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Analysis of the interaction of influenza A virus with the PI3K pathway in different avian hosts

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Abstract

The PI3K/Akt signalling pathway has been identified as having a major role in influenza A virus pathogenicity. For influenza virus A, the pathway has been shown to be active in humans and chickens, but inactive in ducks. Furthermore, the p85 β catalytic subunit of PI3K was shown to bind to NS1, a non-structural protein of influenza viruses, in human lung cells (A549). This triggers a downstream effect of activating the PI3K/Akt pathway, which prolongs cell survival, thus working in favour of viral replication. Western blots of lysed infected primary embryonic quail cells showed that the pathway is also active in infected quail cells for up to 24 hours post-infection. A phylogenetic tree was constructed using the amino acid sequences of p85 β originating from different selected species. This showed that avian and mammalian species' PI3K p85 β subunit sequences cluster separately on a phylogenetic tree, indicating a low level of sequence homology. In particular, chicken and quail cluster close together, and appear to be closely related to turkey, whilst duck appears to be very distant from these. Further research is necessary to understand if NS1 interacts with the p85 β of avian species, to better dissect the mechanisms that affect influenza A virus pathogenicity.

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Author's declaration

I declare that the work in this thesis was carried out in accordance with the regulations of the University of Nottingham. The work is original and has not been submitted for any other degree at the University of Nottingham or elsewhere.

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Signature and date:

A handwritten signature in black ink, reading 'Irene Di Lauro' in a cursive script.

16.09.2018

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Abbreviations

APCs	Antigen presenting cells
A549	Human lung cells
bGH	Bovine growth hormone
CD4	Helper T cell
CD8	Cytotoxic T cell
CM	Complete medium
cRNA	Complementary RNA
DC	Dendritic cells
dsRNA	Double stranded RNA
D5	Decoy 5
D7	Decoy 7
GE	Genetically engineered
GFP	Green fluorescent protein
GMO	Genetically modified organisms
HA	Haemagglutinin
HA ₀	Hemagglutinin precursor
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HLN	Haloxanide
HPAI	Highly pathogenic avian influenza
IFIT	Interferon-induced proteins with tetratricopeptide repeats
IFITM3	Interferon-inducible transmembrane 3
IFN	Interferon
IFN-R	Interferon receptor
IL	Interleukin
IM	Infection media
ISG	Interferon stimulated gene
JAK	Janus kinase
JAK-STAT	Janus kinase signal transducer and activator of transcription
LB	Luria Bertrani

LPAI	Low pathogenic avian influenza
MAC	Membrane attack complex
MALDI-TOF MS	Matrix-Assisted Laser Desorption Ionization Time-Of-Flight mass spectrometry
MDA5	Melanoma differentiation-associated protein 5
MDCK	Madin-Derby canine kidney
MHC	Major histocompatibility complex
mRNA	Messenger RNA
M2	Matrix ion channel
NA	Neuraminidase
NAI	Neuraminidase inhibitors
NP	Nucleoprotein
NTZ	Nitazoxanide
NS	Non-structural protein
PA	Polymerase acidic
PAMP	Pathogen-associated molecular pattern
PB	Polymerase basic
p.i.	Post-infection
PKB	Protein kinase B
PKR	Protein kinase R
PMF	Peptide mass fingerprinting
PRR	Pattern recognition receptor
PIP2	Phosphatidylinositol 4,5-biphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
QEC	Quail embryotic cell
RBD	Receptor binding domain
RIG-I	Retinoic acid-inducible gene-I
RNP	Ribonucleoprotein
RT	Room temperature
Ser473	Serine 473
shRNA	Short hairpin RNA
siRNA	Small interfering RNA

ssRNA	Single stranded RNA
TBS	Tris-buffered saline
TBST	Tris-buffered saline-Tween
TCR	T-cell receptor
TLR	Toll-like receptor
vRNA	Viral RNA
WHO	World Health Organisation

1. Introduction

1.1 Influenza virus

1.1.1 Influenza virus

Influenza is a highly contagious disease caused by influenza viruses, which are considered important pathogens in both human and animal health. The disease presents itself as a febrile respiratory illness, with a range of symptoms in humans, ranging from mild respiratory disease to death. Influenza virus belongs to the *Orthomyxoviridae* family. This comprises four different genera of Influenza virus (A, B, C and D), as well as Thogotovirus, Quaranjavirus and Isavirus. Influenza viruses have a segmented genome of negative sense single-stranded RNA, with 8 segments in the A and B genera and 7 segments in the C and D genera (MacLachlan *et al.*, 2016). Influenza A viruses are commonly found in horses, swine, humans and avian species, although they can also cause disease in other species, such as dogs and seals. Influenza B virus is mainly found in humans, however there have been reported cases of infection in seals. Influenza C virus is found in humans and swine, and rarely causes serious disease in other species. The newly discovered Influenza D virus has been found in cows (Ferguson *et al.*, 2016) and pigs (Chiapponi *et al.*, 2018), whilst antibodies against it have been found in horse serum (Nedland *et al.*, 2018).

1.1.2 Influenza A

Avian influenza results from infection by influenza A viruses, which can affect a diverse range of host species (Short *et al.*, 2015). Wild birds, in particular waterfowl, such as ducks, are the natural reservoirs for this virus. Human influenza A infections typically occur as seasonal outbreaks of infectious disease, however zoonotic infection may also occur. Influenza A viruses are able to jump interspecies barriers and infect multiple hosts (figure 1). Humans can either be infected directly by some avian influenza strains (e.g. H5N1, H9N2), or they can contract the disease through an intermediate mammalian host (e.g. pig). Influenza A virus strains are identified by a nomenclature which includes the genus of the virus, host of origin, where it was located, the isolate numerical reference, and the year of isolation (e.g. A/quail/Guangxi/198Q39/2015). Also, the haemagglutinin and neuraminidase of the viral strain is added in the nomenclature (e.g. A/quail/Guangxi/198Q39/2015 (H9N2)).

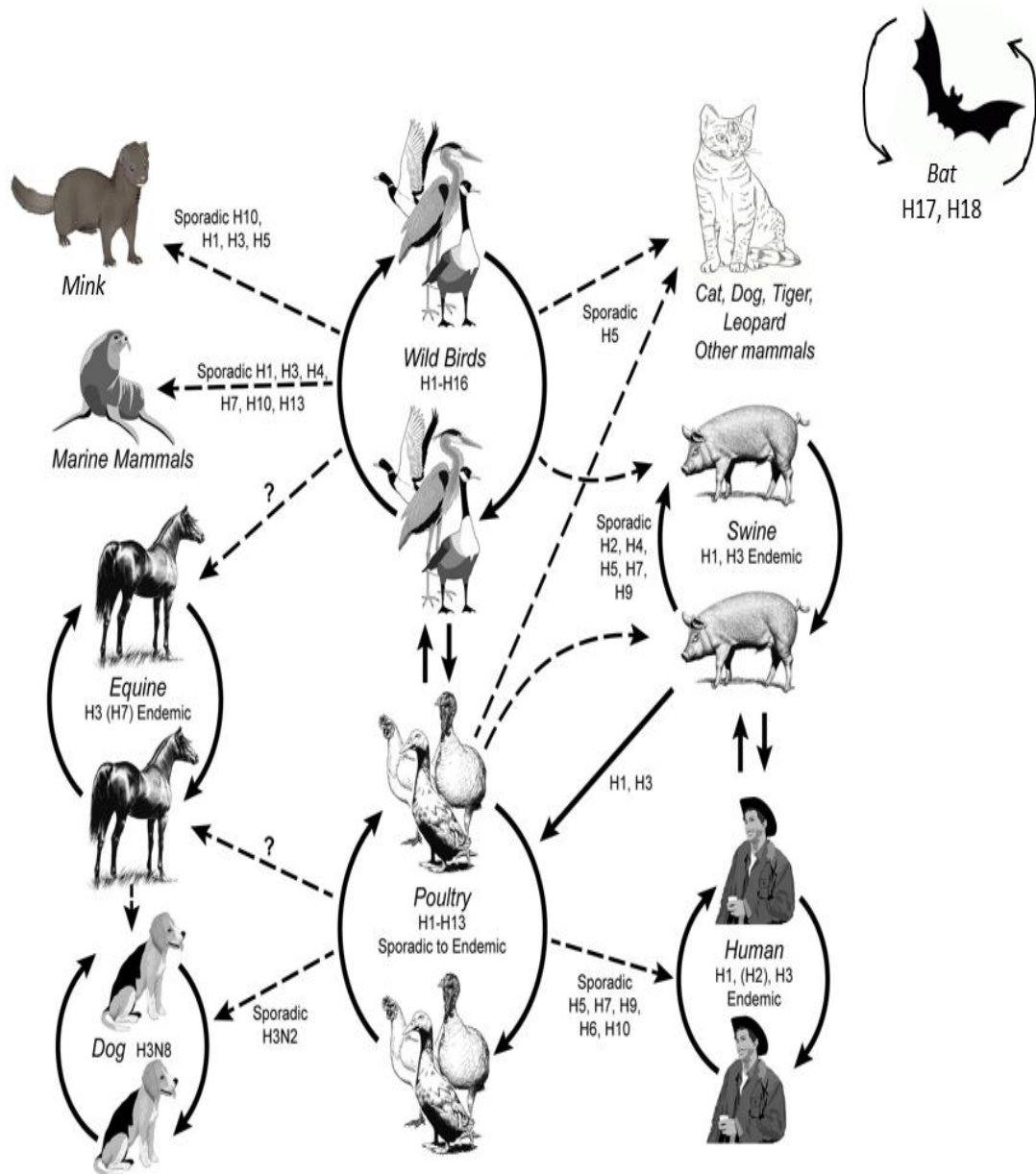


Figure 1: Interspecies transmission of influenza A viruses.

Diagrammatic presentation of the source and migration of influenza A viruses. H, haemagglutinin subtype; N, neuraminidase subtype; those in () were previously common, but not currently. Adapted from MacLachlan *et al.*, 2016.

1.1.3 Genomic organization and viral proteins

Influenza A viruses are either spherical, with a size of around 100nm in diameter, or filamentous, with a length ranging from 300nm up to 50µm (Bouvier and Palese, 2008; Badham and Rossman, 2016). The outer membrane of the influenza A virion is studded with spikes of glycoproteins haemagglutinin (HA) and neuraminidase (NA), with a ratio of approximately four to one. A smaller number of matrix ion channels (M2) cross the lipid membrane. The envelope and its three integral membrane proteins HA, NA and M2 overlay a matrix of M1 protein, which enclose the virion core. Within the M1 matrix is the ribonucleoprotein (RNP) complex, which is made up of the eight negative-sense viral RNA (vRNA) segments, coated with nucleoprotein (NP) and three polymerase proteins: PB1, PB2 and PA (figure 2). These vRNA segments encode 11 major viral proteins: the polymerase basic (PB1, PB2 and PB1-F2), the polymerase acidic (PA), haemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M1 and M2), nuclear export protein (NEP) and the non-structural (NS1) proteins (Samji, 2009). The functions of the proteins encoded by the genomic segments are described in table 1. However, since the original mapping of influenza viral proteins in 1970, several new proteins have been discovered. In 2001 the PB1-F2 protein was identified (Chen *et al.* 2001). Since then, another six viral proteins have been found: PB1-N40 (Wise *et al.*, 2009), PA-X (Jagger *et al.*, 2012), PA-N155 (Muramoto *et al.*, 2013), PA-N182 (Muramoto *et al.*, 2013), M42 (Wise *et al.*, 2012) and NS3 (Selman *et al.*, 2012). The functions of these proteins are detailed in table 2.

1.1.3.1 Haemagglutinin and Neuraminidase

Influenza A viruses are classified into subtypes through the characteristics of their two surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA), which mediate interactions between host and virus (Gamblin and Skehel, 2010). Overall there are 16 HA and 9 NA subtypes found in avian species, with an additional two HA subtypes and NA-like subtypes found only in bats. These influenza A strains found in bats are made up of haemagglutinin H17 and H18: H17N10, which was found in Central American fruit bats, and H18N11, which was identified in flat-faced fruit bats (Tong *et al.*, 2013). Phylogenetic as well as structural and

functional analyses indicate the possibility of bats being a potential reservoir for a diverse pool of influenza virus strains.

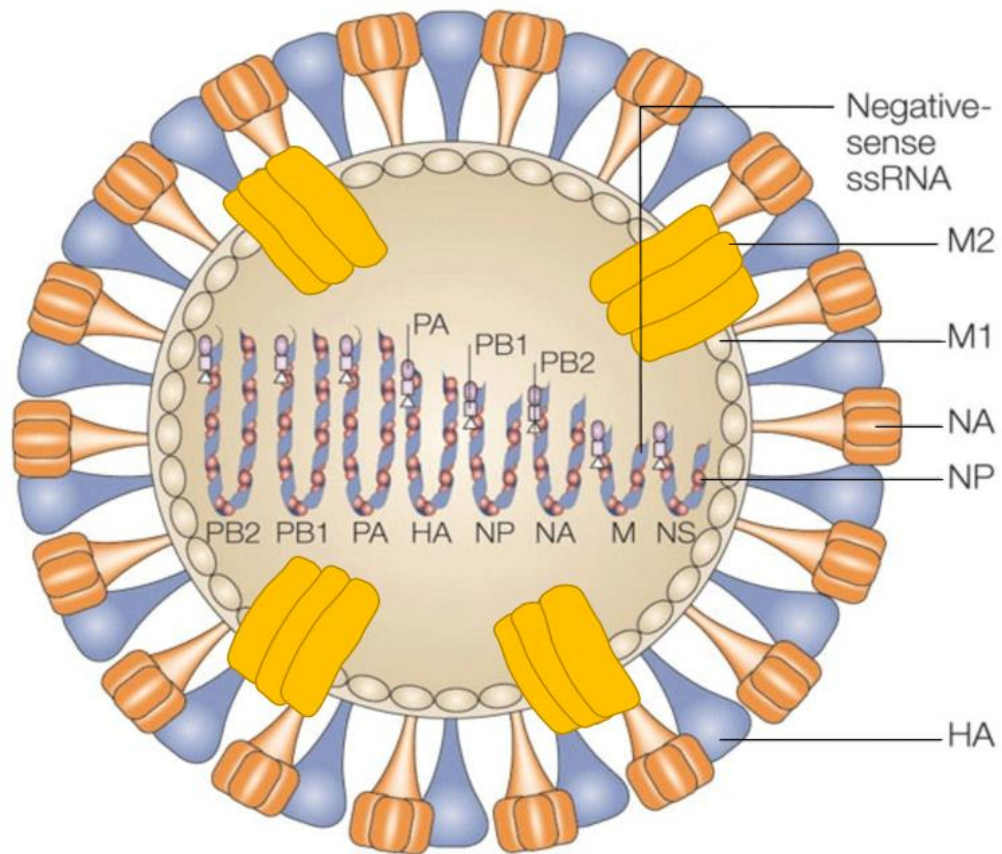


Figure 2: Diagram of the Influenza A virus virion.

Within the lipid bilayer, there are two glycoproteins: Haemagglutinin (HA) and Neuraminidase (NA), and the M2 ion-channel protein, embedded. The M1 protein is found underneath the viral envelope. The nucleoprotein (NP) and the three polymerase proteins (PA, PB1 and PB2) are associated with all eight single-stranded RNA segments. Modified from Horimoto *et al.*, 2005.

Table 1: Classification of genomic segments of influenza A viruses.

Segment	Protein product	Function
1	PB2	Polymerase protein
2	PB1	Polymerase protein
3	PA	Polymerase protein
4	HA	Attachment of virion to host cell surface, major antigen, integral membrane protein
5	NP	Structural protein
6	NA	Aids the release and spread of mature virions, integral membrane protein, major antigen
7	M1, M2	Matrix proteins
8	NS1, NEP	NS1 counteracts the activity of type I interferons; NEP takes part in nuclear transport and viral assembly

This is based on length and description of functions of the major proteins encoded by the segments.

Modified from Koparde and Singh, 2011.

Table 2: Accessory proteins of Influenza A.

Protein	Function
PB1-F2	Induces mitochondria-associated apoptosis and interacts with PB1, which influences polymerase activity
PB1-N40	Maintains the balance in PB1 and PB1-F2 expression
PA-X	Regulates the host response and viral virulence
PA-N115	Function unclear
PA-N182	Function unclear
M42	Acts as an M2 substitute in M2-null viruses
NS3	It is speculated that it is associated with the adaption of influenza A to mouse host, however it is still yet to be demonstrated

This table represents accessory proteins that might appear or be absent in influenza A virus isolates.

Adapted from Vasin et al., 2014.

1.1.3.1.1 Function of HA and NA surface glycoproteins

Multiple HA molecules are required to enable virus binding to sialic acids, which are found on the carbohydrate side chains of the glycolipids and glycoproteins on the surface of host cells. Sialic acids are a family of sugars, made up of a nine-carbon backbone. Infection is initiated by the binding of HA to the sialic acids. Once inside the endosome, before acidification, the HA is activated in order to fuse the viral and endosomal membranes (Gamblin and Skehel, 2010). After viral replication, the NA molecule cleaves sialic acids, from the infected cell surfaces so that the virus can move unimpeded to an uninfected cell. Thereby, viruses are prevented from binding to the already infected cell and from aggregating with one another through binding of HA with sialic acids.

1.1.3.2 M1 and M2

The M1 protein is able to drive virus budding and is the main viral protein component of influenza A viruses, making up the intermediate layer between the viral envelope and the integral viral proteins (Noton *et al.*, 2007). This protein is also able to control the trafficking of viral RNPs. When the virus enters a cell, the M1-RNP interaction has to be disrupted in order to allow the transport of the RNPs to the host nucleus. This is a crucial step required for the release of the viral genome. The M2 protein of Influenza A viruses is a single-pass, type III integral membrane protein that works as a pH-gated proton channel (Manzoor *et al.*, 2017; Schnell and Chou, 2008). The channel is closed and stable at pH 7.5. Once the outer (endosomal) pH decreases to 4.5 – 5.0, the channel is activated, allowing the efflux of cations (K^+ and Na^+) and the influx of protons, which trigger the release of the viral RNP into the host cytoplasm.

1.1.3.3 PB1, PB2 and PA

Each vRNA segment is assembled into a RNP complex, which comprises the vRNA, multiple copies of NP and three polymerase subunits, also called P proteins: PB1, PB2 and PA. PB1 is responsible for the management of the length of the RNA chain in both replication and transcription (Perez and Donis, 2001). This protein contains several amino acid motifs common in RNA-dependent DNA and RNA polymerases. If these motifs present with mutations, they render the viral complex unable to carry out transcription and replication. The RNA polymerase subunit PB2 is a major virulence

factor of avian influenza viruses (Graef *et al.*, 2010). The PB2 protein contributes towards the generation of 5'-capped RNA fragments from cellular pre-mRNA molecules, which act as primers for viral transcription. By interacting with mitochondrial antiviral-signalling proteins, PB2 is able to inhibit anti-viral immune responses. Furthermore, an *in vivo* study (Graef *et al.*, 2010), conducted in mice, demonstrated that viruses that encode PB2 that does not localise to mitochondria are less virulent and are more restricted in replication, compared to viruses with PB2 which shows mitochondrial localisation. Finally, PA works together with PB1 and PB2 to contribute to an effective transcription and replication of vRNA, however further research is required to further understand its role.

1.1.3 Viral Replication

1.1.3.1. Entry

Enveloped viruses, such as influenza virus, can enter the host through two routes (Diehl and Schaal, 2013). The pH-independent route allows the virus to attach to the host cell and, by interacting with its receptors, the viral envelope is able to fuse to the plasma membrane of the target cell. The second route, which is pH-dependent, enables the virus to enter the target cell via endocytosis. Viruses can spread between cells in two different ways, either by diffusion through the extracellular space or by direct cell-to-cell contact (Zhong *et al.*, 2013). Transmission via cell-free virus is less restricted and, therefore, can travel longer distances throughout the body. However, cell-to-cell transmission appears to be more efficient and is achieved by direct contact with the target cell. Viruses are able to manipulate the cellular metabolism of the host and evade defence mechanisms to promote their survival (Agudelo-Romero *et al.*, 2008). Whether the interactions between the pathogen and its host lead to more or less virulent or harmless situations is dependent on genetic and environmental factors. In particular, the viral genome plays a crucial role in the adaptation to the host and environment, as it can mutate through genomic reassortment.

1.1.3.2 RNP release and nuclear import

During infection, after the virus has gained access into the host cell, through endocytosis, and after the viral membrane has fused within the endosome, the RNPs are released into the cytosol, from which they are then transported to the nucleus

(Fig. 3). The NEP protein of influenza A viruses contributes to the nuclear export of viral proteins (O'Neill *et al.*, 1998). The import and export of viral nucleic acids from the nucleus is a crucial step of viral infection. NEP acts as an adaptor between the viral RNP and the host cell nuclear export machinery. Overall, RNPs affect viral infection as they direct the replication and transcription of vRNAs (Zheng and Tao, 2013). The RNPs are then able to enter the host nucleus through active transport. In the nucleus, they are used as active templates for the synthesis of viral messenger RNA (mRNA) and complementary RNA (cRNA). The cRNAs direct the synthesis of nascent vRNAs. Newly translated NP, PB1, PB2 and PA are imported back into the nucleus and are assembled with nascent cRNAs and vRNA encapsidated into an RNP structure.

1.1.3.3 Budding and release

Two viral proteins, M1 and NEP, assist with the nuclear export of the newly assembled viral RNPs. Once they are exported into the cytoplasm, the RNPs are then packaged into budding virions. Both HA and NA are capable of initiating the budding process, through which the virus is able to exit the host cell. These two glycoproteins are bound to lipid raft domains and they are able to alter the curvature of the host cell membrane, which facilitates virus budding (Rossman and Lamb, 2011). These viral membrane glycoproteins work together with the inner structural cellular proteins to form a 'pulling-pushing' system, by which the budding process is driven (Welsch *et al.*, 2007). The overall influenza A virus cycle is illustrated in figure 3.

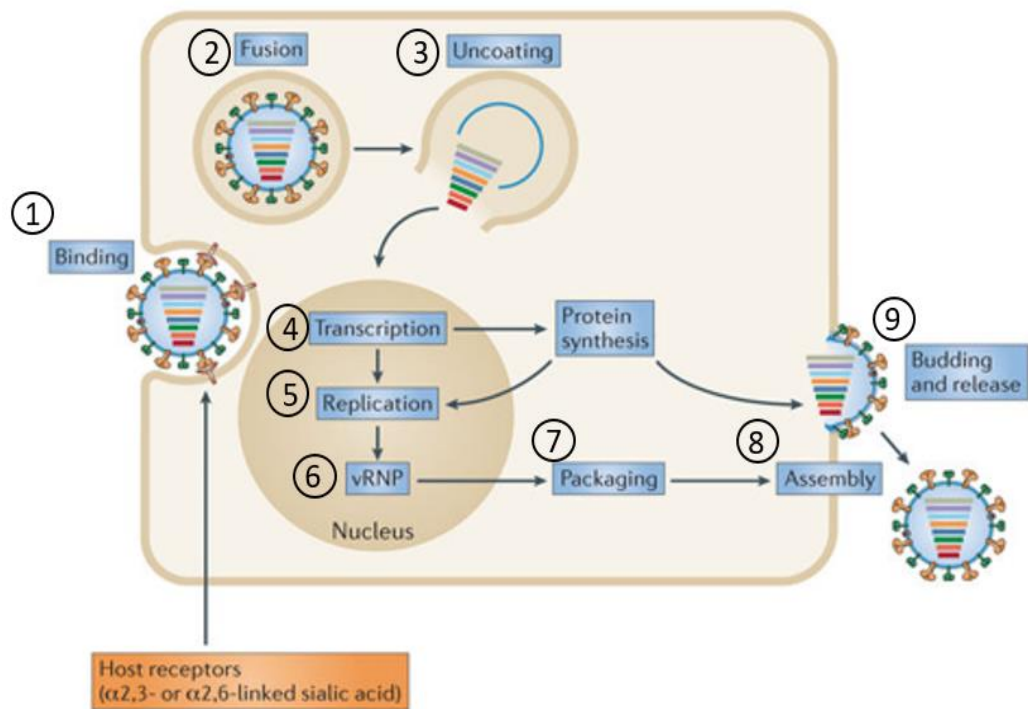


Figure 3: The Influenza A virus life cycle.

The virus begins infection by binding to host receptors (1) and entering the cell through endocytosis. Once inside the host, the fusion (2) of the viral and endosomal membrane, which occurs with low pH, allows for the RNPs to be released (3) into the cytoplasm. The RNPs are actively transported into the nucleus where the transcription (4) and replication (5) are carried out. After the protein synthesis is carried out in the cytoplasm, viral proteins are assembled into viral nucleoproteins (vRNPs) within the nucleus (6). The vRNPs are then exported from the nucleus and packaged (7) into new virions, with the aid of NEP and M1. New viral particles are assembled (8) near the membrane and the new virion exits the cell via budding (9). Modified from Shi et al., 2014.

1.1.4 Viral Pathogenicity

1.1.4.1 LPAI and HPAI

Avian influenza A virus is divided into two groups, based on its virulence: highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) (Rebel *et al.*, 2011). HPAI typically results in high mortality rates, often as high as 100%, in gallinaceous poultry. These viruses are restricted to subtypes H5 and H7, although not all H5 and H7 subtypes are highly pathogenic. LPAI is less threatening as it causes respiratory signs of infection, such as nasal discharge and swollen sinuses in gallinaceous poultry, however, certain strains of LPAI viruses, of subtype H5 or H7,

can transform into HPAI viral strains (Alexander, 2007). The factors that lead to such mutations are unknown, and the LPAI exposure time before turning into HPAI varies. However, as it typically occurs by chance, a greater number of infected poultry would increase the likelihood of its occurrence. One of the major determinants of avian influenza pathogenicity is the HA cleavage site. Studies focusing on nine different HA subtypes in avian species showed that pathogenicity correlated directly with the ability of viruses to produce cleaved HA in infected cells (Steinhauer, 1999). The HA precursor (HA₀) polypeptides are cleaved into two subunits HA₁ and HA₂, linked by a disulphide bond. This is necessary to render the virus infectious, as it activates the membrane fusion potential of HA. Cleavage of the HA allows for the molecule to undergo molecular changes, when triggered by the acidic pH of endosomes, in order to acquire the structure necessary for fusion. Modifications to the haemagglutinin cleavage site cause alterations in the pathogenicity of the virus. HPAI viruses are equipped with multibasic cleavage sites, whereas LPAIs are equipped with monobasic cleavage sites, meaning they are only able to grow in designated areas of the host, as the HA is only cleaved by trypsin-like proteases (Sun *et al.*, 2010). The presence of a multibasic cleavage sites strongly affects viral systematic spread as these cleavage sites are recognised by several different proteases, such as furin, allowing HPAI viruses to spread widely throughout infected host tissues.

1.1.4.2 Host factors

When influenza virus infects susceptible cells, for example, epithelial cells or alveolar macrophages, its single stranded RNA is released and recognized by pattern recognition receptors (PRRs), which include toll-like receptors (TLR) 7 and retinoic acid-inducible gene-I (RIG-I) (Fukuyama and Kawaoka, 2011). The signalling pathways of TLR7 and RIG-I are able to induce the production of type I interferons and activate the host antiviral response. However, the virus is often able to evade this defence mechanism, in particular, with the aid of NS1, which interferes with the RIG-I pathway. In chickens, although RIG-I appears absent (whilst it is present in ducks), IFN type I production is still active, and it is thought that melanoma differentiation-associated protein 5 (MDA5) compensates for the lack of RIG-I (Liniger *et al.*, 2012). The host PRRs are able to interact with the viruses by detecting their pathogen-associated molecular patterns (PAMPs), such as double-stranded RNA (dsRNA), single-stranded

RNA (ssRNA) and viral proteins (Kolli *et al.*, 2013). The host response to viral infections is affected by pre-existing conditions, such as pregnancy, immunosuppression, diabetes mellitus, underlying respiratory conditions or cardiovascular complications (Kash and Taubenberger, 2015). A single nucleotide polymorphism (rs12252-C) in the interferon-inducible transmembrane 3 (IFITM3) gene was identified in patients that presented a more severe reaction to the influenza infections. This genetic variation was also identified in patients with an elevated cytokine expression as well as in patients who died after infection with H7N9 influenza A strain.

