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Use of pseudotyped viruses to study H3 subtype influenza A viruses circulating in the wild duck reservoir

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Abstract

The Mallard (*Anas platyrhynchos*) is the world's most numerous wild duck species and is an important reservoir species for influenza A viruses (IAV). Wildfowl maintain the largest genetic variance of IAV subtypes H1-H16 and N1–N9. The H3 subtype is the most frequently observed low pathogenicity avian influenza (LPAI) and is antigenically and genetically diverse, with the capability to infect mammalian and avian hosts. Previous human IAV pandemics are thought to have originated from avian lineage viruses and are therefore important to monitor. Surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA) are crucial components in interspecies transmission. Current literature on H3 N1–N9 subtypes and the Global Initiative on Sharing All Influenza Data (GISAID) database were examined to determine protein compatibility and to explore the phylogenetic relationship between isolates. Results identified H3N8 as the most frequently observed subtype, H3N2 and H3N6 equally have high prevalence amongst literature and the sequence dataset. All other subtypes were observed at a lower frequency. The phylogeny of the H3 and N1–N9 isolates revealed two clades (Eurasian and North American) which coincided with published studies on IAV lineages. Subsequently, the use of pseudotyped viruses (PVs) to examine the compatibility of the surface proteins and further explore their potential use in serological assays were undertaken. An A/equine/Sussex/1989 (H3N8) PV was generated and tested in conjunction with previously generated PVs of the H3 subtype. The results of MUNANA neuraminidase activity assays concurred with an earlier report, i.e. that the assay was insufficiently sensitive for measuring NA activity in PVs. The pseudotype-based enzyme linked lectin assay (ELLA) was adopted and provided promising results, however demonstrated the need for greater optimisation of PV dilutions to ensure that a suitable curve is generated. The ELLA identified the potential application of a PV generated containing vesicular stomatitis virus envelope glycoprotein (VSV-G) as a replacement for HA, in conjunction with N8 A/equine/Sussex/1989, highlighting the possibility of using VSV-G with any NA combination to measure NA activity and inhibition. Further studies to replicate results are required.

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List of Abbreviations

AI	Avian influenza
D10	DMEM with 10% fetal calf serum
HPAI	Highly pathogenic avian influenza
IAV	Influenza A virus
LPAI	Low pathogenicity avian influenza
PBS	Phosphate buffered saline
PV	Pseudotyped virus

1. Introduction

1.1. Classification

Avian influenza A virus (IAV) belongs to the *Orthomyxoviridae* family of segmented negative-sense RNA viruses. *Orthomyxoviridae* contains Thogotovirus, Isavirus and four types of influenza, A, B, C, and the recently emerged influenza D (Zhai *et al.*, 2017). Influenza type A is considered important as it possesses the ability to infect various avian and mammalian species, and is the most widespread of the influenza subtypes (Chatziprodromidou *et al.*, 2018). Influenza A viruses have two primary mechanisms to enable genetic diversity: genetic reassortment and a high mutation rate (Mostafa *et al.*, 2018). The genome consists of eight segments that are readily available to exchange genetic material when two different viruses replicate in a co-infected cell. This reassortment, coupled with genetic drift are the underpinning mechanisms that facilitate the quick evolution of influenza viruses (Tao, Steel and Lowen, 2014). Due to the ability to rapidly evolve, IAV is considered a significant zoonotic risk. Previous human influenza pandemics are thought to have avian origins (McFee, 2007). The continued study of avian IAV is vital to understand the mechanisms that facilitate reassortment, which may signify a threat to human, livestock and wildlife health.

Influenza A viruses are most commonly divided into subtypes based on the two major surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA). In addition, avian influenza (AI) viruses can be categorised into highly pathogenic avian influenza (HPAI) and low pathogenicity avian influenza (LPAI) viruses (Mostafa *et al.*, 2018). LPAI viruses have the potential to mutate into HPAI viruses through acquisition of multiple basic amino acids in the cleavage site of the HA protein either as a result of genetic drift or possibly also through recombination with another gene segment or host genome (Xu *et al.*, 2017). Due to the nature of HPAI viruses, the potential mortality loss to the poultry industry is substantial, in addition to

significant economic impact and difficulty maintaining control/preventing further spreading of the virus (Xu *et al.*, 2017).

1.2. Transmission

The evolution of IAV is somewhat reliant on interspecies transmission; it is evident that some viral combinations circulate in certain taxa whilst spilling over into other species. Figure 1 details the suspected transmission routes of the haemagglutinin subtypes. There are 16 HA and 9 NA subtypes that can theoretically occur in 144 different combinations, most of which have been identified in aquatic birds (Monamele *et al.*, 2017).

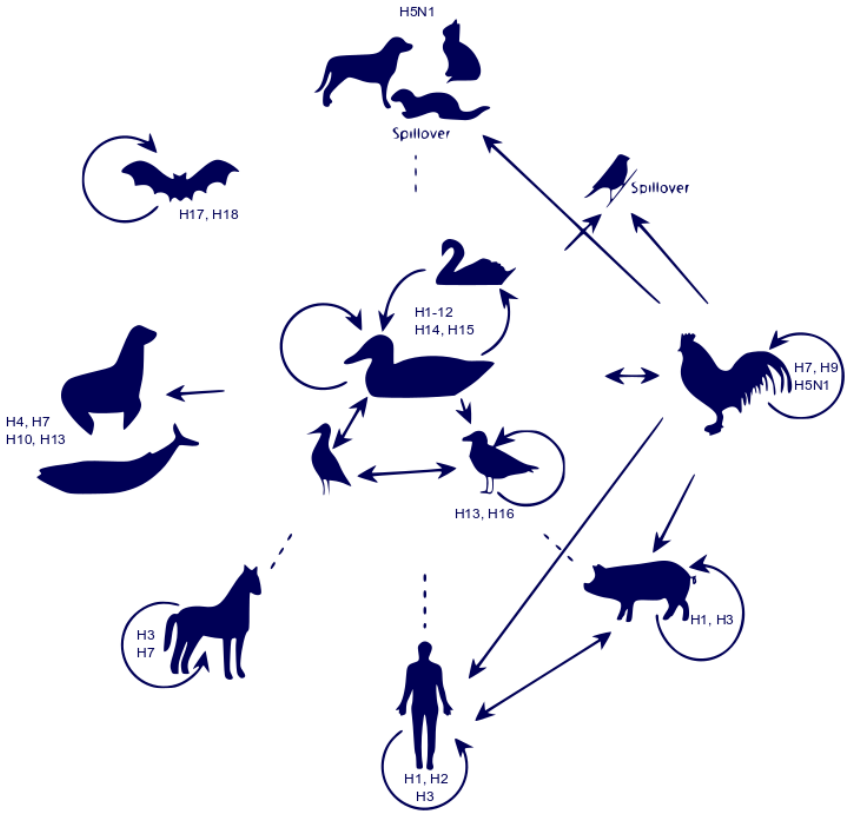


Figure 1: Transmission routes of IAV: image details how IAV may "jump" between species and spill over into other taxonomic groups (modified from Wille et al., 2017)

A further two subtype combinations (H17N10 and H18N11) have recently been identified in bats in South America (Tong *et al.*, 2012, 2013), but how they fit in with the overall ecology of

IAV is unclear at present. Despite spill over, transmission from a natural IAV host to a new host species does not usually result in viral establishment within the new species (Song *et al.*, 2008). However, there have been occurrences where spill over has occurred and establishment within a new host species has been observed. The domestic dog (*Canis lupus familiaris*) has become a new host species within the last 20 years. (Zhu, Hughes and Murcia, 2015). In recent years, an H3N8 subtype IAV became established within canine populations within the US; phylogenetic analysis concluded this strain of canine IAV is of equine origin (Crawford *et al.*, 2005; Dalziel *et al.*, 2014). H3N2 canine IAV has also established circulation within South East Asia. First reported in South Korea it has continued to spread across the continent. This strain is considered to be of avian origin (Zhu, Hughes and Murcia, 2015) and it has spread to the US.

Host specificity is an important factor in viral transmission and is somewhat dependent on the host cell receptor sialic acid. The most common linkages are the α-2,3 and α-2,6 and influenza viruses have strong preferences for either of these sialic acids (Mostafa *et al.*, 2018). Typically α-2,6 linkage is observed in the human upper respiratory tract, while α-2,3 linkage resides in the gastrointestinal tract in avian species (Gagneux *et al.*, 2003). A common theory in the influenza community is that species containing multiple sialic acid receptor types have the potential to act as a "mixing vessel" between different types of influenza (Cong *et al.*, 2010). The domestic pig *(Sus domesticus)* has a high expression of both α-2,3 and α-2,6 linkage and could potentially be simultaneously infected with each applicable virus strain (Cong *et al.*, 2010). The concern is that this would facilitate novel reassortment into a new virus with genes adapted for pig or human replication (Cong *et al.*, 2010). Multiple emergences of canine IAV with both mammalian and avian origin is of great concern and has substantial implications (Daly *et al.*, 2008).

1.3. Influenza A virus morphology

Influenza A viruses are often spherical or filamentous in shape. The virus genome contains eight separate negative-sense RNA segments that encode eight structural proteins (Figure 2) and at least four additional proteins (Calder *et al.*, 2010). Externally, the virus is enveloped in a lipid membrane, studded with glycoprotein spikes of haemagglutinin (HA) and neuraminidase (NA) proteins (Palese and Bouvier, 2008). Located between the lipid layer and internal virion are the matrix 2 (M2) proteins. The M2 proteins act as ion channels and are responsible for triggering the viral uncoating (Mostafa *et al.*, 2018).

