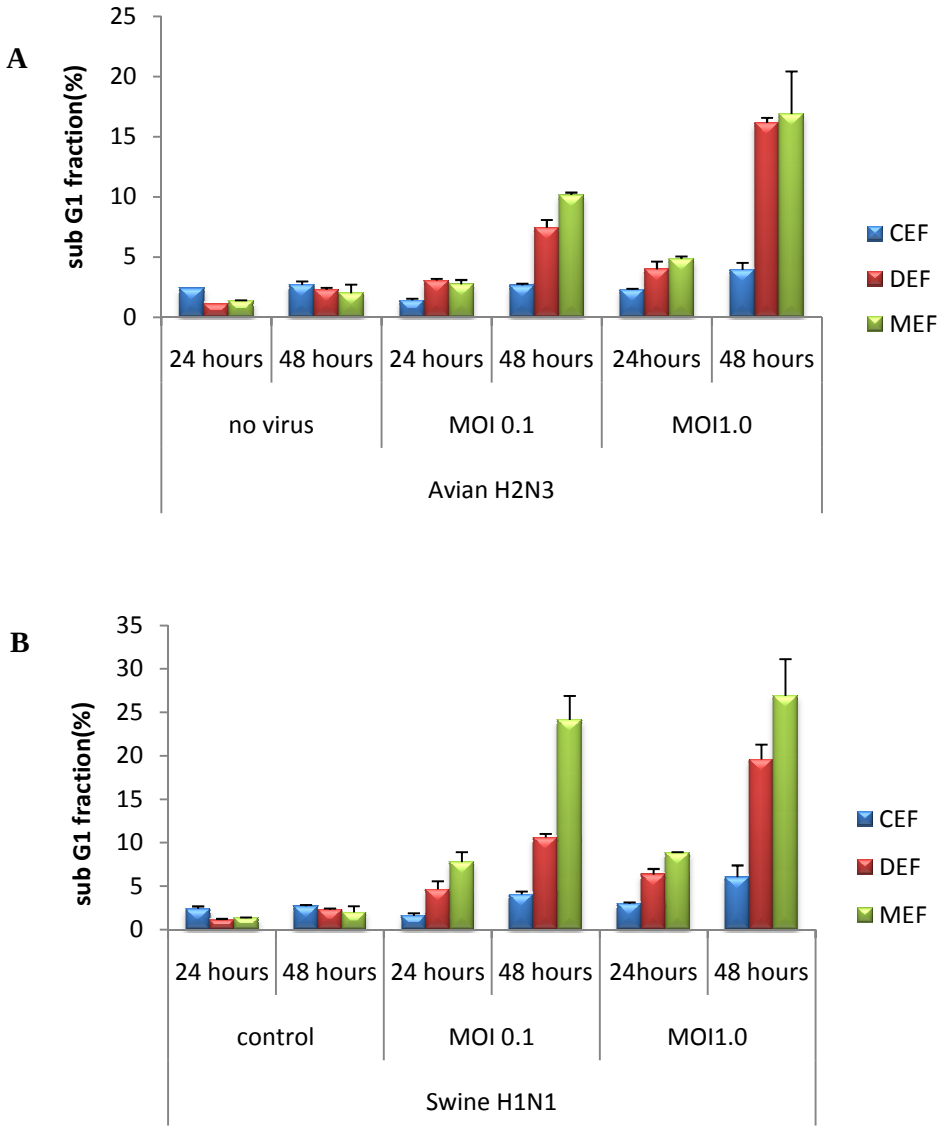


Figure 6.4-4 Quantification of levels of hypodiploid cells in avian embryo cells following LPAI and swine influenza infection

Pekin and mallard duck embryo cells showing significantly higher levels of hypodiploid cells compared to chicken embryo cells following infection with avian H2N3 and swine H1N1 ($p<0.01$ for both viruses) at 24 (A) and 48 hrs (B) post-infection. Data points are the mean of duplicate wells with error bars showing standard deviation.

CEF-chicken embryo fibroblasts; DEF- duck embryo fibroblasts; MEF- mallard embryo fibroblasts

6.4.3 Measurement of caspase 3/7 activation in cells

Duck cells produced significantly higher levels ($p<0.01$ for both viruses and time points) of activated caspase -3 and - 7 compared to chicken cells following infection with avian H2N3 and swine H1N1 at 18 and 40hrs post-infection (Figure 6.4-5). Levels of activated caspase -3 and - 7 in uninfected control cells were found to be low compared to infected cells and were not significantly different between chicken and duck cells ($P>0.05$).

Staurosporine treatment of cells produced high levels of activated caspase -3 and – 7, and was not significantly different between chicken and duck cells ($p>0.05$). A series of measurements of caspase activation made in chicken and duck cells treated with low and high concentrations of Staurosporine revealed no significant differences between the two species (data not shown). No significant differences in activated caspase levels were observed between duck cells infected with avian H2N3 and swine H1N1 ($p>0.05$).

Virus dose had no significant effect on the levels of caspase activation in both chicken and duck cells such that the levels of caspase induction were not significantly increased following infection at an MOI of 1.0 compared with 0.1, with either avian H2N3 or swine H1N1 ($p>0.05$).

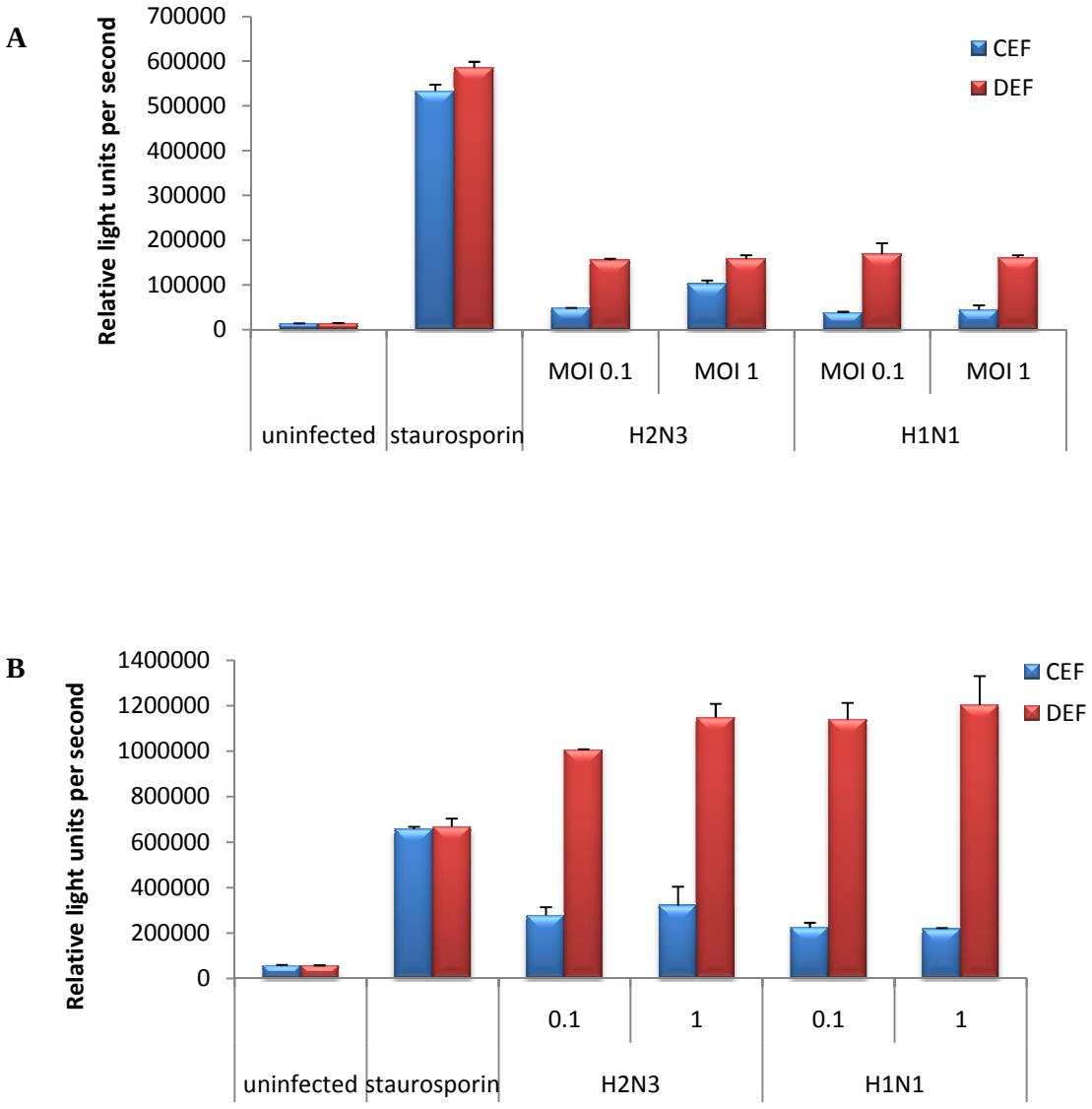


Figure 6.4-5 Measurement of activated caspase3/7 in cells following influenza virus infection

Duck cells showed significantly higher ($p<0.01$) levels of activated caspase -3 and - 7 compared to chicken cells following infection with avian H2N3 and swine H1N1 at 18hrs (A) and 40 hrs (B) post-infection at MOI 1.0 or 0.1. Staurosporine treatment induced comparable levels of activated caspase-3 and -7 in chicken and duck cells with no significant difference between species ($p>0.05$). Data points are the mean of duplicate wells with error bars showing standard deviation. CEF-chicken embryo fibroblasts; DEF- duck embryo fibroblasts.

6.5 Discussion

Due to the rapid induction of cell death following influenza infection in duck cells, the potential role of apoptosis as a cause of this phenomenon was investigated. Dying duck cells exhibited certain morphological features i.e. rounding, pyknosis and karyorrhexis which are characteristic morphological hallmarks of apoptosis (Kroemer et al. 2009). One of the characteristic biochemical features of apoptosis is DNA fragmentation which is very widely used as a marker to study apoptosis (Gopalakrishna & Khar 1995;Ioannou & Chen 1996).

The degree of apoptosis in chicken and duck cells following influenza virus infection was quantified by measuring nuclear fragmentation, using PI assay to detect hypodiploid cells. A significantly greater proportion of hypodiploid cells were found following infection of duck lung cells, compared to chicken lung cells, using with H2N3, swine H1N1 and H5N1 50-92 viruses. These findings further supported the role of apoptosis in the rapid death of duck cells. Higher levels of hypodiploid cells were observed in duck cells at 24 hrs following infection with swine H1N1 compared to avian H2N3 infection, whereas the levels were comparable at 48hrs post-infection. These results suggested more rapid onset of apoptosis in duck cells in response to swine H1N1 compared to avian H2N3. Comparison with HPAI could not be made as the PI assay was performed at only one time point due to restrictions in the containment facility.

Host response to strains of influenza viruses can be different depending on their relative pathogenicity. For example IFN-inducing capacity appears to be different between strains of influenza viruses which are a significant factor in regulating the pathogenesis, virulence, and viral transmission (Cauthen et al. 2007). Despite the differences between the viruses in the dynamics of cell death, duck cells consistently showed more rapid and higher levels of cell death than chicken cells, between 24 – 48 hours following infection with avian H2N3, classical swine H1N1 or with H5N1 50-92 viruses.

Later, the relative effects of infection on chicken and duck cells was studied with a contemporary Eurasian lineage H5N1 subtype, ty-Ty, known to cause severe clinical signs in ducks (Londt et al. 2008). Interestingly, only low levels of hypodiploid cells were detected in chicken and duck lung cells with no significant difference between the species in the level of hypodiploid cells. These finding further strengthened the role of apoptosis in influenza resistance in ducks, as the loss of this cellular response correlated with a more severe outcome of infection in ducks.

Parallel experiments performed using primary cells derived from Pekin duck and White leghorn chicken embryos showed similar differences in cell death as investigated in this project. This was evidenced by higher levels of hypodiploid cells in duck cells compared to chicken embryo cells. The differential rapid death of duck embryo cells was consistent across time points, viruses and doses. This suggests that the phenomenon of rapid cell death may be a species specific response of ducks and not cell type specific.

Aquatic birds are the major reservoir for influenza A, with wild ducks harbouring the majority of virus subtypes (Kim et al. 2009) and typically showing very few clinical signs (Olsen et al. 2006). The infection studies with mallard embryo cells showed much greater levels of hypodiploid cells at 24 and 48 hrs following infection with avian H2N3 or swine H1N1 at MOI of 1.0 or 0.1compared with the results for the chicken and Pekin duck embryo cells. This was supported by the morphological appearance of the cells, with mallard cells showing more severe cytopathic effects (data not shown) and greater hypodiploid cells (Figure 6.4-4) than Pekin duck cells.

Caspases 3 and 7 are the important effector caspases in the terminal phase of both the intrinsic and extrinsic pathways of apoptosis (Kurokawa & Kornbluth 2009;Taylor, Cullen, & Martin 2008). To further strengthen the supportive evidences for apoptosis, enzymatic characterization of cell death was carried out by measuring levels of activated caspases -3 and -7 in avian embryo cells following infection with avian H2N3 and swine H1N1viruses. Significantly higher levels of activated caspase-3 and -7 were found in

duck cells compared to chicken cells at 18 and 40 hrs following infection with these viruses. An earlier study described caspase mediated apoptosis in MDCK cells infected with equine influenza virus (Lin et al. 2002). The levels of activated caspases correlated with the observed differences in hypodiploid cells suggesting that the rapid death of duck cells is at least in part caspase mediated.

Treatment of chicken and duck embryo cells with Staurosporine induced rapid cell death and produced similar levels of caspase-3 and -7 activation, while un-infected chicken and duck cells showed very low levels. This implies that the differential induction of caspase -3 and -7 in duck compared with chicken cells was an influenza virus-specific phenomenon. Activated caspase levels in chicken and duck cells following infection with HPAI viruses could not be performed due to restrictions in the containment facility to carry out such assays.

Apoptosis in cells can also be triggered by caspase independent mechanisms which do not require activation of caspases (Kawahara et al. 1998; Perfettini et al. 2002). Further work is needed to investigate whether HPAI viruses also trigger caspase mediated cell death like LPAI or use alternate methods of apoptosis induction. Especially are caspases activated by 50-92 like viruses but not ty-Ty (as an example of emerging Eurasian lineage viruses)?

It is well established that influenza A viruses can cause apoptosis of virus infected cells, both *in-vitro* (Hinshaw et al. 1994; Takizawa et al. 1993) and *in-vivo* (Mori et al. 1995). However, the role of apoptosis in influenza A infection is complex. Apoptosis appears to be useful for the effective replication of influenza viruses. For example caspase activation is required for effective replication of influenza A viruses specifically for the release of viral ribonucleoprotein from the nucleus (Wurzer et al. 2003). Inhibition of apoptosis by knocking out Bax, a downstream target of Bcl-2, causes diminished virus output (McLean et al. 2009). Influenza A virus infection leads to increased p53 which contributes to apoptosis of infected cells (Turpin et al. 2005). However, recent evidence

suggests that influenza A virus initiates anti-apoptotic PI3k-Akt signalling at early and middle phases of infection to protect cells from fast apoptotic death, whereas both p53-dependent and alternative p53-independent apoptotic pathways are triggered at the late stage of infection (Zhirnov & Klenk 2007). Further, accumulation of p53 occurs in a biphasic pattern during influenza infection with a transient elevation at the beginning phase of infection and a second elevation at the middle-late phase of infection (Shen et al. 2009).

Apoptosis may help to limit virus replication such that the early apoptotic death of virus infected cells could result in impaired virus multiplication. For example Herpes simplex virus type1 (HSV-1) γ 34.5 mutant has been found to induce programmed cell death in neuroblastoma cell lines concomitant with a loss of virus multiplication (Chou & Roizman 1992). It appears that early apoptosis could be antiviral and beneficial to the host while apoptosis at late stages of infection could be beneficial to the virus. As a consequence, influenza viruses have evolved a number of mechanisms to delay apoptosis in infected cells. Since, influenza virus appears to block early apoptosis for its efficient replication, host cellular responses triggering early apoptosis could potentially be antiviral and may confer protection from viral infection.

Caspase mediated apoptosis in duck cells following influenza virus infection *in-vitro* , causing a decreased infective virus production could be a potential resistance mechanism *in-vivo.* The contemporary Eurasian H5N1 viruses that can overcome such response are able to cause severe clinical disease. Early apoptosis followed by phagocytic clearance of apoptotic bodies contributes to host survival. Inhibition of phagocytosis leads to increased host mortality in mice (Hashimoto et al. 2007).

Further investigation is required to determine the molecular mechanism involved in the induction of apoptosis in duck cells which could provide valuable insights in to the role of apoptosis in influenza infection.

Chapter 7

Study of the differences in global gene expression between chicken and duck cells following influenza virus infection

7.1 Summary

Chicken and duck gene expression profiles of primary lung cells infected with one LPAI and two HPAI H5N1 influenza virus subtypes were analyzed using an Affymetrix chicken expression microarray platform to attempt to understand the molecular basis of host susceptibility and resistance. To take into account the specificity of heterologous hybridization (between labelled duck targets on chicken probes), a well established analytical tool was used which involved the hybridization of duck genomic DNA to the chicken chip to establish specific probe binding. Gene expression and ontology analyses revealed that genes involved in vital functions such as protein and nucleic acid metabolism were differentially expressed between chicken and duck lung cells following influenza virus infection. Quantitative RT-PCR analysis of selected genes validated the microarray findings. The differential expression patterns of BCLAF1, AVEN, HSPA9 and BCOR suggest their possible role in the manifestation of rapid cell death in duck cells following influenza infection. Infected duck cells expressed higher levels of interferon-α and IL-18 than infected chicken cells. Infected chicken cells, however, showed higher levels of pro-inflammatory cytokines TNF-α, IL-6 and IL-8 expression than infected duck cells. Transcriptional regulation of STAT-3 gene appears to be important in mediating innate antiviral response and anti inflammatory response in influenza virus infected avian primary cells.

7.2 Introduction

Genomic tools are very useful to study host–pathogen interactions (Kash et al. 2006) and to understand molecular mechanisms in gene regulations. Whole-genome profiling of virus-infected cells, in combination with other high-throughput technology is a powerful tool to understand virus-host interactions and may eventually lead to the discovery of new anti-viral therapies (Tan et al. 2007).

7.2.1 The principle of microarrays

Combination of solid-phase chemistry, photo-labile protecting groups and photolithography have been used to direct the synthesis of oligonucleotides to specific positions on a glass surface by irradiating the surface through a set of patterned photolithographic masks (Fodor et al. 1991). This light directed fabrication method was the basis for the development of high density microarray technology, which is a powerful molecular genetics technique. Microarrays consist of an array of oligonucleotides called probes, each containing a specific DNA sequence attached to an impermeable solid surface like glass or polypropylene (Matson, Rampal, & Coassin 1994) or silicon (Southern 2001).

Microarrays exist in a variety of forms and can be classified based on length of target sequence (long cDNAs or oligonucleotides), commercial or custom synthesis, global or specific design, glass- or membrane material and spotted or *in-situ* (e.g. photolithography) printing. Since microarrays are extensively used across several disciplines, many commercial suppliers have made available standard microarrays and analysis packages. Manufacturing high quality cDNA microarrays depends on some key factors including a printing device, spotting pins, glass slides, spotting solution, and humidity during spotting (Park et al. 2004). Affymetrix, one of the leading commercial manufacturers of microarrays, produces several microarray platforms which are based on *in-situ* synthesis of oligonucleotides on glass. Whereas other companies such as Agilent and BD Clontech use long cDNAs derived from ESTs, cloned cDNA, or PCR products attached to a variety of support structures, including nylon filters, glass slides, or silicon chips (Li, Pankratz, & Johnson 2002).

The GeneChip® array (Affymetrix) use probe-sets rather than single oligonucleotide per gene, comprising between 11 and 20 probe-pairs to quantify abundance for each transcript. Each probe-pair consists of a perfect-match (PM), a 25-base sequence

complementary to the target transcript, and a mismatch (MM) probe which is identical to the PM probe with the exception of a single mismatch at the 13th base.

7.2.2 Gene expression analysis

With the advent of microarray technology gene expression analysis has changed from the traditional notion of "one gene, one experiment" to a completely parallel, automated and miniaturized assay to analyze genome wide changes in expression (Li, Pankratz, & Johnson 2002). Gene expression profiling data from infection studies with a wide variety of pathogens can be compared to understand common trends in the infectious disease process and host-pathogen interactions. This may be useful in developing broad-spectrum therapeutic agents. For example, a common host-transcriptional-response to infection comprising Toll-like receptor and pathogen-mediated signalling pathways was observed by comparing published transcriptional-profiling data from 32 studies that involved 77 different host–pathogen interactions (Jenner & Young 2005).

7.2.3 Host gene expression following influenza virus infection

Gene expression profiling of cells following influenza virus infection has been used to understand the pathogenesis and host response to influenza infection. For example DNA-microarray gene expression profiles of lung cells following influenza virus infection *In-vitro* has helped in understanding the role of NS1 protein in the pathogenesis of influenza infections (Geiss et al. 2002). Infection of human middle-ear epithelial cells with influenza causes significant differential regulation of several genes. These include interferon inducible genes, chemokine and cytokine genes, pro- and anti-apoptotic genes, signal transduction and transcription factors, cellular immune response genes, cell cycle and metabolism genes (Tong et al. 2004). Influenza virus infection causes significant induction of genes involved in the interferon pathway of influenza-infected mice (Kash et al. 2004) and pigtailed macaques (Baskin et al. 2004). Gene expression profiles of

peripheral blood of influenza infected children show a strong up-regulation of interferon-regulated genes, such as IFIT1, IFIT2 and vipirin (Kawada et al. 2006).

Recently, the use of expression microarrays was reported to study avian host response to influenza infection. For example, host gene expression profiles in infected chicken tracheae *in-vivo* are different from infection findings from chicken tracheal organ cultures. In *in-vivo* chicken and tracheal organ cultures (TOC) genes associated with IFN-mediated responses were differentially regulated. However, in TOCs the early immune responses were masked by wound healing response triggered by cutting and processing of tracheal tissue for culturing (Reemers et al. 2009a). Furthermore, differences in gene expression profiles were also observed in different parts of the chicken lung depending on anatomical location and airflow (Reemers et al. 2009b).

7.2.4 Validation of microarray data

Validation of microarray data is an essential step as array results can vary based on several factors like array manufacture, sample preparation and analysis. There are two major approaches for confirmation of microarray data: *in-silico* analysis and laboratory-based analysis. The *in-silico* method involves comparison of array results with information available in the literature and in public or private expression databases. This is useful to validate data without the need for further experimentation. Laboratory-based validation of data is typically done with the same samples that were used for the array experiment(s) which includes techniques like semi-quantitative reverse transcription PCR (RT−PCR), real-time RT−PCR (qRT-PCR), Northern blot, and *in-situ* hybridization or immunohistochemistry using tissue microarrays (Chuaqui et al. 2002). Real-time RT-PCR is well-suited to validate expression array results because it is quantitative, rapid, and requires 1000-fold less RNA than conventional assays (Rajeevan et al. 2001;Walker 2002).

7.2.4.1 Normalizing qRT-PCR data

Real-time PCR is widely used to quantify biologically relevant changes in mRNA levels. However the following factors need to be considered and appropriately addressed to ensure accurate results: the inherent variability of RNA, variability of extraction protocols and different reverse transcription and PCR efficiencies (Bustin 2002). Consequently, it is important that an accurate method of normalisation is chosen to control the error introduced by these factors. A widely used method for normalization involves the measurement of an internal reference or "housekeeping" gene, which has the advantage over other methods that it takes into account many variables such as enzyme efficiency and RNA quality (McCurley & Callard 2008).

An ideal reference/ housekeeping gene should show stable expression in samples from different subjects, different tissues, across developmental and life stages, and after undergoing experimental treatments. Unless a reference gene satisfies these criteria, gene expression analysis based on normalization to a varying reference gene could produce erroneous results (Bustin 2000). Ribosomal RNA (rRNA), the central component of the ribosome is the most conserved (least variable) gene in all cells (Smit, Widmann, & Knight 2007). Among many genes used for normalizing qPCR data, 18S rRNA gene is considered suitable for gene expression studies in avian and other species. For example satisfactory results were obtained by gene expression studies in chicken (Dridi et al. 2005;Wang et al. 2009) and geese (Ding et al. 2007) using *18S rRNA* as a housekeeping gene. In our laboratory, we had extensively evaluated several housekeeping genes, including *β-actin*, *GAPDH*, *18S rRNA* and *ATP5B* for their suitability for qPCR normalization in primary pig and human cells infected by low and high pathogenicity influenza viruses. The least variant gene among these genes was found to be *18S rRNA* (unpublished data).

To understand the molecular mechanisms contributing to host susceptibility or resistance to influenza virus infection in chicken and ducks, gene expression profiling (using Gene

Chip chicken genome arrays from Affymetrix) of primary lung cells from chicken and duck was carried out.