1.2 Avian Influenza

1.2.1 Differences in the response to avian influenza in avian hosts

Ducks and chickens are both major hosts for avian influenza viruses, however they show a marked difference in response to infection. Waterfowl (e.g. mallard ducks), as well as shorebirds, are able to harbour and shed influenza A viruses with limited clinical disease (Achenbach and Bowen, 2011). They are able to shed large amounts of virus without showing any clinical signs and they can travel long distances, allowing them to potentially transport the virus from one region to another. Amongst these, mallard ducks are of great interest, as they act as a reservoir for viral subtypes H1-H12. In contrast, other terrestrial avian species, such as chickens and quails, show a varied spectrum of disease, which for highly pathogenic viruses can be very severe, and often rapidly fatal. For example, when compared with ducks, quails and chickens infected with HPAI H5N1 show a much more rapid deterioration, with sudden death occurring within 2-4 days post-infection (p.i.) (Saito *et al.*, 2009). Studies have shown that pintail and mallard ducks are less susceptible and are more likely to survive and perpetuate the HPAI H5N1 strain (Kim *et al.*, 2009). Experimentally-infected mallard ducks continued to carry the virus for up to 17 days, following which the duck developed humoral immunity as well as antigen variants. This suggests that in order to maintain the virus in nature, it may require as little as one infected waterfowl bird. This has been demonstrated by infecting four ducks with HPAI H5N8, which shed the virus without presenting any debilitating clinical signs, demonstrating the potential of waterfowl to act as long-distance vectors (van den Brand *et al.*, 2018). However,

virus spread to domestic poultry is also caused by the movement of poultry and their products, as well as through virus reintroduction through migrating birds. The ability of some wild ducks, in particular mallards, to shed a greater amount of virus, compared to other avian species, suggests that they are important vectors for transmission of HPAI, whilst others, such as tufted ducks, function as sentinels, as they are more susceptible to the virus (Keawcharoen *et al.*, 2008). Following infection of tufted ducks with HPAI H5N1, neurological signs appeared at approximately 4 days p.i.. However, HPAI H5N1 infections in chicken resulted in widespread oedema and haemorrhage, as well as death, at 2 day p.i.. Such differences are likely explained by differences in tissue tropism between species. However, chickens are only able to survive exposure to LPAI, as they tend to present either with mild or no signs of infection (Smith *et al.*, 2015). The interferon induced transmembrane protein (IFITM) family has the potential to limit the spread of influenza in mammals. Amongst birds, it appears that ducks appear to present with the most developed IFITM1 system, which may play a role in viral resistance. A more recent study highlighted the difference between ducks and chickens infected with LPAI virus, via the PI3K/Akt pathway activation (Kumar *et al.*, 2018). The pathway appeared active in chickens, but not in mallard ducks. Although the pathway promotes cell survival in infected chickens, it promotes the propagation of the infection throughout the organism, thus making the subject more susceptible to influenza infections. On the other hand, since this pathway is inactive in infected ducks, this terminates the infection at a much faster pace, which then allows the subject to show little to no signs of infection.

1.2.2 Importance of quails in the transmission of avian influenza

Avian influenza virus infection is not restricted to major avian species, such as chickens, turkeys and ducks, but it can also spread to minor avian species (Bertran *et al.*, 2014). These include: Japanese quail, bobwhite quail, pearl guinea fowl, chukar partridge, ring-necked pheasant and others. These birds can be found in the wild, live poultry markets and also in outdoor environments (e.g. Farms and wildlife parks), which have shown to be linked to a higher avian influenza exposure. Quails have the potential to act as an interspecies bridge, meaning they are able to contract a viral strain from wild birds and create new reassortant viruses, which have the potential

to cause further more widespread disease in poultry (Thontiravong *et al.*, 2012; Adel *et al.*, 2017).

1.2.3 Recombination of avian influenza strains

As segmented RNA viruses, influenza A viruses of different strains are able swap their genomic segments during co-infection (McDonald *et al.*, 2016). This confers the viral progeny with mixed genetic components, from different predecessors, which can result in an increase (or potentially decrease) in viral fitness. Through virus evolution, a new strain could not only be a threat to avian species, but also to humans. An outbreak within poultry of H9N2 in Egypt caused great economical losses, in particular when complicated by exposure to other pathogens (Adel *et al.*, 2017). This outbreak highlighted the zoonotic potential of the virus, as some viruses isolated from chickens were equipped with amino acid residues H173, H179 and L216 in HA1. Although there were no registered cases of human infections of the H9N2 in Egypt, the residues present in this strain would be able to assist with the binding of the virus to α 2,6 human sialic acid receptors and hence support the virus entry to human epithelial cells. The ability of the HA to bind to a sialic acid is a major determinant towards host specificity and viral tropism. The majority of avian influenza viruses bind to α 2,3-linked sialic acid in birds, however human viruses typically bind to α 2,6-linked sialic acid (Leung *et al.*, 2012). Thus, direct transmission between species is extremely rare. Quails have been shown to carry both α 2,3- and α 2,6- receptors in their trachea and intestine (Wan and Perez, 2006), meaning that they are potentially able to be infected with both human and avian type viruses. The LPAI H9N2 in Egypt (Egy/G1) showed continuous genetic and antigenic evolution leading to virus variants, which appeared in quails vaccinated against the original strain of Egy/G1 virus (Adel *et al.*, 2017). Another H9N2 strain isolated from birds in Hong Kong, in the 1970s, replicated more rapidly in quails than in chickens (Perez *et al.*, 2003). Quails are able to provide a host environment for viruses derived from ducks, and, furthermore, are a suitable host for evolution of the original strain, which can lead to the virus subsequently infecting other avian species. Such characteristics were observed in virus strains isolated from quails in 1988, in which the viruses had acquired molecular markers associated with established viral lineages found in land-based birds. The 1988 H9N2 strain was the first registered case of an H9 virus in land-based poultry (Wan and

Perez, 2006). Overall, outbreaks of avian influenza viruses within poultry flocks cause great disturbances and economical loss within the industry (Capua and Marangon, 2009; Govindaraj *et al.*, 2018). Quails have been found to be effective mixing vessels for influenza viruses, presenting a potential threat to other avian subspecies, as well as other animals (Adel *et al.*, 2017).

1.3 Zoonotic Infections

1.3.1 Zoonosis

A zoonosis is a disease that can be transmitted from animal to human hosts (Hubálek, 2003). These can be classified accordingly to their ecosystem. Synanthropic zoonotic diseases (e.g. urban rabies, zoonotic ringworm) are more common in urban environments, derived from domestic or synanthropic animals; whilst exoanthropic zoonotic diseases (e.g. wildlife rabies, tularaemia, arboviruses) are found in natural environments. However, there are zoonoses that can be found in both environments (e.g. yellow fever). Zoonotic agents can be transmitted through direct contact, airborne or foodborne or waterborne routes, however some can also be rodent-borne or arthropod-borne. One Health is a concept that aims to provide optimal health for animals, humans and environment (Bidaisee and Macpherson, 2014). The World Health Organisation (WHO) estimated that approximately 75% of human emerging diseases are zoonotic. The One Health approach has been implemented to accelerate biomedical research discoveries, improve public health, strengthen the base of scientific knowledge and also enhance medical education and care. Zoonotic diseases can pose a major threat to both human and animal health, therefore it is one of the main points of focus of One Health, together with food safety and health delivery systems. In particular, Influenza A is one of the most challenging zoonotic diseases, as it poses a threat to a vast range of species and it has the ability to evolve and adapt to new hosts. The host tropism of different species restricts infection of hosts and host tissues to specific pathogens. Amino acid changes that affect the receptor binding domain (RBD) of HA, dramatically alter the binding preferences of the virion (Imai and Kawaoka, 2012). In order to jump from one species to another, the HA RBD of influenza viruses must undergo certain changes in order to switch from recognizing α 2,3-linkage of sialic acids to binding to α 2,6-linkaged sialic acids.

However, the specific amino acid requirement variations required vary amongst different HAs. For example, H2 and H3 require substitutions of G226L and G228L in order to form α 2,3- to α 2,6- glycan bindings, whilst HA1 need E190D and D225G mutations. Direct transmission is relatively rare, the virus more commonly passes through an intermediate host such as the pig, which is equipped with both sialic acids linkages, α 2-3- and α 2-6-, in its trachea (Ito *et al.*, 1998).

1.3.2 Interspecies transmissions

Influenza pandemics emerge when an influenza virus with a particular HA and/or NA, against which there is little to no immunity in the human population efficiently passes from animals to humans and, further, from human to human. Influenza H1N1 was first isolated from swine in 1930, which shared similarities with a human H1N1 strain from 1918, meaning that there is a possibility that they share a common ancestor (Garten *et al.*, 2009). The outbreak of the 2009 H1N1 influenza A virus of swine-origin raised concern as it can be readily transmitted to humans. Furthermore, this strain is equipped with antigenically and genetically divergent HA. In 2013 the first H7N9 outbreak of avian influenza virus amongst humans was recorded (Gao *et al.*, 2013). The three registered patients presented with rapidly progressing lower respiratory tract infections. Although human infections with H7 had been observed in previous years, this was the first documented case of human infections with an N9 influenza virus subtype. Figure 4 illustrates the hypothetical genetic re-assortment required to make up the re-assortant avian-origin H7N9 strain that is able to infect humans. The spread of this strain demonstrated that it is possible for avian viruses to re-assort in order to infect other species.

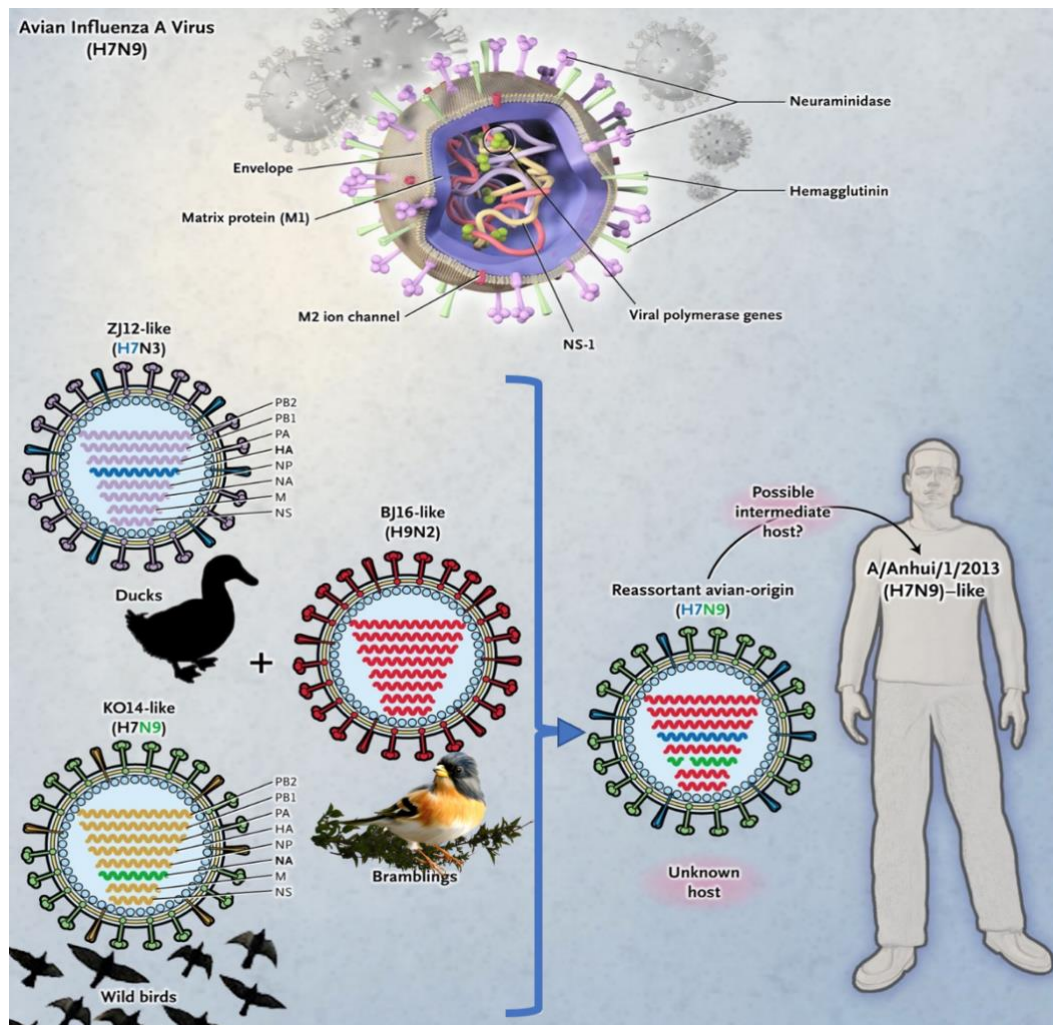


Figure 4: Hypothetical genetic re-assortment of H7N9

The colour of the genetic segments within the virions indicate their origin. BJ16: A/brambling/Beijing/16/2012; ZJ12: A/duck/Zhejiang/12/2012; KO14: A/wild bird/Korea/A14/2011. Modified from Gao et al., 2013.

1.3.3 H5N1

The H5N1 strain poses a major threat to humans by direct transmission from infected poultry (Peiris *et al.*, 2007). However, the virus is yet to gain the ability to transmit from human-to-human. The threat of a HPAI viral infection across different species raises concerns of a possible pandemic outbreak, which could potentially result in a high fatality rate in humans. Strict infection control measures, such as good hygiene and flock monitoring, are required to minimise the threat to human health. In 1997, an outbreak of the H5N1 avian influenza strain caused serious disease in both humans and poultry in Hong Kong (Chan, 2002). In all 18 human cases registered in 1997 the patients had been in close contact with birds; they presented with different clinical

manifestations, ranging from asymptomatic with mild upper respiratory infection to fatal pneumonitis and organ failure. Overall, the disease was fatal for 6 of the 18 patients and more than a million chickens were slaughtered to prevent further disease spread. Following this, prevention measures took place; intensive monitoring and tests are now obligatory in order to reduce disease risk. Reported cases of H5N1 infections from outbreaks that occurred in Thailand, Vietnam and Cambodia in 2004-2005 presented with an overall lower death toll, however the number of infant fatalities was increased from previous years (WHO, 2005). It has been shown that, when compared to other viral strains, H5N1 infections often lead to high fatality rates (Neumann, 2015).

1.3.5 H7N1

H7 avian influenza viruses are recognized as a potential pandemic threat (Sutton *et al.*, 2014). These viruses are often transmitted as low pathogenic viruses from wild waterfowl to domestic poultry. Upon infection in chickens, the H7 virus can mutate generating mutations at the HA cleavage site causing the virus to become highly pathogenic, leading to high mortality rates. When these strains are transmitted to humans, they usually cause mild conjunctivitis. However, there have been reported cases where human hosts also present with respiratory infection. In 1999 an outbreak of H7N1 HPAI in Italy caused the death of over 13 million birds, causing significant losses in the poultry industry (Capua and Marangon, 2000). The severity of the outbreak was caused by the absence of preventative measures to eradicate the infections of an LPAI H7N1 virus, which caused the strain to spread until a HPAI virus of the same subtype emerged. The high mortality rates were a result of ineffective prevention policies to avoid further spread. In order to prevent severe outbreaks within livestock in rural areas, farmers and staff in direct contact should be provided with an appropriate education on biosecurity, and precautionary measures must be enforced within the poultry industrial system. Studies conducted on ferrets, the most reliable animal model for human influenza virus infections, showed that the H7N1 subtype can evolve and infect co-housed ferrets probably by airborne contact (Sutton *et al.*, 2014). This highlights the threat of a possible H7N1 pandemic amongst humans.

1.4 Immune response to viral infection

1.4.1 Innate Immunity

The immune system works as a defence barrier, in humans and other animals, against pathogen invasions. The system includes a variety of host defences that vary from the non-specific barrier function of epithelia, to the highly selective recognition of pathogens through germline-encoded receptors (figure 5). Innate immunity provides an immediate, non-specific response to infection. This inherent response causes a pro-inflammatory reaction to the invading pathogens (Mogensen, 2009). The inflammation is caused by PRRs, which are germline-encoded host receptors (e.g. toll like receptors (TLR)), detecting PAMPs. Upon PAMP recognition, PRRs alert the cell either by presenting at its surface or by sending an intracellular signal. This triggers the activation of pro-inflammatory and antimicrobial responses, with the help of several intracellular pathways, such as kinases, transcription factors and adaptor molecules.

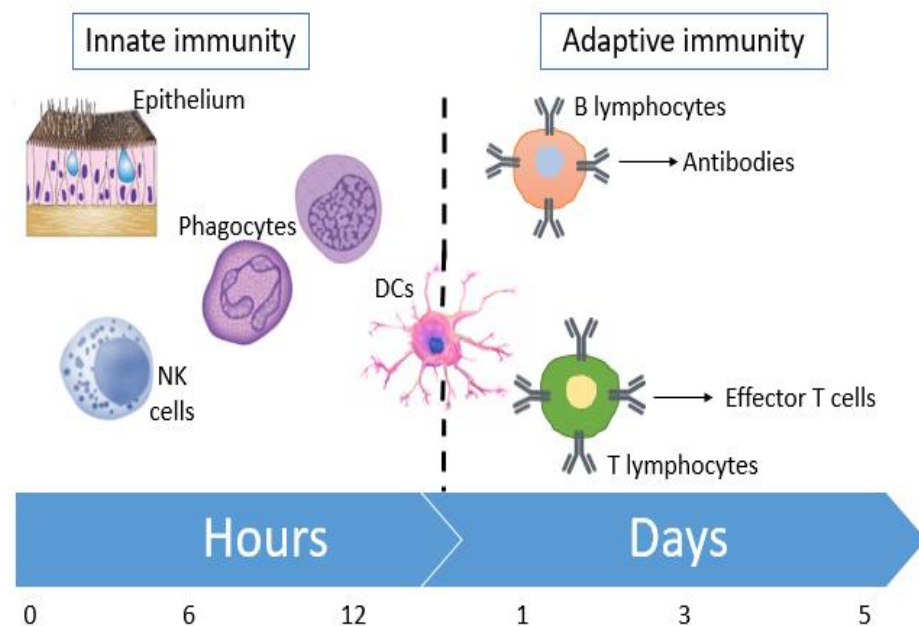


Figure 5: Immune response timeline.

The diagram illustrates the cells involved in the innate and adaptive immunities. DCs = dendritic cells; NK = natural killer (Adapted from Hogg, 2007).

1.4.2 Interferons

Interferons (IFNs) are proteins produced by virus-infected lymphocytes, fibroblasts and macrophages, and they play a crucial role in the innate immune response, providing a strong first line of defence against viral and microbial infections (Tortora and Derrickson, 2011). Once released, IFNs travel to uninfected neighbouring cells, where they trigger the synthesis of several antiviral proteins, which are able to interfere with viral replication. IFN receptors (IFN-R) are made up of two chains, one with low affinity and one with high affinity, essential for IFN binding (Schneider *et al.*, 2014). These signalling complexes are classified into three groups: type I IFNs (IFN- α , - β , - ϵ , - κ , - ω), type II IFNs (IFN- γ) and type III IFNs (IFN- λ 1, - λ 2, - λ 3, - λ 4). As the IFN binds to cell surface receptors, a signal is transmitted through the outer membrane and into the cell, causing alterations of its cellular properties. All IFNs signal through the Janus kinase signal transducer and activator of transcription (JAK-STAT) pathway. This signalling pathway regulates IFN signalling by phosphorylating IFN-Rs through active Janus kinases (JAKs) and, furthermore, by regulating the expression of IFN-stimulated gene (ISG) expression with the assistance of STAT proteins as transcriptional activators. Of the ISGs, several have been identified to have specific anti-viral activity against influenza viruses (Killip *et al.*, 2015). These include the Mx family of GTPases, which are able to inhibit viral nuclear import; viperin, which blocks viral budding, therefore limiting the release and proliferation of virions; and the IFITM family members that aim to block viral entry by interfering with the interaction between viral and endosomal membranes.

1.4.3 Dendritic Cells

Dendritic cells (DCs) are a main component of both innate and adaptive immune response. These cells develop within the bone marrow and then migrate to tissues whilst still immature, but with highly phagocytic abilities (Clark and Kupper, 2005; Banchereau *et al.*, 2000). Maturation of DCs occurs once the cells are exposed to a number of factors, including PAMPs, cytokines and other tissue factors. Once mature, DCs become effective T cells stimulators, as they transport antigens to antigen-specific T cells. DCs present the antigen to T helper (CD4⁺) cells, which help regulate the immune system's effector cells, such as NK cells, CD8⁺ cytotoxic T cells, B cells and eosinophils.

1.4.4 Adaptive Immunity

Activated DCs lead to induction of humoral and cellular immune response by activation of antigen specific B- and T- cells respectively after approximately 4–7 days. Other than being the defence mechanism in the later stages of infection, the adaptive immune system is responsible for long-term immunological memory. It consists of a cellular response, which is mediated by T lymphocytes, as well as a humoral response, represented by specific antibodies produced by B lymphocytes. These originate from different lymphatic organs (Bonilla and Oettgen, 2010). The T cells originate from the thymus, whilst the B cells originate from hematopoietic cells in the bone marrow in mammals. However, the origin of the B cells differs in avian species, as they originate from the Bursa of Fabricius, within the cloaca (Sharma, 1991). Once the pathogen has been recognized, the lymphocytes proliferate and differentiate into effector cells that target that particular pathogen.

1.4.4.1 *T lymphocytes*

1.4.4.1.1 Helper T cell activation

Cell-mediated immunity is initiated by the activation of several T cells through antigen recognition, carried out by interactions between T-cell receptors (TCRs) and antigen-presenting cells (APCs), such as dendritic cells (Tortora and Derrickson, 2011). Once activated, T cells proliferate and differentiate into effector and memory cells (figure 6). Cells that display CD4 develop into helper T (CD4) cells. These are activated through interactions with exogenous antigen fragments associated with major histocompatibility complex II (MHC-II) molecules, which are found on the surface of APCs. The CD4 protein assists with the T cell and APC interaction, further activating the helper T cells. Once active, the cells undergo clonal expansion, resulting in the production of active helper T cells and memory helper cells. Active CD4 cells secrete a variety of cytokines, including interleukin-2 (IL-2), which enhances T cell, B cell and NK cell proliferation, whilst memory helper T cells are able to assist if the same initial antigen enters the body, thus accelerating immune response by producing active and helper memory T cells.

1.4.4.1.2 Cytotoxic T cell activation

Cytotoxic T (CD8) cells initiate activation by recognising foreign antigenic peptides coupled with the major histocompatibility complex I (MHC-I) on infected cells, as well as tumour and tissue transplant cells (Tortora and Derrickson, 2011). However, the full activation requires additional co-signals including IL-2, produced by helper T cells. Clonal expansion results in the production of a large population of cytotoxic T cells and cytotoxic memory T cells specific for a particular antigen. The active CD8 cells are able to attack infected cells by releasing granzymes, protein-digesting enzymes that trigger apoptosis, as well as granulysin and perforin, which further assist in the destruction of infected cells. Memory CD8 cells are able to trigger a more rapid response if the same antigen enters the body in the future. Although all T cells share the basic principles of activation and development, there is great variation of effector functions (Bonilla and Oettgen, 2010). T cells can acquire direct roles in the removal of pathogens or can function as helper cells, which help enhance B and T effector cell functions through cognate receptors or the release of cytokines. Finally, T cells can also assist with the regulation of the immune response, which includes limiting tissue damage in the host.

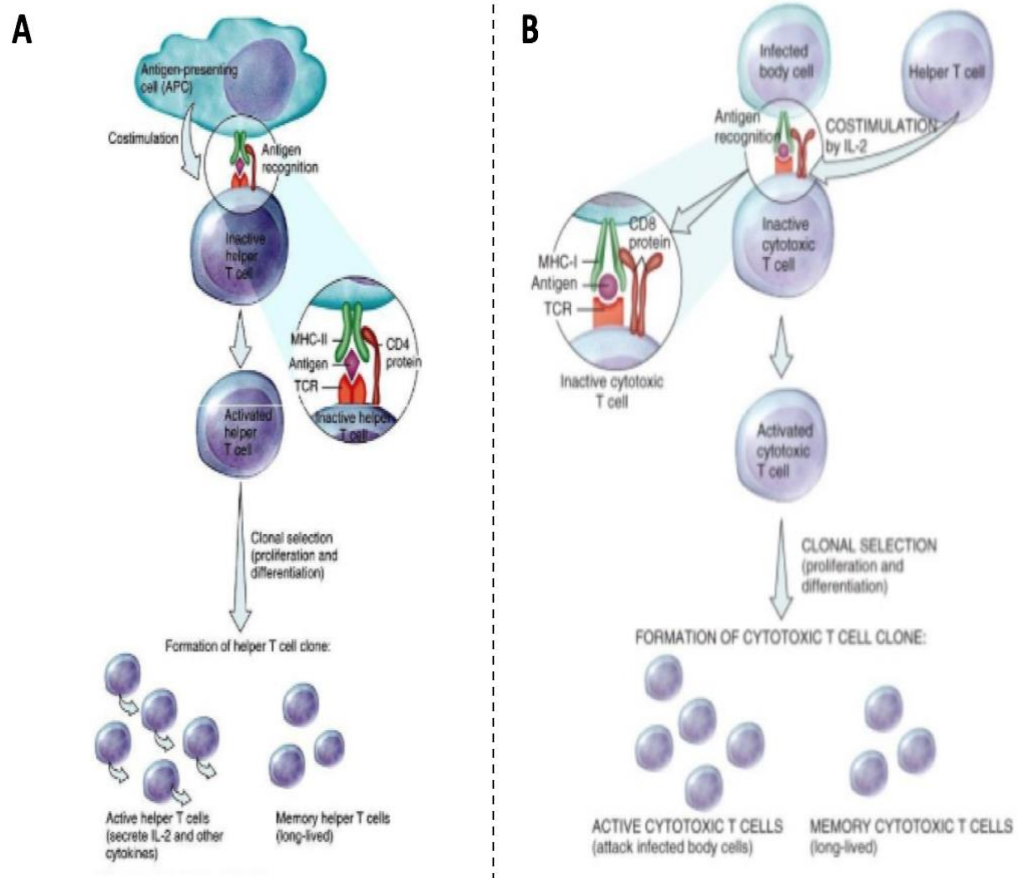


Figure 6: Comparison of activation and clonal selection of memory and cytotoxic T cells.

The T helper cells are identified by displaying CD4 (A) (Adapted from Tortora and Derrickson, 2011). These are activated through the interaction with exogenous antigen fragments associated with MHC-II, which are found on the surface of APCs. CD4 assists with the interaction between the inactive T helper cell and the APC, which triggers the activation of the T helper cell. Once active, it generates clones of itself as well as memory helper T cells. On the other hand, activation of cytotoxic T (CD8) cells (B) is initiated through the interaction of infected cells and MHC-I. The cells are fully activated once they are triggered by IL-2, which is presented by active CD4 cells. Clonal selection results in clones of the original CD8 cell and memory CD8 cells. Both CD4 and CD8 memory cells are able to trigger a quicker response if the same antigen presents itself again.