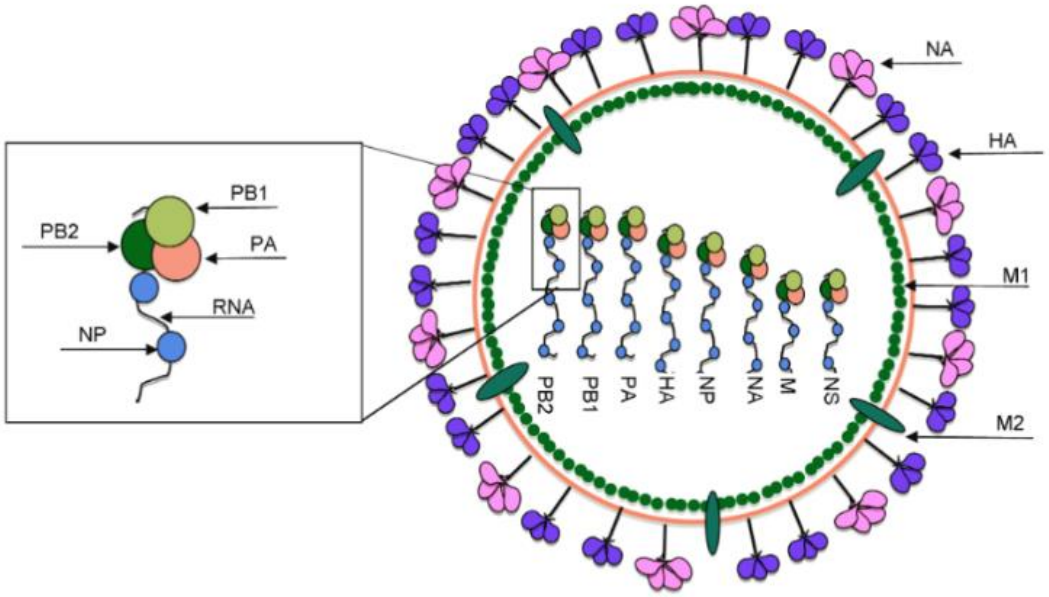


Figure 2: Structure of the influenza A virus, demonstrating the eight gene segments in the centre, surface proteins NA, HA, M1 structural layer and M2 ion channel. Image modified from Grant et al., (2014)

Inside the envelope is a layer of Matrix 1 (M1) protein, this layer is considered to give the virus structure and is the most abundant protein within the virus (Calder *et al.*, 2010). The M1 protein surrounds the eight ribonucleoprotein (RNP) structures. Each RNP consists of one RNA segment covered in nucleoprotein (NP) and is associated with three polymerase proteins,

composed of the acidic polymerase (PA), basic polymerase 1 (PB1) and basic polymerase 2 (PB2) which are necessary for viral replication (Murphy, 1999). Two additional non-structural proteins are produced by the virus RNA - interferon-antagonist NS1 protein and the NEP/NS2, which is involved in viral RNP export from the host cell nucleus (Palese and Bouvier, 2008).

1.4. Influenza A virus replication cycle

Viral attachment occurs, resulting in IAV binding to the host cell. This is dependent on the preferential sialic acid binding of the virus strain by the HA as described above. The HA is synthesised as a precursor (HA0), which must be cleaved into HA1 and HA2 for the virus to be infectious (Lim *et al.*, 2019)[SC1]. HA containing a monobasic cleavage site (e.g. HA of an LPAI virus) is cleaved by a protease called TMPRSS2 in the Golgi apparatus or HAT at the cell surface (either during attachment and entry or assembly and budding of new virus). In the case of an HPAI virus, the HA0 is cleaved in the Golgi apparatus by furin. HA1 is considered to contain the receptor binding and antigenic sites, while HA2 is thought to meditate the fusion of the viral envelope (Palese and Bouvier, 2008).

Following the viral attachment to sialic acid, the virion is endocytosed into an endosome. Low pH triggers a structural change in the HA within the endosome, exposing a fusion peptide that allows the merge of the endosomal membrane and the viral envelope. This consequently opens a pore through which the IAV RNPs are expelled into the host cell cytoplasm. Once detachment has taken place, the RNPs are trafficked to the host cell nucleus, here synthesis of viral RNA occurs (Figure 3). The negative-sense RNA is copied into positive-sense polyadenylated messenger RNA (mRNA) and is actioned by the polymerase complex, including PB1, PB2, PA and NP. The IAV uses the host proteins to begin the mRNA synthesis, including RNA polymerase ii, using the PB2 protein to cap snatch the required 5′ primer. The positive-sense mRNA migrates from the nucleus to the cytoplasm to initiate viral protein translation. The positive-sense full length RNA (cRNA) serves as a template for the synthesis

of the negative-sense viral RNA (vRNA) for virion replication. Furthermore, the two proteins, M1 and NEP/NS2 are imperative in trafficking the viral proteins in and out of the nucleus. The HA, NA and M2 proteins are synthesised, entering the endoplasmic reticulum, where they are folded and glycosylated. Once all the vRNA and proteins are synthesised and assembled at the host cell surface, the virion reaches the budding and release stage. This requires the NA to cleave the virus away from the host sialic acid (Noda, 2012).

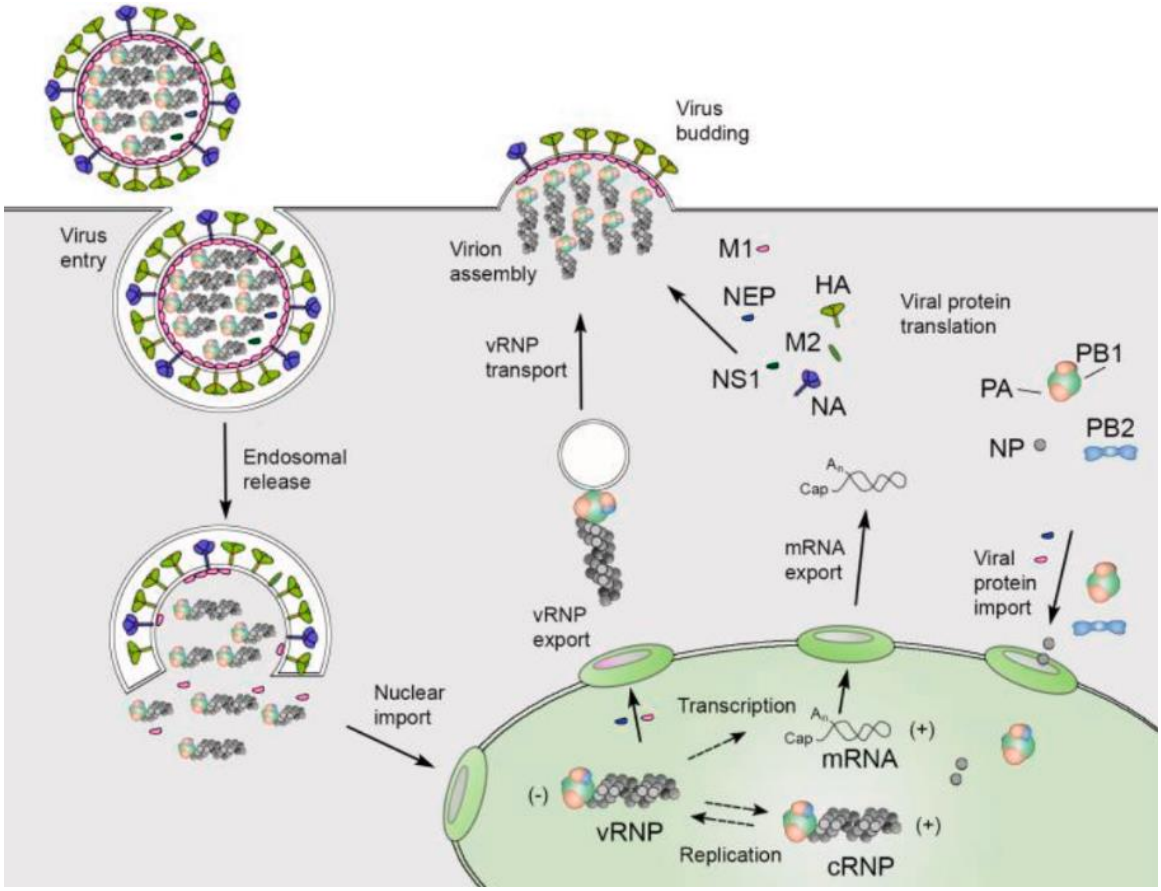


Figure 3: Influenza A virus replication cycle, detailed view of host entry and synthesis of viral RNA, followed by budding. Image modified from Te Velthuis and Fodor (2016).

1.5. Prevalence of the H3 subtype

Some subtypes and strains of IAV evolve and adapt at faster rates than others, this could be why some strains are more often seen in multiple species and more frequently. The H3 subtype of IAV is both antigenically and genetically diverse, and can infect various mammalian hosts including sea mammals and swine, canine and equine hosts, in addition to many avian taxa (Bailey *et al.*, 2016). Waterfowl species are considered to be the major natural reservoir for IAV, particularly those from the Anseriformes order (ducks, geese, and swans) and the Charadriiformes order (gulls, terns, and waders) (Bailey *et al.*, 2016). The mallard *(Anas platyrhynchos)*, is thought to be the major natural reservoir or spill over species for LPAI viruses. It is also considered to be the location where the largest genetic and antigenic variations occur (Wille, Latorre-Margalef and Waldenström, 2017). The H3 subtype is the most predominant within LPAI and equally they possess a similar pattern of seasonal variation as the human H3 subtype (Peng *et al.*, 2011). As some H3 outbreaks are known to emerge from avian lineage and H3 subtypes are often observed circulating within avian species, it is evident the requirement for surveillance is high. To explore the relationship of the H3 subtype and their associated NA surface protein, an examination of current literature of each subtype was undertaken to assess the compatibility of the surface proteins and consider the evidence for occurrence of interspecies transmissibility of each subtype.