7.3 Materials and methods

7.3.1 Experimental design

Chicken and duck lung cells were used to study the transcriptome following infection with LPAI H2N3 or HPAI H5N1 50-92 or H5N1 ty-Ty viruses. Three wells of each cell type were used for each of the three viruses. Negative controls were performed in triplicate wells for each cell type without virus infection. Total RNA extracted from two wells from each of the treatment and control groups were hybridized to one GeneChip® chicken genome array (Affymetrix) and a total of 16 array chips were used.

7.3.2 Virus infection and total RNA extraction

Primary lung cells derived from White Leghorn chicken (*Gallus gallus*) and Pekin ducks (*Anas platyrhynchos*) were grown in 6 well cell culture plates (Costar), and were infected with avian H2N3 or H5N1 50-92 or H5N1 ty-Ty at MOI of 1.0 as described in Chapter 4. Twenty-four hrs following infection, total RNA from each well was extracted as described in Chapter 2.

7.3.3 Microarray expression analysis

Microarray expression analysis was carried out using GeneChip® chicken genome array (Affymetrix) which contains 32,773 transcripts corresponding to over 28,000 chicken genes and 689 probe sets for detecting 684 transcripts from 17 avian viruses (Figure 7.3-1). GeneChip arrays consist of 25-mer oligonucleotide probes and a set of 11 oligonucleotide probe pairs for each transcript. Each feature on the array measures 11µm

and the detection sensitivity of the array is 1:100,000 which is the lowest concentration of probe at which signal to background ratio exceeds a threshold value (for example 3). In this project, services for RNA target preparation, hybridization and scanning were provided by the Nottingham Arabidopsis Stock Centre (NASC, Sutton Bonington, UK).

Figure 7.3-1 GeneChip chicken genome array

Photograph of GeneChip chicken (*Gallus gallus*) genome array used in the study is shown. This chip contains 32,773 transcripts corresponding to over 28,000 chicken genes and 689 probe sets for detecting 684 transcripts from 17 avian viruses

7.3.4 Preparation of RNA target

For microarray expression analysis, RNA targets for labelling were prepared using the GeneChip® 3' IVT Express Kit (Affymetrix) which is based upon linear RNA amplification and employs T7 *in-vitro* transcription technology. This method is also known as the Eberwine (Phillips & Eberwine 1996) or reverse transcription-IVT (RT-IVT) method, which is considered the 'gold standard' for target preparation for gene expression analysis.

In the GeneChip® 3' IVT Express protocol, total RNA undergoes reverse transcription to synthesize first-strand cDNA. This cDNA is then converted into a double-stranded DNA template for transcription. *In-vitro* transcription synthesizes anti-sense aRNA (cRNA is

also known as amplified RNA or aRNA) and incorporates a biotin-conjugated nucleotide. The aRNA is then purified to remove unincorporated NTPs, salts, enzymes, and inorganic phosphate. Fragmentation of the biotin-labelled aRNA prepares the sample for hybridization onto GeneChip 3' expression arrays. All the steps involved in preparation of sample to hybridization were summarized in Figure 7.3-2 and a detail description of each step is provided in Appendix IV.

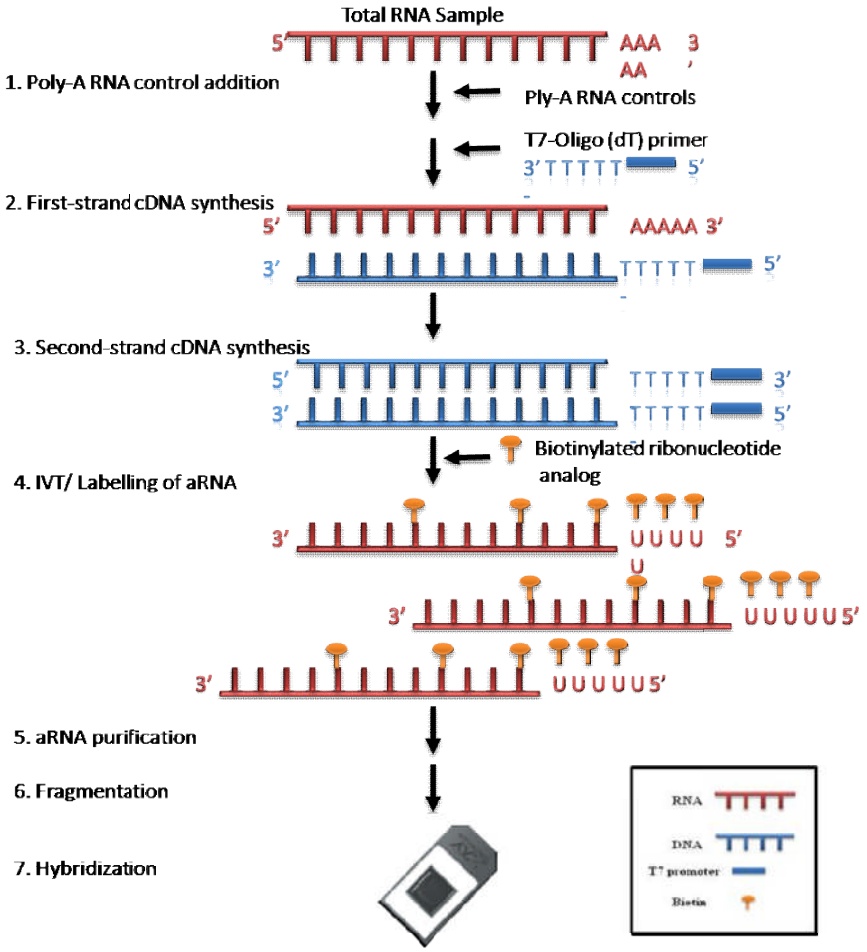


Figure 7.3-2 Overview of the GeneChip® 3' IVT Express Kit Labelling Assay

The different steps involved in the preparation of RNA target for hybridization to Genechip arrays using GeneChip® 3' IVT Express Kit.

7.3.5 Cross species hybridization

Although the high-density GeneChip arrays are highly useful for transcript profiling, their usefulness is limited because they are currently available for only a limited number of plant and animal species. A genomic DNA (gDNA) based probe-selection method was developed at the NASC to study the transcriptome of plant or animal species by hybridization on to GeneChip arrays of heterologous species (Hammond et al. 2005). As there is no high-density microarray platform available for duck, chicken gene chip was utilized for duck transcriptome analysis. The method of gDNA based probe selection for increasing the sensitivity of chicken GeneChip to study the transcriptome of duck is briefly described here. Genomic DNA from Pekin duck (*Anas platyrhynchos*) cells was biotin-labelled and hybridized to the Chicken *(Gallus gallus)* GeneChip® array and a probe intensity data file (*cel*) was generated as described in the section 7.3.3. Probe-sets on the chicken chip were selected for subsequent duck transcriptome analyses if the probe-set was represented by PM probes with duck gDNA hybridization intensities above an experimentally set threshold. Selection was performed using a *cel* file parser script written in the Perl programming language (X-species Version 2.1, http://affymetrix.arabidopsis.info/xspecies/**)**. After installing Active Perl software for windows (Active Perl version 5.10.1.1007 for Windows), *CDF_masking.zip* was downloaded and unzipped to a chosen location on the computer (http://affymetrix.arabidopsis.info/xspecies/CDF_masking.zip). Original CDF file for chicken chip downloaded from Affymetrix website (http://www.affymetrix.com) and duck gDNA hybridization *cel* file were copied to CDF-masking folder. In the CDF-masking folder *easy_script.pl* was run to generate a series of probe mask (CDF) files for duck with a range of threshold values. After executing *easy_script.pl* desired gDNA hybridization intensity threshold value need to be mentioned to generate a probe masking file with a particular intensity threshold. Using this method 20 probe mask files were generated with gDNA hybridization intensity thresholds ranging from 20 to 2000.

7.3.6 Microarray data analysis

Microarray expression analysis was carried out using the GeneSpring GX10 expression analysis software (Agilent Technologies). The *Advanced Workflow* option was used for data analysis in the GeneSpring GX 10, which provides many options for summarization algorithms, normalization routines etc, depending upon the technology used. After creating a new project, a new experiment was created by choosing *Affymetrix expression* from the dropdown menu. In the next step data files (*cel* files) were uploaded.

7.3.6.1 Probe summarization

Probe summarization was carried out by Robust Multichip Averaging (RMA) summarization algorithm (Irizarry et al. 2003a; Irizarry et al. 2003b). The RMA algorithm conducts background correction, followed by Quantile normalization and probe summarization. Subsequent to probe-set summarization, baseline transformation of the data was performed with the option of baseline to median of all samples. The software calculates the log summarized values from all the samples for each probe and calculates the median and subtracts from each of the samples.

7.3.6.2 Experimental grouping

Experimental grouping was done by defining four groups which are un-infected control, H2N3 infected, 50-92 infected and ty-Ty infected with replicates arrays in each group.

7.3.6.3 Creating interpretation

An interpretation was created to specify grouping of samples based on treatment as the experimental condition using the *create interpretation* function.

7.3.6.4 Quality Control on all samples

Quality control check on all samples was carried out by *Principal Component Analysis* (PCA) and the scores were visually represented in a 3D scatter plot. The scores were used to check data quality. One point per array coloured by the experimental factors was shown allowing viewing of separations between groups of replicates. Ideally, replicates within a group should cluster together and separately from arrays in other groups. The *Correlation Plots* shows the correlation analysis across arrays carried out by the Pearson correlation coefficients. Correlation coefficients for each pair of arrays were calculated and the results were displayed in visual form as a heatmap. The *Internal Controls* view depicts RNA sample quality by showing 3′/5' ratios for a set of specific probe sets which include the actin and GAPDH probe sets. The 3′/5' ratio is output for each such probe set and for each array. For good quality samples the ratios for actin and GAPDH should be no more than 3.

The *Hybridization Controls* view depicts the hybridization quality. Hybridization controls are composed of a mixture of biotin-labelled cRNA transcripts of *bioB, bioC, bioD,* and *cre* prepared in staggered concentrations (1.5, 5, 25, and 100 pm respectively). This mixture is spiked-in into the hybridization cocktail. *bioB* is at the level of assay sensitivity and should be present at least 50% of the time whereas *bioC*, *bioD* and cre must be present all of the time and must appear in increasing concentrations. The *Hybridization Controls* show the signal value profiles of these transcripts (only 3' probe sets are taken) where the X axis represents the biotin labelled cRNA transcripts and the Y axis represents the log of the normalized signal values.

7.3.6.5 Statistical analysis

Statistical analysis was carried out by analysis of variance (ANOVA) with a p value cut-off of 0.05 by asymptotic p-value computation algorithm with no multiple testing correction. The entities satisfying the significance analysis were passed on for the fold

change analysis. *Fold Change Analysis* was used to identify genes with expression ratios of treatment and control samples that are outside of cut-off of 1.3. Fold change was calculated between control and each treatment separately and the ratio between treatment and control was calculated (Fold change = treatment/ control). Fold change gives the absolute ratio of normalized intensities (no log scale) between the average intensities of the samples grouped.

7.3.7 Analysis of cross species hybridization data

Using the create technology function in Genespring Gx10, a technology was created using the duck CDF file for each of the gDNA intensity thresholds tested. The probes were annotated using the chicken GeneChip annotation file. The *cel* files obtained by hybridizing duck total RNA on to chicken GeneChip were analyzed as described in the previous section using the technology created for duck. A series of 11 analyses were performed for each gDNA intensity threshold from 40-450. Selection of the gDNA intensity threshold for subsequent analysis was made based on the number of differentially regulated genes.

7.3.8 Validation of microarray data

7.3.8.1 Quantitative real-time PCR (qRT-PCR)

For validating microarray data, a set of genes were analyzed by quantitative PCR assay using the original total RNA samples from infected cells that were used for microarray experiment. The selected genes for qRT-PCR analysis were BCL2-associated transcription factor 1 (*BCLAF-1*), apoptotic peptidase activating factor 1 (*Apaf-1*), interleukin-8 (*IL-8*), interleukin-6 (*IL-6*) genes along with interferon alpha (*IFN-α*), and tumor necrosis factor alpha (*TNF-α*).

7.3.8.2 qPCR primer and probe design

Oligonuclotide primers and hydrolysis probes for TaqMan assay were designed based on published sequences of selected genes using Primer Express software version 2.0 (Applied Biosystems). Typically probes and primers were between 20-30 deoxynucleotide residues in length, with a base comparison of 50-60% guanine (G) or cytosine (C) where possible avoiding repeats of long nucleotides. Primer and probe sequences used in this study were provided in Table 7.3-1. All primers were provided by Eurofins MWG Operon (London, UK) and all probes were supplied by Sigma Aldrich.

7.3.8.3 Housekeeping gene

Chicken 18S rRNA gene was used as a house keeping gene in the present study for relative expression analyses. Primers and TaqMan probe were designed using published sequence of chicken 18SrRNA (GenBank: AF173612.1). Crossing point (CP) values of control and infected samples were analyzed by two sample *t* test using *Minitab* statistical software version 15.

Table 7.3-1 Primer and probe sequences used in the study

Gene	GenBank acc.No	Primers	Probe
Chicken 18S rRNA	AF173612.1	Fwd :TGTGCCGCTAGAGGTGAAATT Rev: TGGCAAATGCTTTCGCTTT	5' (6FAM) TTGGACCGGCGCAAGACGAAC 3' (TAMRA)
Chicken *IL-6*	EU170468	Fwd :CACGATCCGGCAGATGGT Rev: TGGGCGGCCGAGTCT	5' (6FAM)ATAAATCCCGATGAAGTGGTCATCC 3' (TAMRA)
Chicken *IL-8*	NM_205018.1	Fwd :CCCTCGCCACAGAACCAA Rev: CAGCCTTGCCCATCATCTTT	5' (6FAM)CCCAGGTGACACCCGGAAGAAACA 3' (TAMRA)
Duck *IL-6*	AB191038	Fwd :CCAAGGTGACGGAGGAAGAC Rev: TGGAGAGTTTCTTCAAGCATTTCTC	5' (6FAM)TGTCTCCTGGCTGGCTTCGACGA 3' (TAMRA)
Duck *IL-8*	AB236334.1	Fwd :AGCCTGGTAAGGATGGGAAAC Rev: GGGTGGATGAACTTCGAGTGA	5' (6FAM)AGCTCCGGTGCCAGTGCATAAGCA 3' (TAMRA)
Chicken *BCLAF-1*	XM_419728.2	Fwd : CCACCCCGCCTAGTCAGA Rev: TGAGTGGGCCGTACTAAGCAA	5' (6FAM)TTCATCTTGCTCTGATGC CCC3' (TAMRA)
Chicken *Apaf-1*	DR764820.1	Fwd : TCCTTATCAAAAGTAGCAGATTGCA Rev: GAGGATGTCAGAAACAGAGATCCA	5' (6FAM)TCACACAGTGAACCCAGCTCATGT3' (TAMRA)
Chicken *IFN-α*	EU367971	Fwd :CTTCCTCCAAGACAACGATTACAG Rev: AGGAACCAGGCACGAGCTT	5' (6FAM)CCTGCGCCTGGGAACACGTCC 3' (TAMRA)
Chicken *TNF-α factor*	AY765397	Fwd :CCCTTCTGAGGCATTTGGAA Rev: CAGCCTGCAAATTTTGTCTTCTT	5' (6FAM)AGCCCACTCCCGAACGCTG 3' (TAMRA)
Duck *IFN-α*	DQ861429	Fwd :AACCAGCTTCAGCACCACATC Rev: TGTGGTTCTGGAGGAAGTGTTG	5' (6FAM)TGCTTCCCAGCCGACGCC 3' (TAMRA)
Duck *TNF-α*	EU375296	Fwd :GCCAACAAATAACCCCGTTACA Rev: CTGGGCGGTCATAAAATACCA	5' (6FAM)CAGGTTGCTGCACATACACCGTCTGAA 3' (TAMRA)

7.3.8.4 First strand cDNA synthesis

First strand cDNA was synthesized from chicken and duck total RNA samples using the Superscript III First-strand synthesis system (Invitrogen) as described in Chapter 2.

7.3.8.5 qRT-PCR conditions

Quantitative PCR for the relative expression analysis of selected genes was carried out using the *LightCycler* ® 480 Probes Master mix (Roche) and all the reactions were carried out using the LightCycler® 480 (Roche). A master mix was prepared for each target gene comprising 10µl of Roche master mix, 0.6µl each of forward and reverse primers (each at 300nM), 0.4µl of probe (200nM) and 3.4 µl of nuclease free water. Five micro litres of cDNA diluted at 1:200 was used per reaction in a total reaction volume of 20µl. The following programme was used for the qPCR reaction and crossing point (CP) values were calculated in the absolute quantification mode. Denaturation at 95°C for 10min followed by 45 cycles of 95°C for 15s, 60°C for 30s, 72°C for 1s followed by cooling at 40°C for 10s.

7.3.8.6 qRT-PCR calculations

A standard curve was generated for reference gene (18s) and each target gene for each species, using serial dilutions of cDNA sample (pooled infected and control). Slope of the standard curve plotted against Cp value and log cDNA concentration was calculated in Microsoft Excel (2007). Reaction efficiency for reference gene and each target genes was calculated using the following formula.

$$\text{Efficiency} = -1+10^{(-1/\text{slope})}$$

7.3.8.7 Relative expression analysis

Relative expression analysis of target genes was carried out using the relative expression software tool (REST©), which compares two groups, with up to 16 data points in a sample and 16 in a control group, for reference and target genes. The mathematical model used in the software was based on the PCR efficiencies and the mean crossing point deviation between the sample and control group (Pfaffl, Horgan, & Dempfle 2002). Relative expression of target genes was calculated based on comparison of control and treatment groups and the data was normalized to *18S rRNA* expression. The expression ratios of the transcripts were tested for significance by a randomization test with a pair-wise reallocation using REST. Comparison of each treatment group (i.e. H2N3 infected, H5N1 50-92 infected and H5N1 ty-Ty infected) for chicken and duck against control was performed separately in REST.

7.4 Results

7.4.1 Cross species hybridization

Perfect match (PM) probes of chicken genome array hybridized extensively to the *Anas platyrhynchos* genomic DNA (Figure 7.4-1). When the gDNA hybridization intensity threshold was increased from 20 to 2000, probe pairs retention in the probe mask files decreased rapidly. However, the retention of whole probe-sets, representing transcripts, was less sensitive to the increase in gDNA hybridization intensities during probe mask file generation. This is because only a minimum of one probe pair is required to retain a probe-set. For example probe mask file generated using a gDNA hybridization intensity threshold of 20 retained 100% *Gallus gallus* probe-pairs and probe-sets (i.e. 423199 and 38473 respectively). Whereas the probe mask file generated with a gDNA intensity threshold of 100, masked over 50 % of probe pairs, while only 2.5% of *Gallus gallus* probe-sets were masked (retaining 97.5 % probe sets).

7.4.1.1 Selection of gDNA intensity threshold for duck expression analysis

Number of differentially regulated genes in duck cells was analyzed by comparing the treatment group against control using technologies created with each of the 11 gDNA intensities from 40-450. Out of the 11 gDNA intensities analyzed, a threshold of 200 gave the highest number of genes with a significant differential regulation ($p<0.05$) (Figure 7.4-2A). Similarly, a threshold of 200 gave the highest number of genes regulated at a fold change of ± 2($p<0.05$) (Figure 7.4-2 B & C). Based on these findings, technology created with a gDNA threshold of 200 was used for further transcriptome analysis of all the duck samples. Technology created for duck represented 32896 transcripts out of the total 38535 transcripts represented in the original chicken GeneChip technology.

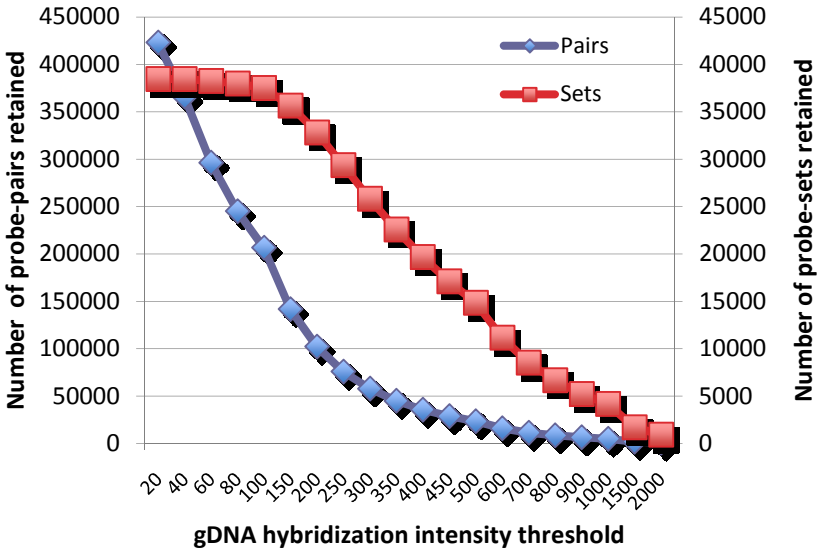


Figure 7.4-1 Probe masking based on gDNA hybridization intensities

Anas platyrhynchos gDNA hybridization intensity thresholds used to generate the probe mask files is shown. Data were obtained by hybridizing duck genomic DNA on chicken Genechip. Number of *Gallus gallus* probe-pairs and probe-sets from the chicken GeneChip® array retained at various levels of intensity threshold is shown. Probe- pairs retained (data in blue) is scaled to the left hand *y*-axis, while number of probe-sets retained (data in red) are scaled to the to the right-hand *y*-axis).

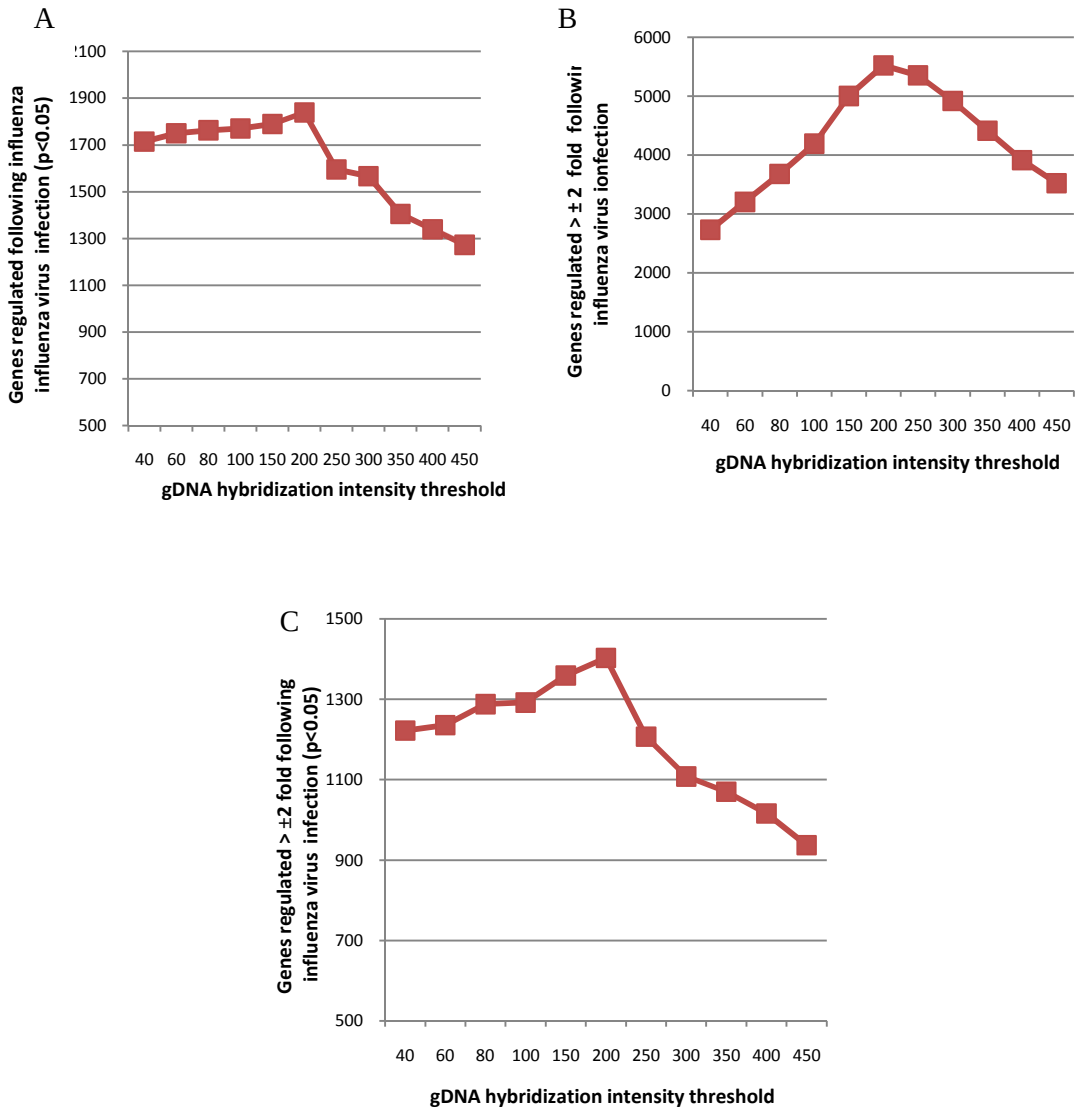


Figure 7.4-2 Genes regulated following influenza infection in *Anas platyrhynchos*

Genes regulated in *Anas platyrhynchos* as a function of gDNA hybridization intensity thresholds used to generate the probe mask files for the transcriptome analysis. Intensity threshold of 200 gave highest number of genes differentially regulated following 24 hrs of infection with influenza viruses (H2N3, 50-92 and ty-Ty) compared to uninfected controls. A) All the genes differentially regulated ($p<0.05$), B) Genes regulated ±2 fold (all) following infection and C) Genes significantly regulated ±2 fold ($p<0.05$).