1.4.4.2 B lymphocytes – humoral immunity

B cells provide antibody mediated immunity, also known as humoral immunity, moderated by specific B-cell receptors that bind to antigens (Tortora and Derrickson, 2011). Interleukin-2 as well as other cytokines secreted by T helper cells provide co-stimulation for the activation of B cells. Once activated, the cells undergo clonal selection, mature and proliferate to plasma cells, and memory cells. The plasma cells

secrete antibodies, also known as immunoglobulins (Ig), which bind to the epitope of the antigen that triggered its activation. There are five main classes of Igs: IgG, IgA, IgM, IgD and IgE. However, birds, amphibians and reptiles have IgY in place of IgG (Warr *et al.*, 1995), which is further discussed in 1.4.6. Each class is able to bind the antigen and effect removal by different mechanisms including: neutralizing the antigen, immobilizing bacteria, agglutinating and precipitation antigens, activating the complement system and enhancing phagocytosis. Memory B cells do not secrete antibodies, but they are able to proliferate and differentiate into more plasma cells, as well as memory B cells, if the antigen were to re-appear in the near future. Development mainly occurs in the bone marrow in mammals or in the Bursa of Fabricius in birds, after which the immature cells are released into circulation and undergo differentiation (Hoffman *et al.*, 2016).

1.4.5 Complement system

The complement system is made up of about 30 proteins that contribute to host defence and inflammation during innate and adaptive responses (Sarma and Ward, 2011). The activation of the system occurs through three different pathways (alternate, classical and lectin) (figure 7) which trigger a cascade of enzymatic reactions (Noris and Remuzzi, 2013). This results in the formation of potent anaphylatoxins, C3a and C5a, which are strong pro-inflammatory molecules that interact with cognate G-protein-coupled receptors C3aR and C5aR, to attract and activate leukocytes. Other products of the system include: opsonins C3b, iC3b and C3d, which facilitate transport and removal of target cells or immune complexes; and the terminal membrane attack complex (MAC, C5b-9), which directly lyses targeted pathogens or damaged self-cells.

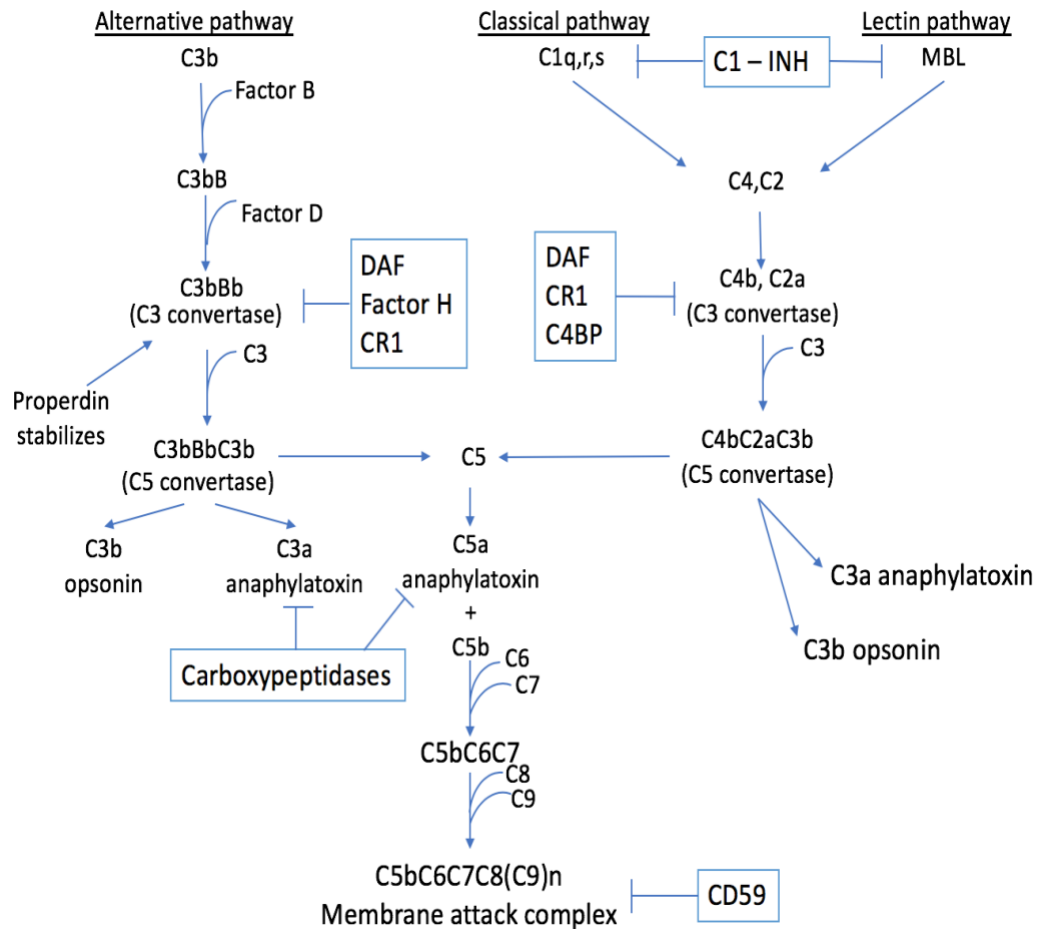


Figure 7: Complement system and its three different pathways of activation: alternative, classical and lectin

Factors that are able to inhibit the pathway are highlighted in boxes. Modified from Sarma and Ward, 2011.

1.4.6 Unique aspects of avian immunity

Avian species present a smaller immune gene component, compared to humans. Birds lack two TLRs: TLR8 and TLR9 (Magor *et al.*, 2013). However, TLR9 has been functionally compensated by another receptor found in birds, TLR21. The lack of TLR8 in birds suggests a possible explanation around their susceptibility to RNA viruses, such as influenza virus, and intracellular bacterial infections. There is also a lack of interferon-induced proteins with tetratricopeptide repeats (IFIT) genes within birds. Overall the IFIT family contributes towards the diverse antiviral activities of interferons as well as towards the cellular innate immune response. The only identifiable IFIT gene in avian species is IFIT5. Birds share many common aspects with the mammalian immune system; however, there are certain aspects that

differentiate them. Three classes of immunoglobulins have been identified in avian species: IgM, IgY (IgG) and IgA (Tirziu and Seres, 2010). IgY is the homologue of the mammalian IgG (figure 8). The main difference between IgY and its mammalian homologue is the longer heavy chain found in the chicken molecule (Härtle *et al.*, 2012). IgY is also made up of five domains (V, C1-C4), compared to the four found in IgG, and it is not equipped with a genetically encoded hinge. Instead, its mobility is restricted by “switch regions” (Cu1-Cu2 and Cu3-Cu4) with limited mobility. Chickens are able to pass the serum onto their offspring through the egg yolk, instead of transfer by colostrum (Amro *et al.*, 2018). This also makes it easier, as well as less stressful for the animal, to retrieve antibodies from chickens than from other animals. The lack of IgD or any homologs, could affect the birds immune response, as it is responsible for the binding of basophils and stimulates the triggering factors of antimicrobial, inflammatory and B lymphocyte responses in humans (Chen *et al.*, 2009). Another aspect to differ from other species, is the limitation of the genome of the major histocompatibility complex (MHC) in chickens (Kaufman *et al.*, 1995). This consequentially affects the ability of the chicken to defend itself from viral pathogens. Birds are also equipped with heterophils, the predominant granulated leukocyte that is found in gallinaceous birds (Harmon, 1998). These white blood cells substitute the mammalian neutrophils and assist during the early stages of acute inflammatory responses, where they carry out phagocytosis.

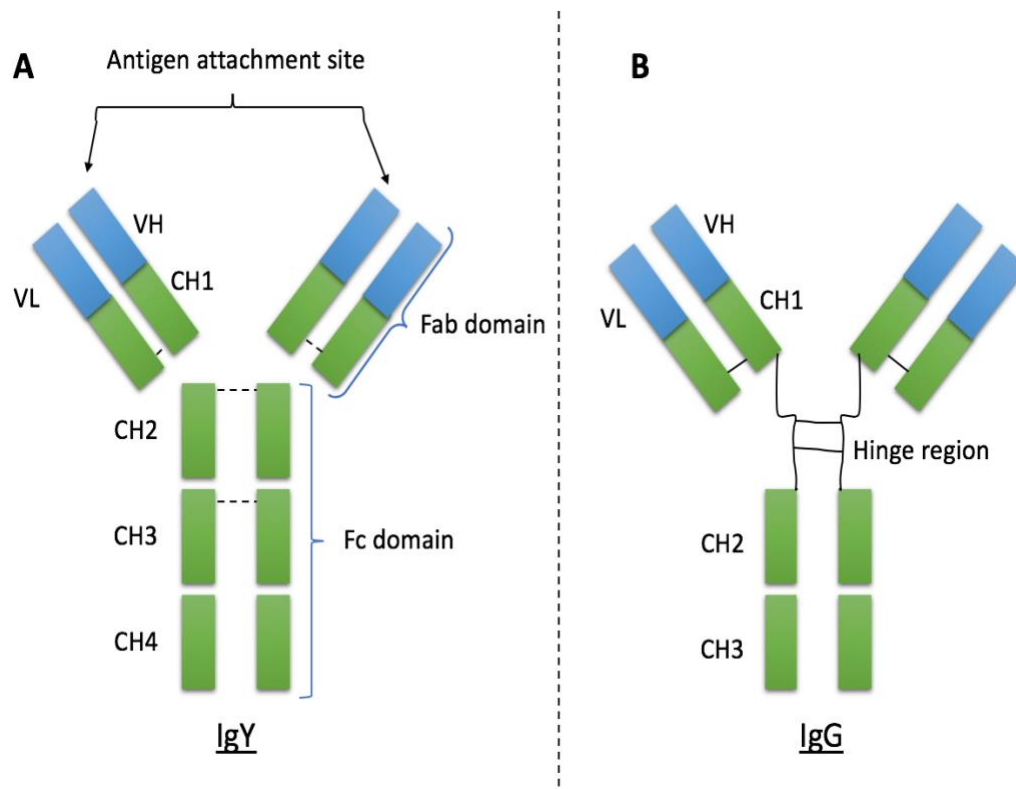


Figure 8: Comparison between IgY from birds and IgG from mammals

Both immunoglobulins contain a heavy and a light chain, with two variables (VH and VL) and constant domains, although birds possess an extra domain. The hinge region in the IgG (B) makes it more flexible than IgY (A). Modified from Müller et al., 2015.

1.4.6.1 Innate response differences between ducks and chickens

Chickens lack the gene encoding RIG-I, a cytoplasmic recognition receptor responsible for the production of IFN type I, DDX58; however, there is a functional RIG-I in ducks (Magor *et al.*, 2013). The gene is also absent in turkeys and partridges, which suggests that the gene is lacking in all galliformes. The lack of the gene likely contributes to the increased susceptibility of chickens, compared to ducks, to influenza A virus. MDA5, a PRR for intracellular RNA related to RIG-I, is responsible for partially compensating for the absence of RIG-I in chickens, to produce interferons. The RIG-I receptor has been identified as the target of NS1 protein of influenza A viruses, in human infections (Gack *et al.*, 2009). This renders the receptor unable to function during infection. TRIM25 plays an essential role in the ubiquitination of the N-terminal caspase recruitment domains, consequently enabling RIG-I to elicit anti-viral signal transduction. It has been shown that NS1 targets TRIM25 in cells infected or transfected with influenza viruses of human, avian and swine origin. This indicates

that the TRIM25 target is a conserved function of NS1. Several genes appeared to be downregulated in chickens infected by the H5N1 virus whilst ducks appear more upregulated (Kuchipudi *et al.*, 2014). In particular, IL-18 has been shown to have an important role in the development of protective immunity against several pathogens (e.g. mycobacterium tuberculosis). Recombinant vaccines have been proven to potentially present more positive results if antigens are used along with IL-18 (Chen *et al.*, 2011). Furthermore, essential genes of the JAK-STAT pathway were also downregulated in chickens, but upregulated or unaltered in duck cells, up to 24 hours post-infection. This pathway is essential for the recruitment of interferons and if it malfunctions, it could negatively affect the antiviral response. Age has been found to be a common factor that affects the immune response, as it broadens with age in swans (Hill *et al.*, 2016). Therefore, juvenile birds, which lack the appropriate antibodies against avian influenza, are more susceptible to infection and are more likely to act as vectors for a longer period of time. This study suggests that swans, as well as possibly other waterfowl avian species, are equipped with a long-lasting immune memory following influenza A infection.

1.4.7 Antigenic drift and shift

Influenza viruses often display genetic variation, which contributes towards their survival. Such variations can result in antigenic shift or drift due to changes in the antigenic structure of the surface glycoproteins (figure 9) (Parham, 2015). Antigenic drift causes mild and restricted epidemics. These are a result of a change of the viral glycoproteins, HA and NA, which completely or partially reduce the binding to HA of neutralizing antibodies. On the other hand, antigenic shifts lead to the emergence of novel viral strains and as such they have a bigger impact, with the potential to cause pandemic disease. These novel strains arise due to genome re-assortment following coinfection of two viruses (figure 4). It is thought that such reassortant viruses are more likely to arise in hosts such as pigs, or even quails, which are often termed 'mixing vessels' (Ma *et al.*, 2009; Thontiravong *et al.*, 2012). These hosts are infected simultaneously by different viral strains and re-assort their genetic combinations, thereby generating new strains, which can be potentially hazardous.

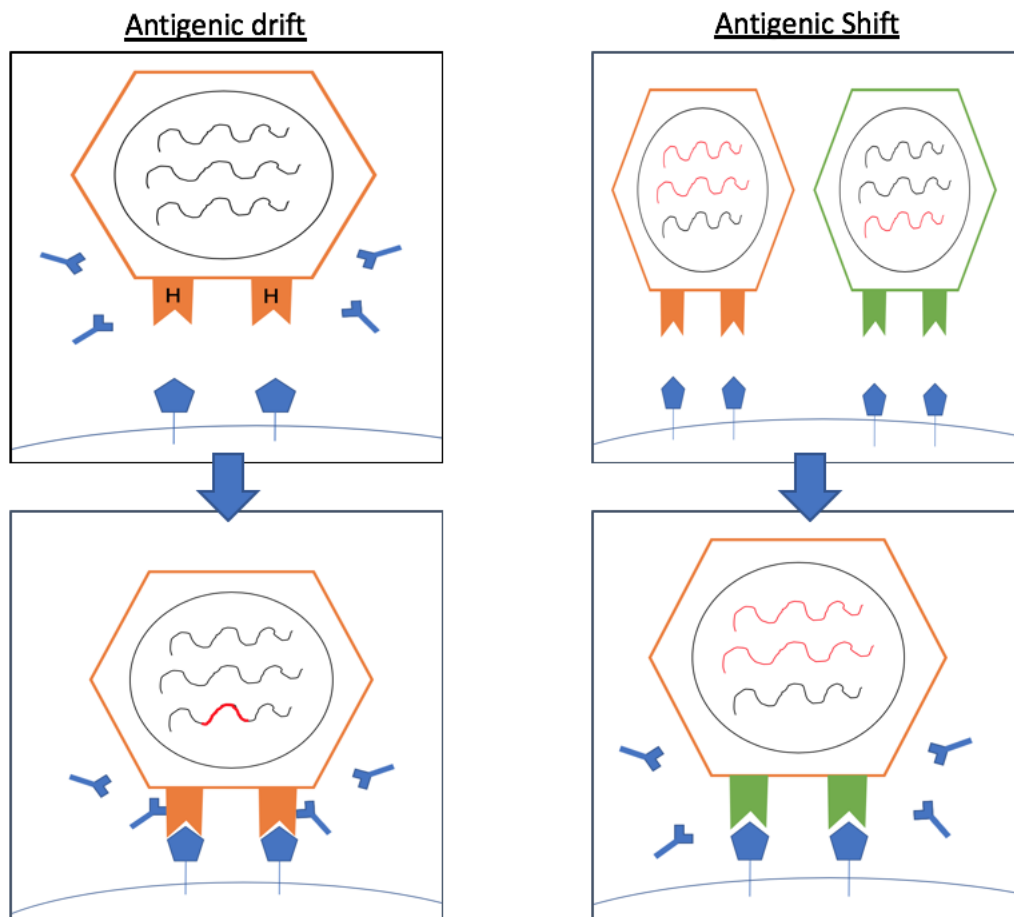


Figure 9: Two types of genetic variation allow repeated viral infections.

Variations of HA can inhibit the neutralizing antibody activity. This can be achieved through either genetic drift or shift (Modified from Murphy and Weaver, 2016). An antigenic drift causes a slight alteration of the genome, which consequently alters the HA or NA of the virus. On the other hand, antigenic shift is caused by the interaction of two, or more, different viruses, which cause the generation of new viruses with a mixed genome. Both of these genetic alterations allow the virus to defeat defence barriers presented by the immune system of the host. Modified from Murphy and Weaver, 2016.

1.5 Drug therapy for influenza infection

1.5.1 Influenza viral inhibitors

The ability of influenza viruses to undergo marked genetic variation can lead to enhanced transmission, transmission across species or escape from vaccine mediated protection. This presents a threat to human health, as there have been numerous avian influenza cases registered in humans. The most recent is an outbreak of H7N9, which occurred throughout China, with imported cases in Malaysia and Canada (Fao.org, 2018). Overall there have been 1625 registered human cases, out of which

623 ended fatally, since February 2013. Although vaccines are the preferred preventive strategy, it is inevitably challenging to produce vaccines with current strains in time to protect against newly emerging strains. Therefore, drug treatment might be required where patients present with severe symptoms, which need immediate treatment. There are currently two types of licenced drugs against influenza viruses: M2 channel inhibitors and neuraminidase inhibitors (NAI) (McKimm-Breschkin, 2013). The M2 channel inhibitors, Amantadine and Rimantadine, are cheaper, however they are less effective as they are able to protect only against influenza A viruses, and viruses form resistance against them rapidly. Influenza B have a BM2 protein, which is similar to the M2 protein found in influenza A, however, it is not sensitive to the M2 inhibitors.

1.5.2 Neuraminidase inhibitors

The NAIs, of which zanamivir and oseltamivir are most frequently used, can be used to treat both influenza A and B infections. Influenza virus NA is able to cleave the sialic acids, which are found on the host cell receptors, in order to release new virions; this process is blocked by NAIs (Reece, 2007). Zanamivir has a guanidine group within position four on the transition state of sialic acid. Zanamivir is delivered to the infected lungs through a powder that can be inhaled. Oseltamivir is a carbocyclic analogue of the sialic acids, which is administered orally. Peramivir is also a carbocyclic analogue, which, as well as another drug called laninamivir, is only approved for use in certain countries (Samson *et al.*, 2013).

1.5.3 Viral resistance to neuraminidase inhibitors

The increasing usage of NAIs raises concern towards the possibility of drug resistance. There are generally three levels of viral resistance that correlate with the way that resistance can be detected or hindered: genotypic, phenotypic and clinical resistance (EMA, 2007). Genotypic resistance is revealed through sequencing the viral genome of interest, which allows for the detection of genetic patterns that are present in resistant strains. Phenotypic resistance is observed through *in vitro* drug tests, whilst clinical resistance is evidenced through observational studies of patients affected by viral infections. NAIs have proven to be very effective against influenza viral infections, however there have been several reports of oseltamivir-resistant strains, such as the seasonal H1N1 that circulated between 2007 and 2009 (Pizzorno *et al.*, 2011). The

emergence of drug-resistant viral strains is of great concern as it poses a threat to the efficacy of certain treatments. Resistance to NAIs could be due to either mutations of the HA, which would cause resistance to both oseltamivir and zanamivir, or of the NA, which would only affect the efficiency of oseltamivir-based treatments (Nitsch-Osuch and Brydak, 2014).

1.5.4 Novel drugs against influenza virus

Recently it has been shown that the second-generation thiazolide haloxanide (HLN) could potentially be used as a drug treatment against avian influenza viruses *in vitro* (La Frazia *et al.*, 2018). The previously tested thiazolide anti-infective nitazoxanide (NTZ) also showed significant results as it inhibited the maturation of viral HA, however, HLN, as well as its precursor (p-haloxanide), appeared to be more effective. HLN acts in a similar way to NTZ, as it blocks the avian HA terminal glycosylation, which precedes the stage of resistance of endoglycosidase-H digestion. By targeting the HA maturation stage, the *in vitro* treatments were able to disrupt the creation, and propagation, of viral particles.

1.6 Molecular interactions between IAV and host cell signalling pathways

1.6.1 PI3K/Akt pathway

Viruses are able to directly disrupt healthy body functions by damaging target cells and organs or by inducing a pathogenic response in the host. The virus can affect the cell directly through different mechanisms, such as shutting down the synthesis of certain proteins (e.g. interferons) and using up the cell's energy. Upon viral infection by influenza A viruses, several antiviral and virus-supportive signalling pathways are induced.

1.6.1.1 Overview of the PI3K/Akt pathway

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is one of the pathways activated by influenza virus. PI3K is composed of an enzymatic subunit, p110, and a regulatory subunit, p85. The PI3K is part of a family of heterodimeric enzymes involved in cellular functions, such as cell growth, proliferation and survival. The activation of PI3K is thought to result not only in the promotion of viral entry, but also inhibition of premature apoptosis (Ehrhardt and Ludwig, 2009). The p85 subunit can act as a stabilizer and inhibitor for p110 subunits. PI3K is activated by the binding of

Src homology (SH) domain, in the p85 subunit, to auto-phosphorylated tyrosine kinase receptors, non-receptor tyrosine kinases or viral proteins such as the non-structural protein 5A, which has been found to interact with the PI3K in hepatitis C patients (Shin *et al.*, 2007; Street *et al.*, 2004). Following activation, the p110 subunit phosphorylates the substrate phosphatidylinositol 4,5-bisphosphate (PIP₂), which produces phosphatidylinositol 3,4,5-trisphosphate (PIP₃) (Figure 10). This molecule works as a second messenger and acts as a regulator for the phosphorylation of several kinases, including Akt. The serine/threonine kinase Akt, also known as protein kinase B (PKB), comprises of three closely related isoforms: PKB α /Akt1, PKB β /Akt2 and PKB γ /Akt3 (Song *et al.*, 2005). Akt1 is able to delay apoptosis, thus promoting cell survival. Akt2 plays a critical role in glucose homeostasis, as marked hyperglycaemia and high loss of β cells were detected in mice with Akt2 deficiency (Garofalo *et al.*, 2003). All kinases have been found to be involved in many types of cancer (Cheung and Testa, 2013). Although the Akt3 is not well known, the overall PKB plays an important role in multiple cellular processes, such as cell migration, proliferation and apoptosis. Akt is activated through the phosphorylation at Thr308 and Ser473. It is able to enhance cell survival by blocking pro-apoptotic proteins and processes (Manning and Cantley, 2007). The signalling cascade can be triggered by the interaction of the receptors found within the cell membrane, such as cytokines (CY) and growth factors (GF). Activation of the PI3K/Akt pathway ensures cellular survival, as well as virus propagation, whilst its inhibition results in apoptosis (Diehl and Schaal, 2013). During infections, apoptosis presents itself as a drastic, but effective method to stop cell infection by a range of pathogens. Thus, in order to replicate, the virus must keep the pathway active and prevent apoptosis.

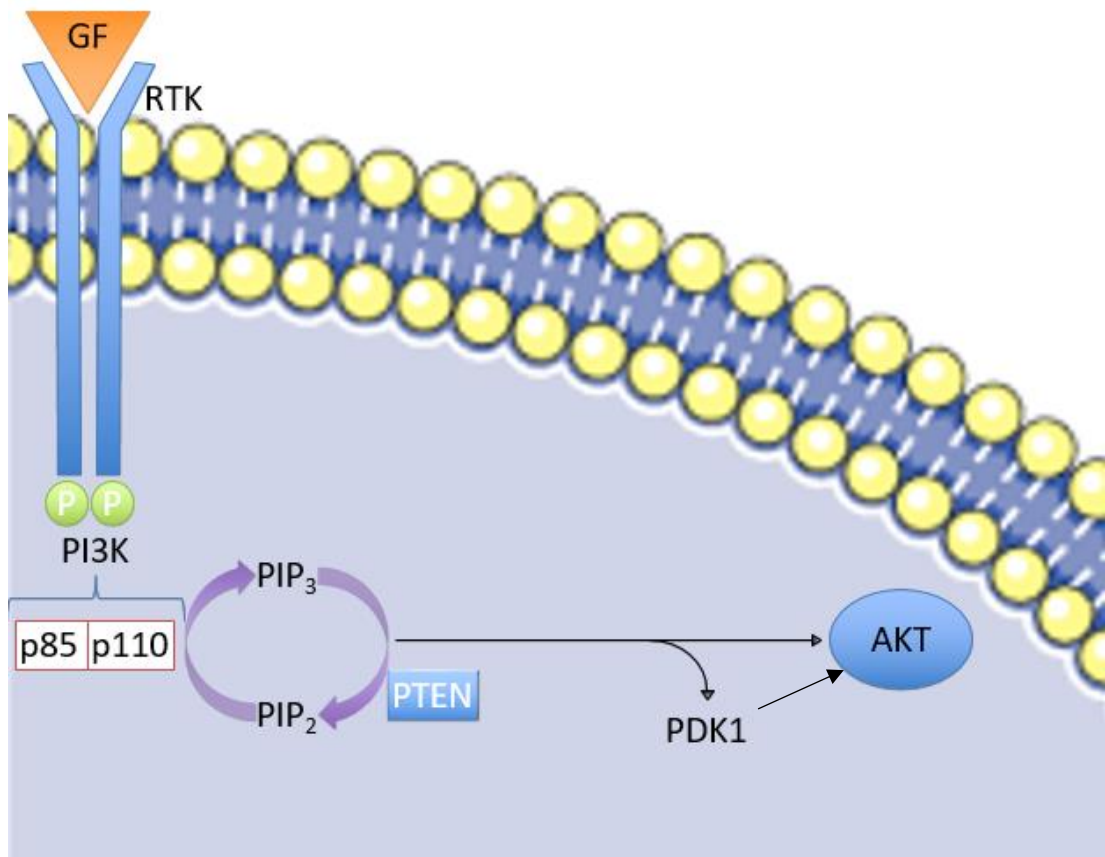


Figure 10: PI3K/Akt Pathway.

Receptor Tyrosine Kinase (RTK), can be activated by growth factors (GF), or cytokines. This results in the activation of Phosphatidylinositol-3 kinase (PI3K), which triggers the phosphorylation of PIP₂ into PIP₃. Protein kinase B (Akt) and phosphoinositide-dependent kinase 1 (PDK1) bind to PIP₃, and PDK1 phosphorylates the activation group of Akt at T308. The signalling cascade can be terminated by phosphatase and tensin homolog (PTEN), which will dephosphorylate PIP₃ (Adapted from Dienstmann et al., 2014).