1.5.1. H3N1

H3N1 in recent decades has made an appearance, reported as a novel reassortant between the H3N2 and H1N1 subtypes (Lekcharoensuk *et al.*, 2006; Shin *et al.*, 2006; Moreno *et al.*, 2009). H3N1 is primarily considered to be a swine IAV, containing reassortants that have acquired the haemagglutinin gene from an H3 human-like virus (Shin *et al.*, 2006). However, research by Song *et al*. (2012) signified the reassortant between pandemic H1N1 and canine H3N2 influenza viruses in Korea. Phylogenetic analysis of the H3N1 canine IAV revealed that the HA and NA proteins were related to those of avian origin H3N2 canine IAV currently

circulating in Asia and pandemic H1N1 virus strains, respectively. The significant changes with this reassortant enabled H3N1 to circulate with a lack of clinical signs in dogs, unlike the related H3N2 canine IAV (Song *et al.*, 2012). In more recent findings, novel H3N1 has been shown to replicate successfully in avian and mammalian cells and showed enhanced replication in human lungs cells (Thontiravong *et al.*, 2017).

1.5.2. H3N2

H3N2 is a well-known subtype of IAV, particularly prolific in interspecies transmission and has mutated into many strains. H3N2 is a subtype known to circulate in the human population. Internationally, prevalence is often dictated by seasonality and is responsible for human influenza epidemics (Yang *et al.*, 2017). H3N2 is also a global subtype of swine influenza with a high capacity to mutate. Triple reassortants of human, swine, and avian influenza viruses (H1N2, H3N2, H1N1) are endemic in the swine populations of America and Korea and has caused significant respiratory illness in the affected animals (Kim *et al.*, 2013). As already mentioned, H3N2 has become established in dog populations in south east Asia and has recently been detected in the USA (Zhu, Hughes and Murcia, 2015; Voorhees *et al.*, 2017). In addition to the recent establishment of canine IAV in the US, it has been detected in shelter cats in the US (Jeoung *et al.*, 2013). In 2010, H3N2 had also transmitted to cats in South Korea (Song *et al.*, 2011; Park *et al.*, 2013). It would appear H3N2 surface proteins are highly compatible and H3N2 viruses can easily exchange genetic information to facilitate multiple reassortants.

1.5.3. H3N3

Subtype H3N3 has been observed from as early as 1978, it is clear from observation this subtype has been found in swine, poultry, waterfowl, wild birds and marine mammals (Markwell and Shortridge, 1982; Callan *et al.*, 1995; Liu *et al.*, 2003; Karasin *et al.*, 2004; Groth *et al.*, 2014). It is reported to have evolved from avian origin virus in other species (Karasin *et*

al., 2004; Groth *et al.*, 2014). Cross-species transmission is evident and human infection has been reported for this subtype, displaying respiratory failure and other severe clinical signs (Zeb and Alegria, 2014). This information suggests the subtype's HA and NA proteins are compatible but possibly to a lesser extent than is observed in other subtypes such as H3N2 and H3N8.

1.5.4. H3N4

This subtype is infrequently observed. According to the information available on the influenza research database (GISAID), there are seven recorded strains, all of which were from waterfowl, primarily mallards, and geographically have originated in America, with one occurrence in Sweden (Elbe and Buckland-Merrett, 2017). This is the subtype of choice being used as a vaccine by turkey breeders in America, however literature suggests this may not be effective at preventing infection (Yassine *et al.*, 2008). It is clear this subtype is present in waterfowl, however there is a lack of evidence supporting cross species transmission and the subtype is rarely reported. This could suggest a lack of HA/NA compatibility or ability to swap genetic material.

1.5.5. H3N5

H3N5 is an avian IAV that has been recorded in avian waterfowl species throughout America, Europe and Asia (Gronesova *et al.*, 2008; Bui *et al.*, 2011; Girard *et al.*, 2012). In a 2008 study by Gronesova *et al,* out of their 19 samples taken from waterfowl and terrestrial birds in the Parizske Wetlands area of Eastern Slovakia, half contained the H3N5 subtype, all of these samples were found in Passerine species that have an association with wetland areas. Currently, there are no records of H3N5 subtype occurring outside of avian species.

1.5.6. H3N6

Current literature on subtype H3N6 suggests it is primarily an avian virus and is frequently recorded in wildfowl species (Li *et al.*, 2013). There is much concern related to this subtype, it has been successfully experimentally replicated in wild caught house mice (*Mus musculus*) (Shriner *et al.*, 2012), and has naturally occurred in species belonging to the order Pelecaniformes, without any obvious clinical signs (Simulundu *et al.*, 2009). Recent phylogenetic analysis of a novel H3N6 reassortant suggest it was derived from H5N6, this subtype has possible zoonotic potential for human infection (Liu *et al.*, 2015; Li *et al.*, 2016). Recent studies suggest the importance of increasing surveillance of H3N6 due to this potential reassortant risk. Although this subtype currently is not frequently observed in other species, it evidently possesses the potential for transmission to other species and appears highly successful in avian species.

1.5.7. H3N7

There is limited literature on subtype H3N7. According to phylogenetic analysis this subtype is of avian lineage and is considered to be of North American/USA origin (Okuya *et al.*, 2015). In 2008 Wilcox *et al*. (2011) found 2 instances of H3N7 infection, out of 438 birds sampled that season, samples came from Agassiz National Wildlife Refuge study site in the USA. Another study reported an avian isolate of H3N7 was retrieved from Toksook Bay, Alaska (Ramey *et al.*, 2016). It appears to be circulating in America however, to a lesser extent than other subtypes.

1.5.8. H3N8

We have substantial knowledge relating to subtype H3N8. This particular subtype is known to infect multiple species and has been able to establish circulation within them. The H3N8 virus has long been a causative subtype of equine influenza and is well established within equine populations. Similar to human influenza, equine IAV causes respiratory disease in equids, and

historical literature suggests equine IAV has been circulating in equine populations for centuries (Morens and Taubenberger, 2010). In addition, equine H3N8 IAV is considered to be the likely source of the 1889 human pandemic (Xie *et al.*, 2016). An initial transcontinental pandemic of equine H3N8 IAV surfaced in Florida in 1963, transferring to Europe by 1965, and was frequently observed in immunologically naïve animals. Later outbreaks and epidemics have continued to occur, even in areas with high vaccination uptake (Murcia, Wood and Holmes, 2011). Equine H3N8 IAV appears to be highly adaptable to mammalian species, observed in dogs in the UK (Daly *et al.*, 2008) and Australia (Kirkland *et al.*, 2010), pigs (Tu *et al.*, 2009), camels (Yondon *et al.*, 2014) and cats (Su *et al.*, 2014). Furthermore, recent transmission to canines (as already mentioned) has resulted in canine IAV of equine lineage (Crawford *et al.*, 2005). Studies comparing the relatedness and biological properties of canine and equine H3N8 IAV have demonstrated that there are subtle changes in the virus biology, however many of the characteristics/traits and functions remain the same (Feng *et al.*, 2015). Phylogenetic analysis of canine IAV in the US suggested the evolution of canine H3N8 IAV into two separate linages, based on geographical location (Pecoraro *et al.*, 2014). The rapid evolution of canine IAV is concerning, particularly in the US where both H3N2 and H3N8 may be circulating in dogs. Vaccines have been developed and continue to be adapted, in an effort to control the viral progression (Nogales *et al.*, 2017).

Avian H3N8 IAV is also well established intercontinentally within reservoir populations of waterfowl. H3N8 of avian origin has caused significant respiratory disease in seals and most recent outbreaks have caused high mortality in these populations (Anthony *et al.*, 2012). Most concerningly, analysis of avian-like H3N8, obtained from the most recent outbreak in seals, has confirmed adaptation of the virus, binding to the α-2,6 sialic acid receptor (Anthony *et al.*, 2012; Karlsson *et al.*, 2014). Furthermore, experimental infection of H3N8 virus of various lineages in pigs has produced some intriguing results. Not only did the avian and seal isolates cause severe respiratory disease within pigs, it also replicated substantially, with no detectable antibodies against haemagglutinin (Solórzano *et al.*, 2015). Moreover, this lack of

immunological protection was also exhibited with experimentally-infected mice (Solórzano *et al.*, 2015). Thus, this virus appears to have an increased likelihood of cross-species transmission.

1.5.9. H3N9

As with H3N7, there is very little literature on the appearance of H3N9. This subtype is of avian origin and has been found in Asia and Canada (Wan, 2012; Krauss *et al.*, 2013).

1.6. Wild birds in avian influenza ecology

Wild birds are thought to play an important role in the evolution of influenza ecology, particularly the mallard *(Anas platyrhynchos).* Wild aquatic bird populations sustain the largest genetic variance of IAV subtypes and are considered a natural host reservoir for IAV. A combination of ideal ecological and biological factors have enabled IAV to exist within species belonging to the taxonomic groups of Anseriformes and Charadriiformes, with occasional spill over into other groups such as Passeriformes (Wille, Latorre-Margalef and Waldenström, 2017). Dugan *et al* (2018) described the evolutionary changes in avian AIV as having “genome constellations” that are continually reshuffled by reassortment in contrast to the limited number of stable genome constellations that characterizes the evolution of mammalian-adapted IAV. The extensive geographic spread of potential hosts coupled with migratory behaviour are potential contributory factors facilitating the continual reshuffling of the avian IAV genomic information and intercontinental distribution. It is thought that the global mallard population is approximately 19 million individuals and they are thought to be thriving as a result of successful introductions to New Zealand, Australia, Peru, Brazil, Uruguay, Argentina, Chile, the Falkland Islands, South Africa and Hawaii (Wille, Latorre-Margalef and Waldenström, 2017). Occupying almost every water-based habitat in the world, with the exception of Antarctica, it is evident the species is highly efficient at adaptation, much like IAV. Interestingly, mallards are seemingly unaffected by the viral burden, most individuals within these

populations show few clinical signs of LPAI infection (Wille, Latorre-Margalef and Waldenström, 2017). However, almost every combination of HA and NA currently known has been identified in mallards. Table 1 demonstrates common combinations.