7.4.2 Quality Control on all samples

Quality control on all samples was carried out by the *Principal Component Analysis* (PCA) and the replicate arrays in each treatment and control group were clustered together indicating good quality of the samples and hybridization (Figure 7.4-4). Correlation analysis across arrays carried out by the Pearson correlation coefficients showed high correlation between the replicates in each group. Correlation coefficients of each pair of arrays were between 0.98 to 1.0 and the results were displayed in visual form as a heatmap (Figure 7.4-3).

The *Internal Controls* analysis showed ratios for actin and GAPDH less than 3, for all the samples, indicating good sample quality. *Hybridization Controls* showed the signal value profiles as expected indicating good hybridization quality.

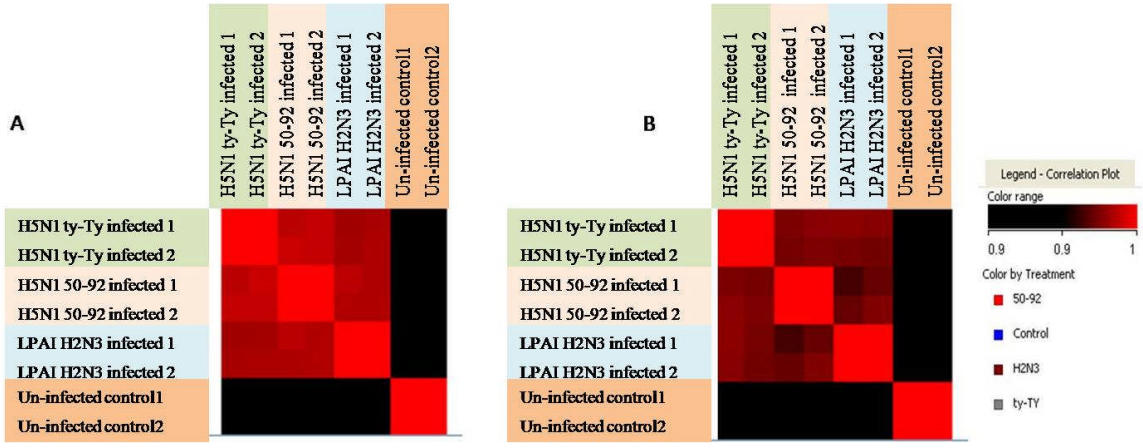
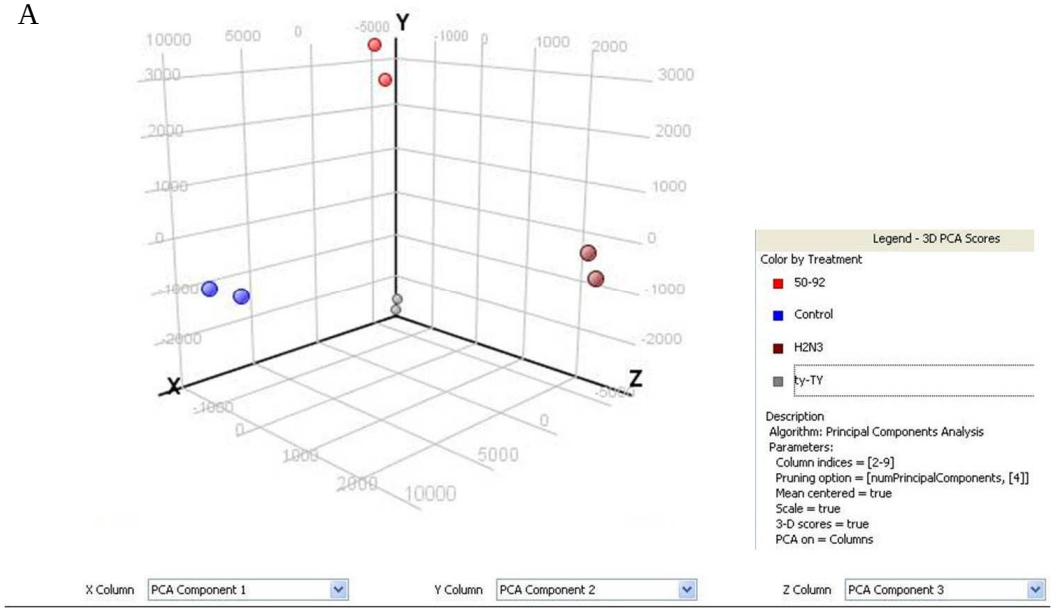


Figure 7.4-3 Heat maps showing correlation analysis of arrays

Correlation analysis of chicken (A) and duck (B) samples showing high degree of correlation between each pair of arrays in treatment and control groups (correlation coefficient values ranging from 0.98 to 1.0).

A



B

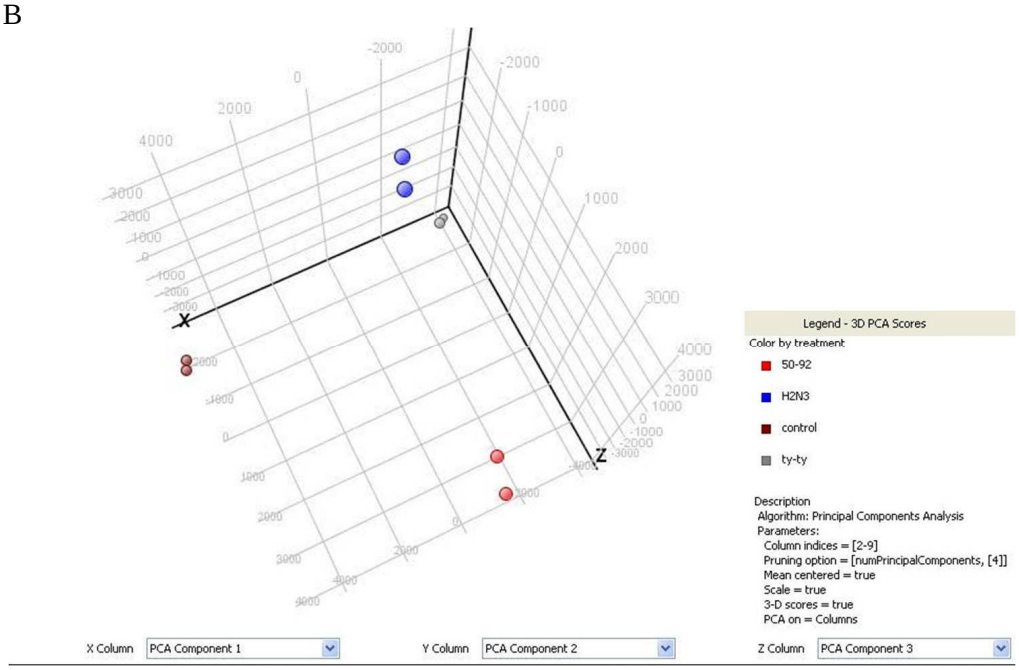


Figure 7.4-4 Principle component analysis of arrays

3D PCA plots showing arrays hybridized with chicken (A) and duck (B) infected and control samples. Each point representing one array with replicate samples in each group represented by the same colour clustered together.

7.4.3 Microarray expression analysis

7.4.3.1 Experimental grouping

Treatment was used as an interpretation to group the arrays. Duplicate arrays in each treatment and control group were normalized by the RMA algorithm (Figure 7.4-5 & Figure 7.4-6).

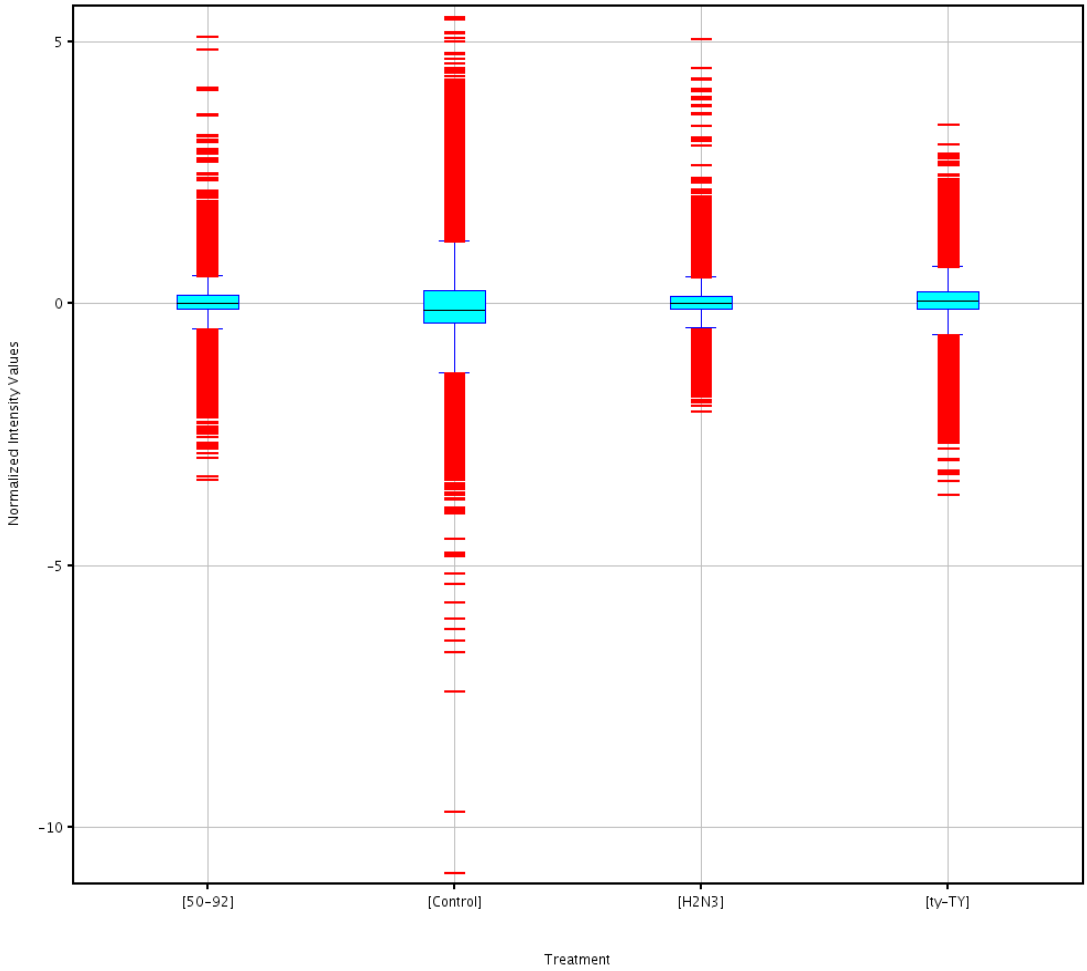


Figure 7.4-5 Box Whisker plot showing experimental grouping of chicken samples

Box whisker plot of normalized intensity values for each of the treatment and control groups are shown. Median values are marked in the centre with a black line.

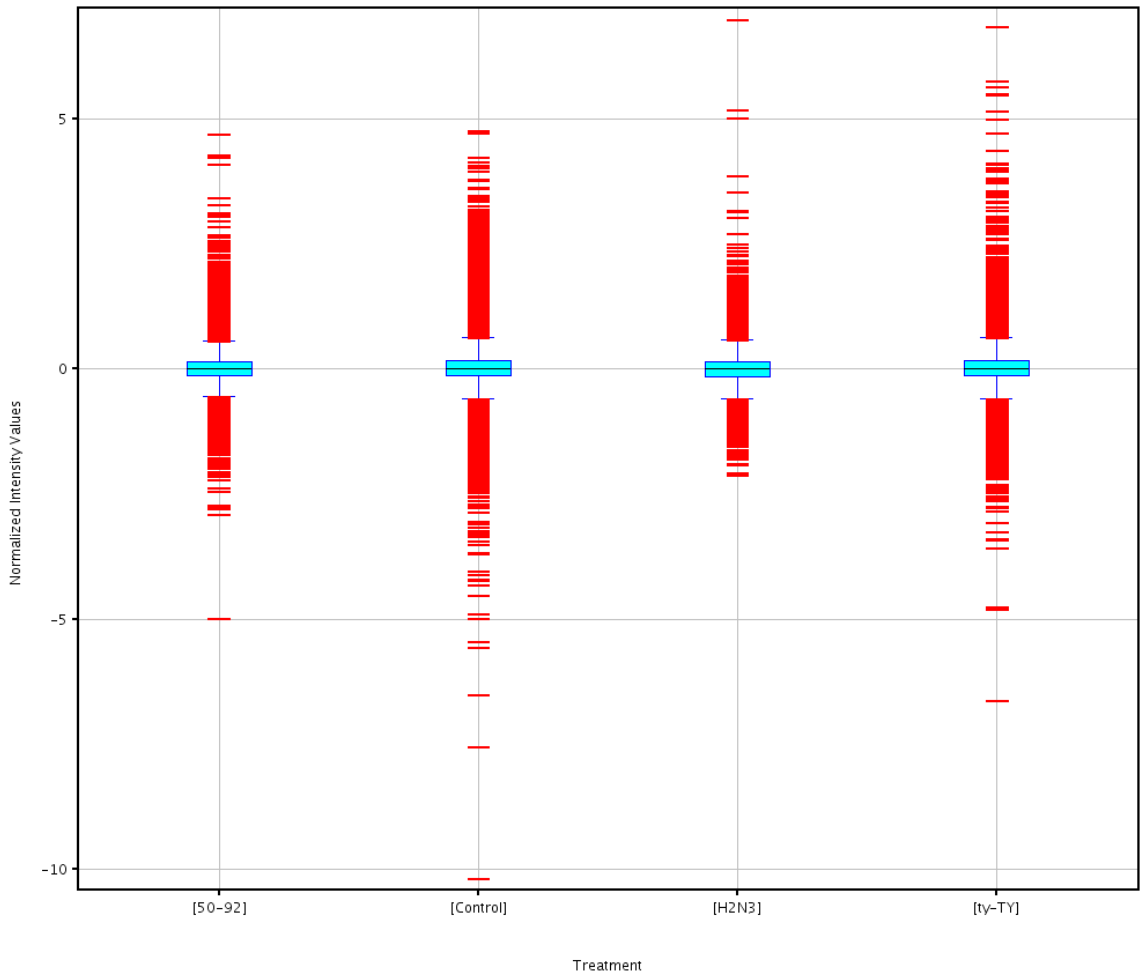


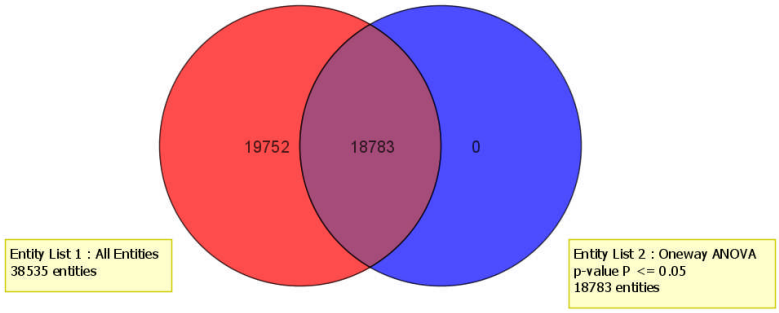
Figure 7.4-6 Box Whisker plot showing experimental grouping of duck samples

Box whisker plot of normalized intensity values for each of the treatment and control groups are shown. Median values are marked in the centre with a black line.

7.4.3.2 Gene expression analysis

Differentially regulated genes were filtered based on ANOVA with a p value cut-off of 0.05. In chicken, 18783 out of 38535 transcripts (48.74%) were found to be differentially regulated ($p<0.05$) following influenza virus infection compared with control (Figure 7.4-7A). However in duck, only 7686 out of 32896 transcripts (23.36%) were differentially regulated ($p<0.05$) following influenza virus infection compared with control (Figure 7.4-7B). These significantly differential gene lists were generated based on the comparison of control group with treatment group comprising arrays from all three virus infections.

A



B

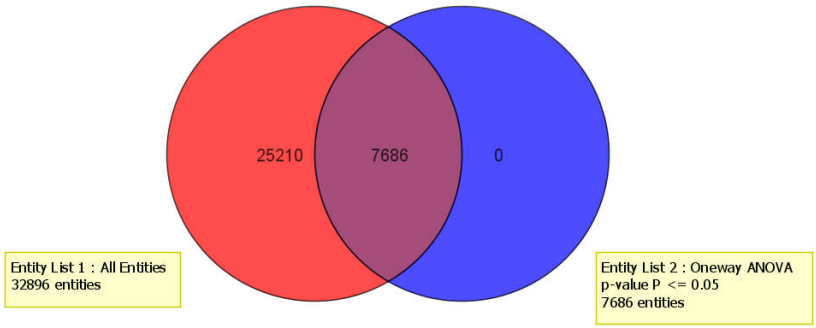
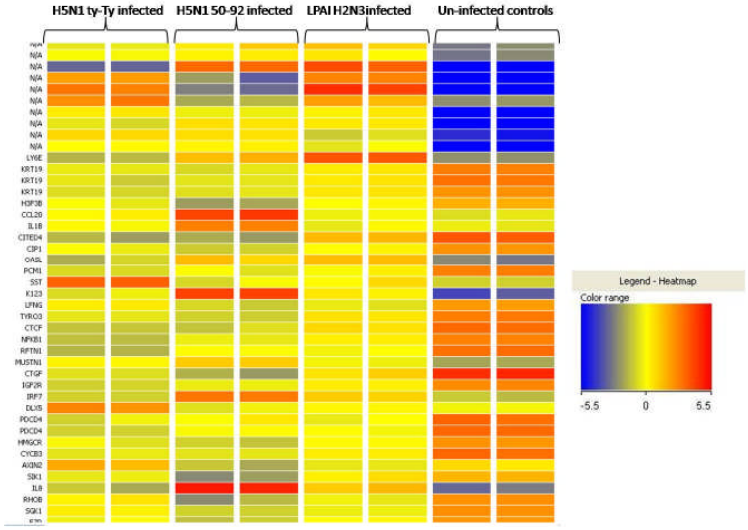


Figure 7.4-7 Differential regulation of transcripts following influenza virus infection

Differentially regulated genes following influenza virus infection in chicken (A) and duck (B) are shown. In chicken 48.74% transcripts were differentially regulated whereas in duck, 23.36% of the transcripts were differentially regulated compared to control. Red circles represent all the entities (transcripts) on the array, blue circles represent significantly ($p<0.05$) differentially regulated genes derived by ANOVA.

Changes in gene expression can be visually displayed in the heat maps, which depict expression changes of each transcript as colour changes.

A



B

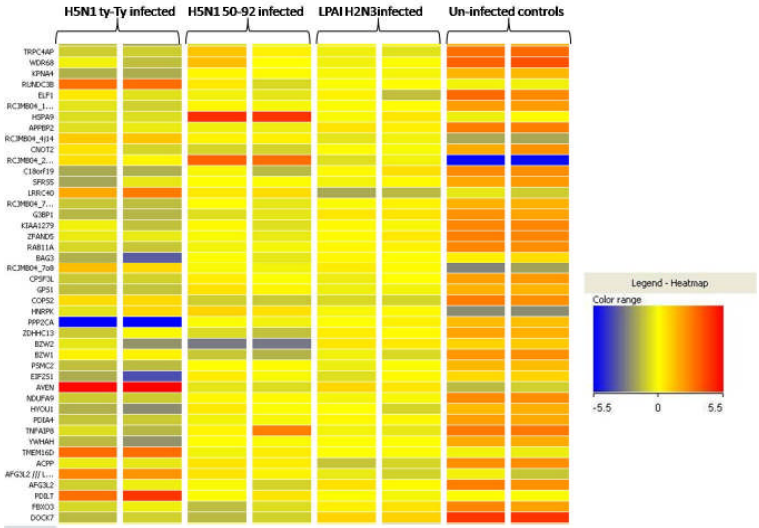


Figure 7.4-8 Heatmap views of the arrays showing differentially regulated genes

Heat map view of the arrays showing a portion of the differentially regulated entities (transcripts) in chicken (A) and duck (B) cells, following influenza virus infection. An increase in intensity of probes is depicted by the red colour while a decrease is depicted by the blue colour. Yellow indicates expression that is around the median distribution.

Following statistical analysis, genes that were significantly differentially regulated ($p<0.05$) were filtered on fold change with a cut-off value of 1.3. List of differentially regulated genes with a fold change difference of ≥ ±1.3 were generated for each treatment group in relation to control for chicken, and duck. Comparison of gene expression profiles of H5N1 50-92 virus infected chicken and duck cells was made to identify genes that were differentially regulated between chicken and duck. Five hundred and thirty two genes that were significantly up-regulated ≥1.3 fold in duck cells were down regulated ≥ 1.3 fold in chicken cells ($p<0.05$). Similarly 549 genes that were significantly up regulated ≥ 1.3 fold in chicken cells were down-regulated ≥1.3 fold in duck cells ($p<0.05$). These genes could provide valuable insights into the molecular mechanisms underlying the contrasting outcome of HPAI infection between chicken and ducks.

B

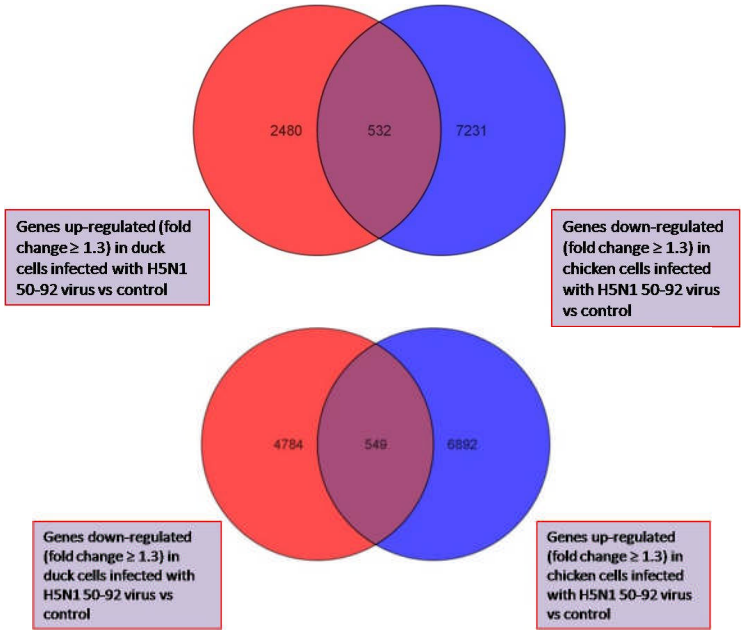
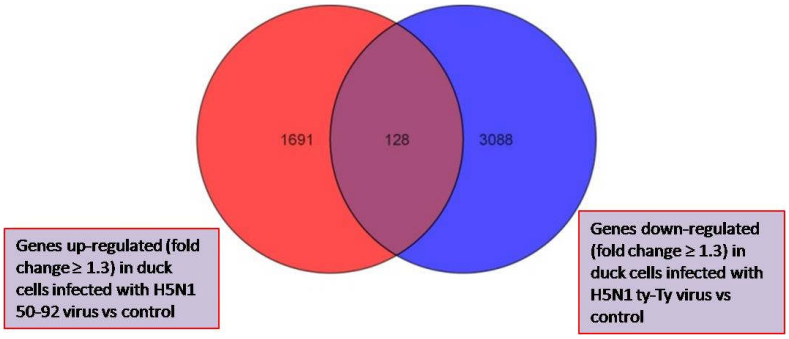


Figure 7.4-9 Differentially regulated genes in chicken and duck cells following H5N1 50-92 virus infection

Comparison of gene expression profiles of H5N1 50-92 virus infected chicken and duck cells to find biologically important genes. This function helped to identify common genes that were differentially regulated in opposite directions in chicken and ducks cells.

Gene expression comparison of H5N1 50-92 virus and H5N1 ty-Ty virus infected duck cells was made to identify genes that are differentially regulated in duck cells following infections with these two HPAI viruses. This comparison of the two viruses in duck cells is important because the differences in transcriptional regulation in duck cells between these two viruses provide valuable insights in to the underlying molecular mechanisms for the contrasting phenotype in ducks infected with these two viruses *in-vivo*. One hundred and twenty eight genes that were significantly up regulated ≥ 1.3 fold in H5N1 50-92 virus infected duck cells were down regulated by ≥ 1.3 fold in H5N1 ty-Ty virus infected duck cells ($p<0.05$). Similarly 146 genes that were significantly up regulated ≥ 1.3 fold in H5N1 ty-Ty virus infected duck cells were down regulated ≥ 1.3 fold in H5N1 50-92 virus infected duck cells ($p<0.05$). Further analysis of these genes could provide valuable insights into the molecular mechanisms underlying the innate resistance of ducks to influenza virus.