1.6.1.2 PI3K/Akt/mTOR pathway

Much of our knowledge of these signalling pathways comes from cancer research, as the PI3K/Akt pathway is part of a larger pathway, which also involves the mammalian target of rapamycin (mTOR), making up the PI3K/Akt/mTOR pathway (figure 11). The activation of this pathway results in excessive growth, angiogenesis and resistance to cancer treatments (Porta *et al.*, 2014). The phosphatase and tensin homolog, PTEN, is often found mutated in recorded cancer cases, as well as in patients with Cowden syndrome, a familial cancer condition. The absence of PTEN activity leads to uncontrolled PI3K/Akt pathway activation, which promotes the excessive

proliferation of cells. Current anti-influenza drugs target viral proteins, in particular the M2 ion channels and NA (Murray *et al.*, 2012), although these are not always effective, as viruses can often be resistant to such treatments. Through gene trap studies and siRNA validation screens, the PI3K/Akt/mTOR pathway is involved in the replication of several DNA and RNA viruses, such as influenza A, thus highlighting the potential of the pathway for treatment of viral infections. Although the roles of PI3K/Akt pathway have been identified in influenza A infections, the overall downstream role of Akt and mTOR has not been established so far. At present no drugs that target this pathway have been tested to treat influenza infections. However, inhibitors for all components of the pathway have already been developed for potential cancer treatment (Porta *et al.*, 2014). Although some drugs were discontinued following clinical trials (e.g. Perifosine), others are registered and used in certain countries (e.g. Miltefosine) or going through early clinical or pre-clinical development studies (e.g. MK2206, oleandrin).

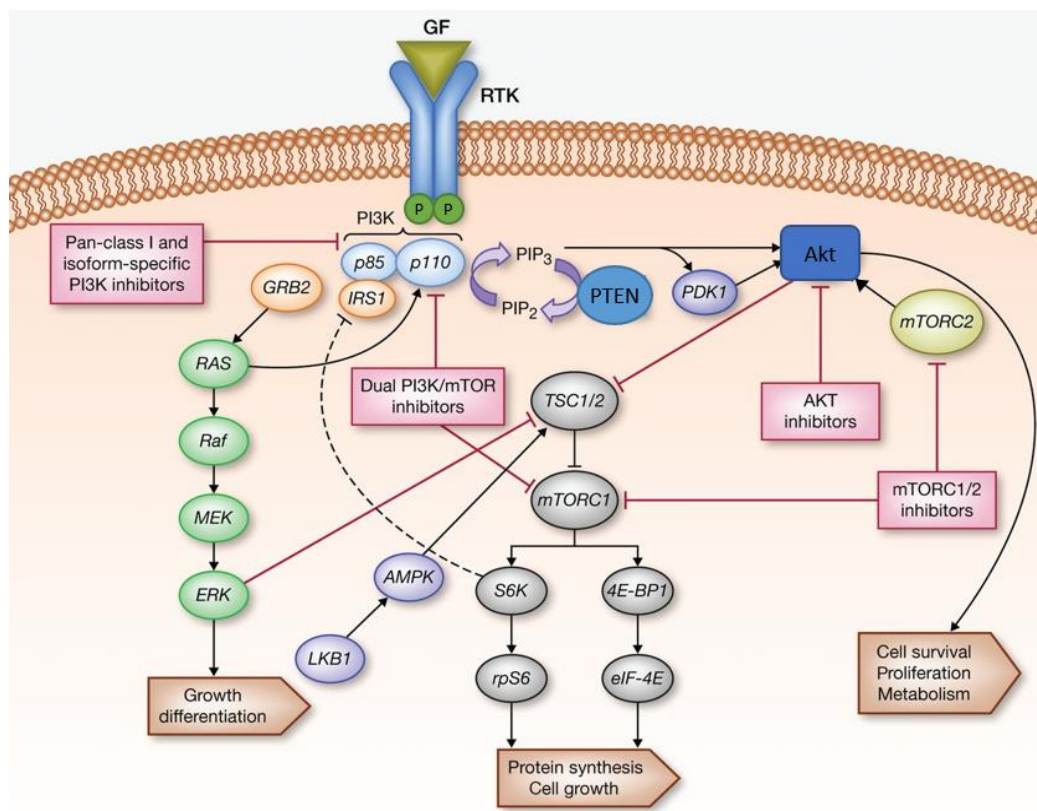


Figure 11: Overview of the PI3K/Akt/mTOR pathway and its targets

Pathway inhibitors are represented in pink boxes, whilst pathway outcomes are in brown block arrows. Modified from Dienstmann *et al.*, 2014.

1.6.2 NS1

1.6.2.1 Interaction of influenza NS1 with host signalling pathways

The non-structural 1 (NS1) protein of influenza A virus is a multifunctional protein, and, during infection, it is capable of modulating various host-cell processes by interacting with cellular factors. Through this, the protein is able to antagonize the host immune response, favouring the survival of the virus. NS1 is made up of an N-terminal RNA binding domain, for interactions with RNA and a C-terminal effector domain, used to mediate interactions with various cellular proteins (Jiang *et al.*, 2016). Some key functions of NS1 include the regulation of viral protein synthesis, through splicing and translating mRNAs, as well as inhibition of the antiviral type I IFN response and suppression of NLRP3 inflammasome-mediated IL-1 β secretion. By directly interacting with Rac1, a multifunctional GTPase cellular protein involved in several vital cellular functions, NS1 is able to facilitate the viral replication process. This is carried out by downregulating Rac1 via post-translational modifications, SUMOylation and ubiquitination. Furthermore, NS1 is able to induce a higher expression of vault RNA, which is non-coding RNA found in cytoplasmic ribonucleoprotein particles (vaults), causing the inhibition of Protein kinase R (PKR)-mediated immunity (Li *et al.*, 2015). PKR is an IFN-induced protein kinase and a key component of antiviral response. The increased production of vault RNAs in infected cells promotes viral replication as they attenuate PKR activity, thus suppressing IFN expression. Several viral strains lacking the NS1 gene, such as lab-generated A/PR8/34, are able to replicate only in hosts with IFN- α/β deficiencies (Wang *et al.*, 2000). IFN- α/β is induced hours post-infection and aids prevention of replication and spread of pathogens. NS1 is able to bind to NF- κ B, a family of transcription factors, and inhibit induction of IFN- α/β . It has been demonstrated that NS1 is able to bind to p85 β , one of the regulatory subunits of PI3K, but not to the related p85 α subunit, in human cells (Hale *et al.*, 2006; Ehrhardt *et al.*, 2007). This binding causes the activation of PI3K, as well as the recruitment and phosphorylation of Akt. This suggests that PI3K confers a virus-supporting activity at certain stages during the infection cycle, as it prevents premature apoptosis and promotes viral proliferation.

Further research is necessary to identify the role of NS1 within infected avian species, thus gaining a better understanding of the pathogenicity of influenza A amongst birds.

1.7 Hypotheses

The PI3K/Akt pathway is activated following infection of embryonic quail cells with avian influenza virus rendering the cell permissive to viral replication. Quail cells will respond to infection in a similar manner to chicken cells.

1.8 Objectives

1. Investigate and establish whether PI3K/Akt pathway signalling is activated within quail cells after infection with Influenza A.
2. Investigate whether inhibition of the PI3K/Akt pathway in quail cells leads to inhibition of virus replication.
3. Analyse p85 β genes to identify any sequence motifs that might predict their ability to interact with NS1 in different avian hosts.

2 Materials and methods

2.1 Ethics

This project was approved by the ethics committee of the School of Veterinary Medicine and Science of the University of Nottingham.

2.2 Cell cultures

Complete cell culture medium was prepared using Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific; Waltham, Massachusetts, United States) by adding 50ml (10%) of One Shot fetal bovine serum (FBS;), 5ml of 100x penicillin-streptomycin (5000units/mL penicillin and 5000µg/mL streptomycin) and 5ml of 200mM L-glutamine (all Thermo Fisher Scientific) to 500ml of DMEM. All complete medium (CM) components were pre-warmed at 37°C, before use all supplies to end.

2.2.1 Preparation of quail embryo fibroblasts from fertilised eggs

Primary quail embryo cells (QEC) were cultured from 8½ day old quail embryos obtained from fertilised Bob White quail eggs, provided by a commercial supplier. The embryo extraction and handling was carried out in a sterilised laminar flow hood. Once the egg had been washed with 70% ethanol, it was placed upright on an egg tray with the air sac upwards. The top of the shell was removed with scissors and discarded. The embryo was then removed with forceps, placed on a Petri dish and decapitated. The limbs and any visible internal organs were removed with the assistance of forceps and scissors. The embryo torso was then moved to a 50ml tube containing 15–20ml of 1x sterile phosphate-buffered saline (PBS; Thermo Fisher Scientific). The embryo was washed twice with 10–15ml of PBS before being transferred to a Petri dish, where it was minced with sterile scalpels. The macerated tissue was pipetted into a 50ml falcon and 10–20ml of 0.25% trypsin (Thermo Fisher Scientific), was added. The solution was incubated for 15–30minutes at 37°C, with intermittent agitation. CM was added to the solution to neutralise the trypsin. Cells were then pelleted by centrifugation at 1200g in a large bench centrifuge (Sorvall Legend RT Plus, Thermo Fisher Scientific), using a BIOShield™ 4 x 250mL Swinging-Bucket Rotor (Thermo Fisher Scientific), for 10 minutes. After removing the supernatant, the pellet was re-suspended in about 20ml of CM. Cell numbers were

determined by applying 20 μ l to a haemocytometer and counting the cells in the centre square, as shown in figure 12. Cell count as cells per ml correspond to number of cells in the central square $\times 10^4 \times$ dilution factor, if applicable. In order to have an accurate cell count, the measure was repeated three times and the average cell count was used. The embryo-derived cells were either plated at a density of $1.5 \times 10^5/\text{cm}^2$ or cryopreserved, for later use.

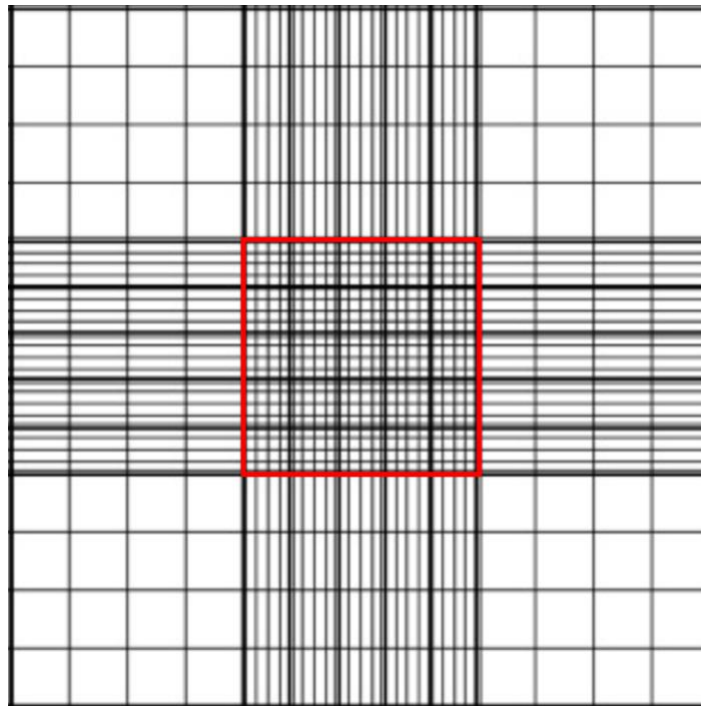


Figure 12: Counting cells with a haemocytometer.

In order to plate cells at an appropriate seeding density, the cells from the central square, highlighted in red, were counted.

2.2.2 Cryopreservation and thawing of cells

Cells were cryopreserved by adding 10% dimethyl sulfoxide (DMSO) to the CM and divided amongst 1.8ml Nunc cryotubes (Nunc, Sigma Aldrich; St. Louis, Missouri, United States). Cells were frozen at a density of 1.5×10^6 cells/ml. The cells were frozen overnight in a Nalgene cryogenic freezing container (Thermo Fisher Scientific) at -80°C , before being transferred to liquid nitrogen storage. To recover cryopreserved cells, vials were removed from liquid nitrogen and thawed rapidly at 37°C . Freshly thawed cells were transferred to a 50ml falcon tube, topped up with fresh media and spun down at 1200g in a centrifuge (Sorvall Legend RT Plus, Thermo

Fisher Scientific), with a BIOShield™ 4 x 250mL Swinging-Bucket Rotor (Thermo Fisher Scientific), for 5 minutes. The supernatant was discarded, the pellet re-suspended in 10 – 15ml of CM, plated in a T75 or T25 flask and incubated at 37°C. Madin-Darby canine kidney (MDCK) cells and human lung cells (A549) were thawed from stocks kept in liquid nitrogen then cultured as described.

2.2.3 Maintenance of cell cultures

Cell cultures were observed daily with an inverted microscope (Leica; Wetzlar, Germany). If there were a few cells floating, the media was changed by removing the current media, and adding fresh, pre-warmed (37°C) media. If cells were 90–100% confluent, they were split 1:2 or 1:3. This was done by removing the media, washing firstly with 10 ml of PBS, then with 3ml of 1x versene (Gibco, Thermo Fisher Scientific), which acts as a gentle non – enzymatic cell dissociation reagent. After this, 3.5ml of 0.25% trypsin (Thermo Fisher Scientific) was added, in order to detach the cells from the bottom of the flask. After trypsin had been added, the cell detachment was monitored using an inverted microscope. Once the cells had detached, CM was added to the flask to neutralise trypsin and the cell suspension centrifuged at 1200g in a centrifuge (Sorvall Legend RT Plus, Thermo Fisher Scientific), equipped with a BIOShield™ 4 x 250mL Swinging-Bucket Rotor (Thermo Fisher Scientific), for 5 minutes. The supernatant was removed and the pellet was re-suspended in 10ml of fresh media. This solution was then divided amongst the new flasks and topped up with fresh media. Typically cells were split three times per week.

2.3 Virus infection

2.3.1 Virus

In this study, the low pathogenic avian influenza virus A/mallard duck/England/7277/06 (H2N3) was used (Kindly supplied by Dr Ian Brown, APHA, Weybridge). Virus was stored in aliquots at -80°C .

2.3.2 Preparing cell plates for infection

Cell were trypsinised as detailed in 2.1.3 and re-suspended with 10ml of CM to inactivate trypsin. The solution was centrifuged at 1200g in a centrifuge (Sorvall Legend RT Plus, Thermo Fisher Scientific), with a BIOShield™ 4 x 250mL Swinging-Bucket Rotor (Thermo Fisher Scientific), for 5 minutes. The supernatant was decanted

and the pellet was re-suspended in 15–20ml of media. Cells were counted with a haemocytometer (Hawksley; Lancing, Sussex, United Kingdom) and diluted to the desired concentration for plating:

- For 96-well plate: 1.25×10^5 cells/well
- For 24-well plate: 1.5×10^5 cells/well
- For 6-well plate: 2.25×10^4 cells/well

2.3.3 Infection of cells with LPAI virus

Cells were infected at a confluence of 70–80%. The virus was rapidly thawed at 37°C and added to the cell cultures at a multiplicity of infection (MOI) of 1.0 in infection media (IM), which was made in aliquots of 50ml of DMEM with 50µl of 2µg/mL tosyl phenylalanyl chloromethyl ketone (TPCK) trypsin (Thermo Fisher Scientific) and 500µl of 100x penicillin-streptomycin (5000units/mL penicillin and 5000µg/mL streptomycin). An MOI of 1 was used to infect cells, as it was proven to be effective in a previous study (Kumar, 2018). Once the virus was added, the cells were incubated for 2 hours at 37°C, before washing and changing their media. Following this, plates of infected cell cultures were incubated for 6, 8 or 24 hours. Infections were performed in triplicate.

2.4 Measuring virus output in MDCK cells

2.4.1 Inhibition of PI3K/Akt pathway

The solutions of LY942002 (Sigma Aldrich) and Wortmannin (Sigma Aldrich) were dissolved in the required amount of DMSO (Sigma Aldrich) in order to reach 1mg/ml, as per manufacturer's instructions. Based on previous optimisation, obtained from treated chicken cells (Kumar, 2018), a 20µM concentration of LY942002 and 10nM concentration of Wortmannin were prepared by diluting in infection media (IM). These were used separately to treat QECs, which had been plated in a 6 well plate, as detailed in 2.2.2. The plate arrangement is shown in figure 13. After the infected cells were exposed to the treatment for one hour, the supernatant was removed, and the wells were washed twice with PBS, before incubating at 37°C for 8 or 24 hours. After removing the supernatant, the cells were washed twice with PBS and lysed with RIPA buffer. The lysates were used to carry out a Western blot to verify whether the

concentrations had worked effectively to inhibit the PI3K/Akt pathway during infection.

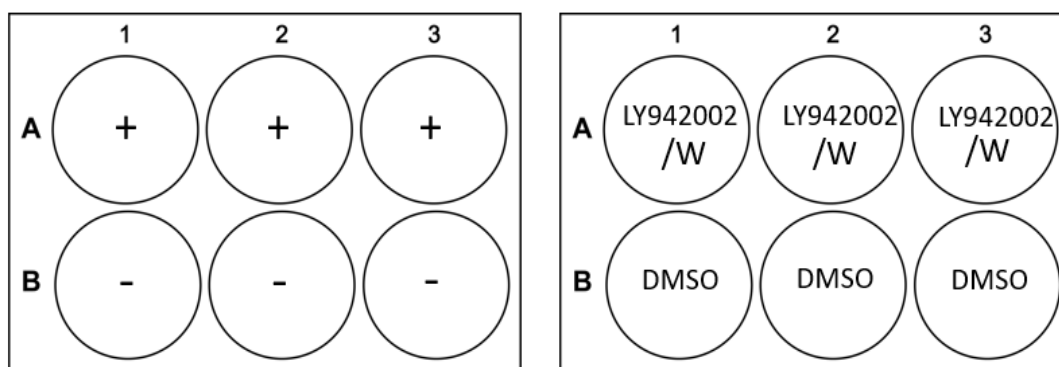


Figure 13: Plate arrangement for treatment protocol

2.4.2 Preparing viral supernatant

The supernatant used to measure virus output was harvested from infected quail cells that had been treated with Wortmannin and LY294002. Supernatants from controls harvested from uninfected cells and infected cells that were not subjected to treatment were also collected. These were stored at -80°C before titration of infectious virus on MDCK cells.

2.4.3 Virus titration on MDCK cells

MDCK cells were plated into a 96-well plate. Cells were plated with a density of 1.25×10^5 /ml. The following day, the media was removed by inverting the plate over a plastic container. Following media removal, 100µl of infection medium was added to all wells, excluding those in the first column, where 146µl of supernatant was added. No infection media was added to the last row in order for it to act as a negative control. A multi-channel pipette was used to sequentially transfer 46µl from all rows in column one to wells in column two in order to create $\frac{1}{2}$ log₁₀ dilutions. This was repeated across all columns in the plate. The aliquot was pipetted gently up and down, without disturbing the monolayer of cells, prior to each dilution step. The plate was incubated, at 37°C for 2 hours, before removing the supernatant and washing the wells twice with PBS and fresh IM was added. After another 4 hours incubation at 37°C, the supernatant was once again removed, and the wells were washed three times with PBS. Culture plates were then incubated at 37°C for 5 days.

2.4.4 Fixation of infected MDCK cells

The MDCK cells were fixed with a 1:1 ratio of pre-chilled acetone:methanol for 10 minutes at RT. Once the solution had been removed, the cells were washed with PBS and fresh PBS was added to the plate which was stored at 4°C prior to immunostaining.

2.4.5 Immunostaining MDCK cells for influenza A nucleoprotein

In order to assess MDCK cells for expression of viral nucleoprotein, a Dako Envision plus kit with anti-mouse IgG (Agilent Technologies; Santa Clara, California, United States) was used. Cells were incubated for 5 minutes with TBS at RT, after which the fluid was removed and enough peroxidase block to cover the cells was added then incubated for 10 minutes. The wells were then rinsed gently with TBS. The excess buffer was removed and the diluted primary monoclonal antibody mouse anti-influenza A nucleoprotein IgG, added (HB65) (ATCC; Manassas, Virginia, United States), diluted 1:30 with TBS. The last two rows worked as negative controls, as one row had untreated MDCK cells, whilst the last row was left unplated to check for contamination from the external environment. Fifty microliters of the solution were added to each well and incubated at RT for 40 minutes. The wells were rinsed with TBS from a wash bottle, without directing the flow onto the cells. Excess buffer was tapped off onto tissue paper and enough labelled polymer was added to cover the wells. The plate was incubated for a further 40 minutes and then washed again with TBS. In order to make up substrate-chromagen, 1 drop of DAB chromagen was added to 1ml of substrate buffer. The freshly made up solution was then applied to cover the cell layer and incubated for 10 minutes. The plate was given a final wash with distilled water, or TBS, before photographing with the inverted microscope to count the stained infected cells. A TCID₅₀ calculator (Binder, 2017; Hierholzer and Killington, 1996) was used to estimate virus titre.

2.5 Detection of protein expression in quail embryo fibroblasts

2.5.1 Preparation of cell lysate

Cells were washed twice with PBS and lysed by adding 115µl of complete RIPA buffer. This consisted of 10ml of RIPA buffer (Sigma Aldrich) with a tablet of cOmplete ULTRA, a mini, EASYpack protease inhibitor cocktail (Roche; Basel, Switzerland) and one of

PhosSTOP EASYpack phosphatase inhibitor cocktail (Roche). Cells were detached with a cell scraper, collected with a pipette and placed in 1.5ml Eppendorf tubes. The lysates were gently agitated and placed on ice for 30 minutes, allowing complete cellular disruption. The lysates were then centrifuged at 14000 $\times g$ for 15 minutes, at 4°C. The supernatant was removed to a clean microcentrifuge tube, avoiding the pellet, and stored in aliquots at -80°C until further use.

2.5.2 Protein quantification

Five dilutions of a protein standard stock were made up using bovine serum albumin (BSA, Sigma Aldrich). The protein standard concentrations were 2, 1.0, 0.5, 0.25 and 0.125 mg/ml. Biorad dye reagent (BioRad; Hercules, California, United States) was diluted at a 1:5 ratio in ultrapure water before use. Protein quantification was carried out in a Costar 96-well plate (Sigma Aldrich). Five microliters of protein lysate or protein standard were added to each well in triplicate. Once the samples were plated, 150 μ l of diluted protein assay reagent were added to each sample and the plate was incubated for 5–10 minutes at room temperature (RT). Spectrophotometric absorbance was measured at 590–596nm (Fluostar Optima, BMG Labtech; Ortenberg, Germany) and protein concentration determined using a standard curve created from the BSA standards.

2.5.3 Polyacrylamide gel electrophoresis

Samples were prepared by mixing a total of 10 μ g of protein lysate with 1.5 μ l NuPAGE sample reducing agent (10x, Thermo Fisher Scientific) and 3.5 μ l of NuPAGE LDS sample loading buffer (4x, Thermo Fisher Scientific). The total volume was brought up to 15 μ l by adding nuclease-free water. The samples were heated in a PCR machine at 70°C for 10 minutes. A precast NuPAGE 4-12% Bis-Tris gel (Invitrogen, Thermo Fisher Scientific; Carlsbad, California, United States) of 1mm thickness and with 12 wells was used to load the samples. The gel was assembled and clamped down in the gel tank slot. In order to make a 1x concentration of running buffer, 50ml of the 20x NuPage 3-N-morpholino propane sulfonic acid (MOPS) sodium dodecyl sulphate (SDS) gel running buffer was diluted in 1L ultrapure water. Two hundred millilitres of 1x running buffer was aliquoted and poured into the inner core of the assembled tank, and 500 μ l of Bolt antioxidant (Invitrogen) was pipetted and mixed with it. The area

surrounding the gel was surrounded by approximately 600ml of 1x running buffer. Each sample was loaded into separate lanes and 10µl of a pre-stained protein marker ranging between 3 and 198kDA (Invitrogen) was loaded in a separate lane. Gel electrophoresis was performed at 200V for 45 minutes.

2.5.4 Protein transfer to PVDF membrane

A 1x NuPage transfer buffer was prepared by diluting 50ml of 20x NuPage transfer buffer stock (Thermo Fisher Scientific) with 100ml of methanol and 850ml of ultrapure water. The polyvinylidene fluoride (PVDF) membrane (Amersham; Little Chalfont, United Kingdom) and filter papers (Whatman; Maidstone, United Kingdom) were prepared by cutting into appropriate sizes for the gel. The PVDF membrane was charged by dipping in methanol for 30 seconds, then washed in ultrapure water for 5 minutes and left to soak in transfer buffer until needed. The filter papers and blotting pads were pre-soaked in transfer buffer. Once the gel had finished running, the gel casting was disassembled and the gel was moved to sandwich between PVDF membrane, filter papers and blotting pads. The arrangement is illustrated in figure 14. The sandwich was clamped down tightly in the transfer apparatus and transfer buffer was poured around it. The transfer was run at 30V for 1 hour.

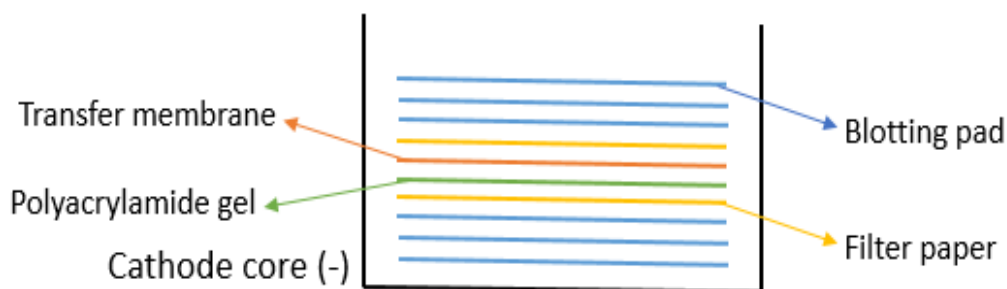


Figure 14: Transfer of agarose gel to PVDF membrane

Once the gel had run, the gel was disassembled from its cast and sandwiched as shown, in the order of: blotting pad/ blotting pad/ blotting pad/ filter paper/ polyacrylamide gel/ transfer membrane/ filter paper/ blotting pad/ blotting pad/ blotting pad. This was done from the cathode core side of the sandwich cast.

2.5.5 Membrane blocking

Tris-buffered saline-Tween (0.1%) (TBST) was made up by adding 1ml of Tween-20 (Merck; Watford, United Kingdom) to 1L of 1:10 tris-buffered saline (TBS). The 5%

BSA blocking solution was prepared by adding 2.5g of bovine serum albumin (BSA; Fisher BioReagents, Thermo Fisher Scientific) to 50ml of TBST. Once the proteins had been transferred to the PVDF membrane, this was removed from the cast and submerged in 10–15ml of 5% BSA blocking solution and incubated on a shaker at RT for an hour. Once the membrane had been blocked, it was cut into strips in order to stain different sections of interest with different antibodies.

2.5.6 Antibody staining

After blocking, the membrane was washed twice with 25ml TBST, shaking gently at RT for 5 minutes. The concentrations used for each primary and secondary antibody are shown in table 3. Primary antibodies, diluted in 5% BSA, were incubated overnight on a shaker at 4°C. Before incubating the membrane with secondary antibodies, the membrane was washed 3–5 times with TBST, each wash lasting 5 minutes. The incubation with secondary antibodies was carried out at RT for one hour. After the incubation with the secondary antibody, the membrane was washed once more with TBST.

Table 3: Details of antibodies.

Antigen – host species	Dilution	Manufacturer
<u>Primary</u>		
PB1 (GTX125923) - rabbit	1:20000	Santa Cruz Biotechnology
Akt (9272s) - rabbit	1:1000	Cell Signalling Technology
pAkt (ser473) - rabbit	1:1000	Cell Signalling Technology
β-actin (4970) - rabbit	1:10000	Cell Signalling Technology
NS1 (sc-130568) - mouse	1:500	Santa Cruz Biotechnology
p85β (MCA1170G) - bovine	1:1000	BioRad
<u>Secondary</u>		
goat IgG (sc2768) - rabbit	1:10000	Santa Cruz Biotechnology
mouse IgG (7076s) - horse	1:10000	Cell Signalling Technology

2.5.7 Chemiluminescent development

Amersham ECL prime Western blotting detection reagent (GE Healthcare; Chicago, Illinois, United States) was prepared according to the manufacturer's instructions. The solution was made up by mixing 300µl of solution A and 300µl of solution B. The membrane was probed by adding the freshly made-up reagent and incubated for up to a minute. The membrane was drained of any excess reagents using forceps and wrapped in Sarogold cling film (Thermo Fisher Scientific), avoiding folds. Photographs of the membrane were taken with the ChemiDoc imaging system (BioRad) and chemiluminescence was observed.