Table 1: Haemagglutinin and Neuraminidase subtypes.The frequency of IAV observed in mallards at Ottenby Bird Observatory in Sweden, 2002–2009. Green highlight over H3 subtype represents those (modified from Latorre-Margalef et al., 2014).

Haemagglutinin	Neuraminidase									
	N1	N2	N3	N4	N5	N6	N7	N8	N9	total
H1	94	6	5	0	1	4	0	1	0	16.6% (111)
H2	6	5	58	0	2	1	1	6	7	4.2% (28)
H3	2	14	2	1	1	13	0	32	0	9.7% (65)
H4	6	35	5	1	5	198	1	2	1	38% (254)
H5	1	53	27	0	0	1	0	0	8	13.5% (90)
H6	9	53	6	2	14	3	0	8	1	14.4% (96)
H7	0	2	1	1	0	3	32	0	1	1.2% (8)
H8	1	1	0	12	0	0	0	0	0	2.1% (14)
H9	1	5	0	0	0	0	0	0	1	0.3% (2)
total	18% (120)	25.3% (169)	6.9% (46)	2.5% (17)	3.4% (23)	33.4% (223)	0.3% (2)	7.3% (49)	2.8% (19)	

Several questions arise, including why are mallards the ideal hosts for LPAI viruses? Why do they generally not show any clinical signs of disease? What role does mallard ecology play in the transmission of IAV? To fully understand how avian IAV evolves and sustains the ecological niche within the global mallard population, consideration must be given to several factors including the host's life cycle, behaviour, environment and immune function enabling avian IAV to persist.

1.6.1. Influenza A virus circulation in mallards

The main route of LPAI transmission in waterfowl is believed to be the faecal-oral route, bird-to-bird or from the environment such as water contamination and shared feeding sites. This occurs frequently, although the prevalence of IAV in mallards follows a seasonal pattern; it peaks during the autumn migration and has low prevalence during late winter, spring and summer months (Latorre-Margalef *et al.*, 2014). There are several events or factors that result in an increase in the prevalence of IAV in ducks. The extrinsic factors are often related to, but not exclusive to, migratory, moulting, foraging and reproductive behaviour. Figure 4

demonstrates intrinsic and extrinsic factors, and how they directly and indirectly influence IAV transmission.

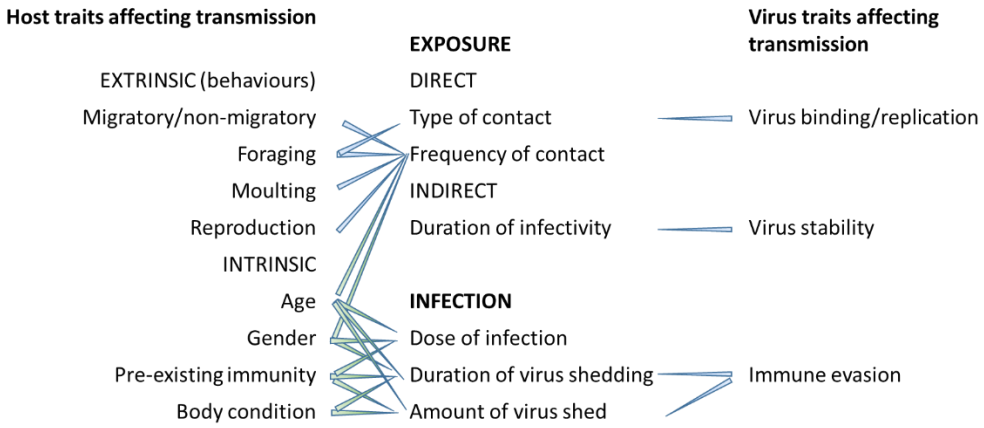


Figure 4: Host and virus traits: exposure and susceptibility to low pathogenic influenza A virus, modified from van Dijk et al. (2018)

Mallard migration has great variation and flexibility, a population in one geographical location may permanently reside there or only partially migrate, whereas others embark on much more extensive journeys to acquire new habitat and mating opportunities (figure 5). It is thought many may switch between these migratory states depending on conditions such as food availability and temperature. In addition there are migratory differences between the sexes, females exhibit a higher level of philopatry to natal sites than male (Wille, Latorre-Margalef and Waldenström, 2017). Migration may in fact lead to greater susceptibility to infection due to limited body resources from the exertion (Bauer and Hoye, 2014). Pair formation occurs during late winter, subsequently breeding sites become densely populated during a short period in the year when food availability is high and peaks in avian IAV occur (van Dijk *et al.*, 2018). Foraging behaviour plays a significant role in the exposure of birds to IAV with a significant proportion of a mallard's time is spent feeding either in water or at water's edge. Influenza A virus adopts an environmental persistence strategy whereby it can survive and remain infectious outside a host for 30–35 days at 4°C and for 7 days at 20°C (De Marco *et al.*, 2016). In a wetland habitat, this strategy provides the virus with a high probability of

acquiring a host. Additionally, at certain points throughout the year the mallard undergoes a seasonal moulting. This involves a gathering of mallards to shed their plumage for the forthcoming season, which is yet another event causing many birds to congregate together, enhancing the chances of IAV acquiring a new host. It is thought the shedding of plumage could increase the individual mallard susceptibility to infection, through insufficient thermal protection and stress-induced immunological suppression (van Dijk *et al.*, 2018). Additionally, the gender of the individual can play a role in the susceptibility to avian IAV infection. A recent study in the wild swan population (*Cygnus olor*) suggested that male birds are more likely to be infected that female birds because the male birds consistently had lower levels of IAV nucleoprotein antibodies (Pybus *et al.*, 2016). This may be due to a reproductive or immunological trade off known to affect many species, as a result of sexual selection (Norris and Evans, 2000).

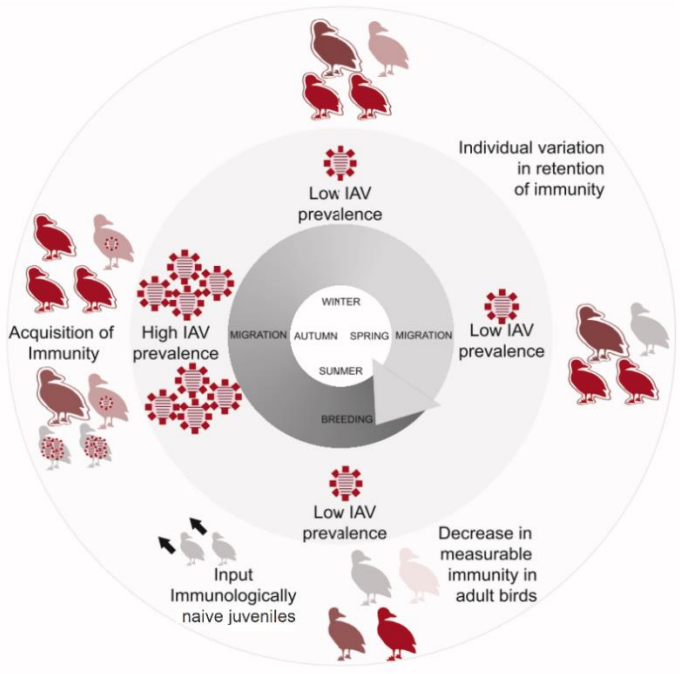


Figure 5: Seasonal dynamics of avian IAV in mallards, demonstrating the annual acquisition and immunity cycle, modified from Wille et al. (2017)

Multitudes of intrinsic traits affect the likelihood of transmission. It is well documented that the juvenile mallards are immunologically naive and as a result are infected most frequently (Latorre-Margalef *et al.*, 2009; Jourdain *et al.*, 2010; Hoye *et al.*, 2011; Van Dijk *et al.*, 2014). It is thought that those that survive their first infection and overwintering will continue to be infected by several different strains of avian IAV but will likely have a broader immune response to further infections, a way of coping with multiple or simultaneous avian IAV infections (van Dijk *et al.*, 2018). Figure 5 demonstrates the cycle of seasonal infection. The acquisition of an HA subtype means the individual is unlikely to be re-infected with the same HA subtype during that same year, this is known as homosubtypic immunity. It is also thought that homosubtypic immunity to one subtype also builds immunity to closely related subtypes, this is known as heterosubtypic immunity (Latorre-Margalef *et al.*, 2013; Avril *et al.*, 2016).

1.7. The application of pseudotyped viruses to study the avian H3 IAV subtype

1.7.1 Pseudotyped virus structure

Pseudotyped viruses (PVs) are chimeric virions that are produced to present a desired enveloped virus and measure immune response in vitro. PVs do not produce viral progeny, the core omits the necessary genes vital to allow the virus to reproduce. Disabled retroviruses expressing the HIV-1 gag/pol gene to facilitate the viral self-assembly can be used as the core for producing PVs (figure 6) (Mather *et al.*, 2013). The core encodes a reporter gene, which is expressed/produces a signal on successful entry to the target host and can thus be measured. Surface glycoproteins of the target virus are replicated and transfected to complete the virion.