A



B

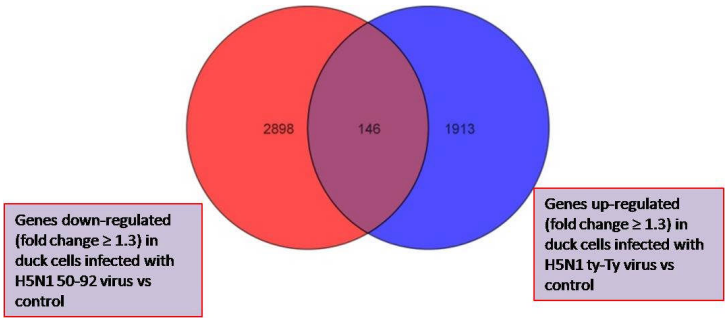


Figure 7.4-10 Differentially regulated genes between H5N1 50-92 virus and H5N1 ty-Ty virus infected duck cells

Comparison of gene expression profiles of H5N1 50-92 and H5N1 ty-Ty virus infected duck cells to find biologically important genes. This function helped to identify common genes that were differentially regulated in opposite directions between the two HPAI viruses in duck cells.

A sample of relevant and important genes involved in immunity, signal transduction and apoptosis that are differentially transcribed between infected chicken and duck cells are shown in Table 7.4-1.

These genes can be broadly grouped based on their biological significance like immune related genes(eg.*IL-6, IL-8, IL-18, Il-10, IL-1β, IL-10, MX1, SOCS5, CX3CR1, BCLAF-1*etc), genes involved in apoptosis (*BCoR, AVEN, HSPA9,ING-3, CASP3* etc), genes involved signal transduction (*STAT-3, JAK-1, MAP3K7 etc*) , genes coding for enzymes (*B4GALNT3, DNPEP* etc) along with other functions.

Table 7.4-1 Selected list of genes differentially regulated in chicken and duck cells following HPAI infection

Gene Symbol	Gene Title	Entrez Gene ID	Chicken 50-92 infected		Chicken ty-Ty infected		Duck 50-92 infected		Duck ty-Ty infected		Biological significance
			fold change	regulation	fold change	regulation	fold change	regulation	fold change	regulation	
GPS1	G protein pathway suppressor 1	417382	2.86	down	3.01	down	2.21	up	3.20	up	GTPase inhibitor activity
ACOX1	acyl-Coenzyme A oxidase 1, palmitoyl	417366	4.33	down	2.88	down	-	unchanged	3.24	up	Lipid metabolism production of ROS
RREB1	Ras responsive element binding protein 1	395920	3.41	down	2.26	down	2.73	up	7.31	up	transcription factor
PSAT1	phosphoserine aminotransferase 1	427263	4.38	down	3.90	down	11.44	up	11.69	up	catalytic activity (vit B6 metabolism)
IARS2	isoleucyl-tRNA synthetase 2, mitochondrial	421346	17.58	down	22.92	down	14.52	up	38.82	up	isoleucyl-tRNA aminoacylation
MMP28	matrix metallopeptidase 28	417523	2.66	down	1.93	down	3.31	up	10.38	up	peptidolysis IL-4 biosynthesis
B4GALNT3	beta-1,4-N-acetyl-galactosaminyl transferase 3	418150	2.07	down	1.33	up	2.58	up	2.08	up	enzyme
DNPEP	aspartyl aminopeptidase	424200	2.38	up	1.30	up	-	unchanged	1.44	down	Enzyme
IPO7	importin 7	423046	-	unchanged	2.21	down	4.83	up	2.99	down	signal transduction
STAT3	signal transducer and activator of transcription 3	420027	2.33	down	2.72	down	1.39	up	-	unchanged	Signal transduction Antiviral immunity
MAP3K7	mitogen-activated protein kinase kinase kinase 7	421808	2.35	up	2.78	down	1.56	up	2.01	down	signal transduction
PRKAR2A	protein kinase, cAMP-dependent, regulatory, type II, alpha	416062	2.27	up	4.59	down	3.96	down	5.86	down	signal transduction
JAK1	Janus kinase 1 (a protein tyrosine kinase)	554219	2.79	down	3.21	down	-	Removed*	-	Removed*	signal transduction
BCOR	BCL6 co-repressor	418574	2.92	down	2.23	down	2.72	up	2.07	down	Pro-apoptotic
ING3	inhibitor of growth family, member 3	417762	4.63	down	3.31	down	-	unchanged	1.43	down	pro-apoptotic
HSPA9	heat shock 70kDa protein 9 (mortalin)	416183	2.17	down	3.03	down	20.15	up	1.44	down	apoptosis
AVEN	apoptosis, caspase activation inhibitor	395127	2.02	up	4.05	up	-	unchanged	99.33	up	anti-apoptosis
BCLAF1	BCL2-associated transcription factor 1	421691	14.05	down	4.09	down	-	unchanged	2.55	down	Pro-apoptotic immune system function

CASP3	caspase 3, apoptosis-related cysteine peptidase	395476	4.46	down	2.35	down	-	Removed*	-	Removed*	**apoptosis**
CDC5L	CDC5 cell division cycle 5-like (S. pombe)	422052	2.99	down	1.34	down	2.96	up	4.64	up	**cell cycle regulator**
IFNB	Interferon beta	554219	3.04	up	4.15	up	-	Removed*	-	Removed*	**Immunity (antiviral)**
CCL4	chemokine (C-C motif) ligand 4	395551	36.82	up	6.00	up	1.64	up	1.60	up	**immunity**
IL18	interleukin 18 (interferon-gamma-inducing factor)	395312	4.70	down	4.14	down	3.02	up	2.40	up	**immunity**
AIFM3	apoptosis-inducing factor, mitochondrion-associated, 3	416999	-	unchanged	-	unchanged	1.73	up	1.70	up	**immunity**
IL8	interleukin 8	395872	232.80	up	2.96	up	1.69	up	3.07	up	**immunity**
IL1B	interleukin 1, beta	395196	8.79	up	1.32	up	-	not detected	-	not detected	**immunity**
IL6	interleukin 6 (interferon, beta 2)	395337	131.08	up	10.66	up	2.92	up	-	unchanged	**immunity**
IL10	interleukin 10	428264	1.39	up	1.60	up	1.39	down	-	unchanged	**immunity**
SOCS5	suppressor of cytokine signalling 5	428570	8.80	down	3.91	down	7.54	down	9.70	down	**immunity**
CX3CR1	chemokine (C-X3-C motif) receptor 1	420719	-	unchanged	1.34	up	5.81	up	2.73	up	**Immunity**
MX1	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	395313	13.64	up		unchanged		not detected		not detected	**Immunity**

* Genes that are removed during probe masking experiment and are not represented in duck microarray platform

7.4.3.3 Gene ontology analysis

All the genes that were significantly regulated by a fold change difference of ± 1.3 ($p<0.05$) were grouped into gene ontology (GO) terms using the *GO analysis* function in GeneSpring software. Genes were grouped into cellular component, biological process and molecular function GO terms. Percentage of differentially regulated genes that fit into each of these GO terms was determined from the gene expression profiles of chicken and duck cells. Following infection with LPAI at 24 hrs, genes relating to cellular metabolic and biosynthetic process were grouped under the GO term biological process in chicken cells (Figure 7.4-11). GO analysis of duck expression profiles from H2N3 virus infected cells revealed no changes in genes involved in biological process.

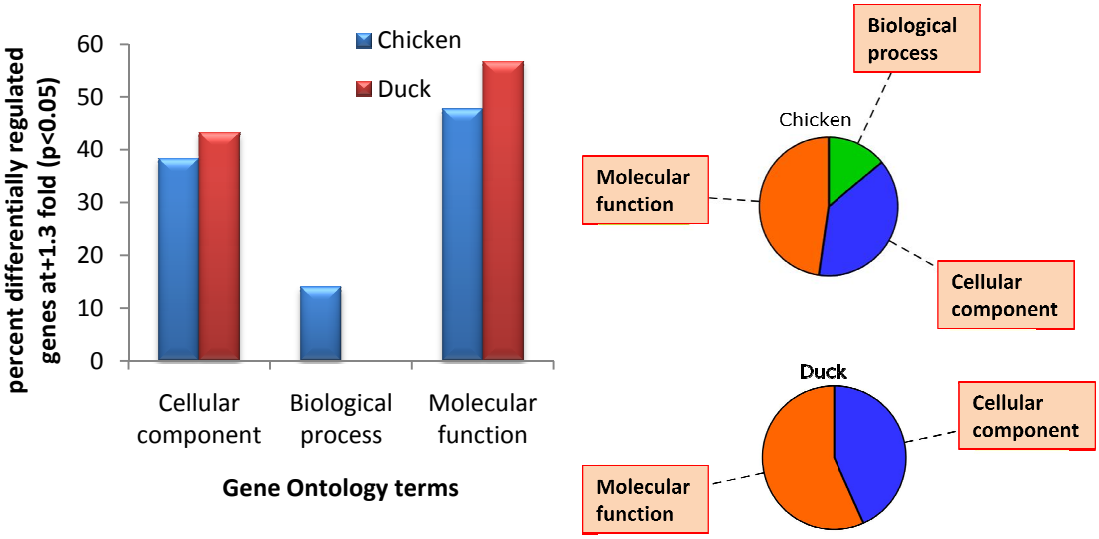


Figure 7.4-11 Gene Ontology analysis of chicken and duck gene expression profiles at 24hrs post-infection with LPAI H2N3 virus

Genes that that differed significantly ($P<0.05$) with a fold change of ±1.3 between infected and control chicken and duck samples at 24 hrs post-infection with LPAI H2N3 virus are categorized into three major gene ontology terms. Each data point represents percentage of all the differentially regulated genes that fits into the particular ontology term.

The number of genes grouped under each GO term was found to be different between H5N1 50-92 virus infected chicken and duck cells. Out of all the differentially regulated genes 16.85% of the genes in chicken and 5.16% of genes in duck were grouped under the GO term biological process (Figure 7.4-12). This group in chicken comprised genes involved mainly in protein and nucleic acid metabolism; while in duck cells this GO group comprised genes involved mainly in cellular macromolecular complex organization.

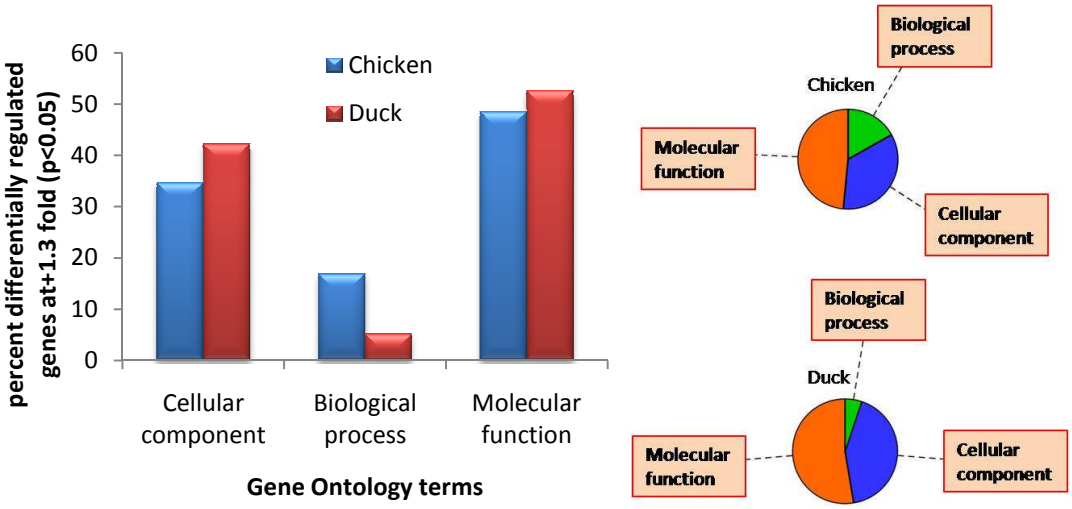


Figure 7.4-12 Gene Ontology analysis of chicken and duck gene expression profiles at 24hrs post-infection with H5N1 50-92 virus

Genes that that differed significantly (P<0.05) with a fold change of ± 1.3 between infected and control chicken and duck samples at 24 hrs post-infection with H5N1 50-92 virus are categorized into three major gene ontology terms. Each data point represents percentage of all the differentially regulated genes that fits in to the particular ontology term.

The number of genes grouped under each GO term was found to be comparable between H5N1 ty-Ty virus infected chicken and duck cells (Figure 7.4-13). However, sub-groups of genes relating to specific biological function under the broad GO term were different between chicken and duck cells. For example 9.03% of the genes in chicken and 7.09% of genes in duck were grouped under the GO term biological process. However, this group in chicken included genes involved in nucleic acid metabolism, whereas genes involved in cellular macromolecular complex organization were included duck.

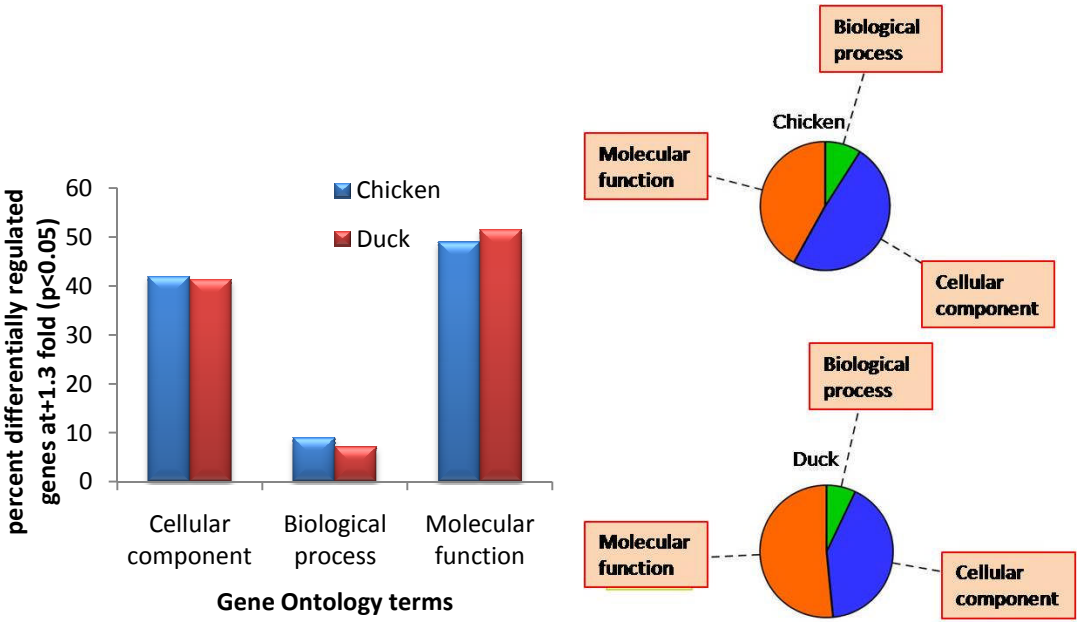


Figure 7.4-13 Gene Ontology analysis of chicken and duck gene expression profiles at 24hrs post-infection with H5N1 ty-Ty virus

Genes that were differentially regulated with a fold change of ± 1.3 following H5N1 ty-Ty virus infection compared to control were grouped in gene ontology terms. Each data point represents percentage of all the differentially regulated genes that fits in to the particular ontology term.

7.4.4 Validation of microarray results by qPCR

7.4.4.1 *18S rRNA* gene expression

Standard curves were generated by plotting CP values against the log cDNA values (Figure 7.4-14). Efficiency of *18s rRNA* qRT-PCR was calculated as 99.083% and 98.663% respectively for chicken and duck.

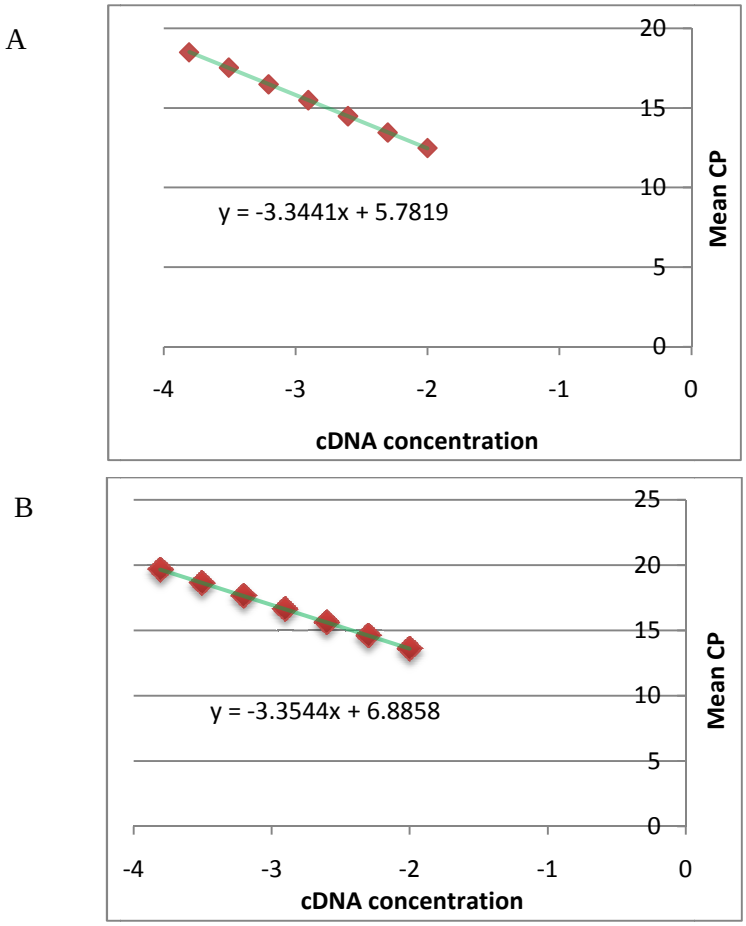


Figure 7.4-14 *18sr RNA* standard curves

Standard curves for *18S r RNA* gene expression in chicken (A) and duck (B) cDNA samples. Efficiency was calculated using the slope of the curve based on the formula, Efficiency = $-1+10^{(-1/slope)}$

Expression of *18S rRNA* gene was found to be consistent across control and virus infected chicken and duck samples. There was no significant difference in the RNA levels of *18S rRNA* gene following infection with LPAI or HPAI virus in chicken and duck cells ($p>0.05$) (Figure 7.4-15). Five microlitre of input cDNA was used for each reaction and the cDNA samples were derived from 2.0 µg total RNA from the cells.

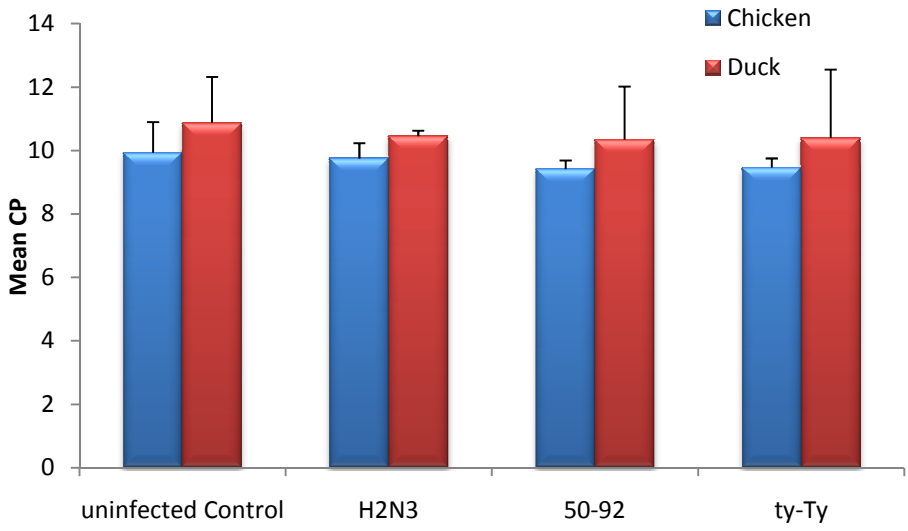


Figure 7.4-15 Expression of *18S rRNA* gene does not vary significantly in infected chicken and duck cells

18S rRNA gene expression in control and virus infected samples is shown. The mean CP values were not significantly different between control and infected samples in both chicken and duck ($p>0.05$). Each cDNA sample used for qRT-PCR was derived from 2.0 µg total RNA.

7.4.4.2 Relative expression analysis of selected target genes

Relative expression analysis of *BCLAF-1*, *Apaf-1*, *IL-8* and *IL-6* genes was performed by qRT-PCR for validating the microarray analysis data (Table 7.4-2). Microarray results were constant with qRT-PCR results for the selected genes with no exceptions. Microarray analysis provided fold change values whereas qRT-PCR analysis provided an expression value and the two methods use different approaches to generate the numerical value for gene expression. Hence, a direct comparison with reference to the fold change between both the methods could not be made. However, the transcriptional regulation of these genes was consistent in both analyses.

Table 7.4-2 Comparison of gene expression by microarray and qRT-PCR (18S rRNA normalized)

	LPAI H2N3 infected vs control				H5N1 50-92 infected vs control				H5N1 ty-Ty infected vs control			
	Microarray		qRT-PCR		Microarray		qRT-PCR		Microarray		qRT-PCR	
	regulation	fold change	regulation	expression value	regulation	fold change	regulation	expression value	regulation	fold change	regulation	expression value
Chicken genes												
IL-6	**UP**	**22.3**	**UP**	**1.6**	**UP**	**131.0**	**UP**	**48.4**	**UP**	**10.6**	**UP**	**5.4**
IL-8	**UP**	**19.7**	**UP**	**15.5**	**UP**	**232.0**	**UP**	**2.8**	**UP**	**2.9**	**UP**	**5.5**
BCLAF-1	**DOWN**	**4.5**	**DOWN**	**1.0**	**DOWN**	**14.0**	**DOWN**	**0.1**	**DOWN**	**4.0**	**DOWN**	**1.0**
APAF-1	**DOWN**	**2.6**	**DOWN**	**0.1**	**DOWN**	**3.4**	**DOWN**	**0.1**	**DOWN**	**1.9**	**DOWN**	**0.4**
Duck genes												
IL-6	unchanged	-	unchanged	-	**UP**	**2.9**	**UP**	**6.3**	unchanged	-	unchanged	
IL-8	**UP**	**2.9**	**UP**	**4.2**	**UP**	**1.6**	**UP**	**11.7**	**UP**	**3.0**	**UP**	**5.7**

7.4.5 Cytokine gene expression analysis

mRNA expression of representative anti-viral and pro-inflammatory cytokine genes were assessed in control and virus infected samples using real-time qRT-PC which are *IFN-α* , *IL-8, IL-6* and *TNF-α* genes. The expression of selected genes was normalized to *18S rRNA* expression using REST.

7.4.5.1 Cytokine expression following infection with LPAI H2N3 virus

Expression profiles of anti-viral and pro-inflammatory cytokine genes in chicken and duck lung cells at 24 hrs following LPAI H2N3 virus infection were found to be different (Figure 7.4-16).

IFN-α was significantly up-regulated in chicken and duck cells ($p<0.05$). However, the expression value in duck cells was 286.6, which is much higher compared to the expression value of 2.2 in chicken cells.

In chicken cells, *IL-6* gene was significantly up-regulated ($p<0.05$) with an expression value of 1.6, whereas IL-*6* expression was not changed significantly in duck cells ($p>0.05$).

IL-8 gene was found to be significantly up-regulated in chicken cells with an expression value15.5 ($p<0.05$). In duck cells, *IL-8* was significantly up regulated ($p<0.05$) with an expression value of 4.2.

TNF-α gene was significantly up-regulated in infected chicken cells with an expression value of 18.6 ($p<0.05$). In duck cells, *TNF-α* expression was not significantly different between infected and control samples ($p>0.05$).