2.6 In silico comparison of p85β sequences of selected mammalian and avian species

2.6.1 Phylogenetic tree of p85β

Before constructing the phylogenetic tree, amino acid sequences were retrieved from the NCBI website. Most sequences were unpublished and were thus predicted by an automated computational analysis tool, Gnomon. The evolutionary history of the PI3K p85β subunit of selected species was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). Amino acid sequences were retrieved from the NCBI database from the selected species of interest. The accession numbers for these sequences are shown, in table 4. The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) were shown next to the branches (Felsenstein, 1985). Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2018).

Table 4: NCBI accession numbers of selected species' p85 β amino acid sequences.

Species	Species common name	Accession number
<i>Equus caballus</i>	Horse	XP_023481250.1
<i>Homo sapiens</i>	Human	NP_005018.1
<i>Canis lupus familiaris</i>	Dog	XP_852406.2
<i>Bos Taurus</i>	Cattle	NP_777001.1
<i>Sus scrofa</i>	Pig	XP_020939165.1
<i>Taeniopygia guttata</i>	Zebra finch	XP_004174640.1
<i>Cuculus canorus</i>	Common cuckoo	XP_009561114.1
<i>Chaetura pelagica</i>	Chimney swift	XP_009996094.1
<i>Melopsittacus undulates</i>	Budgerigar	XP_005140568.2
<i>Anas platyrhynchos</i>	Mallard duck	XP_021133555.1
<i>Columba livia</i>	Rock dove	PKK19499.1
<i>Lonchura striata domestica</i>	Society finch	OWK51426.1
<i>Corvus cornix cornix</i>	Hooded crow	XP_010409819.1
<i>Corvus brachyrhynchos</i>	American crow	XP_008639226.1
<i>Coturnix japonica</i>	Japanese quail	XP_015741981
<i>Meleagris gallopavo</i>	Turkey	XP_010723359.1
<i>Gallus gallus</i>	Chicken	XP_001233341.3
<i>Haliaeetus leucocephalus</i>	Bald eagle	XP_010560473.1
<i>Falco peregrinus</i>	Peregrine falcon	XP_005244191.1

2.6.2 Comparison of susceptibility of avian species when exposed to avian influenza

Table 5 illustrates the susceptibility of the selected avian species to avian influenza, which was determined from various sources, referenced in the table.

Table 5: Comparison of susceptibility of selected avian species to avian influenza strains

Avian species	Susceptibility to avian influenza	Reference
Zebra finch	High susceptibility (H5N1)	(Perkins and Swayne, 2003)
Society finch	Low susceptibility (H7N9)	(Jones <i>et al.</i> , 2015)
Hooded crow	Unknown	(Ferrazzi <i>et al.</i> , 2007)
American crow	Unknown	N/A
Bald eagle	Unknown	(Goyal <i>et al.</i> , 2010)
Peregrine falcon	High susceptibility (H7N3)	(Manvell <i>et al.</i> , 2000)
Domesticated turkey	High susceptibility	(Tumpey <i>et al.</i> , 2004; Alexander, 2007)
Japanese quail	High susceptibility	(Makarova <i>et al.</i> , 2003)
Chicken	High susceptibility	(Tumpey <i>et al.</i> , 2004; Alexander, 2007)
Chimney swift	Unknown	N/A
Common cuckoo	Unknown	N/A
Rock dove	Low susceptibility	(Kaleta and Hönicke, 2004)
Mallard duck	Low susceptibility	(Jourdain <i>et al.</i> , 2010)
Budgerigar	Unknown	(Matsuoka <i>et al.</i> , 1979)

2.7 Analysis of Influenza A NS1 and p85 β interaction in quail embryo fibroblasts

2.7.1 Growth of bacterial cultures with NS1 influenza A protein

A recombinant plasmid coding for the NS1 protein of influenza A/Udorn/72 (H3N2) (kindly provided by Holly Shelton, The Pirbright Institute) was used to transform bacterial DH5 α cells and plated on selective agar plates. This was carried out by thawing DH5 α cells on ice, to which 1 μ l of plasmid encoding NS1 was added. These were mixed by gently pipetting up and down. Cells were further held on ice for 20 minutes, after which they were heat shocked in a 42°C water bath for 30-45 seconds. The cells were returned to ice for 2 minutes. Five hundred microliters of Luria Bertrani (LB) medium was added to each tube and mixed by inversion. Tubes were incubated at 37°C for 1 hour after which they were spun at 16000g, on a bench top centrifuge, for 2 minutes. Media was removed, and pellet was re-suspended in 100 μ l of fresh LB

medium. One hundred microliters of each transformation solution were added to an agar plate, with 100µg/ml ampicillin (Sigma-Aldrich) resistance and spread either in a flow hood or on a bench near a Bunsen burner. Plates were left to set for 5 minutes, after which they were incubated at 37°C overnight.

2.7.2 Miniprep with NS1 plasmid

Single colonies were selected and grown overnight in 3-5ml of LB medium with a 1:500 of 100µg/ml ampicillin (Sigma-Aldrich) on a shaker at 37°C with vigorous shaking. The solution was used to prepare plasmid minipreps (QIAGEN), according to the manufacturer's instructions. This was carried out by spinning down the bacterial overnight culture at 6800xg for 3 minutes at RT (15-25°C). The supernatant was removed and the pellet was re-suspended in 250µl of buffer P1 and moved to a clean micro-centrifuge tube. Two-hundred and fifty microliters of buffer P2 was added and the tube was inverted 4–6 times. The solution will turn blue if LyseBlue reagent is used, otherwise it will stay clear. Following this, 350µl of buffer P3 were added and the tube was inverted 4–6 times. The solution will turn from blue to clear if using LyseBlue reagent. The tube was centrifuged for 10 minutes at about 17900g on a table-top micro-centrifuge. Eight-hundred microliters of the supernatant were moved to a QIAprep 2.0 spin column and the tube was briefly centrifuged for 30-60 seconds and the flow-through was discarded. The QIAprep column was washed twice with 500µl of PB buffer by centrifuging for 30-60 seconds per wash. Flow-through was discarded and the column was washed with 750µl of PE buffer by centrifuging for 30-60 seconds, after which the flow-through was discarded. The tube was centrifuged again to remove any remaining wash buffer. The QIAprep column was moved to a clean 1.5ml micro-centrifuge tube and 50µl of EB buffer were added to the centre of the column to elute the plasmid. The tube was left to stand for 1 minute and then washed for a further 1 minute. The nucleic acid concentration of the mini prep samples was estimated using a Nanodrop (Thermo Fisher Scientific). Samples that presented with suitable concentrations were Sanger sequenced through Source Bioscience (Nottingham, United Kingdom) sequencing services.

2.7.2 Transfection of human lung A549 cells with Udorn NS1 plasmid

Of the samples which contained the complete NS1 sequence, a maxi prep was carried out, according to manufacturer's instructions (QIAGEN; Hilden, Germany). Furthermore, a transfection was carried out on Human lung A549 carcinoma cells with NS1, using jetPRIME reagent (PolyPlus; Berkeley, California, United States) in a six well plate. The day before transfection, A549 cells were seeded at a 1×10^5 density into each well of a six well plate and topped up to 2ml with CM. Transfection was carried out on a six well plate, as displayed in figure 15. Two hours before transfection, the CM was replaced with serum-free medium. One microgram of plasmid was diluted into 200 μ l of JetPrime buffer and mixed with a vortex. Two microliters of JetPRIME was added to the solution, vortexed for 10 seconds and incubated at RT for 10 minutes. A plasmid encoding green fluorescent protein (GFP), pT7 GFP, was used to monitor cell transfection. The GFP control was made up by first mixing 200 μ l of JetPrime buffer and 2.38 μ l of pT7 GFP, then adding 2 μ l of JetPRIME. The transfection mix was added dropwise onto the cells and the plate was gently rocked back and forth to spread the solution. The plate was incubated at 37°C for 2 hours then gently rocked before a further 2 hours of incubation. Following incubation, the supernatant was replaced with CM. Cells were harvested at 48 hours and observed with an inverted microscope and a green fluorescent light filter.

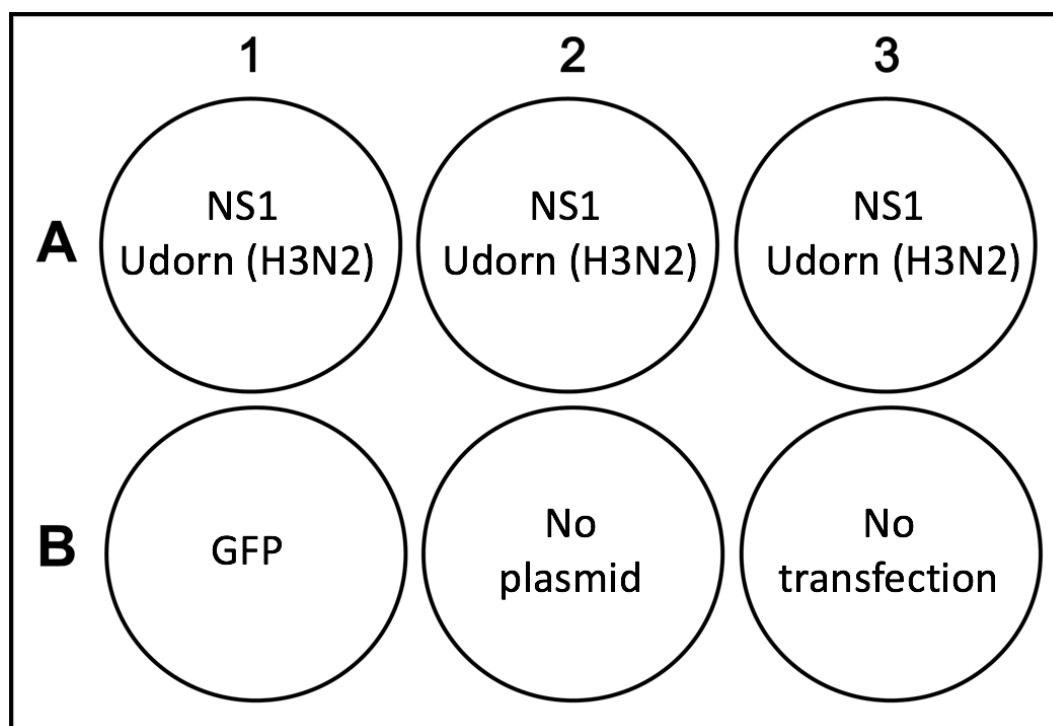


Figure 15: Transfection plate arrangement for NS1 Udorn H2N3

Display of how treatments were administered on a six well plate. Three wells contained A549 cells transfected with the NS1 plasmid from Udorn H2N3 strain. One well was used for green fluorescent protein (GFP) transfection, to monitor cell transfection progression. Negative controls included jetPRIME reagent alone and untreated cells.

2.7.3 H2N3 NS1 plasmid

Plasmids containing H2N3 NS1 from the influenza A/Udorn/72 strain were modified using previously created chicken cDNA, as a template with H2N3 NS1 specific primers. PCR was performed on a Bioer LifeECO Thermal Cycler (Alpha laboratories; Eastleigh, United Kingdom), running 40 cycles following an initial denaturation of 1 minute at 95°C. This consisted of: denaturing for 1 minute at 94°C, annealing for 15 seconds at 52°C and extending for 4 minutes at 72°C, with a final extension of 10 minutes at 72°C. The PCR samples were then run on a 1.5% agarose gel and those amplicons purified. The NS1 containing segment 8 of H2N3 was amplified and cloned into pCR2.1, using TOPO-TA cloning (Invitrogen), with site specific primers for the extreme termini of segment 8, external to the NS1 sequence. The oligonucleotide primers H2N3/NSF (5'-AGCAAAACCAGGGTGACA-3') and H2N3/NSR (5'-AGTAGAAACAAGGGTGT TTT-3) were used to amplify the sequence shown in figure 16.

1	10	20	30	40	50

AGCAAAAGCAGGGTGACAAAAACATA**ATG**GATTCCAACACTGTGTCAAGC
 TTTCAGGTAGACTGCTTTCTTTGGCATGTCCGCAAACGATTTGCAGACCA
 AGAACTGGGTGATGCCCCATTCCTTGACCGGCTTCGCCGAGATCAAAAAT
 CTCTAAGAGGAAGAGGCAGCACTCTTGGTCTGGATATCGAAACAGCCACT
 CGCTCTGGAAAGCAGATAGTGGAGAGGATTCTGGAGGAAGAATCCGACGA
 GGCACCTCAAAATGACTATTGCCTCTGTACCTGCTTCACGCTACCTAACTG
 ACATGACTCTTGAAGAGATGTCAAGAGACTGGTTCATGCTCATGCCCAAA
 CAAAAAGTGGCAGGTTCCCTCTGTATCAGAATGGACCAGGCGATCATGGA
 TAAGAATATTATACTGAAAGCGAATTTTCAGTGTGATCTTCGATCGGCTGG
 AGACACTAATACTACTCAGAGCTTTCCTGAAGAAGGAGCAATTGTCCGC
 GAAATTTACCATTTGCCTTCTCTTCCAGGACATACTGATGAGGATGTCAA
 AAATGCAATTGGGGTCCTCATCGGAGGACTTGAATGGAATGATAACACAG
 TTCGAGTCTCTGAAACTCTACAGAGATTCGCTTGGAGAAGCTGTAATGAG
 GATGGGAGACCTCCACTCCCTCCAAAGCAGAAACGGAAAATGGCGAGAAC
 AATTGAGTCAGAAGTT**TGA**GGAAATAAGGTGGCTAATTGAAGAGGTGCGA
 CATAGACTAAAGGTTACAGAGAATAGTTTTGAACAAATAACATTTATGCA
 AGCCTTACAACCTACTGCTTGAAGTGGAGCAAGAGATAAGAACTTTCTCGT
 TTCAGCTTATTTAATGATAAAAAACACCCTTGTTTCTACT

Figure 16: Influenza A virus (A/mallard/ALB/226/1998(H2N3)) segment 8, complete sequence

(NCBI GenBank: CY003980.1) The start and stop codons of the sense strand are highlighted in red. The complete NS1 sequence (cyan) annealing sites of PCR cloning primers H2N3/NSF (5'-AGCAAAAGCAGGGTGACA-3') and H2N3/NSR (5'-AGTAGAAACAAGGGTGTTTT-3') for TOPO-TA cloning in pCR2.1 are underlined.

The TOPO cloning reaction was carried out by gently mixing 2µl of PCR product with 1µl of salt solution, 2µl of ultrapure water and 1µl of TOPO vector (TOPO TA cloning kit, Invitrogen). The solution was incubated for 5 minutes at RT, after which it was moved to ice to carry out a One Shot transformation. Two microliters of the TOPO cloning reaction were added to a vial of One Shot Chemically Competent *E. coli* (Invitrogen) and mixed gently, without pipetting up and down. The solution was incubated for 30 minutes on ice and then heat-shocked for 30 seconds at 42°C in a water bath. The vial was then transferred immediately to ice and 250µl of RT S.O.C.

medium was added to the solution. The solution was placed horizontally on a shaker at 37°C for 1 hour. Two prewarmed agar Petri dishes, with 100µg/ml ampicillin (Sigma-Aldrich) concentration, were plated with of 20µl or 100µl of transformation solution. The plates were incubated at 37°C for 16 hours. Six colonies were picked to grow in LB medium, containing 50µg/ml of 100µg/ml ampicillin (Sigma-Aldrich), overnight. Minipreps were performed with a QIAGEN miniprep kit (QIAGEN) and correct constructs were determined by restriction enzyme digest with EcoRI. Following digestion they were run on a 1.5% agarose gel.

3 Results

3.1 LPAI H2N3 infection induces activation of PI3K/Akt pathway in primary quail embryo cells

Activation of the PI3K/Akt pathway was determined in QECs using Western blot analysis of LPAI H2N3-infected or uninfected cell lysates. As shown in figure 17, phosphorylated Akt (pAkt) was not expressed in uninfected QECs at 6, 8 and 24 hours post-infection (p.i.). However, its expression was observed in infected cells at 6, 8 and 24 hours p.i. through chemiluminescence imaging. The presence of native Akt was detected in both infected and uninfected lysates, at all time points. Both PB1 and NS1 were expressed in infected samples at 6, 8 and 24 hour p.i., and showed no significant variation. β -actin was used as a loading control for all samples.

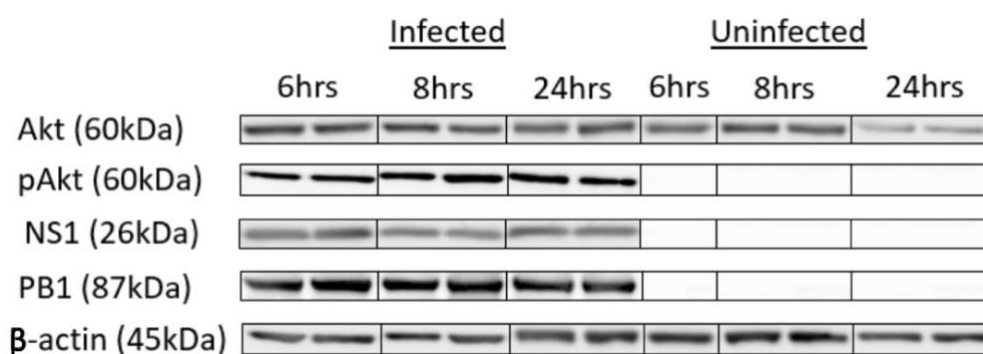


Figure 17: Western blot of primary quail embryo cells

Image shows results of Western blot run with lysates retrieved at 6, 8 and 24 hours from LPAI H2N3 infected or mock infected cells. PB1 and NS1 were used for as controls for virus infection, whilst β -actin was used as a loading control.

3.2 QEC treatment with LY492002 and Wortmannin

QEC cells were treated with LY492002 and Wortmannin separately to assess whether phosphorylation of Akt could be inhibited. Following infection and treatment, QEC cells were lysed at 8 and 24 hours p.i.. Control samples consisted of QEC lysate collected at 8 hour after infection with influenza virus and infected cells treated with DMSO alone (the diluent for Wortmannin and LY492001). However, the Western blot showed that by comparison with the controls, the inhibition by Wortmannin was inefficient, as phosphorylated Akt was detected in all infected samples, including those that had been subjected to Wortmannin treatment (figure 18). No variation

was detected in the expression of PB1 or NS1 in the infected or infected and Wortmannin treated samples. The uninfected samples show a higher expression of native Akt at 8 and 24 hours.

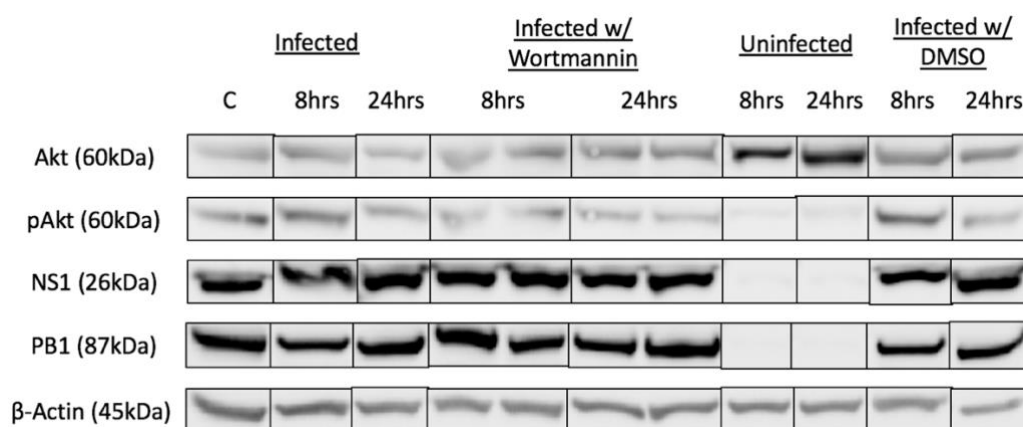


Figure 18: QEC with Wortmannin and controls

Lysates of cells were retrieved at 8 and 24 hours p.i.. PB1 and NS1 were used to confirm virus replication in infected cells, whilst β-actin was used as a loading control. As a control (C), an 8 hour infected QEC lysate sample was run alongside other samples.

The results obtained from LY942002-treated, influenza infected QEC cells (figure 19) also showed expression of phosphorylated Akt at 8 and 24 hours p.i., but very weakly. One of the treated 8 hour samples failed to run properly, as the band is not as defined as the others. Both PB1 and NS1 appear to have a uniform expression across all infected samples, at 8 and 24 hour p.i.. Samples of infected cells treated with DMSO showed similar levels of phosphorylated Akt to untreated infected cells.

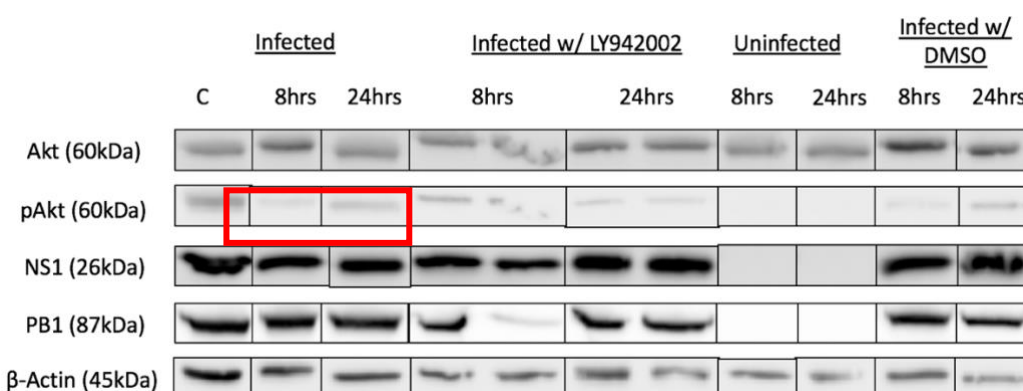


Figure 19: QEC treated with LY942002 and controls

Cell lysates were retrieved at 8 and 24 hours p.i.. PB1 and NS1 were used as controls for virus infection and β-actin was used as a loading control. A previously harvested 8 hour infected QEC sample was used a control (C). Presence of weaker pAkt is highlighted by a red box.

3.2.1 MDCK virus titration with LY942002

In order to assess the effects of LY492002 inhibitor treatment on LPAI H2N3 replication, in MDCK cells, infected cells were counted. This was done across all rows of the 96 well plate. Infected cells were identified by the presence of red-brown chromagen (figure 20). Results showed that there was no significant difference when assessing MDCK cells treated with LY492002 or without treatment.

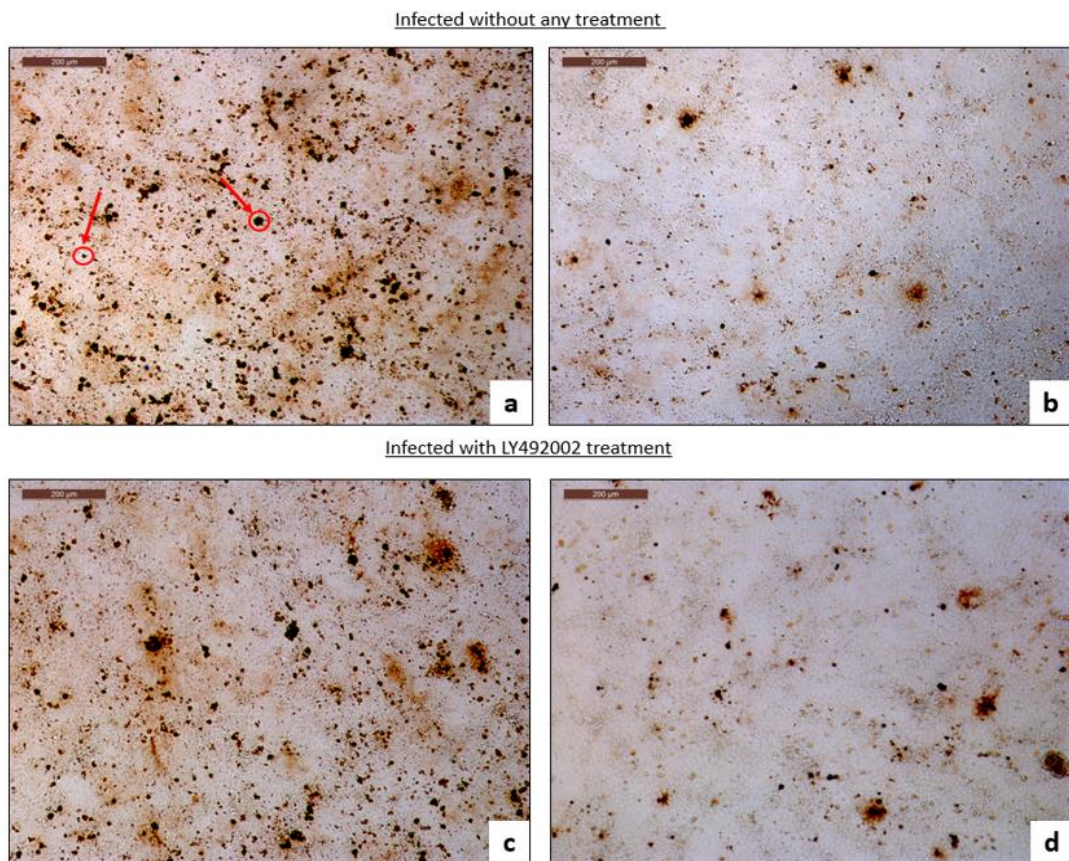


Figure 20: MDCK virus titration with and without LY942002 treatment

Expression of influenza A nucleoprotein in MDCK cells following addition of supernatant from LPAI H2N3 infected quail embryonic cells as detected by immunohistochemistry. NP positive cells are coloured brown. a) and b) untreated; c) and d) LY492002 treated. a) and c) neat supernatant. b) and d) 0.5 log₁₀ dilution of supernatant.

3.3 Phylogenetic tree of p85 β

By carrying out the molecular phylogenetic analysis with 500 bootstrap repetitions, a final phylogenetic tree of p85 β was produced. Figure 21 shows the bootstrap consensus tree of several species, based on the similarity of their amino acid sequence for p85 β , one of the catalytic subunits of PI3K. The relationship between the p85 β sequences from different species could potentially be closely related to the

response of the host to influenza A infections. Results show that avian species clustered separately from mammals. In particular, chicken clustered together with quail and turkey, whilst the duck was separate. A further comparison was made amongst the selected avian species in order to determinate the birds which were most susceptible to avian influenza (figure 22). Here, the different responses to avian influenza infections, both LPAI and HPAI, are highlighted with different coloured circles. The more susceptible species are defined as those that have recorded cases of infections with severe symptoms, often with fatal outcomes; whilst less susceptible species are defined as registered cases of infection with mild symptoms or asymptomatic. Finally, the neutral species are defined as no registered cases of influenza infections. The results overall show that land-based poultry is more susceptible to LPAI, as well as HPAI. In contrast, it is well known that waterfowl birds are more likely to counteract LPAI virus infections. The whole genome phylogenetic tree (figure 23) shows a more detailed tree regarding avian species. Here the (Pekin) duck clusters closely with chicken and turkey (Zhang *et al.*, 2014), whilst the mallard duck does not appear as closely related in the bootstrap consensus tree. This could potentially indicate a higher genetic correlation between p85 β sequences in land-based poultry and certain waterfowl birds.