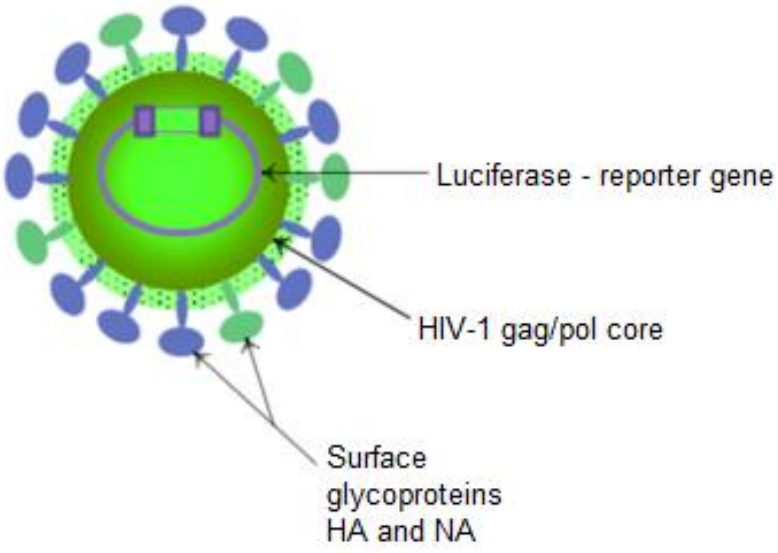


Figure 6: Pseudotyped viruse structure displaying surface glycoproteins HA and NA, HIV-1 gag/pol core and luciferase reporter gene (image modified from Scott et al., 2012)

1.7.2 Pseudotyped virus advantages and applications

The main aim of this project was to develop methods and reagents for the generation of PVs to study H3 IAVs circulating in the wild duck reservoir. PVs could be instrumental in understanding virus ecology and reassortment events. Pseudotyped viruses can have several advantages and applications:

- The production and use of PVs are relatively safe. While wild-type viruses may require a laboratory biosafety containment level of 3 or 4. PVs make excellent tools for safe study without the associated requirements and costs of high containment and can be handled at level 2 containment (Bentley, Mather and Temperton, 2015).
- Novel PVs can be generated and applied in serological assays to predict potential transmission events. For example, if the antibodies fail to recognise the selected PV it could suggest a lack of immunological protection of the host, it could also suggest a possible mutation in the genes or an entirely new subtype. This makes pseudotyping appealing to public and animal health agencies as serosurveillance tools.
- PVs can be generated using HA and NA sequences reported in the literature or available from sequence databases by having the genes synthesised, therefore there is no requirement to have a virus isolate.
- PVs can be used to assess novel HA/NA combinations. The compatibility of the HA and NA depends on the balance between the strength of binding of the HA to its receptor and the enzyme activity of the NA it is paired with (Xu *et al.*, 2012). If a particular HA and NA combination is not compatible, it may be more difficult to generate a PV expressing this combination, providing a read-out for HA/NA compatibility.
- PVs provide a more tractable system for measuring the contribution of antibodies against the NA to immunity in avian species. It is only recently that the importance of antibodies to NA have been realised (Memoli *et al.*, 2016). To isolate the contribution of NA antibodies from HA antibodies, reassortant viruses with an 'irrelevant' HA have been generated. PVs also offer the opportunity to generate virus particles with no HA on the surface.
- PVs have been used successfully for therapeutic agent screening. PVs have the ability to rapidly generate key data has been critical for viral outbreaks of Middle Eastern Respiratory syndrome (MERS) and Ebola (Zhao *et al.*, 2013; Long *et al.*, 2015). In

these circumstances PVs helped the identification of potential antivirals, as well as advancing the study of the virus life cycle and virus receptor interaction.

- PVs capable of expressing different proteins have the potential to be used as vaccines. vesicular stomatitis virus envelope (VSV-G) based PV vaccine bearing the hantavirus envelope glycoproteins have been shown to elicit neutralising antibodies in mice (Lee *et al.*, 2006). Lentiviral vector PV vaccines have proven to be less toxic than VSV-G based PV vaccines in immunised mice against spleen focus forming virus (SFFV) (Lopes *et al.*, 2011).

2. Materials and Methods

2.1. Cell Culture

An aliquot of human embryonic kidney (HEK) 293T cells was initially thawed from liquid nitrogen by emersion in a 37°C water bath for 1–2 minutes and resuspended in Dulbecco"s Modified Eagle Medium (Gibco® Thermo Scientific™ Altrincham UK) supplemented with 1% L-glutamine (Gibco® Thermo Scientific™ Altrincham UK), 1% penicillin-streptomycin and 10% fetal bovine serum, also referred to as D-10. The cells were centrifuged at 300g for 5 minutes, the D-10 and freezing media were removed and cells resuspended in fresh D-10. The 239T cells were used as both the producer and target cells in PV production and titration. Cells were cultured in T75 filtered Nunc™ EasYFlask™ (Thermo Scientific™ UK) at 37°C in 5% CO_2 and sub-cultured by removing D-10, washed with PBS before detaching the cells from the T75 flask with 10% trypsin solution and resuspending in fresh D-10 media. Seeding was determined based on how quickly the cells grew and when the cells were to be used. Madin-Darby canine kidney (MDCK) cells were also used intermittently for PV titration and were cultured identically to the 293T cells.

2.2. Molecular Biology

2.2.1. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Influenza subtype A/mallard duck/England/7277/06 (H2N3) was selected to clone because this isolate originated from a wild duck so was of interest to study. The aim was to amplify the NA gene for pseudotyping purposes. The RNA was extracted using the Qiagen viral RNA extraction kit (Manchester UK) according to the manufacturer’s recommendations. The purified RNA sample was measured using the nanodrop to determine the concentration and stored at -20°C. Following the extraction, the RNA was reverse transcribed into cDNA (complementary DNA) using AMV reverse transcriptase (New England Biolabs (NEB) Hitchin,

UK). As per AMV reverse transcriptase product protocol, 2µg of RNA template was added with the reverse primer. The reverse primer bound to the complementary sequence and thus enable a higher rate of correctly reverse transcribed product. The reaction included RNasin® ribonuclease inhibitors (Promega, Southampton UK), deoxynucleotides (dNTPs) solution mix (NEB UK) and the required amount of RNA-free water up to the total reaction volume of 20µl. The cDNA was amplified via polymerase chain reaction (PCR) using forward and reverse primers. PCR reaction was cycled in a Bioer LifeECO™ Thermal Cycler (Alpha Labs, Brighton UK) on the following quantities and cycle:

10X Standard *Taq* Reaction Buffer	2.5 µl
10 mM dNTPs	0.5 µl
10 µM Forward Primer	0.5 µl
10 µM Reverse Primer	0.5 µl
Template cDNA	500ng
Taq DNA Polymerase	0.125 µl
Nuclease-free water	to 25 µl
Total reaction volume	25 µl

Initial denaturation	94°C	1 min
Denaturation	94°C	30 seconds
Annealing	60°C	30 seconds
Extension	72°C	3 minutes
Cycle end	72°C	5 minutes

Denaturation, annealing and extension were cycled 30 times.

2.2.2. Primer design

Primers were designed using the A/mallard/ALB/201/1996 (H2N3) full sequence (accession number CY003946), as described in (Feeney, Murphy and Lopilato, 2014). Restriction enzyme sites, *BamH*I and *Xho*I (indicated in bold) were included for cloning into a pI.18 plasmid (GenScript, Leiden Netherlands). The following primers were synthesised (Sigma-Aldrich, Gillingham UK) and used for reverse transcription and PCR:

Forward = 5'- GCG CGC **GGA TCC** ATG AGC AAA AGC AGG -3'

Reverse = 5'- GCG CGC **CTC GAG** TTA AGT AGA AAC AAG GTG -3'

2.2.3. Gel electrophoresis

Gel electrophoresis was used to visualise the PCR product, prior to cloning. A 1% agarose gel with 1x TAE (Tris-acetate-EDTA) buffer and Nancy-520 fluorescent stain was prepared. The set gel was placed into a tank containing 1x TAE buffer, and samples are loaded into the gel with purple X6 loading dye (NEB, UK). A 1KB ladder (NEB, UK) was used to determine the base pair size of the amplified cDNA. The tank was supplied with 70V for 1 hour. Once complete the gel was placed in a UV light box and imager to acquire an image.

2.2.4. DNA concentration

A NanoDrop™ 1000 Spectrophotometer was used to measure the concentration of the DNA. Adhering to manufacturer's guidelines detailed in the V3.7 User's Manual. The sample was measured at the absorbance ratio of 260/280 nm and blanked with nuclease-free water. Figure 7 represents an example of a nanodrop reading, taken from cDNA produced from RT of H2N3.