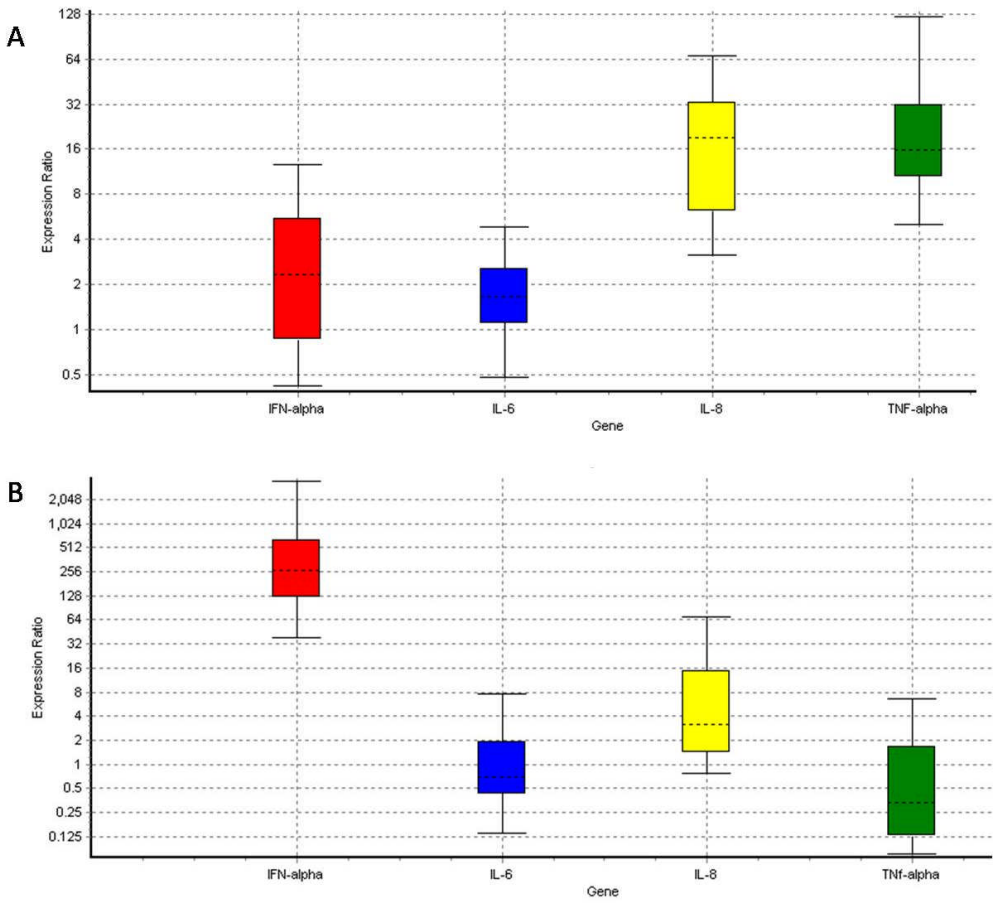


Figure 7.4-16 Cytokine mRNA expression profiles of chicken and duck cells at 24 hrs following infection with LPAI H2N3 virus

Box Whisker plots showing differential cytokine expression profiles of chicken (A) and duck (B) lung cells at 24 hrs following infection with LPAI H2N3 virus. The data were derived from biological replicates of 3 total RNA samples and the data points were expression ratios of infected to control samples normalized to 18S r RNA expression. Median expression value is depicted with a dotted line in the box.

7.4.5.2 Cytokine expression following H5N1 50-92 virus infection

Cytokine expression profiles of chicken and duck cells at 24 hrs following infection with H5N1 50-92 virus are shown in Figure 7.4-17. *IFN-α* gene expression was not significantly different between infected and control chicken cells ($p>0.05$), whereas *IFN-α* was significantly up-regulated ($p<0.05$) in duck cells with an expression value of 34.9.

IL-6 was significantly up-regulated in infected chicken ($p<0.05$) cells with an expression value of 48.4 and duck cells ($p<0.05$) with an expression value of 6.3. Similarly *IL-8* was significantly up-regulated in chicken cells ($p<0.05$) with an expression value of 283.2 and in duck cells ($p<0.05$) with an expression value of 11.7. *TNF-α* was significantly up-regulated in infected chicken cells with an expression value of 14.9 ($p<0.05$). Whereas *TNF-α* was significantly down regulated in infected duck cells with control with an expression value of 0.13 ($p<0.05$).

7.4.5.3 Cytokine expression following infection with H5N1 ty-Ty virus

Cytokine expression profiles of chicken and duck cells at 24 hrs following H5N1 ty-Ty virus infection are shown in Figure 7.4-18. *IFN-α* expression was not significantly changed in chicken cells ($p>0.05$). Whereas, *IFN-α* was significantly up-regulated ($p<0.05$) in duck cells with an expression value of 9.1. In infected chicken cells, *IL-6* gene was significantly up-regulated compared to control in chicken ($p<0.05$) samples with an expression value of 5.4. In duck cells, *IL-6* expression was unchanged in infected samples compared with control ($p>0.05$). *IL-8* gene was significantly up-regulated in chicken cells with an expression value of 5.5 ($p<0.05$) and in duck cells with a mean factor of 5.7($p<0.05$). *TNF-α* gene was found to be significantly up-regulated in chicken cells compared to control with a mean factor of 20.9 ($p<0.05$). In duck cells, *TNF-α* was significantly down regulated in infected samples with an expression value of 0.352 ($p<0.05$).

7.4.5.4 Summary of qRT-PCR observations of cytokine gene expression

Pro-inflammatory cytokine genes *TNF-α, IL-6* and *IL-8* were highly up-regulated in chicken cells following infection with LPAI and two strains of HPAI viruses used in this study. In contrast, down regulation of *TNF-α* and up-regulated *IL-6* and *IL-8* genes was observed in infected duck cells. Although *IL-6* and *IL-8* genes were up-regulated in chicken and duck cells, the expression levels were much higher in chicken cells compared with duck cells. Similarly, *IFN-α* expression was up-regulated in infected chicken and duck cells. However, the expression levels of *IFN-α were* much higher in duck cells compared with chicken cells. In summary cytokine gene expression analysis revealed a strong pro-inflammatory and a weak anti-viral cytokine gene expression in chicken cells. In contrast, duck cells exhibited a very strong anti-viral and a weak pro-inflammatory cytokine gene expression.

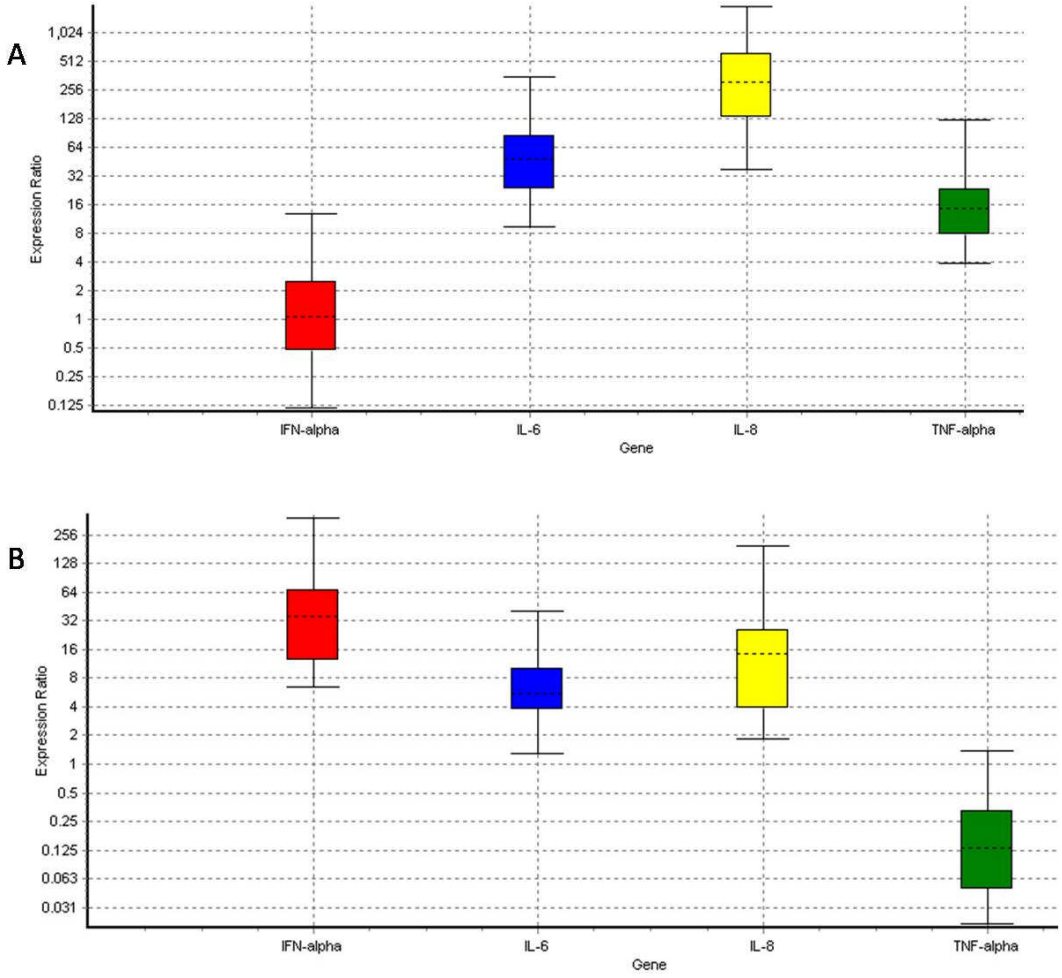


Figure 7.4-17 Cytokine mRNA expression profiles of chicken and duck cells at 24 hrs following infection with H5N1 50-92 virus

Box Whisker plots depicting differential cytokine expression profiles of chicken (A) and duck (B) lung cells at 24 hrs following infection with H5N1 50-92 virus. The data were derived from biological triplicates of total RNA samples and the data points were expression ratios of infected to control samples normalized to 18S r RNA expression. Median expression value is depicted with a dotted line in the box.

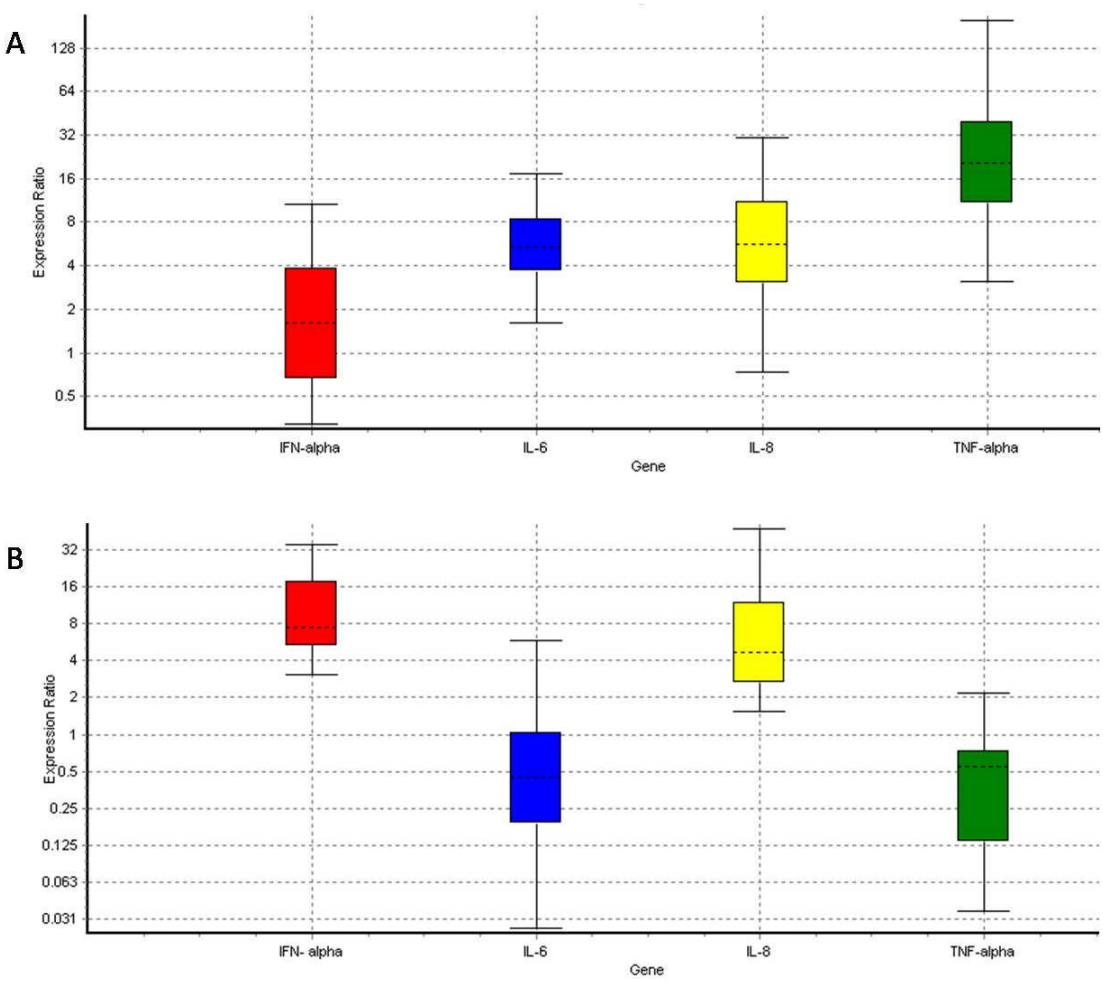


Figure 7.4-18 Cytokine expression profiles of chicken and duck cells at 24 hrs following infection with H5N1 ty-Ty virus

Box Whisker plots showing differential cytokine expression profiles of chicken (A) and duck (B) lung cells at 24 hrs following infection with H5N1 ty-Ty virus. The data was derived from triplicate RNA samples and the data points were expression ratios of infected to control samples normalized to 18S r RNA expression. Median expression value is depicted with a dotted line in the box.

7.5 Discussion

Cellular host transcriptional responses to influenza virus infection in chicken and duck lung cells were studied using DNA microarrays. Using a genomic DNA-based probe-selection method the sensitivity of chicken GeneChip arrays was improved for hybridizing duck RNA for the transcriptome analysis. Based on the number of differentially regulated genes in infected cells compared with un-infected control cells, a genomic DNA hybridization intensity threshold of 200 was found to be the best for duck data analysis. The technology created for analysis of duck expression profiles in GeneSpring retained 85.36% of the total genes represented in chicken GeneChip.

Several studies have reported the usefulness of oligonucleotide microarrays for hybridizing RNA from heterologous species for gene expression analysis. For example, hybridization of woodchuck RNA on human microarrays (Anderson, Tennant, & Lee 2006) and pig RNA on human nylon microarrays (Moody, Zou, & McIntyre 2002) was carried out with high reproducibility of the results. In the present study, hybridization of duck RNA samples on to chicken oligonucleotide microarray was described. Using this method high throughput data for the gene expression analysis of ducks was generated and the results were validated by real time PCR assay. This method was very useful for the study of global gene expression in duck cells, as there are no high-density microarray platforms available for duck currently.

Influenza infection of primary lung cells caused differential regulation of greater number of genes in chicken cells compared to duck cells. In chicken cells, 48.74% genes were differentially regulated ($p<0.05$) following influenza virus infection, where as 23.36% gene were differentially regulated ($p<0.05$) in duck cells. Duck technology created in this study for data analysis did not represent all the genes present in chicken chip, as around 5000 genes were removed during probe masking experiment. It is likely that detection of lower level of differentially regulated genes in duck cells compared to chicken cells could be due to the absence of some of the important genes in duck microarray.

However, several genes that were differentially regulated in infected chicken cells were present in the duck microarray but unchanged in duck cells following influenza infection. In summary influenza virus infection caused differential regulation of more genes in chicken cells compared to duck cells.

Gene expression profiles of chicken and duck cells were grouped in to three major gene ontology terms. Genes involved in vital functions such as protein metabolism and nucleic acid metabolism were differentially regulated in chicken cells, while very few genes in this category were differentially regulated in duck cells. Influenza virus infection inhibits host protein synthesis. For example infection of chicken embryos with influenza virus is associated with detectable alterations in the peptides and free amino acids of the chorio allantoic membranes (Shulls & Rights 1958). Influenza virus infection of chicken lung cells caused differential transcriptional regulation of many genes involved in protein metabolism. Such changes *in-vivo* could potentially contribute to alterations in the function of infected cells and to the pathogenesis of influenza in chicken. In contrast changes in very few genes in this category in duck cells might explain their relative resistance to influenza infection. Gene ontology analysis was helpful to broadly group the differentially regulated genes based on their function, cellular location etc, however, individual genes and their function need to be studied in each of these groups to determine their significance in outcome of influenza virus infection. In addition these *in-vitro* findings need to be correlated with similar studies using *in- vivo* systems.

Many genes involved in the regulation of apoptosis were differentially regulated in both chicken and duck primary lung cells following influenza virus infection. A number of genes that could potentially explain the rapid cell death response in duck, compared to chicken cells, were identified in this study from chicken and duck gene expression profiles.

Transcriptional regulation of BcL-6 co-repressor (*BCoR*), a pro-apoptotic gene (Yamochi et al. 1999) was found to be consistent with the differential cell death response in chicken

and duck primary lung cells following influenza infection observed in this study (Chapter 5). Array data showed that this gene was up regulated in H5N1 50-92 infected duck cells whereas it was down -regulated in H5N1 50-92 infected chicken cells, H5N1 ty-Ty virus infected chicken and duck cells.

Inhibitor of growth family member 3 (*ING-3*) is a tumour suppressor family protein that is involved in apoptosis independent of p53 and the normal function of *ING-3* is important in apoptosis. *ING-3* plays a role in apoptosis by increasing the cleavage of Bcl-2 family member Bid, caspases-8, -9, and -3(Wang & Li 2006). Expression of *ING-3* was down regulated in H5N1 50-92 infected chicken cells, H5N1 ty-Ty virus infected chicken and duck cells. Notably, the expression of this gene was not changed in H5N1 50-92 infected duck cells where the result of infection is more rapid cell death.

Differential transcriptional regulation of heat shock 70 kDa protein 9 (*HSPA9* or *Mortalin*) a tumour marker that is important in apoptosis (Yi et al. 2008) was observed in chicken and duck cells. *HSPA9* was down-regulated in chicken cells while it is up-regulated in duck cells infected with H5N1 50-92 virus. Notably HSPA9 was down regulated in both chicken and duck cells following infection with H5N1 ty-Ty virus. Although the significance of this gene in influenza virus induced apoptosis is not known, the transcriptional regulation of this genes raises the possibility of its role in rapid death of duck cells following influenza virus infection.

Apoptosis caspase activation inhibitor (*AVEN*) gene, an inhibitor of caspase activation, that binds to Bcl-xL and Apaf-1 (Chau et al. 2000;Figueroa, Jr. et al. 2004) also showed differential transcriptional regulation between chicken and duck cells. Expression of *AVEN* was up regulated in H5N1 50-92 infected chicken cells, H5N1 ty-Ty virus infected chicken and duck cells. Notably, the expression of this gene was not changed in H5N1 50-92 infected duck cells. Absence of a rapid cell death response in chicken lung cells and H5N1 ty-Ty virus infected duck lung cells correlated to high up regulation of *AVEN* gene expression that could potentially block apoptosis signalling.

The transcriptional regulation of these genes in chicken and duck cells following infection with classical and contemporary Eurasian H5N1 viruses used in this study, may explain at least in part the contrasting cellular response observed in this study. However, further studies are necessary to evaluate the casual relationship of these molecules to post-influenza infection apoptosis observed in duck primary cells such as gene knock down studies, changes at protein level etc.

Interferons are pleiotropic cytokines produced in response to double-stranded RNA during viral infection which initiate an intracellular antiviral state to suppress multiple steps of the viral life cycle (Stark et al. 1998). Interferons play key roles in mediating antiviral responses and in modulating immune response to many viral infections. Interferon- alpha expression was analyzed in chicken and duck cells at 24hrs following influenza virus infection by qRT-PCR.

Up regulation of *IFN-α* gene was found in influenza virus infected chicken and duck cells compared to control. However, the levels of *IFN-α* expression were quite different between chicken and duck cells such that duck cells exhibited very high levels of *IFN-α* compared to chicken cells. Microarray results show that *IFN-β* is also up regulated in chicken cells following infection with LPAI and HPAI viruses used in this study. In contrast to this finding, an earlier study found that infection of primary chicken embryo fibroblasts with certain H5N1 strains induced *IFN-α* gene expression, whereas *IFN-β* was not significantly induced (Sarmento et al. 2008). Although chicken embryo fibroblast cells are capable of producing both interferon alpha and beta and the predominant type of interferon responses can be different between virus strains (Schwarz et al. 2004).

IFN-β expression in duck cells was not determined in this study as the gene was not represented in the duck microarray technology. Due to un-availability of interferon-beta sequence in ducks, real time PCR analysis to compare the relative expression levels of this cytokine in chicken and duck could not be performed. However, *IFN-α* expression

provided valuable insights in to the differences in type-I interferon response between chicken and duck cells.

Microarray results showed an up-regulation of *IL-18* in duck cells, where as *IL-18* was down regulated in chicken cells following influenza virus infection. Interleukin-18 (*IL-18*) plays a critical role in the development of protective immunity against various intracellular pathogens including *Mycobacterium tuberculosis*, *Yersinia enterocolitica*, *Cryptococcus neoformans* and herpes simplex virus (Bohn et al. 1998;Fujioka et al. 1999;Kawakami et al. 1997;Sugawara et al. 1999). During influenza virus infection, IL-18 improves the early defence system by augmenting natural killer cell-mediated cytotoxicity. Further IL-18 is involved in controlling influenza virus replication in the lung, especially at an early stage of infection, through activation of the innate immune mechanisms such as IFN and NK cells (Liu et al. 2004). The summary of these evidence suggested that *IL-18* could be another important factor in the defence mechanism to influenza viruses and the differential transcriptional regulation of *IL-18* in chicken and duck lung cells explains at least in part the basis for the contrasting outcome of influenza infection in these avian hosts.

Pathogenesis of avian influenza has been attributed to an exaggerated host cytokine response often referred as 'cytokine storm'. *TNF-α* plays a major role in the development of clinical signs like fever and contributes to the lung lesions in humans (Kaiser et al. 2001) and pigs (Kim et al. 2009a) during influenza virus infections. To understand the basis for the contrasting clinical disease between ducks and chickens following influenza virus infection and the factors contributing to the severe clinical symptoms in chickens, *TNF-α* expression was analyzed by qRT-PCR. Virus infected chicken cells expressed very high levels of *TNF-α* while *TNF-α* gene was down-regulated in infected duck cells. Due to absence of a 'conventional' *TNF-α* gene sequence in chickens, a lipopolysaccharide induced TNF alpha factor (*LITAF*) sequence was used to design the qRT-PCR assay. This gene was earlier shown to be very highly up regulated along with other pro-inflammatory cytokines following treatment with *E.coli* and *Salmonella*

endotoxins (Hong et al. 2006). It is likely that this gene product might have a similar function as *TNF-α* in other species. However, in ducks *TNF-α* gene sequence which is available in the public database was used for designing qRT-PCR assay and the results of this study clearly indicate negative transcriptional regulation of this gene in duck cells. Although *TNF-α* genes tested are not exactly the same in chicken and duck, the differential regulation of these genes suggest that the differences in pro-inflammatory cytokine response between these two species may, in part, determine the difference in clinical outcome following virus infection.

Along with *TNF-α*, expression of other pro-inflammatory cytokines namely *IL-6* and *IL-8* was analyzed in chicken and duck lung cells by qRT-PCR. While, *IL-6* and *IL-8* genes were up-regulated in chicken and duck cells, the expression levels were much higher in chicken cells compared with duck cells, suggesting a very strong pro-inflammatory response compared to duck cells.

Cytokine gene expression analysis of influenza virus infected chicken and duck lung cells suggested a strong antiviral cytokine response in duck cells in contrast to the strong pro-inflammatory cytokine gene response in chicken cells following influenza virus infection.

Signal transducer and activator of transcription 3 (*STAT-3*) can be activated by many cytokines, and its activation has been implicated in cell growth and regulation. In addition *STAT-3* also plays a very critical role in the IFN signalling pathways and its activation is very important for efficient IFN-induced antiviral or anti-proliferation responses (Yang, Murti, & Pfeffer 1998). An interesting correlation between this contrasting cytokine response and transcriptional regulation of *STAT-3* was observed in chicken and duck gene expression profiles. In the present study, *STAT-3* gene expression was up regulated in H5N1 50-92 infected duck cells whereas it is down regulated in H5N1 50-92 infected chicken cells, H5N1 ty-Ty virus infected chicken and duck cells. The transcriptional regulation of *STAT-3* in chicken and duck cells *in-vitro* is in

correlation with the outcome of influenza infection *in- vivo* such that down regulation of *STAT-3* is associated with a severe outcome of the infection whereas up regulation is associated with disease resistance.

Another important function of *STAT-3* is its antagonistic effect on the inflammatory response. Activation of *STAT-3* signaling pathway promotes a strong anti-inflammatory response (AIR) there by blocking the inflammatory cytokine response (El Kasmi et al. 2006). In addition to its role in IFN signaling and anti-inflammatory functions, *STAT-3* has been shown to have direct antiviral activity thorough inhibitory action on virus replication. In human liver cells, *STAT-3* induces anti hepatitis viral activity and inhibits virus replication (Zhu et al. 2004). The sum of this evidence suggests that transcriptional regulation of *STAT-3*, as observed *in-vitro* following influenza virus infection, could play an important role in the outcome of virus infection *in-vivo*.

Activation of *STAT-3* in human T-cells and monocytes is dependent up on interleukin-10 (IL-10), a pleiotropic cytokine (Finbloom & Winestock 1995; Moore et al. 1993). In the present study, in chicken cells, IL-10 was up regulated while it was down regulated in duck cells IL-10 is a strong anti-inflammatory cytokines and has a suppressive effect on macrophages through activation of *STAT-3* (Ji et al. 2005). In the present study, transcriptional regulation of *IL-10* and *STAT-3* were found to be inversely related such that up-regulated *STAT-3* and down-regulated of *IL-10* was observed in duck cells where as down-regulated *STAT-3* and up-regulated *IL-10* was observed in chicken cells. However, activation of *STAT-3* does not necessarily require *IL-10* signaling and can be independent of *IL-10* activation. For example *Toxoplasma gondii* activates *STAT-3* independent of *IL-10* in mouse macrophages to prevent LPS-triggered IL-12 and TNF-α production (Butcher et al. 2005). These findings suggest that activation of *STAT-3* in duck cells following influenza virus infection appears to be independent of *IL-10* activation. It will be interesting to further explore the mechanism underlying *STAT-3* activation in duck cells and the role of *IL-10* in influenza virus infection in avian species.