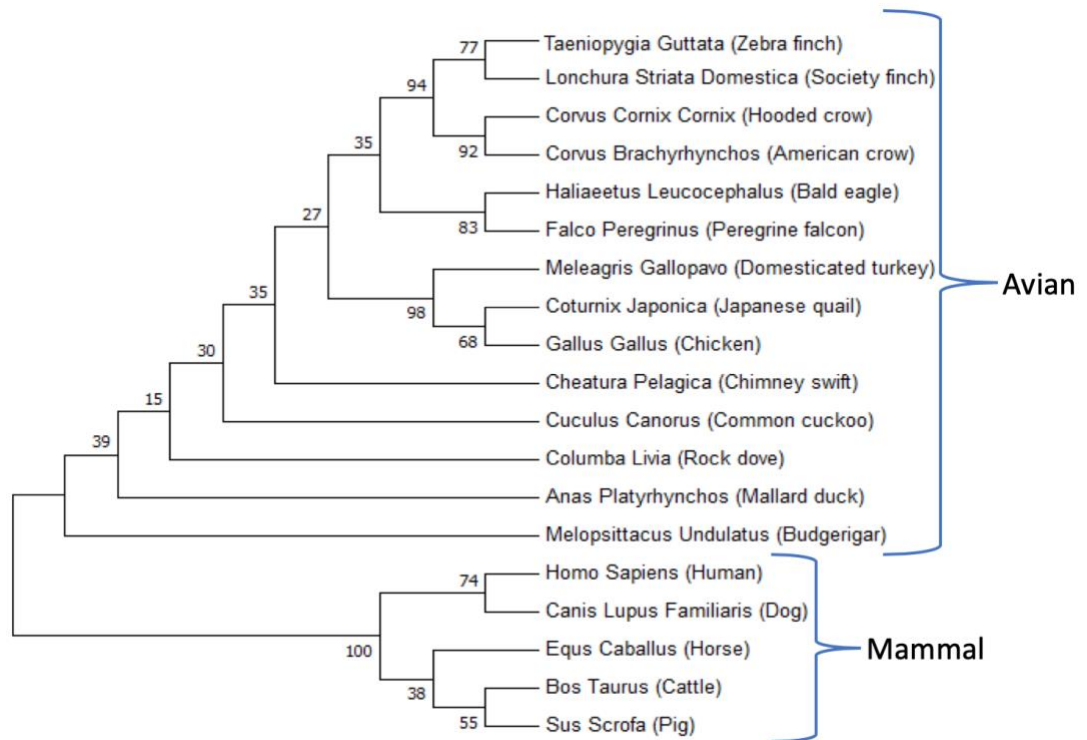


Figure 21: Molecular phylogenetic of p85β through Maximum Likelihood method, bootstrap consensus tree

The p85β phylogenetic tree shows how the selected avian species clustered separately from the mammals, which in turn clustered together. In particular, chickens and quails appeared to be very closely related, and share similarities with turkeys.

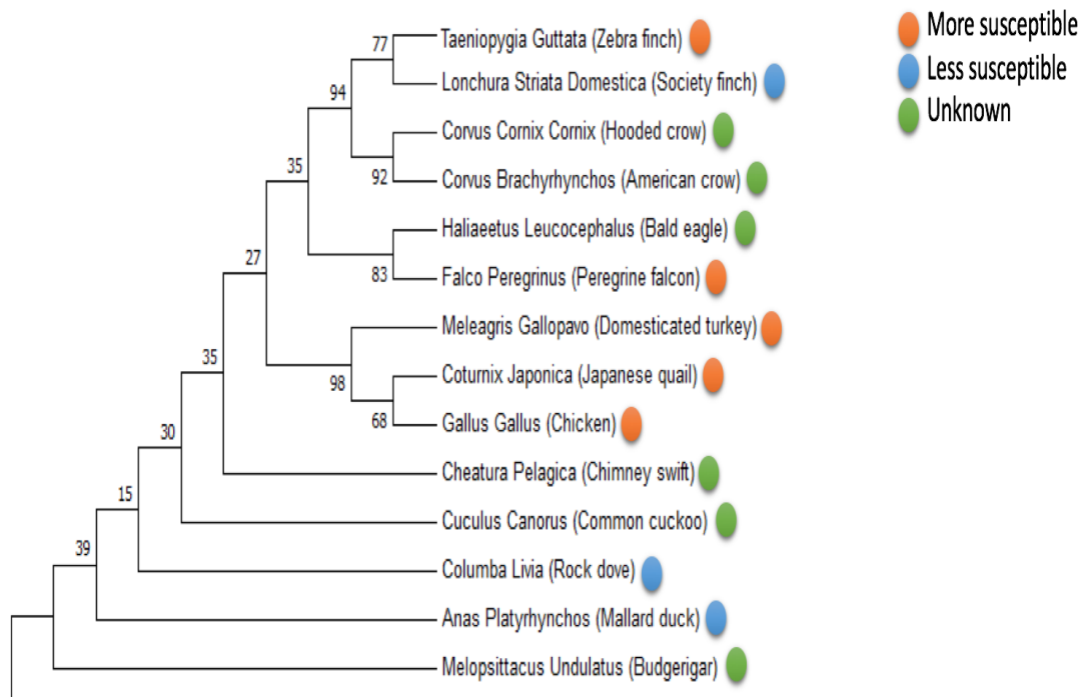


Figure 22: Comparison of selected avian species when exposed to avian influenza

Results obtained from comparing susceptibility of selected avian species when exposed to avian influenza. Different colored circles indicate which avian species are more or less susceptible to exposure to influenza virus, as well as those which have yet to present registered deaths caused by influenza A. The definitions of severity are: more susceptible show severe signs or death; less susceptible show mild signs or asymptomatic. Unknown mean no outcome of infection is registered. Whilst the more susceptible subjects are more likely to succumb to both LPAI and HPAI viral strains, the less susceptible ones are at risk only when exposed to certain HPAI viral strains.

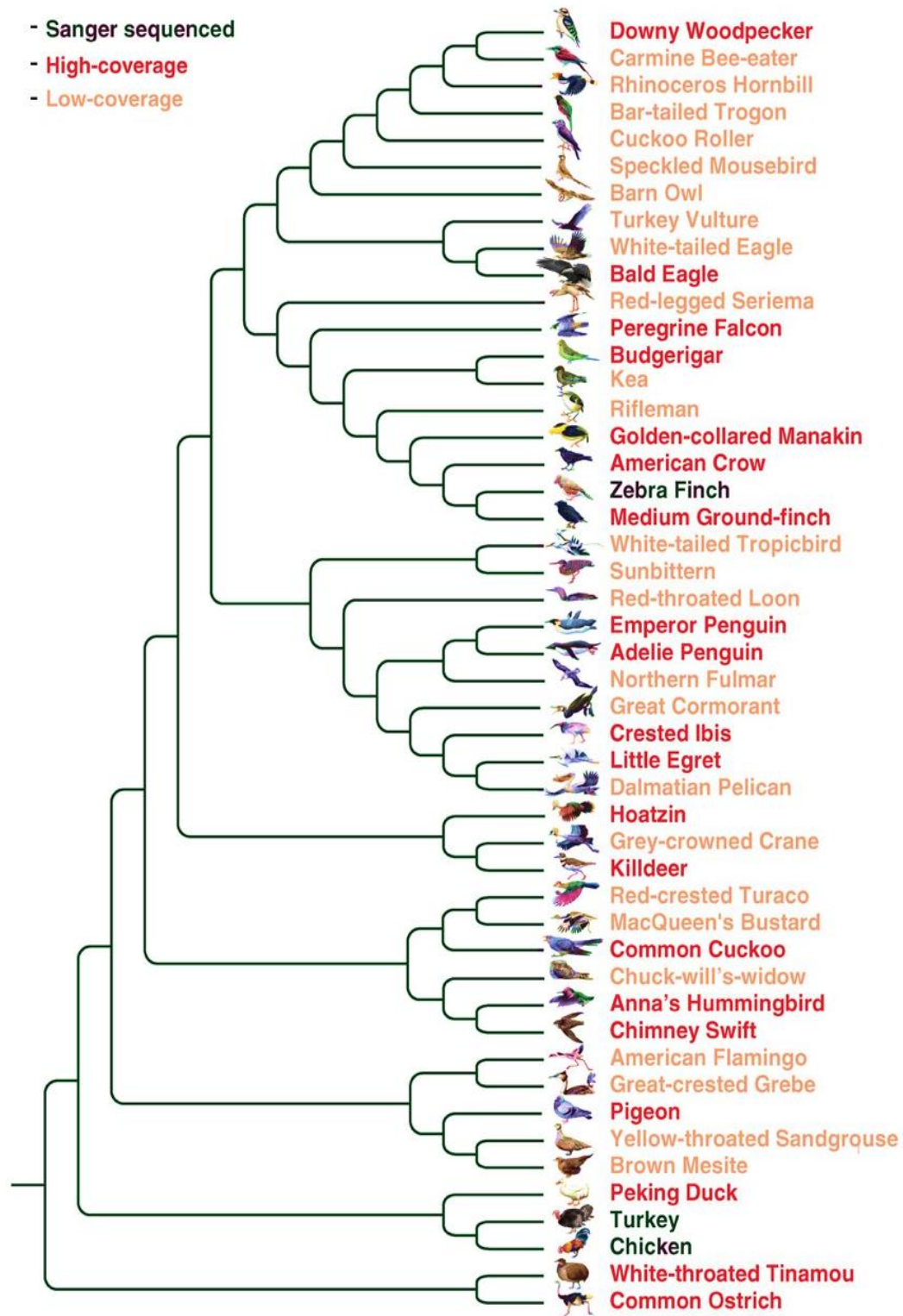


Figure 23: Phylogenetic tree of whole genome analysis of 48 avian species

The sequences have been retrieved with Sanger sequencing (black), high coverage (bright red) and low coverage (light red). Modified from Zhang et al., 2014.

3.4 Variation of p85 β in selected sequences

After retrieving PI3K – p85 β sequences of different species from the NCBI database, and aligning them, observations were carried out in order to detect any variations amongst different species. There was remarkable consensus between mammals and birds but also marked differences in selected regions. Furthermore, variations between different bird species were detected. Examples of these were multiple amino acid variations, such as the two amino acid variation detected in quails at 250-251, and various single amino acid variations, like substitution of glutamine with lysine at position 430 on the quail sequence. Domains identified in the *Bos taurus* sequence are highlighted in the figure 24.

Duck

-----MGSTDGFOYRALYPYRKEREEDIDL**L**PGDILVVSKGALOALGFKDGDEONPNOIGW 56

Chicken

MAERMGSTDGFQYRALYPYRKEREEDIDLVPGDILVVSKGALQALGFKDGDEQNPNQIGW 60

Ouail

MAERMGSTDGFQYRALYPYRKEREEDIDLPGDILVVSKGALOALGFKDGDEONPNOIGW 60

Human

----MAGPEGFOYRALYPFRRERPEDLELLPGDVLVVSRAALOALGVAEGGERCPOSVGW 56

Cow

----MAGPEGFOYRALYPFRRERPEDLELLPGDVLVVSRAALOALGVAEGNERCPOSVGW 56

* . * * * * * * * * * . * * * * * * * * : . * * * * * * * : * * * * * * * : * * * * * * * : * * * * * * * : *

SH3

Duck

ILGVNERTKOKGDFPGTYVEYVGPVKISVPSHRPRG-----PGGCRLOEAPEOGSP 107

Chicken

ILGVNERTKOKGDFPGTYVEYVGPVKISVPSHRPRV**ORPLPATPG**ASGCRLOEALEOGSP 120

Ouail

ILGVNERTKOKGDFPGTYVEYVGPVKISVPSHRPRVORPLPATPGASGCRLOEALEOGSP 120

Human

MPGLNERTROBGDFPGTYVEFLGPVALARPGPRPRG**PRPLPARPRD**G-----APEPGLT 110

COW

MPGLNERTRORGDFPGTYVEFLGPVALARPGPRPRGPRPLPARPRDG-----PPEPGLT 110

• * . * * * * . * . * * * * * * * * . . * * * * . . * * * * * * *

SH3

Duck

LPDLQEQFSPPETAPPLLVKLVEAIEKKGLDSEMLYRTSGSELRQALRTDWSLGDLEPFD 167
 Chicken
 LPDLQEQFSPPEVAPPLLVKLVEAIEKKGLDSEMLYRTSGSELRQALRTDWSLGDLEPFD 180
 Quail
 LPDLQEQFSPPFVAPPLLVKLVEAIEKKGLDSEMLYRTSGSELRQALRTDWSLGDLEPFD 180
 Human
 LPDLPEQFSPPDVAPPLLVKLVEAIIERTGLDSESHYRPE----LPAPRTDWSLSDVDQWD 166
 Cow
 LPDLPEQFSPPDVAPPILVKLVEAIIERTGLDS---YRPE----PPAVRTDWSLSDVEQWD 163

Rho – GAP

Duck
 VHSLGEALRGYLQDLPSVVPVAVHGDILRVLQEVQPENCRKQLLLALESFAVPLQNTL 227
 Chicken
 VHSLSEALRGYLQDLPSVVPVSVHGDILRILQEIQPENCRKQLLLALESFATPLQNTL 240
 Quail
 VHSLSETLRGYLQDLPSVVPVSVHGDILRILQEIQPENCRKQLLLALESFATPLQNTL 240
 Human
 TAALADGIKSFLALPAPLVTPEASAEARRALREAAGPVGP-----ALEPPTLPLHRAL 220
 Cow
 AAALS DGVKGFLLALPAPLVTPEAAAEAHRALREAAGPVGP-----ALEPPTLPLHHAL 217
 . :*: :*

Rho – GAP

Duck
 TLQFLLRHLGKVSQQAESNNLHPALGEIFGPLLRLPGA-----SPELSPDFS 276
 Chicken
 TLQFLLRHLGKVSQQAESNSLHPALGEIFGPLLRLPGA-----SPELSPDFS 289
 Quail
 TLQFLLRHLRKVSQQAESNSLHPALGEIFGPLLRLPGA-----SPELSPDFS 289
 Human
 TLRFLQHLGRVARRAPALGPAVRALGATFGPLLRLRAPPPPSSPPPGGAPDGSEPSPDFP 280
 Cow
 TLRFLQHLGRVAGRAPALGPAVRALGATFGPLLRLRAPPPP-SPPPGGAPDGTEPTPDFP 276
 :*:*** :*: :* : . :*** :***** * . * :***

Rho – GAP

Duck
 VLFLEMLLQIKELEPEQLPPALPPKPPKPKPSAASLANGNSVP-LQDAEWYWGDISREEV 335
 Chicken

VLFLEMLLQTKKELEPEQLPPALPPKPPKPKPSAASLANGNSVP-LQDAEWYWGDISREEV 348
 Quail
 VLFLEMLLQTKKELEPEQLPPALPPKPPKPKPSAASLANGNSVP-LQDAEWYWGDISREEV 348
 Human
 ALLVEKLLQEHLEEQEVAPPALPPKPPKAKPAPTVLANGGSPPSLQDAEWYWGDISREEV 340
 Cow
 ALLVEKLLQEHLEEQEVAPPALPPKPPKTKPAPTGLANGGSPPSLQDAEWYWGDISREEV 336
 .*:~* ~*~ : ~*~ ***** **: : ~*~.* ~* *****
 Rho – GAP SH2 1
 Duck
 NEKLRDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKIFHREGKYGFSEPLTFGSVVE 395
 Chicken
 NEKLRDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKIFHREGKYGFSEPLTFGSVVE 408
 Quail
 NEKLRDTPDGTFLVRDASSKI~GEYTLTLRKGGNNKLIKIFHREGKYGFSEPLTFGSVVE 408
 Human
 NEKLRDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKVFHRDGHYGFSEPLTFCSVVD 400
 Cow
 NEKLRDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKVFHRDGHYGFSEPLTFCSVVD 396
 *****:*****:~*:~*:***** ~*~:
 SH2 1
 Duck
 LITHYRHESLAQYNAKLDTRLLYPISKYQQDQVVKEDSVEAVGEQLKVYHQQYQDKSWEY 455
 Chicken
 LITHYRHESLAQYNAKLDTRLLYPISKYQQDQVVKEDSVEAVGEQLKVYHQQYQDKSWEY 468
 Quail
 LITHYRHESLAQYNAKLDTRLLYPISKYQQDQVVKEDSVEAVGEQLKVYHQQYQDKSWEY 468
 Human
 LINHYRHESLAQYNAKLDTRLLYPVSKYQQDQIVKEDSVEAVGAQLKVYHQQYQDKSREY 460
 Cow
 LITHYRHESLAQYNAKLDTRLLYPVSKYQQDQIVKEDSVEAVGAQLKVYHQQYQDKSREY 456
 ~*.*****:*****:***** ***** ~*~
 SH2 1
 Duck
 DLLYEEYTRTS---QMKRTAIEAFNETIKIFEEQCQTQEKCSKEYIERFRREGNEKEVQR 507
 Chicken
 DLLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQCQTQEKCSKEYIERFRREGNEKEVQR 528
 Quail

DLLEYEYTRTSQELQMKRTAIEAFNETIKIFEEQCQTQEKCSKEYIERFRREGNEKEVQR 528
Human

DQLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQGQTQEKCSKEYLERFRREGNEKEMQR 520
Cow

DQLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQGQTQEKCSKEYLERFRREGNEKEMQR 516
* ***** ***** *****:*****:*

Duck
ILMNSEKLKSRITEIHDSKMKLEQDLKKQASENREIDKRMNSLKPDLMLRKLRLDQYLVW 572
Chicken

ILMNSEKLKSRITEIHDSKMKLEQDLKKQASENREIDKRMNSLKPDLMLRKLRLDQYLVW 588
Quail

ILMNSEKLKSRITEIHDSKMKLEQDLKKQASENREIDKRMNSLKPDLMLRKLRLDQYLVW 588
Human

ILLNSERLKSRIAEIHESRTKLEQQLRAQASDNREIDKRMNSLKPDLMLRKLRLDQYLVW 580
Cow

ILLNSERLKSRIAEIHESRTKLEQELRAQASDNREIDKRMNSLKPDLMLRKLRLDQYLVW 576
*:***:*****:***:*: *****:*: ***:*****:*****:*****

SH2 2

Duck
LTQKGARQKKINEWLGIKNETEDQYSMMDDDEELPHHEERTWYVGKINRLQAEEMLCGKR 632
Chicken

LTQKGARQKKINEWLGIKNETEDQYSMMDDDEELPHHEERTWYVGKINRLQAEEMLCGKR 648
Quail

LTQKGARQKKINEWLGIKNETEDQYSMMDDDEELPHHEERTWYVGKINRLQAEEMLCGKR 648
Human

LTQKGARQKKINEWLGIKNETEDQYALMEDEDDLPHEERTWYVGKINRTQAEEMLSGKR 640
Cow

LTQKGARQKKINEWLGIKNETEDQYSLMEDEDDLPHEERTWYVGKINRTQAEEMLSGKR 636
*****:***:***:***** *****:***

SH2 2

Duck
DGTFLIRESSQKGCYACSVVVDGDTKHCVIYKTASGYGFAEPYNLYASLKDVLVHYKHTS 692
Chicken

DGTFLIRESSQKGCYACSVVVDGDTKHCVIYKTATGYGFAEPYNLYASLKDVLVHYKHTS 708
Quail

DGTFLIRESSQKGCYACSVVVDGDTKHCVIYKTATGYGFAEPYNLYASLKDVLVHYKHTS 708
Human



Figure 24: Alignment of p85 β amino acid sequences from five species

Sequences from cattle (*Bos taurus*), human (*Homo sapiens*), quail (*Coturnix japonica*), chicken (*Gallus gallus*) and mallard duck (*Anas platyrhynchos*) were retrieved from the NCBI protein sequence database and aligned on the online software Clustal Omega. Domain regions were retrieved from the cattle p85 β sequence on the UniProt website. These were: SH3 (position 4–80), Rho–GAP (position 109–291), SH2 1 (position 326–421) and SH2 2 (position 618–712). The regions which correspond to the areas where the duck has amino acid deletions are highlighted.

3.5 Analysis of p85 β 3 dimensional protein structure

The amino acid deletions identified in ducks did not correspond to the position of the labelled domains; however, they might still affect the binding properties of the protein. Figure 25 illustrates a 3D picture of the p85 β protein from cattle, retrieved from the UniProt database. Future insights into the deletion sites in ducks might reveal the potential effects that these have regarding the response of this species to influenza A infections.

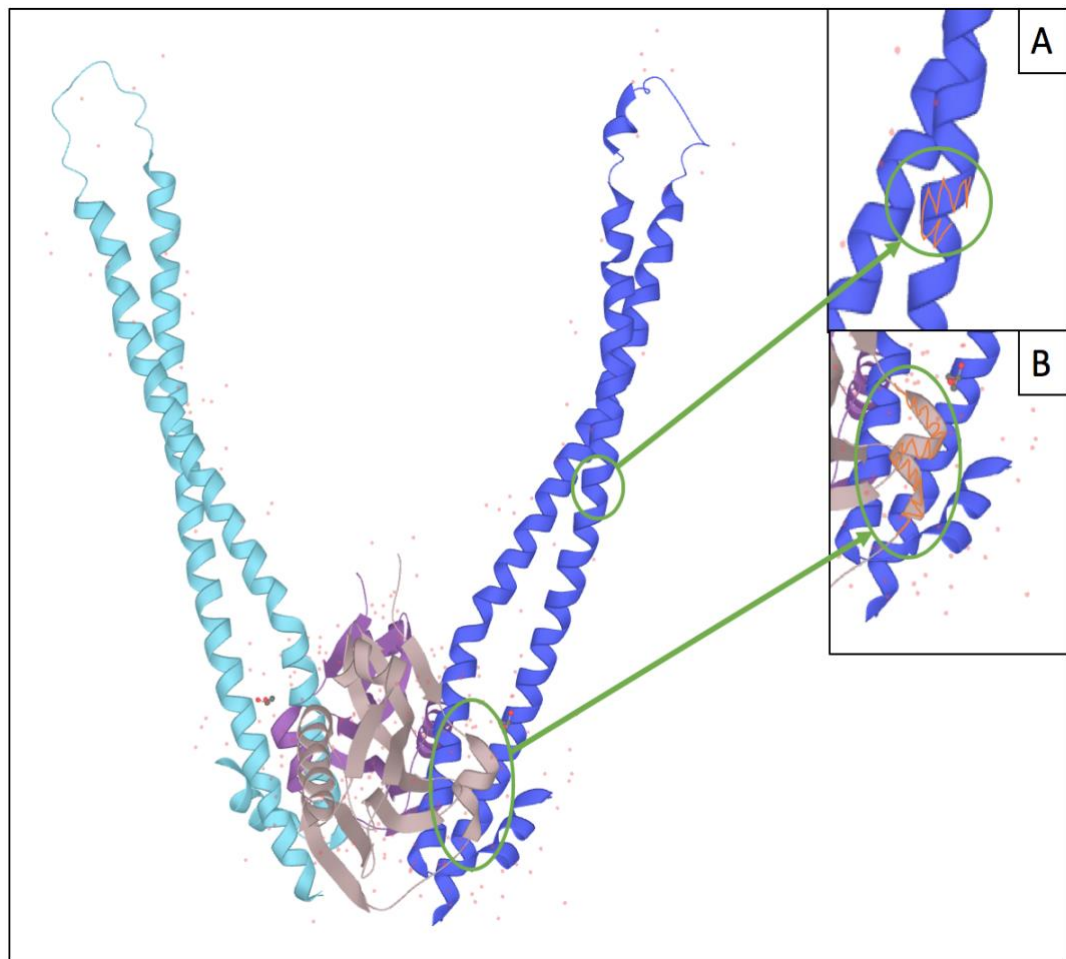


Figure 25: 3D structure of p85 β protein in cattle

The regions which correspond to the areas where the duck has amino acid deletions are highlighted. (A) 467–469 position in the duck sequence and position 468–470 in the cattle sequence. (B) 93–101 deleted region in the duck sequence, which corresponds to the same position in the cattle sequence. Image was retrieved from UniProt website.

3.6 Analysis of interaction between NS1 and p85 β

3.6.1 NS1 plasmid preparation for transfection

3.6.1.1 NS1 plasmid

The potential interaction between influenza A NS1 and avian PI3K regulatory p85 β subunit were studied. The final results obtained from transfection of MDCK cells with the Udorn plasmid were inconclusive. A Western blot using antibodies for NS1 and p85 β showed no detectable expression of NS1 or p85 β in the screened samples.

3.6.1.2 H2N3 NS1 plasmid

Insertion of the H2N3 NS1 segment into the Udorn plasmid was successful, as two bands of approximately 900bp and 3.9Kb were detected (figure 26). This indicated

that the inserted NS1 sequence was present in the vector. Sequencing of two samples showed that one contained NS1.

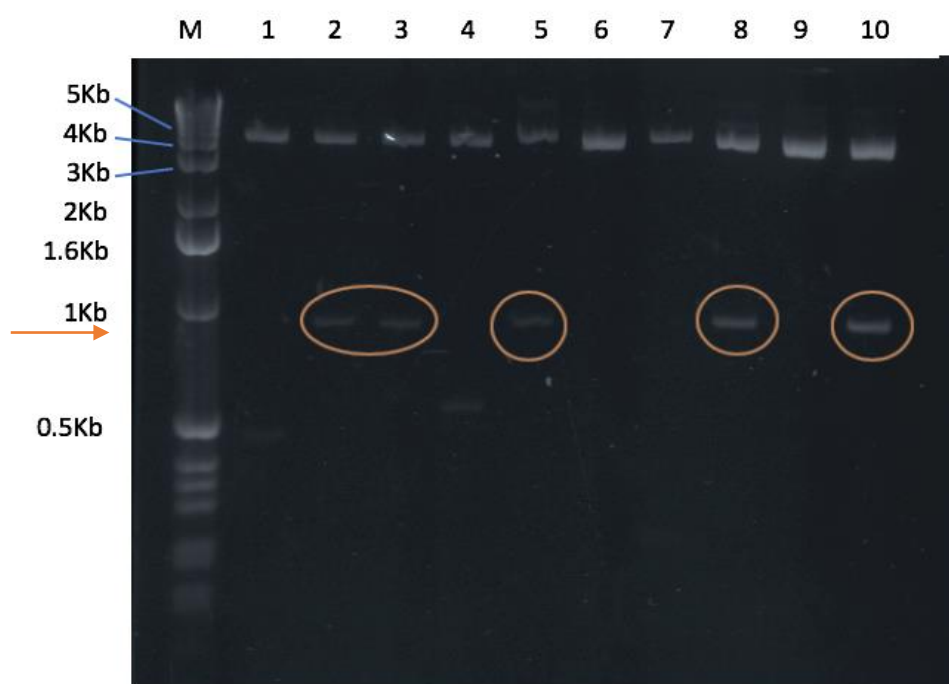


Figure 26: Restriction digest of TOPO-TA vector containing H2N3 segment 8

Agarose gel of recombinant TOPO-H2N3 segment 8 minipreps, digested with EcoRI, to show NS1. The TOPO-TA vector was 3.9kb, whilst the NS1 insert was 890bp. The orange circles highlight the EcoRI digested fragments of the expected size (the marker was a 1Kb ladder).