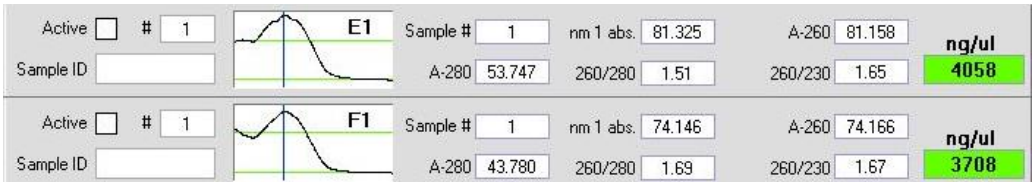


Figure 7: cDNA H2N3 after RT in preparation for PCR

2.2.5. Plasmids

In this study the HIV-1 lentivirus packaging construct was psPAX2. This is a second-generation plasmid encoding the gag-pol genes and enables the arrangement of all components. The pCAGGS-HAT plasmid was used, it expresses the HAT protease which facilitates the cleavage of the HA. The pCSFLW lentiviral vector plasmid expressing firefly luciferase was also used, this provides a robust signal for quantifying the light emitted when target proteins are expressed. The HA and the NA genes were first cloned into a mammalian expression vector pI.18, figure 8 displays the PI.18 plasmid map detailing the restriction enzyme *BamH*I and *Xho*I cloning sites

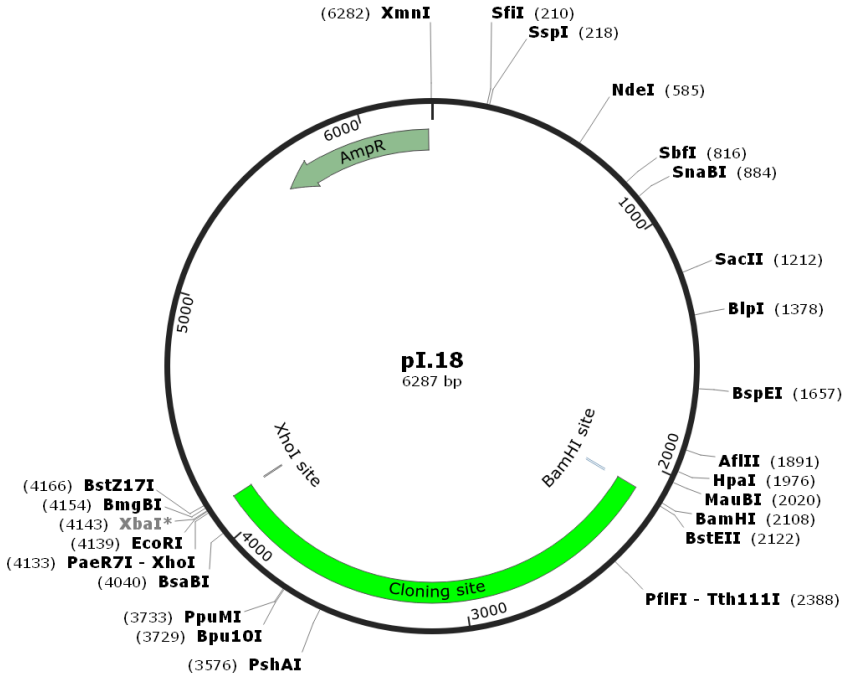


Figure 8: Map of pI.18 mammalian expression plasmid

2.2.6. Restriction enzyme digest and ligation

Time-Saver™ qualified restriction enzymes were obtained from NEB UK. The manufacturer's guidelines were followed to optimise the quality of the digest. T4 DNA Ligase kit (NEB UK) was subsequently used to bind the "sticky ends" using a molar ratio of 1:3 insert:vector and the ligation reaction mix incubated at room temperature overnight.

2.2.7. Bacterial transformation and plasmid purification

Luria broth (LB) agar plates were pre-prepared containing a final concentration of 100ug/ml ampicillin and were stored at 4°C prior to transformation. Sub-cloning Efficiency™ DH5α™ Competent Cells (Thermo Fisher Scientific UK) where thawed on ice before 1µl of DNA was added (dependent on DNA concentration, up to a maximum of 2.5µl), mixed and placed on ice for 20 minutes. Cells were shocked in the water bath for 30 seconds then heated to 42°C exactly. LB broth was added to the DNA and DH5α cell mix and incubated in an air incubator

for 1 hour. Tubes were spun at 16000g for 2 minutes before media was removed and the cells resuspended in fresh LB broth. Agar plates were then streaked with the mixture in a manner that will facilitate selecting a single colony. Plates were moved to an air incubator overnight. A single colony was selected from the plate and placed into a tube with LB broth. The tube was secured onto an incubating shaker (Thermo Forma Orbital Shaker) and the culture grown for 12–16 hours in preparation for plasmid purification using QIAprep Spin Miniprep Kit (QIAGEN, Manchester UK). Manufacturer's guidelines were followed to provide highest yield of purified plasmid concentration. Plasmids were sent for sequencing to Source BioScience (Nottingham) to validate the transformation was successful.

2.2.8 Optimisation of transfection reagent using green fluorescent protein (GFP)

Prior to PV production, transfection reagents Lipofectamine (2000), PEI MAX and JetPRIME were tested for efficiency and toxicity to 293T cells by transfecting PSGFP_NI plasmid (plasmid containing green fluorescent protein) and imaging under an inverted microscope. The 293T cells were grown in a 6-well plate, seeded at a density of 2 x 10^5 cells/ml for 24 hours prior to transfection. Both Lipofectamine and JetPRIME were used following the manufacturer's recommendations. PEI MAX stock solution was used with the concentration of 1 µg/µl and was tested in three different volumes 2µl (1:1),10µl (1:5), 20µl (1:10) with serum-free media. A GFP plasmid was used at 1µl per transfection and one well was void of any reagents as a control. Media was changed 16 hours post transfection to D-10 medium and the transfected cells were harvested/imaged at 48 hours.

2.3. Influenza lentiviral pseudotype virus (PV) production

2.3.1. PV Production

All PVs were produced in 6-well plates with cultured 293T cells, seeded at a density of 3x10^5/well and were transfected at approximately 60 - 70% confluency. Plasmid concentration was measured using the nanodrop and quantities were calculated accordingly (Table 2). Six

plasmids were added to the cells in the well. Each well required one aliquot containing 100µl of OptiMEM™ (reduced serum media) and 7µl of Lipofectamine and a second aliquot containing 100µl of OptiMEM™ and the required DNA with a maximum capacity for DNA at 2500ng per well. Following this, the two aliquots were mixed together and incubated at room temperature for 5 minutes before adding to the well, gently dropping steadily around the well before gently rocking the plate back and forth and from side to side to ensure full coverage. The pseudotyped viruses were harvested after 48 hours of incubation at 37°C and 5% CO_2 and the supernatant filtered through 0.45µm filters. This was stored in aliquots at -80°C until used for further applications.

Table 2: Transfection components and quantities for PV production

Transfection components	**6-well culture plate quantities**
Plasmids	
pI.18-HA	500ng
pI.18-NA	125ng
psPAX2 - core gag/pol	500ng
pCSFLW	1µg
pCAGGS-HAT	125ng
VSV-G	500ng
Transfection reagent	
Lipofectamine 2000	7µl per PV
Transfection medium	
OptiMEM™	100µl x 2

Figure 9 shows the assembly of the PV components via transfection of plasmids into 293T cells, before measuring the efficacy by infecting target cells.

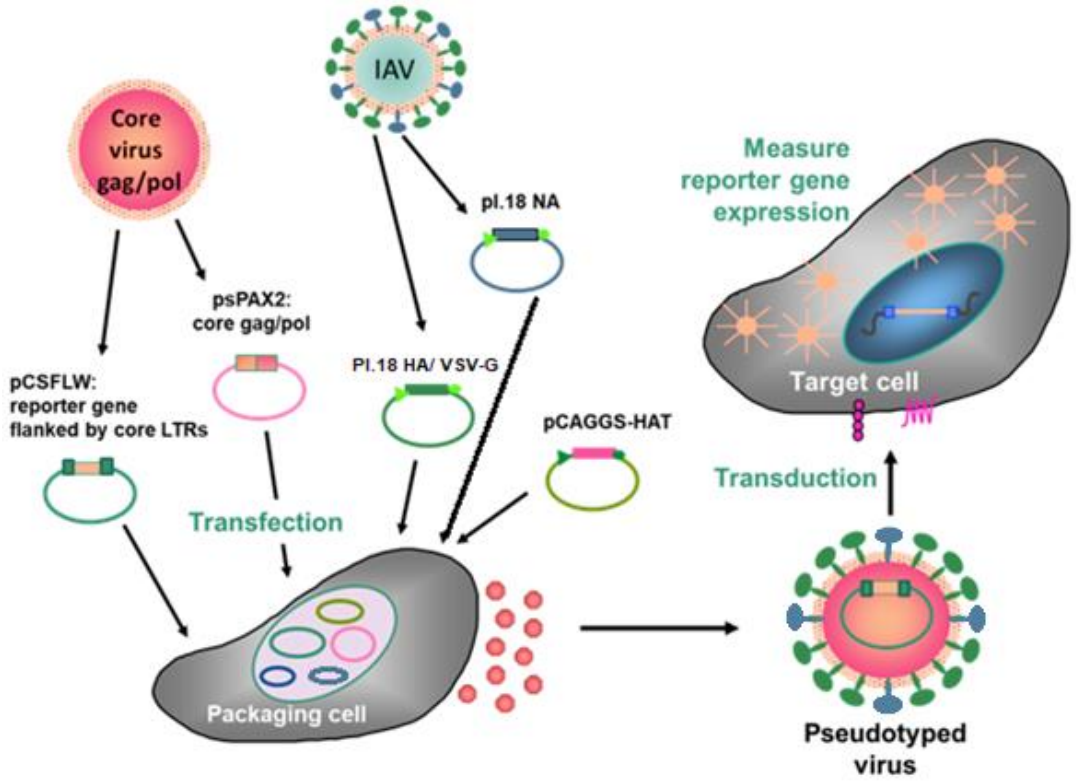


Figure 9: Schematic of production of a pseudotyped virus expressing influenza A virus HA and NA or VSV-G and NA. (image modified from Scott et al., 2012)

2.3.1.1. PV titration

Freshly generated PV viruses were titrated to confirm viral titre. Using a 96-well flat-bottomed cell culture plate, 100µl of neat PV supernatant was added in duplicate on row H. A total of 50µl of D-10 medium was added to all other wells and 50µl of PV supernatant was serially diluted from H to A, discarding the final 50µl into trigene. The end column (12) was reserved for a cell-only control. MDCK cells were added to all wells at $1x10^4$ cells in a volume of 50µl. The plate was then incubated at 37°C 5% CO_2 for 48 hours. Once complete the contents of the plate was removed and 50µl Promega Steady-Glo® (Promega, Southampton UK) added to all wells. After allowing the cells to lyse for 10 minutes at room temperature, the plate was read in a Orion L Microplate Luminometer and the titre reported in relative luminescent units (RLU/ml). The titre was calculated by multiplying the RLU reading by the dilution factors

appropriate for each dilution e.g. 50µl x 20 = 1000µl = 1ml Therefore: RLU reading x 20 = RLU/ml value. The following multiplication factors were applied to the remaining values ranging from least to most titrated (40/80/160/320/640/1280/2560). An average of all the RLU/ml values was calculated to give an overall titre.