Microarray results suggested that BCL2-associated transcription factor 1 (*BCLAF1*), a gene critical for immune system homeostasis and function (McPherson et al. 2009) could also be important in influenza virus resistance in ducks. The transcriptional regulation of this gene was similar in chicken and duck lung cells to *STAT-3* such that an up regulation of this gene correlated to disease resistance while down regulation correlated with development of severe clinical disease. The results of this study indicate that along with other genes, *BCLAF1* gene might play an important role in the homeostasis of immune system and thereby in influenza virus resistance.

Using gene expression analysis, important genes that could potentially explain the contrasting disease outcome in chicken and ducks to influenza infection were identified in this study. However, further studies are essential to elucidate the underlying pathways and their potential role in resistance or susceptibility to influenza virus infections.

The lack of clinical signs in ducks *in-vivo* correlates to high interferon response and low pro-inflammatory cytokine response *in-vitro*. Similarly the high pro-inflammatory cytokines like *TNF-α* response in chicken cells observed *in-vitro* could be contributing to the development of clinical signs in chicken following influenza infection. Rapid cell death (apoptosis) response of duck cells together with reduced production of infectious virus and diminished pro-inflammatory response may explain in part the relative host resistance of ducks compared to chickens. However to qualify this hypothesis, *in-vivo* experimental data on cytokine expression profiles and influenza virus replication in infected chicken and ducks are very much required.

Chapter 8

General Discussion

The aim of this project was to elucidate the innate host mechanisms of susceptibility and resistance in chicken and ducks to avian influenza viruses. Influenza virus infection of cells is initiated by the attachment of viral haemagglutinin to host cell sialic acid (SA) receptors. One of the major determinants of host susceptibility or resistance to influenza virus infection is the presence or absence of the appropriate receptor to which viral haemagglutinin can bind. Consequently, influenza viruses need to acquire the ability to utilize alternate host receptors in order to cross species barrier. Chicken and ducks are valuable natural models of susceptibility and resistance to influenza. An understanding of the differences in receptor distribution between these hosts could provide valuable insights to understand the mechanisms of susceptibility or resistance to influenza infection. This project is the first extensive study of influenza receptor distribution pattern across the major tissues and organs of chicken and ducks using lectin histochemistry and confocal microscopy (Kuchipudi et al, 2009).

Extensive distribution of avian (SA α 2,3-Gal) and mammalian (SA α 2,6-Gal) influenza virus receptors were found in many tissues of chicken and ducks. There was a marked difference in the primary receptor type in the trachea of chickens and ducks. In chicken trachea, SA α 2,6-Gal was the dominant receptor type whereas in ducks SA α 2,3-Gal receptors were most abundant. The differences in receptor expression reported in this study suggest that they may be responsible, at least in part, for some of the differences between ducks and chickens in the pattern of disease following influenza infection. While the presence of a virus receptor is clearly not sufficient to confirm that cells or tissue support efficient virus replication or transmission, the widespread replication of influenza virus in multiple organs has been reported in both chickens (Swayne, 1997) and ducks (Londt et al, 2008) following infection with highly pathogenic viruses.

Genetic studies have revealed that previous pandemic influenza strains were partially or entirely derived from the viruses of avian origin (Webster et al. 1992, Taubenberger et al. 1997, & Horimoto and Kawawoka 2005). Co-expression of avian and mammalian influenza receptors in many organs of chicken and duck suggest that these avian hosts may facilitate entry of both human and avian viruses, with the ensuing danger of virus reassortment.

However, further work is required to confirm that the tissues expressing both receptor types are fully able to support virus replication.

Some recent evidences argued that the requirement of sialic acid receptors for a productive influenza virus infection may not be absolute. A recent study suggested that absence of α2-6 sialic acid does not protect a cell from human H3N2 influenza virus with α2-6- receptor specificity and the presence of high levels of α2-6-sialic acids on a cell surface may not result in productive replication of a virus with α2-6 receptor specificity (Kumari et al. 2007). Infection study using a human H1N1virus (A/New Caledonia/20/99) in mice lacking a major α2-6sialyltransferase found that the virus can infect and grow to high titres in the respiratory tracts of these mice with no detectable levels of α2-6 sialic acid receptors (Glaser et al. 2007). However these studies are limited to a particular virus type and further evidence are required to argue that sialic acid receptors are dispensable for influenza virus infection. It is not clear at present that how much or little sialic acid receptor availability is needed to initiate a productive infection by an influenza virus. Hence, the role of sialic acid receptors in influenza virus infection and the significance of the findings from this study remain important.

The presence of both SA α 2-3-Gal receptor subtypes in chickens suggests that they could be susceptible to a wide range of avian influenza viruses. The dominant presence of SAα2,6 Gal receptor along the chicken tracheal epithelium found in this study, shows some similarities to the prevalence of the same receptor type in mammals such as human and pig. This suggests that chickens could be important intermediate hosts for the transmission of influenza to humans, such as H5N1 influenza which show a respiratory tropism in birds. Whilst much attention has been placed on the role of pigs as “mixing vessels”, the potential importance of chickens in the evolution of humanized influenza viruses should not be overlooked. This potential warrants further investigations to understand the role of chickens in the evolution of influenza viruses with increased pathogenicity to humans.

To evaluate the differences in cellular and molecular responses between chicken and ducks that could explain the contrasting outcome of influenza virus infection, studies were carried out with a range of influenza viruses on primary cells from both species. More rapid and

greater levels of cell death were consistently observed in primary cells derived from inherently resistant Pekin or mallard ducks than cells from susceptible White Leghorn chickens. The difference in cellular metabolic activity was determined by MTT assays, which demonstrated significantly lower metabolic activity in duck cells compared with chicken cells. This relatively rapid cell death in duck cells was mediated, at least in part, by apoptosis, as evident by morphological and biochemical features of dying duck cells which included nuclear condensation, DNA fragmentation, and caspase activation. Notably, duck cells infected with H5N1 ty/Ty, a contemporary Eurasian lineage H5N1 virus, which causes lethal infection in juvenile ducks, did not display accelerated cell death but appeared healthy, much like infected chicken cells.

The accelerated cell death in infected duck cells was accompanied by a reduced output of infectious virus in the culture media compared with chicken cells. However, influenza matrix gene copy number measurement by qPCR of total RNA revealed no significant difference in viral gene replication between chicken and duck cells. Activation of caspases in infected cells results in the cleavage of the influenza virus NP (Zhirnov et al. 1999) and M2 proteins (Zhirnov et al. 2002).

Caspase dependent cleavage of influenza virus NP could play a role in the reduced infective virus production in dying duck cells. For example, NP cleavage prevents incorporation of viral RNP into virus; only uncleaved NP is found to be assembled into virions (Zhirnov & Bukrinskaya 1981) and phosphorylation of NP is necessary for influenza virus replication (Arrese & Portela 1996). Taken together, it is suggested that reduced production of infectious virus *in- vitro* from duck cells, with no apparent alteration in viral RNA released from infected cells, could be due to defective virus assembly rather than RNA replication of the viral genome.

High density DNA microarrays can provide an unparalleled view of the transcriptional events that underlie the host response to virus infection (Manger & Relman 2000). In the present study, global gene expression profiling of chicken and duck lung cells following influenza virus infection was carried out using an Affymetrix chicken oligonucleotides array platform.

Transcriptome analysis of chicken and duck cells revealed that many more genes involved in vital functions, such as protein metabolism and nucleic acid metabolism, were differentially regulated in chicken cells than in duck cells. Such changes *in-vivo* could well account for the alterations in the function of infected cells and the pathogenesis of influenza in chicken.

The relatively less changes in differential expression in infected duck cells are consistent with programmed cellular shutdown and suggest that cellular function is affected to a lesser degree. HPAI viruses like H5N1 cause very severe clinical disease in chickens and have been shown to cause differential transcriptional regulation of many genes in infected cell cultures. For example, comparison of gene expression profiles of chicken embryo fibroblasts infected with two H5N1 virus strains revealed differential regulation of may genes involved in protein metabolism, translation, transcription, host defence/immune response, ubiquitination and the cell cycle (Sarmento et al. 2008).

In this thesis, many genes involved in the regulation of apoptosis were found to be differentially regulated in chicken and duck primary lung cells following influenza virus infection. Out of the many genes, transcriptional regulation of BcL-6 co-repressor (*BCoR*), heat shock 70kDa protein 9 (*HSPA9*), inhibitor of growth family member 3 (*ING3*) and apoptosis caspase activation inhibitor (*AVEN*) appears to be particularly relevant to the contrasting cellular responses of chicken and duck cells. *BCoR*, *HSPA9* and *ING3* were down-regulated chicken cells while they are up-regulated in duck cells following infection with H5N1 50-92 virus. Following infection with H5N1 ty-Ty virus infection *BCoR*, *HSPA9* and *ING3* were down-regulated in chicken as well as duck cells. *AVEN*, a novel caspase activation inhibitor was up-regulated in chicken cells whereas it is unchanged in duck cells following infection with H5N1 50-92 virus. Interestingly *AVEN* was highly up-regulated in ty-Ty virus infected duck cells that did not show rapid cell death. Further studies are required to establish the causal relationship of these genes to the rapid cell death response of duck cells or the lack of such response in chicken cells following influenza virus infection.

Microarray results showed that transcription of *STAT-3*, an important gene involved in cell growth and regulation was found to be differentially regulated in chicken and duck cells

following influenza virus infection. Activation of *STAT-3* signaling plays a critical role in enhancing the efficiency of IFN-induced antiviral or anti-proliferation responses (Yang, Murti, & Pfeffer 1998) and promotes a strong anti-inflammatory response (El Kasmi et al. 2006). In chicken cells, *STAT-3* was down-regulated but was up-regulated in duck cells at 24hrs following infection with LPAI and H5N1 50-92 virus strains. Following infection with H5N1 ty-Ty virus, *STAT-3* expression was down regulated in infected chicken cells whereas its expression is unchanged in infected duck cells. The lack of cell death response along with the absence of *STAT-3* activation in duck cells infected with H5N1 ty-Ty virus *in-vitro* could account for the severe clinical outcome of H5N1 ty-Ty virus infected ducks *in-vivo*. This raises the possibility that *STAT-3* signaling in infected duck cells could provide a strong anti-inflammatory response.

Duck lung cells also showed up-regulation of *IL-18* gene while it was down regulated in chicken cells following infection with all the three influenza virus subtypes used in the study. IL-18 activation is important for the development of protective immunity against various intracellular pathogens (Bohn et al. 1998; Fujioka et al. 1999; Kawakami et al. 1997; Sugawara et al. 1999) and controlling influenza virus replication in the lungs (Liu et al. 2004). In addition to *STAT-3* and *IL-18*, transcriptional regulation of BCL2-associated transcription factor 1 (*BCLAF1*), critical for immune system homeostasis and function (McPherson et al. 2009), also appears to be important in ducks. *BCLAF1* was unchanged in duck cells, while it was down-regulated in chicken cells following H5N1 50-92 virus infection. Notably *BCLAF1* gene was down regulated in both chicken and duck cells infected with H5N1 ty-Ty virus which causes fatal infection in both chicken and duck cells.

Relative expression analysis of the above genes by qRT-PCR could not be performed due to unavailability of nucleotide sequences in duck. However, qRT-PCR analysis of several genes from both chicken and duck were found to be in agreement with the microarray results which supported the validity of the microarray data. Further studies are required to assess the mechanism underlying the ability of these genes in conferring susceptibility or resistance to influenza virus infection.

To assess the anti-viral and pro-inflammatory cytokine responses in chicken and duck cells, expression of *IFN-α, TNF-α, IL-6* and *IL-8* genes was measured by qRT-PCR. Chicken cells showed elevated up-regulation of *TNF-α* gene, whereas *TNF-α* gene expression was down-regulated in duck cells. Up-regulation of *IFN-α* gene was found in both chicken and duck cells; however the expression levels were significantly higher in duck cells compared with chicken cells. These observations showed a strong IFN response in duck cells in contrast to a relatively weak IFN response in chicken cells. *TNF-α* gene was down regulated in duck cells, it is highly up-regulated in chicken cells. Although both *IL-6* and *IL-8* genes were up regulated in chicken and duck cells, the expression levels were relatively much higher in chicken cells.

In summary, influenza virus infection of duck cells appear to trigger a strong type I interferon response as evidenced by strong up–regulation of *IFN-α* gene and an anti-inflammatory response as evidenced by the down-regulation *TNF-α* gene. In contrast, influenza virus infection of chicken cells triggered a highly inflammatory response: highly up-regulated *TNF-α* and a weak IFN-α response. Many studies found the correlation of influenza virus morbidity to a strong pro-inflammatory response while lack of clinical signs to a strong IFN-alpha or beta response. For example, IFN-α pre-treatment of primary lung epithelial cells enhances the expression innate immune modulators TLR and RIG-I signalling pathways especially *RIG-I, TLR3, MyD88, TRIF*, and *IRF7* genes following influenza virus infection (Matikainen et al. 2006). The unusual severity of H5N1 infection in humans has been suggested to be due to high induction of pro-inflammatory cytokines in contrast to low pathogenic H3N2 or H1N1 viruses (Chan et al. 2005; Cheung et al. 2002; Lipatov et al. 2005). Studies in mice suggest that TNF-α may contribute to morbidity during H5N1 influenza virus infection (Szretter et al. 2007), whereas activation of IFN-α/β may reduce the replication of influenza H5N1 viruses both *in-vivo* and *in -vitro* (Szretter et al. 2009).

Based on the findings of my present study that rapid cell death in duck cells, in part mediated by apoptosis, associated with decreased infective virus production, high IFN response and a weak pro-inflammatory cytokine response *in-vitro*, one could hypothesize that these are the basic mechanisms of host survival in ducks *in-vivo*. In contrast, infected chicken cells appear

deceptively healthy but produce large amounts of infective virus accompanied by a strong pro-inflammatory cytokine and a weak IFN response, all of which could explain the high susceptibility and morbidity of chickens to HPAI viruses.

Lack of the rapid cell death response in duck cells *in-vitro* could be associated with severe clinical disease *in-vivo*, as the duck cells infected with a contemporary Eurasian lineage H5N1 strain (ty-Ty) that causes lethal infection in juvenile ducks, did not display accelerated cell death but appeared deceptively healthy, much like infected chicken cells. Ducks infected with virulent H5N1 viruses often display neurological signs prior to death with pathological lesions and presence of viral antigen in the brain and heart at post mortem, along with other vital organs (Antarasena et al. 2006), two major organs not amenable to apoptosis and subsequent regeneration, without detriment to the host. The potential for apoptosis to cause such tissue damage has been recognised for other viral infections of the heart and nervous system, where the degree of apoptosis and cellular destruction are correlated with disease severity (Clarke & Tyler 2009).

A recent study reported that relative susceptibility of chickens compared with ducks to influenza could be due to the absence of retinoic acid inducible gene-I (RIG-I) in chicken, a cytoplasmic RNA sensor that plays a key role in IFN mediated anti-viral responses. Over-expression of duck *RIG-I* in chicken cells marginally enhanced *IFN-β* expression and reduced by about only 2-fold of virus production following influenza virus infection (Barber et al. 2010). However, a reduced *IFN-β* response in chicken cells in comparison with duck cells does not always seem to be consistently found following infection with all influenza viruses. For example, following infection with a low pathogenic H11N9 influenza virus, chicken peripheral blood mononuclear cells (PBMC) expressed a very high up-regulation of *IFN-β* while the levels of *IFN-β* were unaffected in infected duck PBMCs (Adams et al. 2009). In addition, activation of *STAT-3* signalling appears to be important for an efficient antiviral activity of IFN response. Although relative resistance of ducks to influenza due to the presence of *RIG-I* signalling compared with susceptibility of chickens due to its absence seems plausible, the fatal infection of ducks following infection with certain H5N1 strains and the underlying mechanism for a strong pro-inflammatory response in chickens needs to be

addressed. It is possible that other genes like MDa5 could be playing the role of RIG-I in chicken leading to IFN response.

While the pathophysiology of influenza infection in avian species is less well understood, it has recently been shown that pro-inflammatory cytokine expression is up-regulated in the lungs of chickens infected with HPAI H5N1, accompanied by high levels of virus replication (Suzuki et al. 2009). Studies in mice support the role of apoptosis followed by removal of apoptotic bodies by phagocytosis as a crucial mechanism for host survival following influenza A infection; inhibition of phagocytosis leads to increased host mortality (Hashimoto et al. 2007).

Based on the results of this thesis it appears plausible that early apoptotic response of host cells followed by phagocytic clearance of apoptotic bodies during influenza infection as in the case of ducks could result in host survival. In contrast, lack of such early apoptotic response in chickens could lead to continued replication of virus to high titres, leading to spread of the virus to secondary sites of replication and triggering a strong pro-inflammatory cytokine response. In the case of chickens this would lead to the development of severe clinical disease and death of the host (Figure 8-1). However, this tantalising hypothesis needs to be tested with *in-vivo* experiments and hence warrants further studies.

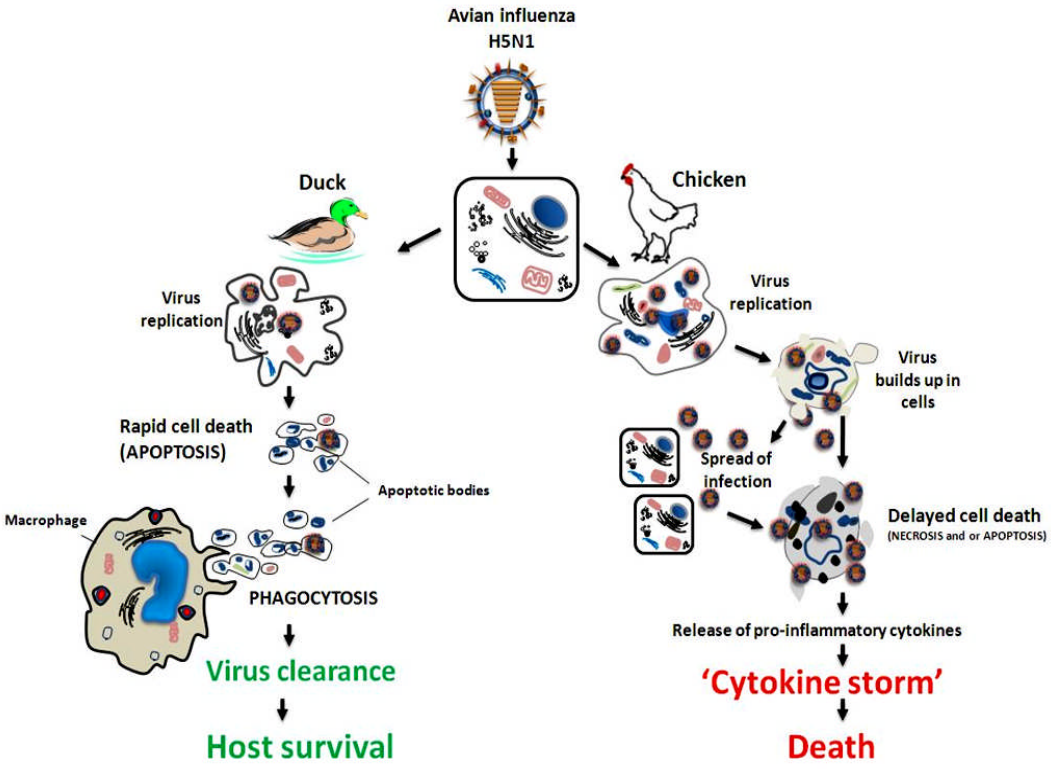


Figure 8-1 1Role of rapid cell death (apoptosis) in host survival following infection with HPAI H5N1 viruses

The potential role of early apoptosis of infected cells in avian host in containing virus infection and there by contributing to host survival is depicted. Lack of such response could lead to increased viral replication leading to infection spreading to secondary sites and eventually causing more severe clinical disease often causing death of the host

Appendix-I: Buffers and solutions

Acid Alcohol (0.3%)

Ethanol ---------- 2800 ml
Distilled water ---------- 1200 ml
Conc. hydrochloric acid --------- 12 ml

Lillie Mayer alum haematoxylin

Aluminium ammonium sulphate ---- 200 g
Haematoxylin (CI 75290) ---------- 20 g
Ethanol ---------- 40 ml
Sodium iodate ---------- 4 g
Acetic acid ---------- 80 ml
Glycerol ---------- 1200 ml
Distilled water ---------- 2800 ml

Paraformaldehyde (4%)

Paraformaldehyde powder --- ------4 gm
distilled water ---------- 5ml , mix the contents and add
0.5to 1M sodium hydroxide---------- 10 drops
heat in a water bath at80ºC for 5min (mix the contents and add)
Phosphate buffered saline (PBS) ---------- 5ml

Scott's tap water substitute

Sodium hydrogen carbonate --- 10 gm
Magnesium sulphate ---------- 100 gm
distilled water ---------- 5 L

Tris Acetate EDTA buffer (TAE) (1x)

Tris base ---------- 242g
Glacial acetic acid ---------- 57.1ml
0.5 M EDTA (pH 8.0) ---------- 100ml
Make up the volume to 1 lt with distilled H2O

Tris borate EDTA buffer (TBE) (1x)

Tris base ---------- 242g
Boric acid ---------- 37.5g
0.5 M EDTA (pH 8.0) ---------- 20ml
Make up the volume to 1 lt with distilled H2O

Tris buffered saline (TBS) (1x)

The following are dissolved in following in 800ml of distilled H2O

Sodium chloride ---------- 8 g
Potassium chloride ---------- 0.2g
Tris base ---------- 3g
Adjust the pH to 7.4 with HCl and make up the volume to 1 lt with distilled H2O.