4. Discussion

4.1 Activation of Akt pathway in LPAI infected embryonic quail cells

The PI3K/Akt pathway has many roles that contribute towards cellular survival. The present study demonstrated the activation of this pathway in LPAI H2N3 infected QECs, as evidenced by the expression of phosphorylated Akt at serine 473 (ser473), where PI3K directly mediates the activation of Akt (Tsuchiya *et al.*, 2015). Overall activation of the pathway promotes cell survival, whilst inhibition of it would cause apoptosis (Diehl and Schaal, 2013). Therefore, activation of the pathway favours virus replication. The results showed that quails have a similar response to chickens; however, chickens do not express pAkt as strongly as quails at 24 hours p.i. (Kumar, 2018). Activation of the pathway in quails and chickens, but not in ducks (Kumar, 2018), suggests that it might be linked to the ability of ducks to show less severe clinical signs, compared to the more severe effects it has on poultry (Carnaccini *et al.*, 2015; Saito *et al.*, 2009).

4.2 Inhibition of PI3K/Akt pathway in infected quail cells

In this study, inhibition of the PI3K/Akt pathway was attempted by treating infected primary quail cells with LY942002 or Wortmannin, in order to determine the pathogenic properties of the viral infections of QECs. Results showed there was a partial inhibition of pAkt, when cells were treated with Wortmannin; conversely, pAkt was absent in infected cells treated with LY942002, so inhibition was not possible.

4.3 Analysis of phylogenetic tree results

4.3.1 Analysis of selected avian species

Birds are the richest species of tetrapod vertebrates, with 10711 existing species and 158 extinct (Gill and Donsker, 2018). The avian genome is made up of fewer genes, compared to other vertebrates (Zhang *et al.*, 2014). Even though birds experience a similar historical radiation to humans, they present less genomic variations, unlike the human genome, which has been exposed to a richer variation and shuffling. The phylogenetic tree for the p85 β subunit of PI3K showed that quails, chickens and turkeys share strong similarities. These species appear to have similar genomes, as they are closely related. Moreover, turkeys appeared to be as susceptible as chickens and quails to influenza A infections (Alexander, 2000). However, compared to the

rate of LPAI infections, there have been hardly any HPAI infections reported in turkeys. Almost all LPAI virus subtypes are present in the avian reservoir, made up of shorebirds and waterfowl (Schrauwen and Fouchier, 2014). These vectors are able to spread the disease across long distances through bird migration. Furthermore, domestic ducks and geese can act as bridge species, transmitting the virus to other domestic poultry. Although birds are recognised as some of the major vectors for all subtypes of avian influenza, the interaction that occurs between birds and other influenza viral hosts is yet to be fully understood. Certain information regarding the susceptibility of avian species to influenza viruses is still lacking. In order to better understand the epidemiology of avian influenza viruses, it is necessary to define the interactions between different hosts, agents and environments.

4.3.2 Correlation between mammal and avian species response to avian influenza infections

Viral subtypes H5 and H7 are able to evolve into HPAI in poultry. These strains present a threat not only to avian species, but also to mammals. The first case of influenza A infection in people was registered in 1997 in Hong Kong (Chan, 2002), when an H5N1 strain infected humans. When humans are exposed to influenza A viruses, they can present with seasonal 'flu symptoms, which occur on a regular basis, or pandemics can arise unexpectedly from re-assorted viral strains. These sudden outbreaks are characterised by increased mortality rates and are able to spread rapidly through naïve immune human populations, which in turn cause a severe clinical and economic impact. Therefore influenza A is classified as a zoonotic disease, as it is able to cross species' barriers. This event triggers a pandemic outbreak, which can cause severe respiratory disease and may even lead to fatalities.

4.3.2.1 Interaction of p85 β with NS1 in human cells

It has already been shown that the influenza A NS1 protein binds to the p85 β subunit, which in turn activates the PI3K/Akt pathway, in human cells (Hale *et al.*, 2006). In particular, analysis of the 1918 Spanish influenza strain (A/Brevig mission/1/18/H1N1) shows that NS1 is able to bind to homology 3 binding motifs Crk/CrkL of p85 β , in human cells (Heikkinen *et al.*, 2007). Through this binding, the PI3K can be stimulated

via the PI3K/Akt pathway, thus promoting viral replication and preventing host cell apoptosis.

4.4 Analysis of the NS1 and p85 β interaction

The experiments carried out in this study were inconclusive in the determination of the relationship between NS1 and p85 β , in infected quail cells. In order to analyse the potential NS1–p85 β interaction in quail cells, the NS1 cDNA alone should be cloned into a eukaryotic expression vector and used to transfect quail embryonic fibroblasts. New site-specific oligonucleotide primers should be designed and used to clone the 693bp NS1 cDNA into a vector with a suitable tag. Upon transfection of QECs the recombinant NS1 protein would be expressed in the fibroblasts, using the vector's eukaryotic promoter. A pulldown assay on the cell lysate could then be used to determine if there is any interaction between NS1 and p85 β , by Western Blot and detection of the tagged protein.

4.5 Future perspectives

4.5.1 Limitations of current study

Throughout the course of this study there have been a number of limitations that have come to light. Namely, some experimental obstacles were difficult to circumvent due to time constraints. For instance, when inhibiting the PI3K/Akt pathway, only a limited number of concentrations were tested. Thus, in order to confirm the lack of inhibition from LY942002 or Wortmannin, the experiment would need to be repeated with a larger range of concentrations and a more reliable positive control. Due to the lack of significant results obtained from the inhibition of LPAI H2N3 viral infection in MDCK cells with LY492002, no immunohistogram was carried out. Therefore, it would be necessary to repeat the experiment, in order to confirm results and test the effects of different inhibitor concentrations. Furthermore, certain products used during the study did not react as expected. In addition, experiments were carried out *in vitro*, so the results may not accurately relate to *in vivo* studies.

4.5.2 Potential experimental studies to investigate NS1 interaction with p85 β

Previous studies (Hale *et al.*, 2006) demonstrated that binding of NS1 to PI3K, through interaction with p85 β , occurs during influenza A infections, in human cells.

However, this same interaction has not yet been demonstrated in birds. In order to analyse this binding interaction, it is necessary to carry out a pull down protocol (e.g. HaloTag Complete Pull-Down System, Promega) using recombinant NS1, cloned in a suitable expression vector. Halo-tagged fusion proteins permit the detection of minimal amounts of interacting proteins due to the presence of a highly sensitive fluorescent tag. This would allow detection of any minimal interactions between NS1 and p85 β via fluorescence of the tagged recombinant protein, once purification and various wash and elution steps have been performed. The protocol shares similarities in methodology with the co-immunoprecipitation assay procedure. However, they differ in methodology and efficacy, as the co-immunoprecipitation utilises immobilised antibodies to attract protein complexes, whilst the pull down assay uses purified and tagged proteins as “bait” for potential interacting proteins (Louche *et al.*, 2017). The “bait” protein is immobilised by an affinity ligand specific to the tag, which allows the formation of a supportive structure to catch and isolate other proteins that interact with the “bait”. Once the protein of interest has been captured, interacting complexes are removed through elution and the protein fractions are analysed through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualised through gel staining or Western blotting. Figure 27 illustrates a generic pull down system with a tag. Following isolation of the tag with the protein of interest, the solution is eluted to remove the tag and a gel is run. Detection of NS1 and p85 β can be achieved through incubation with specific antibodies, following Western blot. If results indicate that the solution contains multiple proteins, the bands are cut out and sent for analysis through Matrix-Assisted Laser Desorption Ionization Time-Of-Flight mass spectrometry (MALDI-TOF MS), followed by peptide mass fingerprinting (PMF). In order to avoid the disruption of natural protein interactions or detection of unnatural interactions, the concentration of the lysis buffer should be chosen carefully. However, other pull down protocols can be adopted to better understand the role of NS1, and its possible interaction with p85 β . These can include viral infection based protocols or transfection with plasmids containing a segment encoding NS1. This protocol was adopted in a study that demonstrated the interaction between NS1 and p85 β in infected human cells (Hale *et al.*, 2006). This was carried out through

immunoprecipitation of V5-tagged NS1, expressed in embryonic kidney cells (293T) cells, with an anti-V5 antibody.

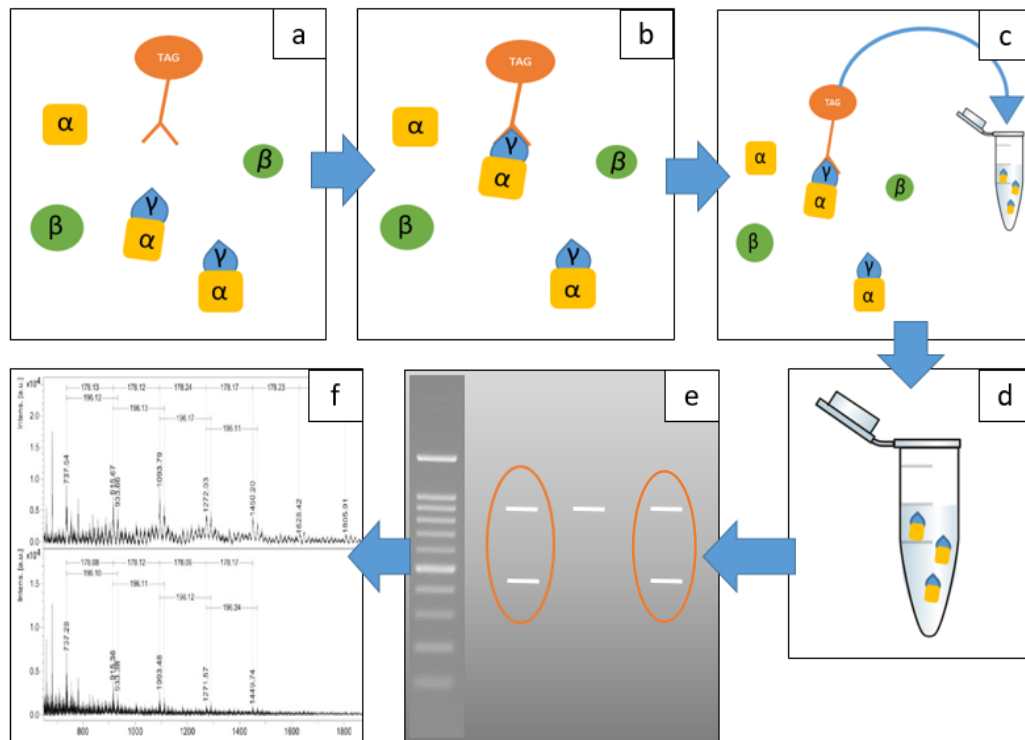


Figure 27: Pull down assay

The assay can be performed to analyse the interaction between two or more proteins. The protein of interest is captured using a “bait” tag, which was added to the solution (a). The tag can bind to the protein of interest (b), which might be attached to one or more proteins. The complex is isolated from the rest of the solution (c) and washed and eluted to remove the tag (d). A gel or Western blot is run (e) and analysis of the bands, to determinate the nature of the protein expressed, is determined by cutting them out and sending for analysis through MALDI-TOFF mass spectrometry (e).

4.6 Potential use of drugs to target the PI3K pathway

4.6.1 Targeting the host

As previously observed in A549 cells, inhibition of the PI3K/Akt pathway during infection is possible (Hirata *et al.*, 2014). The cells were treated with “TCL1- or TCL1b-based *Akt-in*”, which was able to suppress the activity of the Akt kinase, which in turn inhibits viral entry and proliferation. These results suggest that a possible pathway inhibition could be adopted as a treatment against influenza. Most antiviral drugs target viral proteins, such as hepatitis B virus (HBV) inhibitors (Fung *et al.*, 2011), which are made up of nucleotide analogues. Similar treatments are also currently

used to treat influenza infections, as discussed in paragraph 1.5. Viruses are able to thrive by hijacking several cellular functions. An example is the hepatitis C virus (HCV), which appears to depend completely on the host in order to spread. By targeting the host, a stronger resistance barrier can be formed, which provides a broader protection against several virus genotypes, as well as potential infection with multiple viruses (Lin and Gallay, 2013). However, attempts to pursue host targets in drug treatments need care due to concern over potential target toxicity, the uncertainty of predicting effectiveness between cell cultures and animal subjects and the complexity of interactions between the host and the virus. Thus, antiviral drugs are focused on viral targets. There is increased interest in targeting cyclophilins in viral infections, such as HCV cases. Cyclophilins are a group of proteins involved in several functions regarding cellular metabolism and energy homeostasis (Naoumov, 2014). In particular, cyclophilins are involved in the pathogenesis of various liver diseases, thus indicating that the inhibition of this protein would be beneficial. Knock-down of these proteins with inhibitors, such as NIM811, has proven to be effective in inhibiting cyclophilins and protein trafficking (Gaither *et al.*, 2010). Even though HCV differs from IAV, this demonstrates that this novel approach can potentially be adopted for other viral treatments. If the NS1 and p85 β interaction is a crucial factor in influenza A's pathogenicity in birds, drugs could be tailored to inhibit this interaction.

4.6.2 Genetically engineered animals

Genetic engineering uses modern biotechnology and molecular biology, to add new genetic material, or remove or substitute existing sections of the genome. This results in the introduction of new or modification of existing traits. A genetically engineered (GE) animal is obtained by adding or altering genetic material within the subject (e.g. recombinant DNA). Many types of GE animal have been created, such as the AquAdvantage salmon (Du *et al.*, 1992) and the introduction of bovine α -lactalbumin in pig's milk (Murray and Maga, 2016), which in turn favours the survival of the offspring. Infectious diseases are able to affect not only animal welfare, but could potentially also threaten human health and public opinions of livestock production. Genetic modifications offer an alternative method to natural breeding, which can be applied to either species or populations that lack a certain genetic feature as well as

introduce a new engineered gene (Whitelaw and Sang, 2005). In the case of infections, both approaches aim to equip the animal with either a more appropriate immune system or with the ability to block or destroy the pathogen at the entry point. Often certain genetic alleles can be lost from species that have been turned into commercial populations. For example, the *Mx* genes of vertebrates, found in mice, are able to induce a strong immune response, when certain viral infections occur, such as influenza. However, due to a single amino acid substitution, the allele is found to be non-functional in most commercial poultry (Whitelaw and Sang, 2005). Therefore, in order to combat influenza infections within the affected flock, genetic modifications could be applied to enhance the immune response in the target species.

4.6.2.1 GE animals and influenza A

Due to the re-assortant ability of influenza A viruses, achieved through genetic shift or drift, vaccines are partially protective, as they do not always result in a successful sterile immunity. One solution that could control the spread of IAV in domestic poultry, is GE. A study was able to generate transgenic chickens that express a short-hairpin RNA, which is able to interfere with influenza A viral polymerase thus inhibiting viral proliferation (Lyall *et al.*, 2011). The decoy 5 (D5) hairpin comprised a chicken U6 RNA promoter, the decoy sequence and hepatitis δ virus anti-genomic ribozyme. This decoy encodes the conserved 3'- and 5'-terminal sequences of the influenza viral genome segment 8 for the cRNA binding site for viral polymerase and has the ability to disrupt the virus replication and packaging. A second decoy 7 (D7) was also created, with mutations that prevent the binding of the viral polymerase. RNA interference (RNAi) is a natural mechanism through which cells are able to silence specific genes (McColl *et al.*, 2013). RNAi is mediated by small interfering RNA (siRNA) molecules, which are either already present within the cells, or in GE animals they are induced by genetic engineered methods, which start with the production of short hairpin RNA (shRNA). The siRNA interact with the invading viral RNA in order to abrogate its function. This approach is theoretically possible but requires pilot studies on a larger scale to assess, for example, predictability and costs.

5 Conclusion

In a previous study (Kumar, 2018), the PI3K/Akt pathway, which is involved in avian Influenza transmission was active in chickens, but not in mallard ducks. The current study demonstrated that the PI3K/Akt pathway is active in infected primary quail cells, in a similar way to chickens, thus supporting the hypotheses of the study. Since the pathway remains active during infection, this may allow the virus to proliferate more easily. Like chickens, quails are more susceptible to avian influenza viruses in contrast to ducks, which are able to carry the virus without showing any clinical signs. The phylogenetic tree created for p85 β shows the evolutionary relationship between different mammalian and avian species. Chickens' and quails' p85 β amino acid sequences appear to be closely related. However, the mallard duck showed two amino acid deletions. This could potentially indicate molecular variations that render the duck immune to any clinical consequences of avian influenza virus infection. Although the objectives were reached for the most part, the inhibition of the PI3K/Akt pathway in quail cells requires further investigations, using a larger concentration range of inhibitor. This may give a better understanding of the nature of the inhibitory process, potentially by inhibiting viral replication. Further studies are necessary to determinate the potential role of p85 β in avian influenza infections, and whether it interacts with NS1. Insight into these interactions will provide a better understanding of viral pathogenicity of influenza A in avian species.

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Appendix

- List of amino acid sequences used to make up the phylogenetic tree, with their accession NCBI number:

Equus caballus – Horse (XP_023481250.1)

Homo sapiens – Human (NP_005018.1)

Canis lupus familiaris – Dog (XP_852406.2)

Bos Taurus – Cattle (NP_777001.1)

Sus scrofa – Pig (XP_020939165.1)

Taeniopygia guttata – Zebra finch (XP_004174640.1)

Cuculus canorus – Common cuckoo (XP_009561114.1)

Chaetura pelagica – Chimney swift (XP_009996094.1)

Melopsittacus undulates – Budgerigar (XP_005140568.2)

Anas platyrhynchos – Mallard duck (XP_021133555.1)

Columba livia – Rock dove (PKK19499.1)

Lonchura striata domestica – Society finch (OWK51426.1)

Corvus cornix cornix – Hooded crow (XP_010409819.1)

Corvus brachyrhynchos – American crow (XP_008639226.1)

Coturnix japonica – Japanese quail (XP_015741981)

Meleagris gallopavo – Turkey (XP_010723359.1)

Gallus gallus – Chicken (XP_001233341.3)

Haliaeetus leucocephalus – Bald eagle (XP_010560473.1)

Falco peregrinus – Peregrine falcon (XP_005244191.1)

Equus_Caballus	----MAGAEGFQYRALYRFRRRERPEDLELLPGDVLVVSRAALQALGVAEGSERCPQSVGW	56
Homo_Sapiens	----MAGPEGFQYRALYPFRRRERPEDLELLPGDVLVVSRAALQALGVAEGGERCPQSVGW	56
Canis_Lupus_Familiaris	----MAGAEGFQYRALYPFRRRERPEDLELLPGDVLVVSRAALQVLGVAEGNERCPQSVGW	56
Bos_Taurus	----MAGPEGFQYRALYPFRRRERPEDLELLPGDVLVVSRAALQALGVAEGNERCPQSVGW	56
Sus_Scrofa	----MAGAEGFQYRALYPFHRERPEDLELLPGDVLVVSRAALQALGVAEGSERCPQSVGW	56
Taeniopygia_Guttata	----MGSTDGFQYRALYPYRKEREEDIDLLPGDILVVSKGALQALGFKDGDEQTPNQIGW	56
Cuculus_Canorus	-----DGFQYRALYPYRKEREEDIDLLPGDILVVSKGALQALGFKDGDEQTPNQIGW	52
Cheatura_Pelagica	----MGSTDGFQYRALYPYRKEREEDIDLLPGDILVVSKGALQALGFKDGDEQTPNQIGW	56
Melopsittacus_Undulatus	----MGSTDGFQYRALYPYRKEREEDIDLQPGDILVVSKGALQALGFKDGDEQTPNQIGW	56
Anas_Platyrhynchos	----MGSTDGFQYRALYPYRKEREEDIDLLPGDILVVSKGALQALGFKDGDEQNPQNQIGW	56
Columba_Livia	----MGSTDGFQYRALYPYRKEREEDIDLLPGDILVVSKGALQALGFKDGDEQTPNQIGW	56
Lonchura_Striata_Domestica	----MGSTDGFQYRALYPYRKEREEDIDLLPGDILVVSKGALQALGFKDGDEQTPNQIGW	56
Corvus_Cornix_Cornix	----MGSTDGFQYRALYPYRKEREEDIDLLPGDILVVSKGALQALGFKDGDEQTPNQIGW	56
Corvus_Brachyrhynchos	-----DGFQYRALYPYRKEREEDIDLLPGDILVVSKGALQALGFKDGDEQTPNQIGW	52
Coturnix_Japonica	MAERMGSTDGFQYRALYPYRKEREEDIDLLPGDILVVSKGALQALGFKDGDEQNPQNQIGW	60
Meleagris_Gallopavo	----MGSTDGFQYRALYPYRKEREEDIDLVPGDILVVSKGALQALGFKDGDEQNPQNQIGW	56
Gallus_Gallus	MAERMGSTDGFQYRALYPYRKEREEDIDLVPGDILVVSKGALQALGFKDGDEQNPQNQIGW	60
Haliaeetus_Leucocephalus	----MGSTDGFQYRALYPYRKEREEDIDLLPGDILVVSKGALQSLGFKDGDEQTPNQIGW	56
Falco_Peregrinus	----MGSTDGFQYRALYPYRKEREEDIDLLPGDILVVSKGALQALGFKDGDEQTPNQIGW	56

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Equus_Caballus	MPGFNERTRQRGDFPGTYVEFLGPVALARPGPRPRGPRPLPARPRDG-----PPEPGLT	110
Homo_Sapiens	MPGLNERTRQRGDFPGTYVEFLGPVALARPGPRPRGPRPLPARPRDG-----APEPGLT	110
Canis_Lupus_Familiaris	MPGLNERTRQRGDFPGTYVEFLGPVALARPGPRPRGPRPLPARPRDG-----PPEPGLT	110

Bos_Taurus	MPGLNERTQRGDFPGTYVEFLGPVALARPGPRPRGPRPLPARPRDG-----PPEPGLT	110
Sus_Scrofa	MPGLNERTQRGDFPGTYVEFLGPVALARPGPRPRGPRPLPARPRDG-----PPEPGLT	110
Taeniopygia_Guttata	ILGVNERTKQKGDFFPGTYVEYVGPVKIAVPSHRPRVQRPLPATPGASGCRLPEALEQGSP	116
Cuculus_Canorus	ILGVNERTKQKGDFFPGTYVEYVGPVKISVPSHRPRVQRPLPATPGASGCRLQEALEQGPP	112
Cheatura_Pelagica	ILGVNERTKQKGDFFPGTYVEYVGPVKISVPSHRPRVQRPLPATPGASGSRLQEPELEQGSP	116
Melopsittacus_Undulatus	ILGVNERTKQKGDFFPGTYVEYVGPVKIAVPSHRPRVQRPLPATPGAGGCRLPEALEQGSP	116
Anas_Platyrhynchos	ILGVNERTKQKGDFFPGTYVEYVGPVKISVPSHRPR-----GPGGCRLQEAPQEGSP	107
Columba_Livia	ILGVNERTKQKGDFFPGTYVEYVGPVKISVPSHRPRVQRPLPATPGAGGRLPEALEQASP	116
Lonchura_Striata_Domestica	ILGVNERTKQKGDFFPGTYVEYVGPVKIAVPSHRPRVQRPLPATPGASGCRLPEALEQGSP	116
Corvus_Cornix_Cornix	ILGVNERTKQKGDFFPGTYVEYVGPVKIAVPSHRPRVQRPLPATPGASGCRLPEALEQGSP	116
Corvus_Brachyrhynchos	ILGVNERTKQKGDFFPGTYVEYVRPVKIAVPSHRPRVQRPLPATPGASGCRLPEALEQGSP	112
Coturnix_Japonica	ILGVNERTKQKGDFFPGTYVEYVGPVKISVPSHRPRVQRPLPATPGASGCRLQEALEQGSP	120
Meleagris_Gallopavo	ILGVNERTKQKGDFFPGTYVEYVGPVKISVPSHRPRVQRPLPATPGASGCRLQEALEQGSP	116
Gallus_Gallus	ILGVNERTKQKGDFFPGTYVEYVGPVKISVPSHRPRVQRPLPATPGASGCRLQEALEQGSP	120
Haliaeetus_Leucocephalus	ILGVNERTKQKGDFFPGTYVEYVGPVKISVPSHRPRVQRPLPATPGASGCRLQDALEQGSP	116
Falco_Peregrinus	ILGVNERTKQKGDFFPGTYVEYVGPVKISVPSHRPRVQRPLPATPGASGCRLPEALEQGSP	116
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Equus_Caballus	LPDLPEQFSPPDAAPPILVKLVIAIEQTGLDGDLSYRPEP----PAPRTDWSLSDVEQWD	166
Homo_Sapiens	LPDLPEQFSPPDVAPPLLVKLVIAIERTGLDSESHYRPEL----PAPRTDWSLSDVDQWD	166
Canis_Lupus_Familiaris	LPDLPEQFSPPDVAPPLLVKLVIAIERTGLDSESLYRPEL----PAPRTDWSLSDVEQWD	166
Bos_Taurus	LPDLPEQFSPPDVAPPILVKLVIAIERTGLDS---YRPEP----PAVRTDWSLSDVEQWD	163
Sus_Scrofa	LPDLPEQFSPPDVAPPILVKLVIAIERTGLDS---YRPEP----PAARTDWSLSDVEQWD	163
Taeniopygia_Guttata	LPDLQEQQFSPEIAPPLLVKLVIAIEKKGLDSEMLYRTSGSELRQALRTDWTGLDLEPLD	176

Cuculus_Canorus	LPDRQEQFSPPEIAPPLLVKLV	EAEKKGLDSEMLYRTSGSELRQALRTDWT	LGDL	EHFD	172
Cheatura_Pelagica	LLDLQEQFSPPEIAPPLLVKLV	EAEKKGLDSEMLYRTSGSELRQALRTDWT	LGDL	EPFD	176
Melopsittacus_Undulatus	LLELQDQFSPPERAPPLLVKLV	EAEKKGV	DSEMLYRTSGSELRQALRTDWSL	AEELEPFD	176
Anas_Platyrhynchos	LPDLQEQFSPPEIAPPLLVKLV	EAEKKGLDSEMLYRTSGSELRQALRTDWSL	GDLEPFD		167
Columba_Livia	LPDLQDQFSPPELAPPLLVKLV	EAEKKGLDSEMLYRTSGSELRQALRTDWT	LGDL	EPFD	176
Lonchura_Striata_Domestica	LPDLQEQFSPPEIAPPLLVKLV	EAEKKGLDSEMLYRTSGSELRQALRTDWT	LGDL	EPLD	176
Corvus_Cornix_Cornix	LPDLQEQFSPPEVAPPLLVKLV	EAEKKGLDSEMLYRTSGSELRQALRTDWT	LGDL	EPLD	176
Corvus_Brachyrhynchos	LPDLQEQFSPPEVAPPLLVKLV	EAEKKGLDSEMLYRTSGSELRQALRTDWT	LGDL	EPLD	172
Coturnix_Japonica	LPDLQEQFSPPEVAPPLLVKLV	EAEKKGLDSEMLYRTSGSELRQALRTDWSL	GDLEPFD		180
Meleagris_Gallopavo	LPDLQEQFSPPEVAPPLLVKLV	EAEKKGLDSEMLYRTSGSELRQALRTDWSL	GDLEPFD		176
Gallus_Gallus	LPDLQEQFSPPEVAPPLLVKLV	EAEKKGLDSEMLYRTSGSELRQALRTDWSL	GDLEPFD		180
Haliaeetus_Leucocephalus	LPDLQEQFSPPEIAPPLLVKLV	EAEKKGLDSEMLYRTSGSELRQALRTDWT	LGDL	EPFD	176
Falco_Peregrinus	LPDLQEQFSPPEIAPPLLVKLV	EAEKKGLDSEMLYRTSGSELRQALRTDWT	LGDL	EPFD	176