Additional PVs were used throughout this study and these were generated in 2017 by Kinsley, the RLU/ml is shown in table 3. Kinsley (2017) PVs were stored at -80°C until use.

Table 3: PVs generated by Kinsley, 2017. Respective titres obtained in RLU/ml and viral strains used to produce HA and NA.

PVs	Titre (RLU/ml)	HA		NA	
		Isolate	Subtype	Isolate	Subtype
H3N1	2.5×10^8	A/equine/Richmond/1/2007	H3N8	A/chicken/Italy/1067/1999	H7N1
H3N2	$< 10^5$	A/equine/Richmond/1/2007	H3N8	A/Udorn/307/1972	H3N2
H3N3	2.5×10^7	A/equine/Richmond/1/2007	H3N8	A/chicken/Pakistan/34669/1995	H7N3
H3N4	7.1×10^7	A/equine/Richmond/1/2007	H3N8	A/turkey/Ontario/6118/1968	H8N4
H3N8	9.8×10^6	A/equine/Richmond/1/2007	H3N8	A/equine/Richmond/1/2007	H3N8
H3N9	2.9×10^7	A/equine/Richmond/1/2007	H3N8	A/avian/Shanghai/2/2013	H7N9
H5N8	1.1×10^{11}	A/Viet Nam/1194/2004	H5N1	A/equine/Richmond/1/2007	H3N8

2.4. Influenza MUNANA neuraminidase activity assay

The activity of the NA surface protein can be measured using the 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt hydrate activity assay. The methodology was modified from a standard operating procedure available online (Influenza MUNANA neuraminidase

activity and inhibition assay). –Stock solutions, working solutions and buffers used in the MUNANA assay are detailed in Appendix 1.

The assay required a black 96-well plate. A volume of 20µl MES assay buffer was added to each well, followed by 20µl of PV/virus to row A in duplicate and serial diluting in 20µl increments to row G, discarding the final 20µl. The final row (H) contained buffer only as a blank control. MUNANA working stock was added to all wells at 30µl per well. The plate was sealed and kept dark while placed on a shaking incubator at 37°C for 1 hour. The plate was removed from the shaking incubator and the reaction ended by adding 150µl of stop solution to all wells. The plate was read on a Flurostar optima plate reader, at a wavelength of 355/460nm and the 50% effective concentration ($EC_{50)}$ value was calculated using Graphpad prism 7 software.

2.5. Pseudotype-based enzyme linked lectin assay

Designed to be a sensitive alternative the MUNANA measuring assay, the pseudotype-based enzyme linked lectin assay methodology was modified from Prevato *et al.*, (2015).

Assay plates (96-well Nunc maxisorp) were coated with 7.5ug/well of fetuin (prepared in 1x pH 9.6 coating buffer) and incubated at 4°C until use. Plates were washed four times (350µl/well) with PBS and 0.05% Tween (T-PBS). Duplicates for each neat PV/virus were added in 100µl measures to row A. Dulbecco's 1x PBS (DPBS) containing $CaCl_2$, $MgCl_2$ and 1% Bovine Serum Albumin (BSA) was added (50µl) to each well. From row A, 50µl was serially diluted to row G with the final 50µl discarded and row H left as buffer only. Plates were incubated overnight at 37°C. Contents of the plate were carefully discarded and the plates were washed four times with 350µl/well of T-PBS. Then 100µl/well of 1:1000 diluted horseradish peroxidase-labelled peanut agglutinin (HRP-PNA) (conjugate diluent - DPBS containing $CaCl_2$, $MgCl_2$ and 1% BSA) was added and the plates incubated for a further 1 hour. Plates were washed a final four times with 350µl/well of T-PBS. Then 100µl of 3 3' 5 5'-

tetramethylbenzidine (TMB) was added to each well and the plate was incubated at room temperature for 30 minutes. The reaction was stopped with 100µl/well of 0.5M hydrochloric acid and the plate was read at a wavelength of 450nm using a Labtech LT-4000 Plate Reader.

2.6. Analysis of H3 subtype sequences available in the GISAID database

The GISAID initiative influenza database was interrogated to select datasets consisting of isolate criteria “influenza A > avian host species>H3 subtype>all geographical locations”. Data were extracted into an Excel spreadsheet. This was further restricted, removing data that did not specify NA, any data pre-year 2000, data that was clearly a domestic species and any isolates that did not define the host species e.g. “other avian”. Data was further categorised by species order, sample country of origin, each H3 subtype and was organised in pivot tables. Pie charts and graphs detailing occurrences were produced using GraphPad Prism 7. Geographical locations were condensed into three groups and referred to as the following:

Geographical location	Regions included
America	Canada and USA (North America), Central America, South America
Europe	All European countries and Russia
Asia	All countries considered Asia and Oceania (Australia and New Zealand)

Using selected isolates from each geographical location and as many subtypes available, the FASTA files were extracted and uploaded to Mega6. This enabled the formation of phylogenetic trees of the full HA and NA nucleotide sequences, to support critical analysis and support or dispute findings of the literature review of H3 subtypes. The sequences were aligned using MUltiple Sequence Comparison by Log- Expectation (muscle) and phylogeny reconstruction was performed using the maximum likelihood method (Tamura and Nei, 1993; Tamura *et al.*, 2013).

3. Results

3.1. GISAID database analysis

3.1.1. Wild bird dataset

Initially, 2279 isolates were extracted from the GISAID database of which 1882 were selected for analysis. Figure 10 displays the complete data set analysed and categorised as “likely wild origin”. This includes taxa from orders; Anseriformes, Accipitriformes, Charadriiformes, Columbiformes, Gruiformes, Passeriformes and Pelecaniformes. Common species names included in table 4.

These data indicate H3N8 as the primary IAV isolate sampled over the years from 2000 to 2017. The number of isolates identified as this subtype began to increase from 2005, peaking in 2007 with 235 isolates and remaining relatively high until a sudden decrease in 2012. This is probably indicative of either a lesser prevalence of the virus subtype or a bias toward lack of sampling in 2013. Further breakdown into geographical region (Figure 12) demonstrated the high prevalence of H3N8 in each continent, namely Asia 58%, the Americas 71% and Europe 73% respectively. Out of 1882 H3N8 isolates, 1232 of these were species within Anseriformes, 563 of these were identified as *Anas platyrhynchos.* Figure 11 presents other H3 subtypes (excluding H3N8) and their occurrences within the data set. H3N2 and H3N6 were almost equally present within the data set, both reaching peak occurrence in 2008. However in 2011, H3N2 increases while the number of H3N6 samples decreases, this repeats again in 2014 with H3N6 recovering as H3N2 isolates become fewer. Figure 12 demonstrate a similar proportion of both subtypes sampled, irrespective of location. The decrease in both these subtypes appeared to coincide with the increase H3N1 in 2009. Subtypes H3N3, H3N4, H3N5, H3N7 and H3N9 all occurred during this period, although at a much lower rate, disappearing intermittently. With certainty, the most samples were collected within the

Americas (Figure 12, C) and all the subtypes in this study were detected. America was the only location where wild H3N7 isolates occurred in this dataset.

Table 4: Bird species/common names included in the taxonomy of this study

Taxonomic order	**Species**
Anseriformes	Ducks, geese, and swans
Accipitriformes	Hawks, eagles, and vultures
Charadriiformes	Wading birds and gulls
Columbiformes	Doves and pigeons
Gruiformes	Cranes, crakes and rails
Passeriformes	Song birds
Pelecaniformes	Ibises, spoonbills, herons and pelicans