Appendix-II: Media formulations

Collection medium (CM)

Penstrep (Invitrogen) ------- 0.5ml (100 units/ ml penicillin, 100µg/ml streptomycin)
Fungizone (Invitrogen) -------1.5ml (2.5µg/ml amphotericinB)

in 50ml of (DMEM) with Glutamax (Invitrogen)

Cell culture medium (CCM)

Fetal bovine serum (Invitrogen) ----------2.5ml
Chick embryo extract (Biosera) ---------- 1ml
Penstrep (Invitrogen) ------- 0.5ml
Insulin transferring seleniumA supplement (Invitrogen) -----0.5ml

in 50ml of DMEM with Glutamax and Ham's F12 (Invitrogen)

Dissociation medium (DM)

Protease from *Streptomyces griseus* 100x stock (1.4mg/ml)-----0.5ml
Penstrep (Invitrogen) --------- 0.5ml
Fungizone (Invitrogen) ---------1.5ml

in 50ml of DMEM with Glutamax and Ham's F12 (Invitrogen)

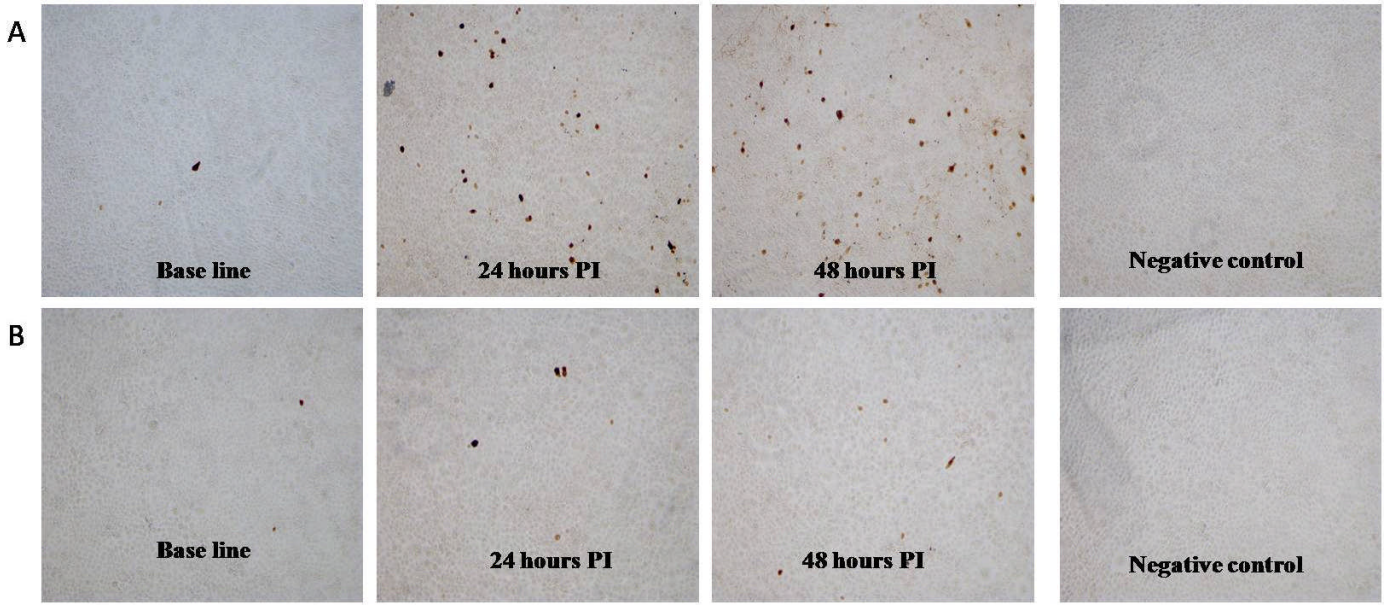
Infection medium (IM)

Ultroser G (Pal Biosepra) ------1ml (2%)
TPCK Trypsin (Sigma) -------- 25ug (500ng/ml)
Penstrep (Invitrogen) ----------0.5ml

in 50ml of DMEM with Glutamax and Ham's F12 (Invitrogen)

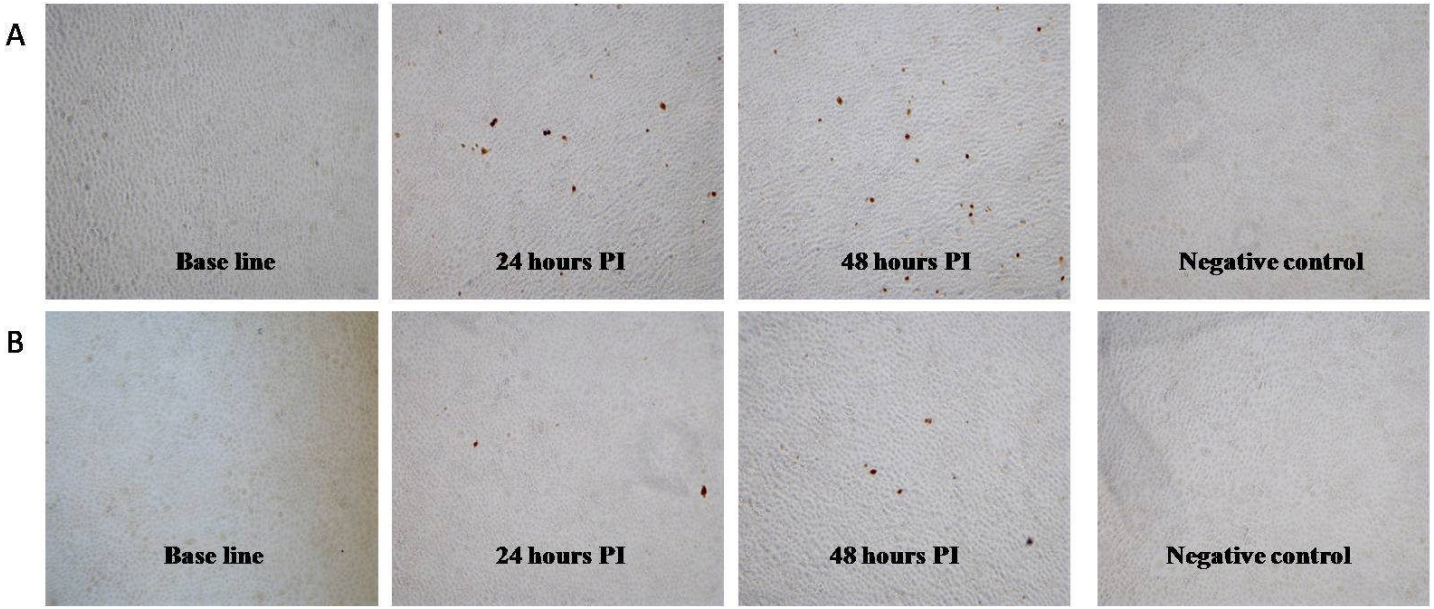
Appendix-III: Supplementary figures for Chapter 5

I



Photomicrograph showing titration of culture supernatants from chicken (A) and duck (A) primary lung cells following infection with avian H2N3 virus in MDCK cells. Immuno-cytochemical staining of influenza nucleoprotein showing significantly lower levels of viral protein in duck cells compared to chicken cells at both time points.

II



Photomicrograph showing titration of culture supernatants from chicken (A) and duck (A) primary lung cells following infection with swine H1N1 virus in MDCK cells. Immuno-cytochemical staining of influenza nucleoprotein showing significantly lower levels of viral protein in duck cells compared to chicken cells at both time points.

Appendix-IV: Supplement for Chapter 7

Hybridization of GeneChip chicken genome arrays

PolyA RNA controls

Set of poly-A RNA controls were used as exogenous positive controls to monitor the entire target labelling process. GeneChip chicken genome array used in this study contains probe sets for *B. subtilis* genes (*dap, lys, phe, thr*) that are absent in the eukaryotic samples. Target RNA samples are mixed with the poly-A RNA controls, which are then amplified and labelled together. Examination of hybridization intensities of these controls on GeneChip arrays helps to monitor the labelling process independently from the quality of the starting RNA samples.

Reverse transcription to synthesize first-strand cDNA

This reaction is primed with T7 oligo (dT) primer to synthesize cDNA containing a T7 promoter sequence. First strand master mix was prepared by mixing 4µl of first strand buffer mix and 1µl of first-strand enzyme mix for each reaction (total volume 5µl for each reaction). To a nuclease free tube, 5µl each of the first-strand master mix and total RNA/poly-A control mixture were added. After mixing and centrifuging the contents, the tubes were incubated for 2 hrs at 42ºC in a thermal cycler. After the incubation, the contents are collected by brief centrifugation and the samples were placed on ice before proceeding to the next step.

Second-strand cDNA Synthesis

This reaction employs DNA polymerase and RNase H to simultaneously degrade the RNA and synthesize second strand cDNA. The second strand master mix was prepared by mixing 5µl of second strand buffer mix, 2µl of second-strand enzyme mix and 13µl of nuclease free water for each reaction (total volume 20µl for each reaction). Twenty micro

litres of second-strand master mix was added to 10µl of the cDNA sample from the previous step. After mixing and centrifuging the contents, the tubes were incubated for 1 hr at 16ºC followed by 10min at 65ºC in a thermal cycler.

In-vitro transcription to synthesize biotin-modified aRNA

This is the amplification step which generates multiple copies of biotin-modified amplified RNA (aRNA) from the double stranded cDNA templates. For in-vitro transcription, an IVT master mix was prepared using 4µl of IVT biotin label, 20µl of IVT labeling buffer and 6µl of IVT enzyme mix for each reaction (total volume 30µl for each reaction). Thirty micro litre of IVT master mix was added to 30µl of the double stranded cDNA sample. After mixing and centrifuging the contents, the tubes were incubated for 4 hrs (for 50-250ng RNA) or16 hrs (for100-500ng RNA) at 40ºC in a thermal cycler.

Purification of aRNA

This step was done to remove unincorporated NTPs, salts, enzymes, and inorganic phosphate to improve the stability of the biotin-modified aRNA. After adding 60µl of aRNA binding mix to each sample, each sample was transferred to a well of a U-bottom plate. The contents were mixed by pipetting up and down several times. After adding 120µl of 100% ethanol to each well, the contents were first mixed by pipetting and later by gentle shaking for 2 min on a plate shaker. The plate was then moved to a magnetic stand and the magnetic beads were captured for 5 min. When the capture is complete the mixture appears transparent and the magnetic beads will form a pellet against the magnets in the magnetic stand. After discarding the supernatant by careful aspiration, the plate was removed from the magnetic stand. After adding 100 µl of aRNA wash solution to each sample, the plate was shaked at moderate speed for 1 min using a plate shaker. The supernatant was collected and discarded by capturing the RNA binding beads as described above. Washing with 100µl of aRNA wash solution was repeated again. After the wash the plate was dried by vigorous shaking for 1 min to evaporate the residual

ethanol from the beads. Finally aRNA was eluted from the RNA binding beads by adding 50 µl of preheated aRNA elution solution to each sample.

Fragmentation

Fragmentation of aRNA target before hybridization onto GeneChip probe array is critical in obtaining optimal assay sensitivity. Fragmentation reaction mixture was prepared by mixing 15µg of aRNA, 8µl of 5x Array fragmentation buffer and the volume was made to 40µl with nuclease free water. After incubating at 94ºC for 35min, the reaction was placed on ice immediately. Fragmentation produced distribution of aRNA fragments measuring 35–200 nt.

Hybridization

Hybridization of labeled target on to GeneChip probe arrays was carried out using GeneChip® Hybridization, Wash, and Stain Kit (Affymetrix) following manufacturer's instructions. Briefly a hybridization cocktail was prepared by mixing 12.5µg of labeled and fragmented aRNA, 4.2µl of control oligonucleotide B2 (3 nM), 12.5µl of 20x Hybridization Controls (*bioB, bioC, bioD, cre*), 12.5µl of 2xhybridization mix, 25 µl of DMSO and 50 µl of nuclease free water. Hybridization cocktail was heated at 99ºC for 5min, in a heat block. Meanwhile probe array was equilibrated at RT and wetted with 200 µl of pre-hybridization mix by filling through one of the septa. Hybridization was carried out using GeneChip® Hybridization Oven 640 (Affymetrix). Probe array filled with pre-hybridization mix was incubated at 45 °C for 10 minutes with rotation. Hybridization cocktail that was heated at 99ºC was transferred to a 45 °C heat block for 5 min.for 5min, in a heat block. Hybridization cocktail was centrifuged at maximum speed to remove any insoluble material. After removing the array from the hybridization oven and the pre-hybridization mix was extracted with a clean micropipette. Array was refilled with the 250 µl of the clarified hybridization cocktail. After placing the probe array in the

hybridization oven, temperature was set to 45 °C and hybridized for 16hrs with rotation at 60rpm.

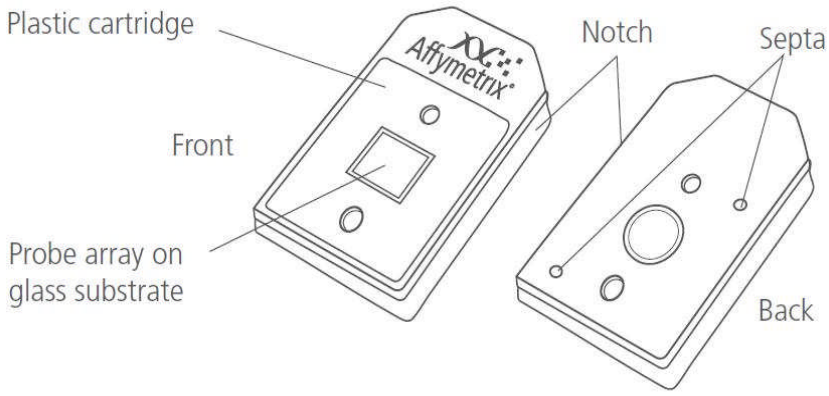


Figure S1: Outline of GeneChip chicken genome array

Washing and staining

After 16hrs hybridization of probe arrays in the oven, the probe array was removed from the oven and the hybridization cocktail was extracted with a micropipette. Washing and staining of probe arrays was carried out using GeneChip® Hybridization Wash and Stain Kit and GeneChip® Fluidics Station 450 (Affymetrix) following manufacturer's instructions.

Scanning

Probe arrays after washing and staining were scanned using GeneChip® Scanner 3000 with AGCC scan control software (Affymetrix). After scanning the software saves the image data aligns a grid on the image to identify the probe cells and computes the probe cell intensity data. The probe intensity data for each array was generated (.cel file) and the data was analyzed using GenespringGx10 software (Agilent).

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Cover page of the Journal of Molecular and genetic medicine showing the receptor distribution images from this thesis

[Composite confocal images of influenza receptors in chicken (top) and duck (bottom) tracheae]

SHORT REPORT

Differences in influenza virus receptors in chickens and ducks: Implications for interspecies transmission

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ABSTRACT

Avian influenza viruses are considered to be key contributors to the emergence of human influenza pandemics. A major determinant of infection is the presence of virus receptors on susceptible cells to which the viral haemagglutinin is able to bind. Avian viruses preferentially bind to sialic acid α2,3-galactose (SAα2,3-Gal) linked receptors, whereas human strains bind to sialic acid α2,6-galactose (SAα2,6-Gal) linked receptors. While ducks are the major reservoir for influenza viruses, they are typically resistant to the effects of viral infection, in contrast to the frequently severe disease observed in chickens. In order to understand whether differences in receptors might contribute to this observation, we studied the distribution of influenza receptors in organs of ducks and chickens using lectin histochemistry with linkage specific lectins and receptor binding assays with swine and avian influenza viruses. Although the intestinal epithelial cells of both species expressed only SAα2,3-Gal receptors, we found widespread presence of both SAα2,6-Gal and SAα2,3-Gal receptors in many organs of both chickens and ducks. Co-expression of both receptors may allow infection of cells with both avian and human viruses and so present a route to genetic reassortment. There was a marked difference in the primary receptor type in the trachea of chickens and ducks. In chicken trachea, SAα2,6-Gal was the dominant receptor type whereas in ducks SAα2,3-Gal receptors were most abundant. This suggests that chickens could be more important as an intermediate host for the generation of influenza viruses with increased ability to bind to SAα2,6-Gal receptors and thus greater potential for infection of humans. Chicken tracheal and intestinal epithelial cells also expressed a broader range of SAα2,3-Gal receptors (both β(1-4)GlcNAc and β(1-3)GalNAc subtypes) in contrast to ducks, which suggests that they may be able to support infection with a broader range of avian influenza viruses.

KEYWORDS: Host receptors, influenza, interspecies transmission, chicken, duck

INTRODUCTION

Influenza A is one of the most important infectious diseases of humans and is responsible for recurrent seasonal disease epidemics. Wild birds are the natural reservoir for all recognized subtypes of influenza A and as such present a potential route for the emergence of new viral strains which can cause human disease. If such newly

emerged viruses acquire the ability to transfer effectively from human to human they may lead to pandemic outbreaks with widespread illness and mortality. The H1N1 influenza pandemic of 1918 caused 20 to 50 million deaths on a global scale, making it the single most devastating disease outbreak in human history (Johnson and Muller, 2002). This pandemic is believed to have been caused by an avian virus that crossed the species barrier to infect humans and underwent subsequent adaptation to a new host (Taubenberger et al, 2005). The resultant virus acquired exceptional virulence with the ability to replicate in the absence of trypsin, induce death in mice and grow rapidly in human epithelial cells (Tumpey et al, 2005). Novel influenza viruses may also arise due to concurrent infection with different virus strains through reassortment of viral RNA segments (Horimoto and Kawawoka, 2005). Reassortment of human and avian influenza A viruses is thought to have generated the pandemic viruses of 1957 and 1968 (Webster et al, 1997). Avian influenza viruses are therefore likely to continue to play a significant role in the emergence of new human influenza strains (Perdue and Swayne, 2005). More recently, human infections have been caused by the emergence of avian H5N1 viruses, initially in Hong Kong in 1997 (Claas et al, 1998; Subbarao et al, 1998). Subsequently, H5N1 has re-emerged as a significant threat to human health, with over 385 confirmed cases and 243 deaths (WHO data, June 2008). These cases have demonstrated that avian viruses can directly infect humans without the need of intermediate hosts such as pigs. However, the precise molecular basis for the efficient transmissibility of avian influenza viruses to mammals is not fully understood.

A major determinant of the ability of influenza viruses to infect cells is the expression of the appropriate host cell receptor to which viral haemagglutinin can bind. Consequently, a crucial hurdle that influenza A viruses need to overcome when crossing a species barrier is the acquisition of the ability to utilize alternate host cell receptors (Ito, 2000). Influenza virus receptors on host cells are glycosylated oligosaccharides that terminate in sialic acid (SA) residues which are bound to glycans through an α2,3 or α2,6 linkage, mediated by sialyltransferases that are expressed in a cell- and species-specific manner (Gagneux et al, 2003). Avian influenza viruses have been shown to preferentially bind to SA receptors that are linked to galactose by an α2,3 linkage (SAα2,3-Gal), while human and classical swine viruses show preference for receptors with an α2,6 linkage (SAα2,6-Gal) (Gagneux et al, 2003; Matrosovich et al, 2004). Among avian influenza viruses, chicken and duck viruses have been shown to further differ in their ability to recognize the structure of the third sugar moiety in SAα2,3-Gal terminated receptors. A collection of avian influenza viruses from chickens and ducks has been studied for their binding affinities to synthetic receptor analogues *in vitro*. A marked contrast in preferential binding was noted, where influenza viruses from chickens preferentially bound to synthetic sialylglycopolymer containing Neu5Ac-α(2-3)Gal-β(1-4)GlcNAc, whereas viruses from ducks displayed a higher affinity for Neu5Ac-α(2-3)Gal-β(1-3)GalNAc containing polymer (Gambaryan et al, 2003).

Ducks and chickens are the major aquatic and terrestrial hosts for a wide variety of influenza viruses and are valuable natural models to study influenza, with ducks displaying resistance to disease, but chickens, in contrast, showing relatively high susceptibility. Receptors are important determinants of virus entry and differences in receptor distribution between hosts could therefore account for variation in susceptibility to infection, including the contrasting outcomes following infection with many strains of highly pathogenic avian influenza (HPAI). Influenza virus receptors in chickens and ducks have previously been studied by virus binding assays using extracted total gangliosides from plasma membranes of respiratory and intestinal epithelial cells (Gambaryan et al, 2002). Based on the binding of human influenza virus with SAα2,6-Gal receptor specificity, it was found that chicken epithelial cells, but not duck epithelial cells, express SAα2,6-Gal receptors. However, the receptor distribution across intact mucosal surfaces and organ systems was not studied. More recently, chicken trachea and duodenum have been studied using lectin binding assays followed by conventional immunohistochemistry and light microscopy (Wan and Perez, 2006). Despite this, detailed information on the distribution of influenza virus receptors in these important avian hosts is lacking. To further evaluate the potential role of receptor distribution in ducks and chickens in determining susceptibility to infection, and the potential of these species to support infection of viruses with tropism for SAα2,6-Gal and SAα2-3-Gal (and therefore act as "mixing vessels") we have examined in detail the anatomical distribution of influenza A receptors in key organs of both species.

MATERIALS AND METHODS

Animal tissues

Animals for this study comprised four 35-40 wk old, commercial layer chickens (Glenrath Farms Ltd, East Lothian, UK), four 4 wk old broilers (PD Hook Hatcheries, Bampton, Oxfordshire, UK), two 3 wk old and four 6 wk old Pekin ducks (Cherry Valley Farms, Rothwell, Lincolnshire, UK). The animals were euthanased and samples from trachea, lungs, heart, kidney, brain, skeletal muscle, small and large intestine collected into buffered neutral formalin.

Lectin histochemistry

Tissue samples were dehydrated and cleared using a histokinette (Leica TP 1020) before being embedded in paraffin wax. After embedding, the tissues were sectioned using a rotary microtome (Leica RM 2255) with a specimen feed of 5µm. Lectin histochemistry using linkage specific lectins was carried out with minor modifications of a method described previously (Shinya et al, 2006). Lectins used in the study were: *Sambucus nigra* agglutinin (SNA) specific for SAα2,6-Gal (Shibuya et al, 1987), *Maackia amurensis* I (MAA I) and *Maackia amurensis* agglutinins (MAA II) which are specific for SAα2,3-Galβ(1-4)GlcNAc and SAα2,3-Galβ(1-3)GalNAc respectively (Konami et al, 1994) (all provided by Vector Laboratories, Burlingame, CA). Sections were pre-soaked in TBS and blocked using a biotin-streptavidin blocking kit (Vector Laboratories) according to manufacturer's

instructions, followed by 4°C overnight incubation with fluorescein isothiocyanate (FITC) labelled SNA or FITC labelled MAA I, and biotinylated MAA II lectin each at a concentration of 10µg/ml. After three washes with TBS, the sections were incubated with streptavidin-Alexa-Fluor594 conjugate (Molecular Probes Inc, Eugene, OR) for 2 hrs at room temperature (RT). The sections were washed and then mounted with ProLong Gold antifade reagent with 4', 6-diamino-2- phenylindole, dihydro-chloride (DAPI; Molecular Probes Inc, Eugene, OR). Negative controls were performed omitting the primary reagents. To rule out nonspecific binding of the lectins, tissue sections were treated, prior to lectin staining, with Sialidase A (N-acetylneuraminate glycohydrolase; Pro-zyme, San Leandro, CA), which cleaves all non-reducing terminal sialic acid residues in the order α(2,6)> α(2,3)> α(2,8)> α(2,9). The sections were imaged using confocal microscopy (Leica TCS SP2 AOBS). Differences in receptor distribution on the mucosal lining of tracheae or intestines were quantified using LCS Lite software. Mean energy values were measured for each fluorochrome in a representative area of the mucosa and corrected by subtracting background energy values.

Receptor binding assays

Receptor binding assays with H1N1 classical swine strain (A/Sw/Iowa/15/30), a subtype closely related to the human 1918 pandemic influenza virus (Tumpey et al, 2004), and a H2N3 low pathogenic avian strain (A/mallard duck/England/7277/06) were performed by a previously reported method, with minor modifications (Couceiro et al, 1993). Briefly, paraffin embedded 5µm sections of chicken and duck tracheae, small and large intestines were deparaffinised in xylene and rehydrated by alcohol. Sections were incubated with avian or swine influenza virus for two hours at 37°C. The sections were washed, blocked with goat serum, and incubated with mouse monoclonal antibody to influenza nucleoprotein (Abcam, Cambridge, UK) at 1:1000 dilution for one hour at RT followed by FITC-labelled goat anti-mouse IgG (Abcam, Cambridge, UK) at 1:500 dilution for two hours at RT. After three further washes with TBS, the sections were mounted with ProLong Gold antifade reagent with DAPI and scanned by confocal microscopy (Leica TCS SP2 AOBS). Negative controls were performed by omitting the initial incubation with virus or primary antibody.

RESULTS AND DISCUSSION

We conducted an extensive examination of influenza virus receptor distribution in a range of tissues from chickens and ducks. No difference in the reported results was observed due to the age or source of animals, and the receptor distribution was consistent between individual animals within each species. Using lectin staining, we found widespread presence of both SAα2,6-Gal (SNA) and SAα2,3-Gal (MAA II) receptors in a range of tissues from each species, suggesting that these organs may be potential targets for both avian and human influenza viruses (Figure 1). The expression of SAα2,6-Gal receptors in duck tissues is in contrast to a previous study using virus binding assays which reported that plasma membranes isolated from duck respiratory and intestinal epithelial cells did not express SAα2,6-Gal-terminated receptors (Gambaryan et al, 2002). The exact reason for this difference is not clear, however the previous study used mallard ducks rather than Pekin ducks used in this study, and the results were based on virus, rather than lectin binding, to isolated cell membranes rather than intact cells. Thus it is possible that a difference in the methodology or choice of duck breed may be responsible.

Although there is widespread distribution of both receptor types in both chickens and ducks, there are clear differences in their spatial distribution within organs between the two species. In the vascular endothelium of the kidney, both SAα2,6-Gal and SAα2,3-Gal receptors were found in duck cells, but only the SAα2,6-Gal receptor type was found in the corresponding region in chicken kidney. The significance of this difference is not clear, however presence of SAα2,6-Gal receptors in the vascular endothelium in both species indicates that these cells can be potentially infected by mammalian-like influenza viruses and possibly play a role in the haematogenous spread of the virus. Tubular cells of duck kidney expressed both SAα2,6-Gal and SAα2,3-Gal receptors, whereas chicken kidney tubular cells expressed either SAα2,6-Gal or SAα2,3-Gal receptors, but no co-expression was observed. The endocardium, meninges and muscle fibres of both species co-expressed both subtypes of receptor (Figure 1). The expression of both host receptors in such tissues in chickens and ducks suggests that these avian hosts could possibly serve as "mixing vessels" for virus reassortment following co-infection by human and avian viruses.

Chicken and duck intestine predominantly expressed the SAα2,3-Gal receptor type across the epithelial lining of villi (Figure 2A), as previously reported (Ito and Kawaoka, 2000 & Wan and Perez, 2006). The measurement of fluorescent mean energy values along the epithelial lining of the mucosa showed no significant presence of SAα2,6-Gal in intestines of either chicken or duck. This finding reaffirms the avian digestive tract as a major predilection site for avian influenza virus replication. The receptor distribution in the large intestine of both avian species was very similar to the small intestine (data not shown).