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Equus_Caballus	AAALSDGVKGFL	LALPAPLVTPEAAAE	AHRALREAAGPV-----	GPAL	EPPTLPL	216
Homo_Sapiens	TAALADGIKSFL	LALPAPLVTPEASAE	ARRALREAAGPV-----	GPAL	EPPTLPL	216
Canis_Lupus_Familiaris	AAALTDGVKSFL	LALPAPLVTPEAAAE	ARRALREAAGPV-----	GPAL	EPPTLPL	216
Bos_Taurus	AAALSDGVKGFL	LALPAPLVTPEAAAE	AHRALREAAGPV-----	GPAL	EPPTLPL	213
Sus_Scrofa	AAALSDGVKGFL	LALPAPLVTPEAAAE	AHRALREAAGPV-----	GPAL	EPPTLPL	213
Taeniopygia_Guttata	VHSLGEALRGYLQD	LPSPVVPVSVHGDILRILQEIP	PARELP	EAAAG--	WPWSRLAVPL	233
Cuculus_Canorus	VHSLGEALRGYLQD	LPSPVVPVSVHGDILRVLQENPQP---	ENCRKQ	LLLALES	PAVPL	228
Cheatura_Pelagica	VHSLGEALRGYLQD	LPSPVVPVSVHGDILRVLQEIPQP---	ENCRKQ	LLLALES	PAVPL	232
Melopsittacus_Undulatus	VHSLCEALRGYLQD	LPSPVVPVAVHGDILRILQETPQP---	ESCRKQ	LLLALEPP	VVPL	232

Anas_Platyrhynchos	VHSLGEALRGYLQDLPSPPVVPVAVHGDILRVLQEVPQP----ENCRKQLLLALESPAVPL	223
Columba_Livia	VHSLGEALRGYLQDLPSPPVVPVAVHGDILRVLQEIPQP----ENCRKQLMLALESPAVPL	232
Lonchura_Striata_Domestica	VHSLGEALRGYLQDLPSPPVVPVSVHGDILRILQEIPQP----ENCRKQLLLALELSAVPL	232
Corvus_Cornix_Cornix	VHSLGEALRGYLQDLPSPPVVPVSVHGDILRILQEIPQP----ENCRKQLQLALESPAVPL	232
Corvus_Brachyrhynchos	VHSLGEALRGYLQDLPSPPVVPVSVHGDILRILQEIPQP----EKCKWQLQLALESPAVPL	228
Coturnix_Japonica	VHSLSETLRGYLQDLPSPPVVPVSVHGDILRILQEIPQP----ENCRKQLLLALESPAIPPL	236
Meleagris_Gallopavo	VHSLGEALRGYLQDLPSPPVVPVSVHGDILRILQEIPQP----ENCRKQLLLALESPAVPL	232
Gallus_Gallus	VHSLSEALRGYLQDLPSPPVVPVSVHGDILRILQEIPQP----ENCRKQLLLALESPAIPPL	236
Haliaeetus_Leucocephalus	VHSLGEALRGYLQDLPSPPVVPVSVHGDILRILQEIPQP----ENCRKQLLLALESPAVPL	232
Falco_Peregrinus	MHSLGEALRGYLQDLPSPPVVPVSVHGDILRILQEIPQP----ENCRKQLLLALESPAVPL	232
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Equus_Caballus	HRALTTLRFLQLHLGRLARRAPAPGPAVRALGAAGFPLLLRAPPP--SPPPGGAPDGTEPG	274
Homo_Sapiens	HRALTTLRFLQLHLGRVARRAPALGPAVRALGATFGPPLLLRAPPPSSPPPGGAPDGSEPS	276
Canis_Lupus_Familiaris	HRALTTLRFLQLHLGRVARRAPAPGPAVRALGATFGPPLLLRAPPP-P-SPPPGGAPDGTEPS	274
Bos_Taurus	HHALTLRFLQLHLGRVAGRAPAPGPAVRALGATFGPPLLLRAPPPP-SPPPGGAPDGTEPT	272
Sus_Scrofa	HRALTTLRFLQLHLGRVARRAPAPGPAVRALSATFGPPLLLRAPPPP-SPPPGGAPDGTEPI	272
Taeniopygia_Guttata	QNTLTQLQFLLRHLGKVSHQAESNSLHPRALGELFGPPLLLRLPGA-----SPELS	282
Cuculus_Canorus	QNTLTQLQFLLRHLGKVSQQAESNNLHPRALGEIFGPPLLLRLPGA-----R-EAD	276
Cheatura_Pelagica	QNTLTQLQFLLRHLGKVSQQAESNSLHPRALGEIFGPPLLLRLPGA-----SPELS	281
Melopsittacus_Undulatus	QNTLTQLQFLLRHLGKVSQQAESNGLHPRALAEIFGPPLLLRLPGA-----SPELS	281
Anas_Platyrhynchos	QNTLTQLQFLLRHLGKVSQQAESNNLHPRALGEIFGPPLLLRLPGA-----SPELS	272
Columba_Livia	QNTLTQLQFLLRHLGKVSQQAESNNLHPRALGEIFGPPLLLRLPGA-----SAELS	281
Lonchura_Striata_Domestica	QNMLTLQFLLRHLGKVSQQAESNNLHPRALGEIFGPPLLLRLPGA-----SPELS	281

Corvus_Cornix_Cornix	QNTLTQLQFLLRHLGKVSQQAESNSLHPRALGEIFGPLLLRLPGA-----SPELS	281
Corvus_Brachyrhynchos	QNTLTQLQFLLRHLGKVSQQAESNSLHPRALGEIFGPLLLRLPGA-----SPELS	277
Coturnix_Japonica	QNTLTQLQFLLRHLRKVSQQAESNSLHPQALGEIFGPLLLRLPGA-----SPELS	285
Meleagris_Gallopavo	QNTLTQLQFLLRHLGKVSQQAESNSLHPQALGEIFGPLLLRLPGA-----SPELS	281
Gallus_Gallus	QNTLTQLQFLLRHLGKVSQQAESNSLHPQALGEIFGPLLLRLPGA-----SPELS	285
Haliaeetus_Leucocephalus	QNTLTQLQFLLRHLGKVSQQAESNSLHPRALGEIFGPLLLRLPGA-----SPELS	281
Falco_Peregrinus	QNTLTQLQFLLRHLGKVSQQAESNSLHPRALGEIFGPLLLRLPGA-----SPELS	281
<pre> .. ***:***:** :: :* : . :**. ***** * </pre>		
Equus_Caballus	PDFPGLLVEKLLQEH-LEEQEVAPPALPPKPPKAKPAPTGVANGGSLPSLQDAEWYWGDI	333
Homo_Sapiens	PDFPALLVEKLLQEH-LEEQEVAPPALPPKPPKAKPAPTVLANGGSPPSLQDAEWYWGDI	335
Canis_Lupus_Familiaris	PDFPVLLVEKLLQEH-LEEQEVTPPALPPKPPKAKPAPAGLANGGSPPSLQDAEWYWGDI	333
Bos_Taurus	PDFPALLVEKLLQEH-LEEQEVAPPALPPKPPKTKPAPTGLANGGSPPSLQDAEWYWGDI	331
Sus_Scrofa	PDFPVLLMEKLLQEH-LEEQEVAPPALPPKPPKAKPAPT--ANGGSPPSLQDAEWYWGDI	329
Taeniopygia_Guttata	PDFSVLFLETLLQTK-ELEPEQLPPALPPKPPKPKPSAASLANGNNAP-LQDAEWYWGDI	340
Cuculus_Canorus	LVLRGITCKELGSLGLTLLQPPSLTALPPKPPKPKPSAASLANGNSVP-LQDAEWYWGDI	335
Cheatura_Pelagica	PDFSILFLEMLLQTK-ELEPEQLPPALPPKPPKPKPSAASLANGNSVP-LQDAEWYWGDI	339
Melopsittacus_Undulatus	PDFSILFLEMLLQTK-ESEPEQLPPALPPKPPXPKPSAASLANGNSVS-LQDAEWYWGDI	339
Anas_Platyrhynchos	PDFSVLFLEMLLQLK-ELEPEQLPPALPPKPPKPKPSAASLANGNSVP-LQDAEWYWGDI	330
Columba_Livia	PDFSVLFLEILLQTK-ELEPEQLPPALPPKPPKPKPSAASLANGNSVP-LQDAEWYWGDI	339
Lonchura_Striata_Domestica	PDFSVLFLETLLQTK-ELEPEQLPPALPPKPPKPKPSAASLANGNNAP-LQDAEWYWGDI	339
Corvus_Cornix_Cornix	PDFSVLFLETLLQAK-ELEPEQLPPALPPKPPKPKPSAASLANGNNAP-LQDAEWYWGDI	339
Corvus_Brachyrhynchos	PDFSVLFLETLLQAK-ELEPEQLPPALPPKPPKPKPSAASLANGNNAP-LQDAEWYWGDI	335
Coturnix_Japonica	PDFSVLFLEMLLQTK-ELEPEQLPPALPPKPPKPKPSAASLANGNSVP-LQDAEWYWGDI	343

Meleagris_Gallopavo	PDFSVLFLEMLLQTK-DLEPEQLPPALPPKPPKPKPSAASLANGNSVP-LQDAEWYWGDI	339
Gallus_Gallus	PDFSVLFLEMLLQTK-ELEPEQLPPALPPKPPKPKPSAASLANGNSVP-LQDAEWYWGDI	343
Haliaeetus_Leucocephalus	PDFSVLLLEILLQTK-ELEPEQLPPALPPKPPKPKPSAASLANGNSVP-LQDAEWYWGDI	339
Falco_Peregrinus	PDFSVLFLEILLQTK-ELEPEQLPPALPPKPPKPKPSAASLANGNSVS-LQDAEWYWGDI	339
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Equus_Caballus	SREEVNEKL RDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKVFHRDGHYGFSEPLTF	393
Homo_Sapiens	SREEVNEKL RDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKVFHRDGHYGFSEPLTF	395
Canis_Lupus_Familiaris	SREEVNEKL RDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKVFHRDGHYGFSEPLTF	393
Bos_Taurus	SREEVNEKL RDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKVFHRDGHYGFSEPLTF	391
Sus_Scrofa	SREEVNEKL RDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKVFHRDGHYGFSEPLTF	389
Taeniopygia_Guttata	SREEVNEKL RDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKIFHREGKYGFSEPLTF	400
Cuculus_Canorus	SREEVNEKL RDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKIFHREGKYGFSEPLTF	395
Cheatura_Pelagica	SREEVNEKL RDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKIFHREGKYGFSEPLTF	399
Melopsittacus_Undulatus	SREEVNEKL RDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKIFHREGKYGFSEPLTF	399
Anas_Platyrhynchos	SREEVNEKL RDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKIFHREGKYGFSEPLTF	390
Columba_Livia	SREEVNEKL RDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKIFHREGKYGFSEPLTF	399
Lonchura_Striata_Domestica	SREEVNEKL RDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKIFHREGKYGFSEPLTF	399
Corvus_Cornix_Cornix	SREEVNEKL RDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKIFHREGKYGFSEPLTF	399
Corvus_Brachyrhynchos	SREEVNEKL RDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKIFHREGKYGFSEPLTF	395
Coturnix_Japonica	SREEVNEKL RDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKIFHREGKYGFSEPLTF	403
Meleagris_Gallopavo	SREEVNEKL RDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKIFHREGKYGFSEPLTF	399
Gallus_Gallus	SREEVNEKL RDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKIFHREGKYGFSEPLTF	403
Haliaeetus_Leucocephalus	SREEVNEKL RDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKIFHREGKYGFSEPLTF	399

Falco_Peregrinus	SREEVNEKLRDTPDGTFLVRDASSKIQGEYTLTLRKGGNNKLIKIFHREGKYGFSEPLTF	399
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Equus_Caballus	GSVVDLITHYRHESLAQYNAKLDTRLLYPVSKYQQDQIVKEDSVEAVGAQLRVYHQYQD	453
Homo_Sapiens	CSVVDLINHYRHESLAQYNAKLDTRLLYPVSKYQQDQIVKEDSVEAVGAQLKVYHQYQD	455
Canis_Lupus_Familiaris	CSVVDLITHYRHESLAQYNAKLDTRLLYPVSKYQQDQIVKEDSVEAVGAQLKVYHQYQD	453
Bos_Taurus	CSVVDLITHYRHESLAQYNAKLDTRLLYPVSKYQQDQIVKEDSVEAVGAQLKVYHQYQD	451
Sus_Scrofa	CSVVDLITHYRHESLAQYNAKLDTRLLYPVSKYQQDQIVKEDSVEAVGAQLKVYHQYQD	449
Taeniopygia_Guttata	GSVVELITHYRHESLAQYNAKLDTRLLYPISKYQQDQVVKEDSVEAVGEQLKVYHQYQD	460
Cuculus_Canorus	GSVVELITHYRHESLAQYNAKLDTRLLYPISKYQQDQVVKEDSVEAVGEQLKVYHQYQD	455
Cheatura_Pelagica	GSVVELITHYRHESLAQYNAKLDTRLLYPISKYQQDQVVKEDSVEAVGEQLKVYHQYQD	459
Melopsittacus_Undulatus	SSVVELITHYRHESLAQYNAKLDTRLLYPISKYQQDQVVKEDSVEAVGEQLKVYHQYQD	459
Anas_Platyrhynchos	GSVVELITHYRHESLAQYNAKLDTRLLYPISKYQQDQVVKEDSVEAVGEQLKVYHQYQD	450
Columba_Livia	GSVVELITHYRHESLAQYNAKLDTRLLYPISKYQQDQVVKEDSVEAVGEQLKVYHQYQD	459
Lonchura_Striata_Domestica	GSVVELITHYRHESLAQYNAKLDTRLLYPISKYQQDQVVKEDSVEAVGEQLKVYHQYQD	459
Corvus_Cornix_Cornix	GSVVELITHYRHESLAQYNAKLDTRLLYPISKYQQDQVVKEDSVEAVGEQLKVYHQYQD	459
Corvus_Brachyrhynchos	GSVVELITHYRHESLAQYNAKLDTRLLYPISKYQQDQVVKEDSVEAVGEQLKVYHQYQD	455
Coturnix_Japonica	GSVVELITHYRHESLAQYNAKLDTRLLYPISKYQQDQVVKEDSVEAVGEQLKVYHQYQD	463
Meleagris_Gallopavo	GSVVELITHYRHESLAQYNAKLDTRLLYPISKYQQDQVVKEDSVEAVGEQLKVYHQYQD	459
Gallus_Gallus	GSVVELITHYRHESLAQYNAKLDTRLLYPISKYQQDQVVKEDSVEAVGEQLKVYHQYQD	463
Haliaeetus_Leucocephalus	GSVVELITHYRHESLAQYNAKLDTRLLYPISKYQQDQVVKEDSVEAVGEQLKVYHQYQD	459
Falco_Peregrinus	GSVVELITHYRHESLAQYNAKLDTRLLYPISKYQQDQVVKEDSVEAVGEQLKVYHQYQD	459
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Equus_Caballus	KSREYDQLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQGQTQEKCSKEYLERFRREGNE	513
Homo_Sapiens	KSREYDQLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQGQTQEKCSKEYLERFRREGNE	515
Canis_Lupus_Familiaris	KSREYDQLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQGQTQEKCSKEYLERFRREGNE	513
Bos_Taurus	KSREYDQLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQGQTQEKCSKEYLERFRREGNE	511
Sus_Scrofa	KSREYDQLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQGQTQEKCSKEYLERFRREGNE	509
Taeniopygia_Guttata	KSWEYDLLYEEYTRTSQVCGHCLRPRAALSPPECWQPPCYPREKCSKGVHERFRREGKE	520
Cuculus_Canorus	KSWEYDLLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQCQTQEKCSKEYIERFRREGNE	515
Cheatura_Pelagica	KSWEYDLLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQCQTQEKCSKEYIERFRRDGNE	519
Melopsittacus_Undulatus	KSWEYDLLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQCQTQEKCSKEYIERFRREGNE	519
Anas_Platyrhynchos	KSWEYDLLYEEYTRTS---QMKRTAIEAFNETIKIFEEQCQTQEKCSKEYIERFRREGNE	507
Columba_Livia	KSWEYDLLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQCQTQEKCSKEYIERFRREGNE	519
Lonchura_Striata_Domestica	KSWEYDLLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQCQTQEKCSKEYIERFRREGNE	519
Corvus_Cornix_Cornix	KSWEYDLLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQCQTQEKCSKEYIERFRREGNE	519
Corvus_Brachyrhynchos	KSWEYDLLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQCQTQEKCSKEYIERFRREGNE	515
Coturnix_Japonica	KSWEYDLLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQCQTQEKCSKEYIERFRREGNE	523
Meleagris_Gallopavo	KSWEYDLLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQCQTQEKCSKEYIERFRREGNE	519
Gallus_Gallus	KSWEYDLLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQCQTQEKCSKEYIERFRREGNE	523
Haliaeetus_Leucocephalus	KSWEYDLLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQCQTQEKCSKEYIERFRREGNE	519
Falco_Peregrinus	KSWEYDLLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQCQTQEKCSKEYIERFRREGNE	519
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Equus_Caballus	KEMQRILLNSERLKSRIAEIHESRTKLEQELRAQASDNREIDKRMNSLKPDLMLRKIRD	573
Homo_Sapiens	KEMQRILLNSERLKSRIAEIHESRTKLEQQLRAQASDNREIDKRMNSLKPDLMLRKIRD	575
Canis_Lupus_Familiaris	KEMQRILLNSERLKSRIAEIHESRTKLEQELRAQASDNREIDKRMNSLKPDLMLRKIRD	573

Bos_Taurus	KEMQRILLNSERLKSRIAEIHESRTKLEQELRAQASDNREIDKRMNSLKPDLMQLRKIRD	571
Sus_Scrofa	KEMQRILLNSERLKSRIAEIHESRTKLEQELRAQASDNREIDKRMNSLKPDLMQLRKIRD	569
Taeniopygia_Guttata	KEVQRILMNSEKLKSRITEIHDSKMKLEQDLKKQASENREIDKRMNSLKPDLMQLRKLIRD	580
Cuculus_Canorus	KEVQRILMNSEKLKSRITEIHDSKMKLEQDLKKQASENREIDKRMNSLKPDLMQLRKLIRD	575
Cheatura_Pelagica	KEVQRILMNSEKLKSRITEIHDSKMKLEQDLKKQASENREIDKRMNSLKPDLMQLRKLIRD	579
Melopsittacus_Undulatus	KEVQRILMNSEKLKSRITEIHDSKMKLEQDLKKQASENREIDKRMNSLKPDLMQLRKLIRD	579
Anas_Platyrhynchos	KEVQRILMNSEKLKSRITEIHDSKMKLEQDLKKQASENREIDKRMNSLKPDLMQLRKLIRD	567
Columba_Livia	KEVQRILMNSEKLKSRITEIHDSKMKLEQDLKKQASENREIDKRMNSLKPDLMQLRKLIRD	579
Lonchura_Striata_Domestica	KEVQRILMNSEKLKSRITEIHDSKMKLEQDLKKQASENREIDKRMNSLKPDLMQLRKLIRD	579
Corvus_Cornix_Cornix	KEVQRILMNSEKLKSRITEIHDSKMKLEQDLKKQASENREIDKRMNSLKPDLMQLRKLIRD	579
Corvus_Brachyrhynchos	KEVQRILMNSEKLKSRITEIHDSKMKLEQDLKKQASENREIDKRMNSLKPDLMQLRKLIRD	575
Coturnix_Japonica	KEVQRILMNSEKLKSRITEIHDSKMKLEQDLKKQASENREIDKRMNSLKPDLMQLRKLIRD	583
Meleagris_Gallopavo	KEVQRILMNSEKLKSRITEIHDSKMKLEQDLKKQASENREIDKRMNSLKPDLMQLRKLIRD	579
Gallus_Gallus	KEVQRILMNSEKLKSRITEIHDSKMKLEQDLKKQASENREIDKRMNSLKPDLMQLRKLIRD	583
Haliaeetus_Leucocephalus	KEVQRILMNSEKLKSRITEIHDSKMKLEQDLKKQASENREIDKRMNSLKPDLMQLRKLIRD	579
Falco_Peregrinus	KEVQRILMNSEKLKSRITEIHDSKMKLEQDLKKQASENREIDKRMNSLKPDLMQLRKLIRD	579

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Cuculus_Canorus	QYLVWLTQKGARQKKINEWLGIKNETE-----DQYSMMDDEEELPHHEERTWYVG	625
Cheatura_Pelagica	QYLVYVLPDPPPSTPLNMWGGLRAQIVGGPILPVLDCSQYSMMDDEEELPHHEERTWYVG	639
Melopsittacus_Undulatus	QYLVWLTQKGARQKKINEWLGIKNETE-----DQYSMMDDEEELPHHEERTWYVG	629
Anas_Platyrhynchos	QYLVWLTQKGARQKKINEWLGIKNETE-----DQYSMMDDEEELPHHEERTWYVG	617
Columba_Livia	QYLVWLTQKGARQKKINEWLGIKNETE-----DQYSMMDDEEELPHHEERTWYVG	629
Lonchura_Striata_Domestica	QYLVWLTQKGARQKKINEWLGIKNETE-----DQYSMMDDEEELPHHEERTWYVG	629
Corvus_Cornix_Cornix	QYLVWLTQKGARQKKINEWLGIKNETE-----DQYSMMDDEEELPHHEERTWYVG	629
Corvus_Brachyrhynchos	QYLVWLTQKGARQKKINEWLGIKNETE-----DQYSMMDDEEELPHHEERTWYVG	625
Coturnix_Japonica	QYLVWLTQKGARQKKINEWLGIKNETE-----DQYSMMDDEEELPHHEERTWYVG	633
Meleagris_Gallopavo	QYLVWLTQKGARQKKINEWLGIKNETE-----DQYSMMDDEEELPHHEERTWYVG	629
Gallus_Gallus	QYLVWLTQKGARQKKINEWLGIKNETE-----DQYSMMDDEEELPHHEERTWYVG	633
Haliaeetus_Leucocephalus	QYLVWLTQKGARQKKINEWLGIKNETE-----DQYSMMDDEEELPHHEERTWYVG	629
Falco_Peregrinus	QYLVWLTQKGARQKKINEWLGIKNETE-----DQYSMMDEEEELPHHEERTWYVG	629
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Equus_Caballus	KINRTQAEEMLSGKRDGTFLIRESSQRCYACSVVVDGDTKHCVIYRTATGFGFAEPYNL	683
Homo_Sapiens	KINRTQAEEMLSGKRDGTFLIRESSQRCYACSVVVDGDTKHCVIYRTATGFGFAEPYNL	685
Canis_Lupus_Familiaris	KINRTQAEEMLSGKRDGTFLIRESSQRCYACSVVVDGDTKHCVIYRTATGFGFAEPYNL	683
Bos_Taurus	KINRTQAEEMLSGKRDGTFLIRESSQRCYACSVVVDGDTKHCVIYRTATGFGFAEPYNL	681
Sus_Scrofa	KINRTQAEEMLSGKRDGTFLIRESSQRCYACSVVVDGDTKHCVIYRTATGFGFAEPYNL	679
Taeniopygia_Guttata	KINRVQAEEMLCGKRDGTFLIRESSQKGCYACSVVVDGDTKHCVIYKTATGYGFAEPYNL	690
Cuculus_Canorus	KINRLQAEEMLCGKRDGTFLIRESSQKGCYACSVVVDGDTKHCVIYKTATGYGFAEPYNL	685
Cheatura_Pelagica	KINRLQAEEMLCGKRDGTFLIRESSQKGCYACSVVVDGDTKHCVIYKTATGYGFAEPYNL	699
Melopsittacus_Undulatus	KINRLQAEEMLCGKRDGTFLIRESSQKGCYACSVVVDGDTKHCVIYKTATGYGFAEPYNL	689

Anas_Platyrhynchos	KINRLQAEEMLCGKRDGTFLIRESSQKGCYACSVVVDGDTKHCVIYKTASGYGFAEPYNL	677
Columba_Livia	KINRLQAEEMLCGKRDGTFLIRESSQKGCYACSVVVDGDTKHCVIYKTATGYGFAEPYNL	689
Lonchura_Striata_Domestica	KINRVQAEEMLCGKRDGTFLIRESSQKGCYACSVVVDGDTKHCVIYKTATGYGFAEPYNL	689
Corvus_Cornix_Cornix	KINRVQAEEMLCGKRDGTFLIRESSQKGCYACSVVVDGDTKHCVIYKTATGYGFAEPYNL	689
Corvus_Brachyrhynchos	KINRVQAEEMLCGKRDGTFLIRESSQKGCYACSVVVDGDTKHCVIYKTATGYGFAEPYNL	685
Coturnix_Japonica	KINRLQAEEMLCGKRDGTFLIRESSQKGCYACSVVVDGDTKHCVIYKTATGYGFAEPYNL	693
Meleagris_Gallopavo	KINRLQAEEMLCGKRDGTFLIRESSQKGCYACSVVVDGDTKHCVIYKTATGYGFAEPYNL	689
Gallus_Gallus	KINRLQAEEMLCGKRDGTFLIRESSQKGCYACSVVVDGDTKHCVIYKTATGYGFAEPYNL	693
Haliaeetus_Leucocephalus	KINRLQAEEMLCGKRDGTFLIRESSQKGCYACSVVVDGDTKHCVIYKTATGYGFAEPYNL	689
Falco_Peregrinus	KINRLQAEEMLCGKRDGTFLIRESSQKGCYACSVVVDGDTKHCVIYKTATGYGFAEPYNL	689
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Equus_Caballus	YGSLRELVLHYQHTSLQHNDAVTLAHPVRAPAPAPAR---	723
Homo_Sapiens	YGSLKELVLHYQHASLVQHNDALTVTLAHPVRAPGPGPPPAAR	728
Canis_Lupus_Familiaris	YGSLKELVLHYQHASLVQHNDALTVTLAHPVRAPGPGPPPATR	726
Bos_Taurus	YGSLKELVLHYQHASLVQHNDALTVTLAHPVRAPGPGPPPAAR	724
Sus_Scrofa	YGSLKELVLHYQHASLVQHNDALTVTLAHPVRAPGPGPPPAAR	722
Taeniopygia_Guttata	YASLKDLVLHYKHTSLVQHNDLSLNVTLAHPVLSQPPA-----	727
Cuculus_Canorus	YASLKDLVLHYKHTSLVQHNDLSLNVTLAHPV-----	716
Cheatura_Pelagica	YASLKDLVLHYKHTSLVQHNDLSLNVTLAHPVLSQPPA-----	736
Melopsittacus_Undulatus	YASLKDLVLHYKHTSLVQHNDLSLNVTLAHPVLSQPPA-----	726
Anas_Platyrhynchos	YASLKDLVLHYKHTSLVQHNDLSLNVTLAHPVLSQPPAR-----	715
Columba_Livia	YASLKDLVLHYKHTSLVQHNDLSLNVTLAHPVLSQPPAR-----	727
Lonchura_Striata_Domestica	YASLKDLVLHYKHTSLVQHNDLSLNVTLAHPVLSQPPAR-----	727

Corvus_Cornix_Cornix	YASLKDLVLHYKHTSLVQHNDSLNVTLAHPVLSQPPA-----	726
Corvus_Brachyrhynchos	YASLKDLVLHYKHTSLVQHNDSLNVTLAHPV-----	716
Coturnix_Japonica	YASLKDLVLHYKHTSLVQHNDSLNVTLAHPVLSQPPAR-----	731
Meleagris_Gallopavo	YASLKDLVLHYKHTSLVQHNDSLNVTLAHPVLSQPPA-----	726
Gallus_Gallus	YASLKDLVLHYKHTSLVQHNDSLNVTLAHPVLSQPPAR-----	731
Haliaeetus_Leucocephalus	YASLKDLVLHYKHTSLVQHNDSLNVTLAHPVLSQPPA-----	726
Falco_Peregrinus	YASLKDLVLHYKHTSLVQHNDSLNVTLAHPVLSQPPA-----	726
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