The major species difference that we observed between chicken and duck in the relative distribution of SAα2,3-Gal and SAα2,6-Gal receptors was along the tracheal epithelium. In chicken tracheal epithelium, SAα2,6-Gal (with SNA binding) was the dominant receptor type whereas in ducks the SAα2,3-Gal receptor (with MAA II binding) was more abundant in the ciliated cells of the tracheal epithelium (Figure 2B). Based on mean fluorescent energy values, it was found that the ratio of SAα2,6-Gal to SAα2,3-Gal in chicken trachea was approximately 10:1 whereas in duck the ratio was 1:20. The tracheal mucous glands of both chicken and duck predominantly expressed SAα2,6-Gal receptor type. The observed difference in dominant receptor type between chickens and ducks was confined to the upper airway (trachea). In the bronchi and bronchioles the clear presence of both receptor types was found in both species. Chicken alveolar cells expressed both receptor types; the

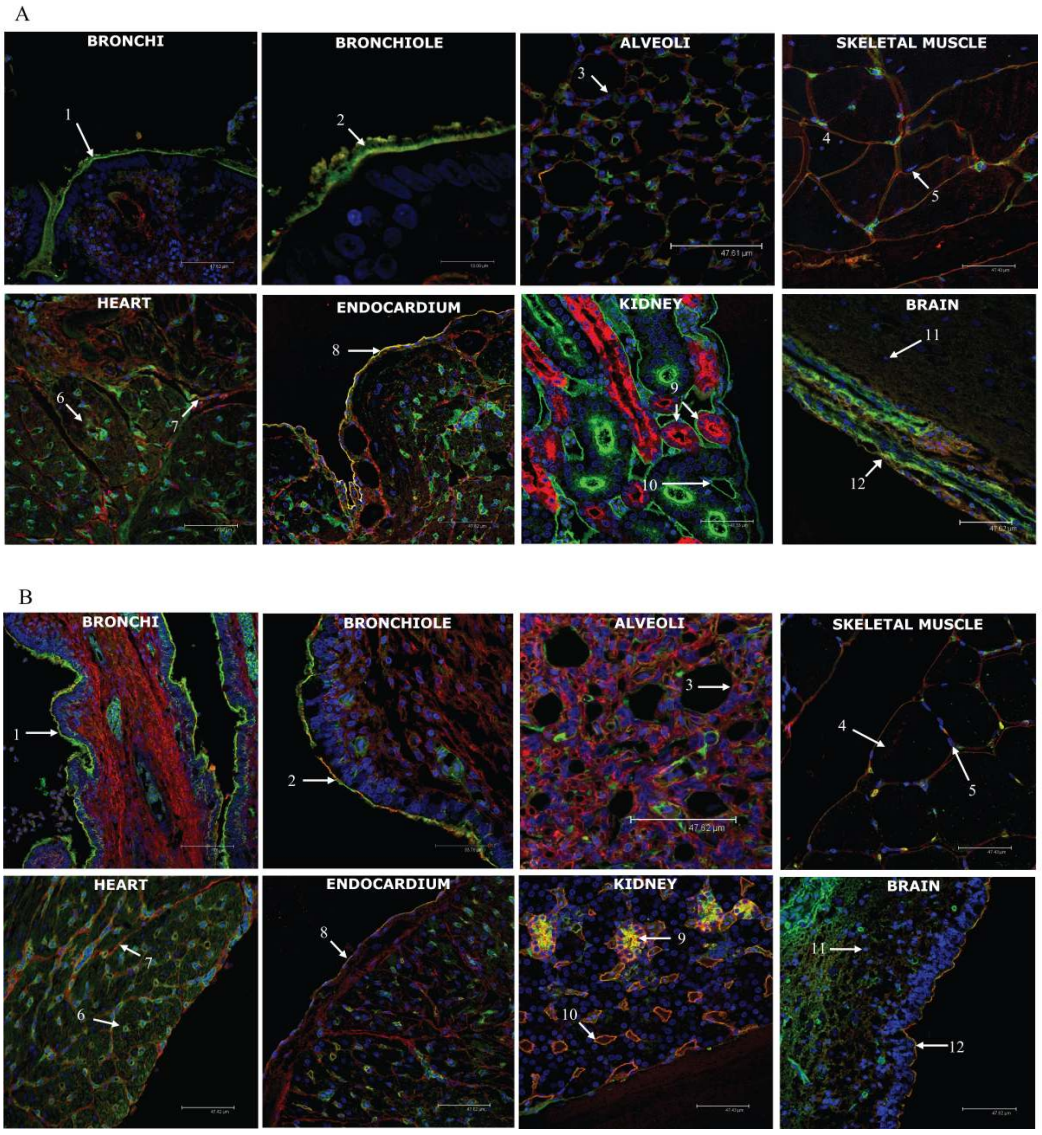

Figure 1. Widespread presence of SAα2,6 Gal (SNA lectin) and SAα2,3 Gal (MAA II lectin) receptors in many of organs of chickens **(A)** and ducks **(B)**. Composite confocal images show distribution of SAα2,6 Gal receptors (green) and SAα2,3 Gal receptors (red) with nuclear staining (blue). 1. bronchial epithelium, 2. bronchiolar epithelium, 3. alveolar lining, 4. muscle fibre, 5. nucleus, 6. myocardium, 7. connective tissue, 8. endocardium, 9. convoluted tubule 10. vascular endothelium,, 11. neuronal tissue, 12. meninges.

precise staining pattern was more difficult to determine in duck alveoli due to the extensive presence of vascular tissue which tended to obscure the alveolar staining, however, both receptors could be seen clearly in some areas of the alveoli (Figure 1). The dominant SAα2,6-Gal receptor expression pattern in chicken trachea was in contrast to a previous study (Wan and Perez, 2006) which, using lectin binding, found that 85% of the epithelial cells in chicken trachea were positive for SAα2,3-Gal receptors, while only 10% were positive for SAα2,6-Gal receptors. However, it is in agreement with the findings of Gambaryan et al (2002), who reported that human influenza viruses with SAα2,6-Gal specificity bound to cell membranes isolated from chicken (but not duck) tracheal cell membranes. A possible explanation for the discrepancy in the reported receptor distribution in chicken trachea could be the source of the lectin used. Lectins from different suppliers may show different binding specificities; in particular the source of MAA has been shown to significantly affect specificity (Nicholls et al, 2007). Glycan microarray screening of lectins from the supplier used for our study confirms that these lectins bind with a high degree of specificity to the appropriate sialic acid linkages (http://www.functionalglycomics.org). It is also possible that this study was able to detect lower levels of receptor expression due to the methodology used (confocal microscopy with fluorescent detection compared with immunohistochemistry). We found that the observed predominance of SAα2,6-Gal in chicken trachea was consistent in different ages and breed of chicken. We also

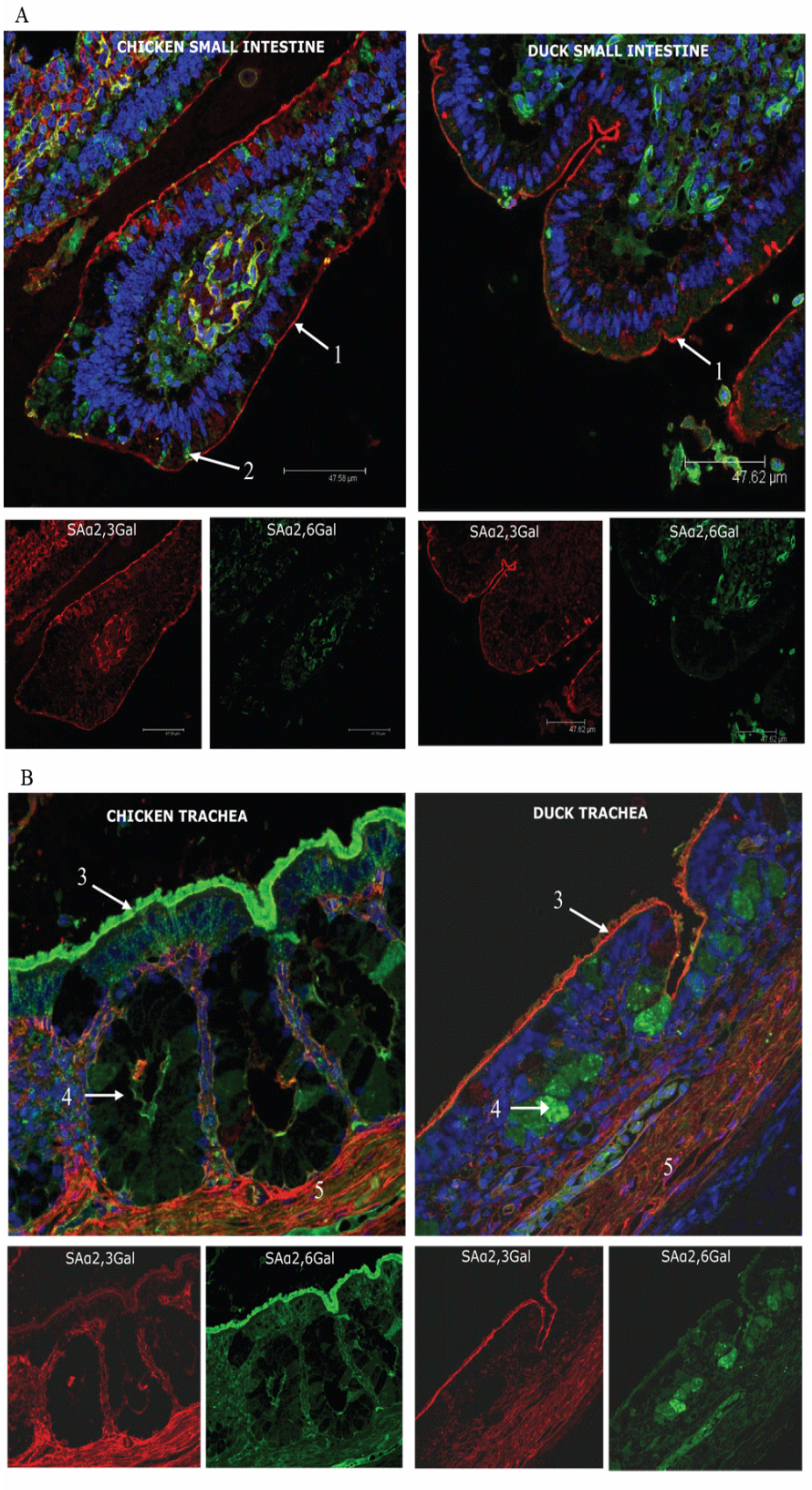

Figure 2. Differential expression of SAα2,6 Gal and SAα2,3Gal receptors in tracheae and small intestine (SI) of chickens and ducks. **(A)** For both avian species, the epithelial lining of the SI shows predominant expression of SAα2,3Gal receptor (red), with little evidence of SAα2,6 Gal (green) expression. SAα2,6 Gal receptors are more apparent in goblet cells of both bird types. Host receptor distribution is similar for both SI and large intestines (data not shown). **(B)** SAα2,6Gal receptor is the more dominant type in chicken tracheal epithelium whereas SAα2,3Gal receptor is the main type in duck tracheal epithelium. 1. epithelial lining of the villus, 2. goblet cell, 3. epithelial cells (ciliated), 4. intra epithelial mucous gland, 5. submucosa.

performed lectin staining on sialidase treated sections, which abrogated all staining confirming that the lectins used in the present study did not bind to non-sialic acid residues (data not shown). The difference in the predominant receptor across the tracheal epithelial lining in chicken and ducks could be an important contributing factor to influenza virus entry via the upper respiratory tract. In particular, such differences could impact on the susceptibility of each species to avian H5N1 influenza with its preferential tropism for infection of the respiratory tract rather than the intestines. The predominant receptor in the human upper airway is SAα2,6-Gal (Shinya et al, 2006; Yao et al, 2007). The high levels of expression of SAα2,6-Gal receptors in chicken tracheal epithelium suggests that this species may be more able to support the evolution of viruses with higher affinity for human SAα2,6-Gal receptors. This supports the suggestion that chickens may the source of emerging H9N2 viruses with a human-virus like receptor specificity (Gambaryan et al, 2002).

We further distinguished SAα2,3-Gal receptor subtypes, based on the third sugar residue, in chicken and duck trachea and intestines, with the use of MAA I (SAα2,3-Gal β(1-4)GlcNAc specificity) and MAA II (SAα2,3-Gal β(1-3)GalNAc specificity). In chicken trachea, both SAα2,3-Gal receptor subtypes were detected in the sub-epithelial region (Figure 3A). However, along the chicken tracheal epithelium, SAα2,3-Gal β(1-4)Glc NAc receptor (MAA I lectin) was more dominant than SAα2,3-Gal β(1-3) Gal NAc receptor (MAA II lectin). In duck trachea, in contrast, minimal MAA I lectin binding was observed along the epithelium; only sub-epithelial mucous glands were MAA I positive. In duck trachea, SAα2,3-Gal β(1-3) Gal NAc receptor (MAA II lectin) was the main subtype detected, with distribution along the epithelial lining and in the mucosa (Figure 3A). In chicken large intestine, both SAα2,3-Gal β1-4 Gal NAc receptor (MAA I lectin) and SAα2,3-Gal β1-3 Glc NAc receptor (MAA II lectin) expression was observed. In duck large intestine, SAα2,3-Gal β1-3 Glc NAc receptor (MAA II lectin) was the main subtype detected, while the goblet cells were positive for SAα2,3-Gal β(1-4) Glc NAc receptor subtype (MAA I lectin) (Figure 3B). The binding pattern of MAA I and MAA II in the small intestines of both avian species was very similar to the large intestine (data not shown). In humans, MAA I shows more widespread binding throughout the upper and lower respiratory tract compared to MAA II (Nicholls et al, 2007). Avian influenza viruses isolated from chicken and ducks have been shown to preferentially bind to SAα2,3-Gal β(1-4) Glc NAc (recognized by MAA I) and SAα(2,3) Gal β(1-3) Gal NAc (recognized by MAA II) respectively (Gambaryan et al, 2003). This reported virus tropism correlates with the observed receptor distribution in chicken and duck trachea and intestinal tissues. The presence of both SAα2-3-Gal receptor subtypes in chickens suggests that they may be susceptible to infection with wider range of avian influenza viruses with broader receptor specificity.

To relate the observed receptor distribution with the ability to bind viruses of avian or mammalian origin, we performed virus binding assays with avian H2N3 and swine H1N1 influenza viruses on tracheal and digestive tract sections. This showed the predicted preferential binding of the avian virus for SAα2,3-Gal receptor and the swine virus for SAα2,6-Gal receptors (Figure 4). The main SAα2,3-Gal receptor type in duck tracheal epithelium showed greater affinity for the avian H2N3 virus. In contrast, the dominant receptor type of SAα2,6-Gal in chicken trachea showed preferential binding of the swine H1N1 virus. The predominant expression of SAα2,3-Gal receptors type along the small and large intestinal epithelia of chickens and ducks showed preferential affinity for the avian H2N3 virus with no significant attachment of the swine virus. Virus-binding specificity was therefore consistent with host receptor type, as determined by lectin staining. The SAα2,6-Gal receptor type expressed by the intestinal goblet cells did not appear seem to be functionally significant as no virus binding was observed with swine H1N1 virus (Figure 4).

The differences in receptor expression reported in this study suggest that they may be responsible, at least in part, for some of the differences between ducks and chickens in the pattern of disease following influenza infection. While the presence of a virus receptor is clearly not sufficient to confirm that cells or tissue support efficient virus replication or transmission, the widespread replication of influenza virus in multiple organs has been reported in both chickens (Swayne, 1997) and ducks (Londt et al, 2008) following infection with highly pathogenic viruses.

Genetic studies have revealed that previous pandemic influenza strains were partially or entirely derived from the viruses of avian origin (Webster et al, 1992 and Taubenberger et al, 1997, Horimoto and Kawawoka 2005). This study suggests that some chicken and duck tissues may facilitate entry of both human and avian viruses, with the ensuing danger of virus reassortment. However, further work is required to confirm that the tissues expressing both receptor types are able to support virus replication. The dominant presence of SAα(2,6)Gal receptor along the chicken tracheal epithelium shows some similarities to the prevalence of the receptor in mammals such as human and pig. This suggests that chickens may be important intermediate hosts for the transmission of influenza to humans, in particular for influenza viruses such as H5N1, which show a respiratory tropism in birds. Whilst much attention has been placed on the role of pigs as "mixing vessels", the potential importance of chickens for the evolution of humanised influenza viruses should not be overlooked and, as such, warrants further study.

CONCLUSIONS

- Both SAα2,3-Gal (avian) and SAα2,6-Gal (human) receptors are expressed in many tissues of chickens and ducks.
- SAα2,6-Gal receptor is the dominant receptor type in chicken tracheal epithelium, whereas in ducks the SAα2,3-Gal receptor is dominant.
- There is greater diversity of SAα2,3-Gal receptor subtypes in chicken than duck. Chicken trachea and

intestinal tissues showed positive binding with MAA I to MAA II whereas positive MAA II binding alone was noted across the epithelial lining in ducks. This suggests that chicken may be susceptible to infection with avian influenza viruses with broader receptor specificity.

• The host receptor distribution pattern in the chicken upper respiratory tract may be functionally significant for the evolution of viruses with a human like receptor specificity and thus for the transmission of influenza from birds and mammals.

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COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

DAPI: 4', 6-diamino-2- phenylindole, dihydrochloride
DAB: diaminobenzidine
FITC: Fluorescein isothiocyanate
MAA: *Maackia amurensis* agglutinin
SA: Sialic acid
SNA: *Sambucus nigra* agglutinin

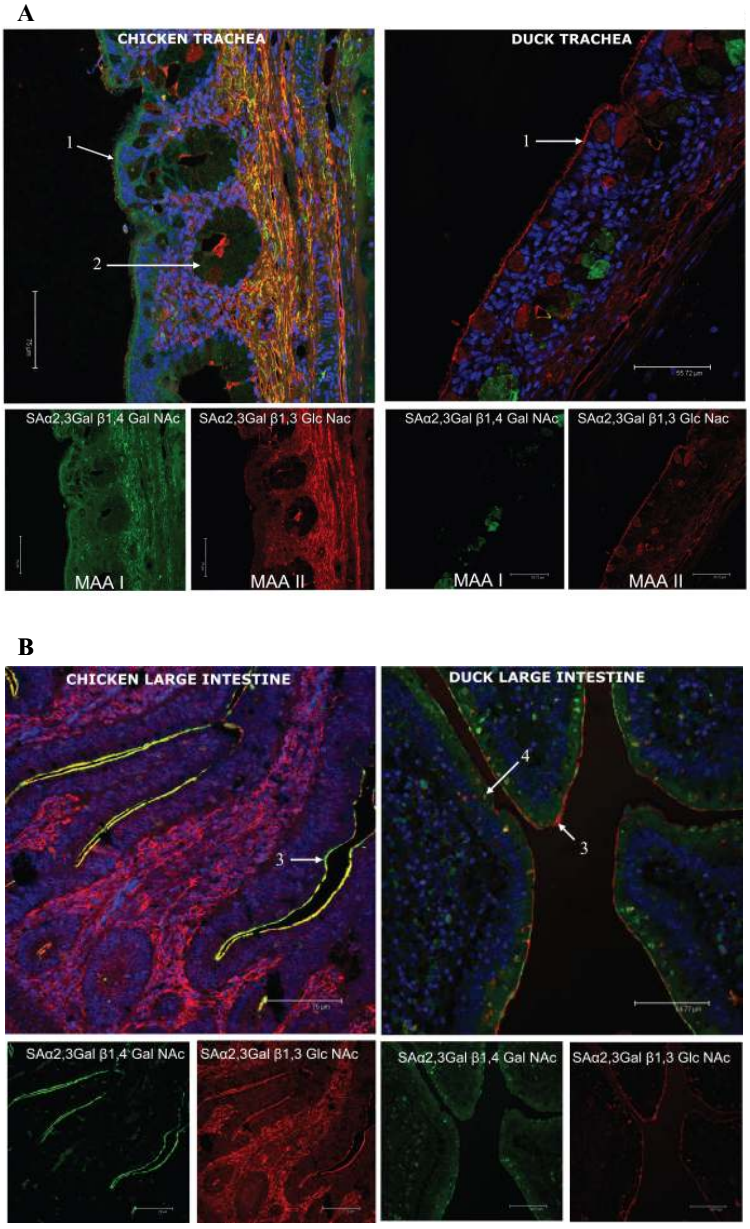

Figure 3. Differential expression of SAα(2,3)Gal receptor subtypes in chicken and duck tracheae and large intestines. MAA I (SAα(2,3)-Gal β(1-4) Glc NAc detection; green) and MAA II (SAα(2,3)-Gal β(1-3) Gal NAc detection; red) were used to distinguish SAα(2,3)-Gal receptor subtypes at the third sugar residue position. **(A)** Chicken trachea expresses SAα(2,3)-Gal β(1-4) Glc NAc receptor (MAA I lectin) more strongly along the epithelial lining than SA α(2,3) Gal β(1-3) Gal NAc (MAA II lectin). Both SAα(2,3)-Gal receptor subtypes are clearly present in the sub-epithelial region (see also MAA II lectin staining in Fig. 2B). In duck trachea, by contrast, minimal SA α(2,3)-Gal β(1-4) Glc NAc receptor subtype (MAA I lectin) is detected along the epithelium. Only the mucosal glands are MAA I positive. In duck trachea, SA α(2,3)-Gal β(1-3) Gal NAc receptor (MAA II lectin) is the main subtype with distribution along the epithelial lining and in the mucosa (see also MAA II lectin staining in Fig. 2B). **(B)** Chicken large intestine expresses similar levels of both SAα(2,3)-Gal β1-4 Gal NAc receptor (MAA I lectin) and SAα(2,3)-Gal β(1-3) Gal NAc (MAA II lectin) along the epithelial lining. In duck large intestine, SA α(2,3)-Gal β(1-3) Gal NAc receptor (MAA II lectin) is the main subtype with distribution along the epithelial lining and in the mucosa. Similar observations were made in small intestines of chicken and duck (data not shown). 1. epithelial lining 2. intra-epithelial mucous gland 3. epithelial lining of the villus 4. goblet cell.

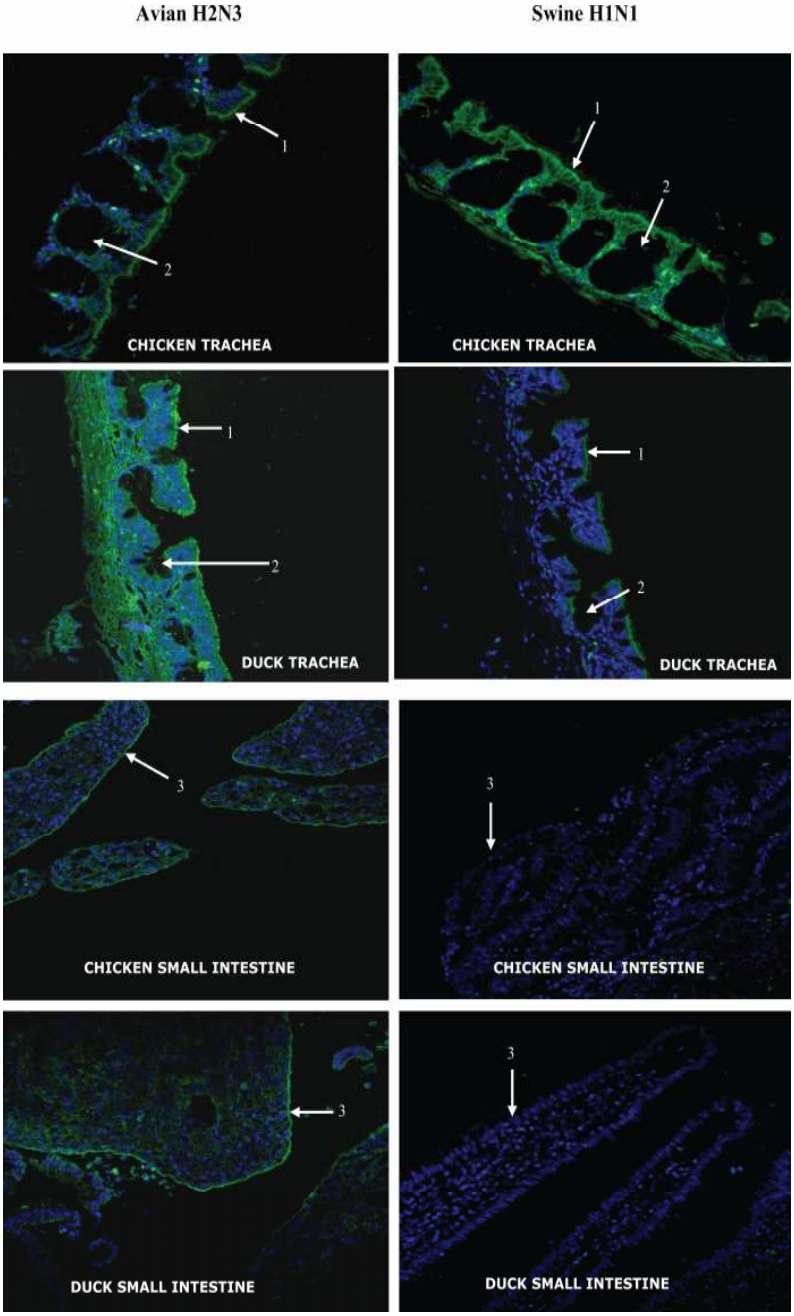

Figure 4. Virus binding assays with avian H2N3 and swine H1N1 influenza viruses on tracheal and intestinal tract sections reflect the observed receptor distribution. The main SAα(2,3)-Gal receptor type in duck tracheal epithelium shows greater affinity for the avian virus than the swine virus. By contrast, the dominant SAα(2,6)-Gal receptor type in chicken trachea shows preferential binding of the swine virus. The primary SAα(2,3)-Gal receptor type along the small intestine (SI) epithelia of both chickens and ducks is associated with preferential affinity for the avian virus, with no significant binding of swine virus. 1. epithelial lining, 2. intra-epithelial mucous gland, 3. epithelial lining of the villus.

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