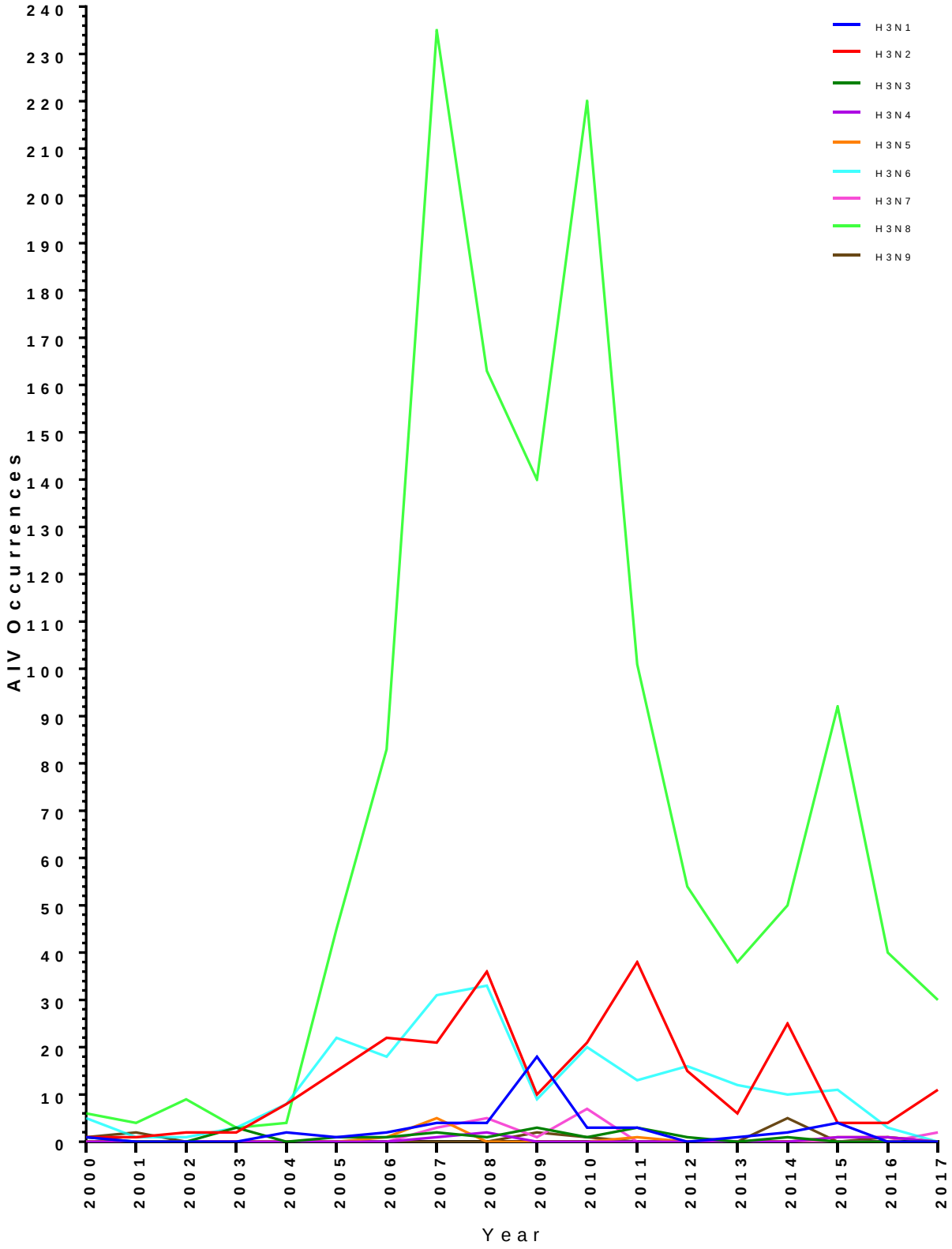


Figure 10: All H3N1 – N9 isolates extracted from GISAID database as "wild" origin, between the period of 2000–2017.

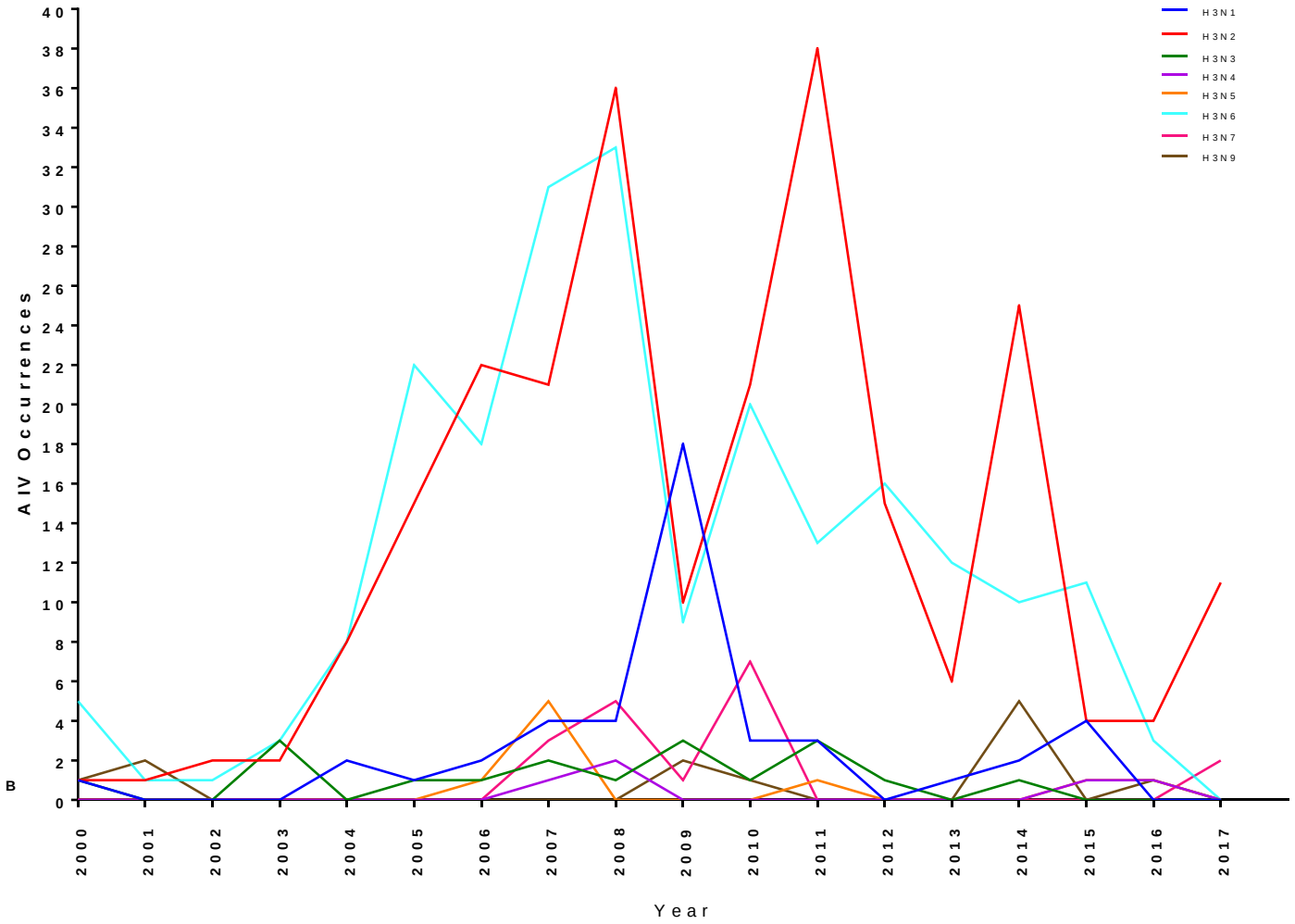


Figure 11: H3N1 – N9 isolates, as per figure 10 but excluding H3N8 to enable a closer view of other subtypes. Extracted from GISAID database as "wild" origin, the period of 2000–2017.

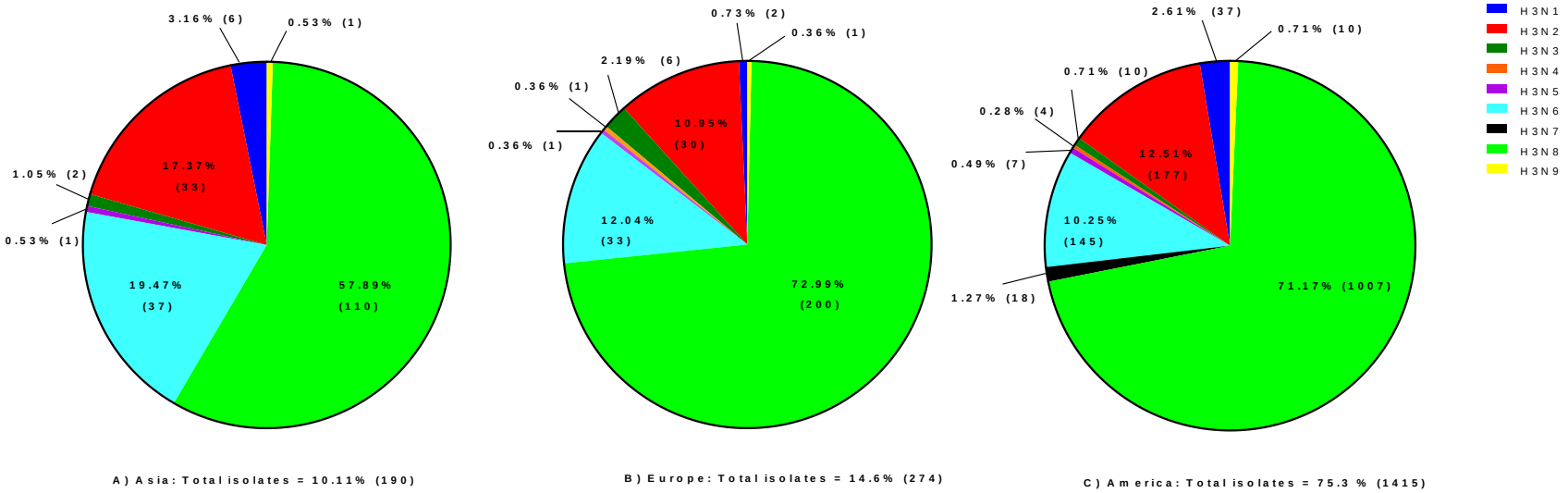


Figure 12: Data extracted from GISAID database displayed in pie charts expressing the percentage of each H3 subtype found within A) Asia, B) Europe, C) America.

3.1.2. Wild bird phylogeny

A selection of 41 isolates were extracted from the dataset and phylogenetic trees generated using Mega6 (Figure 13). These data were selected according to geographical location, subtypes available and if the full HA and NA sequence was present.

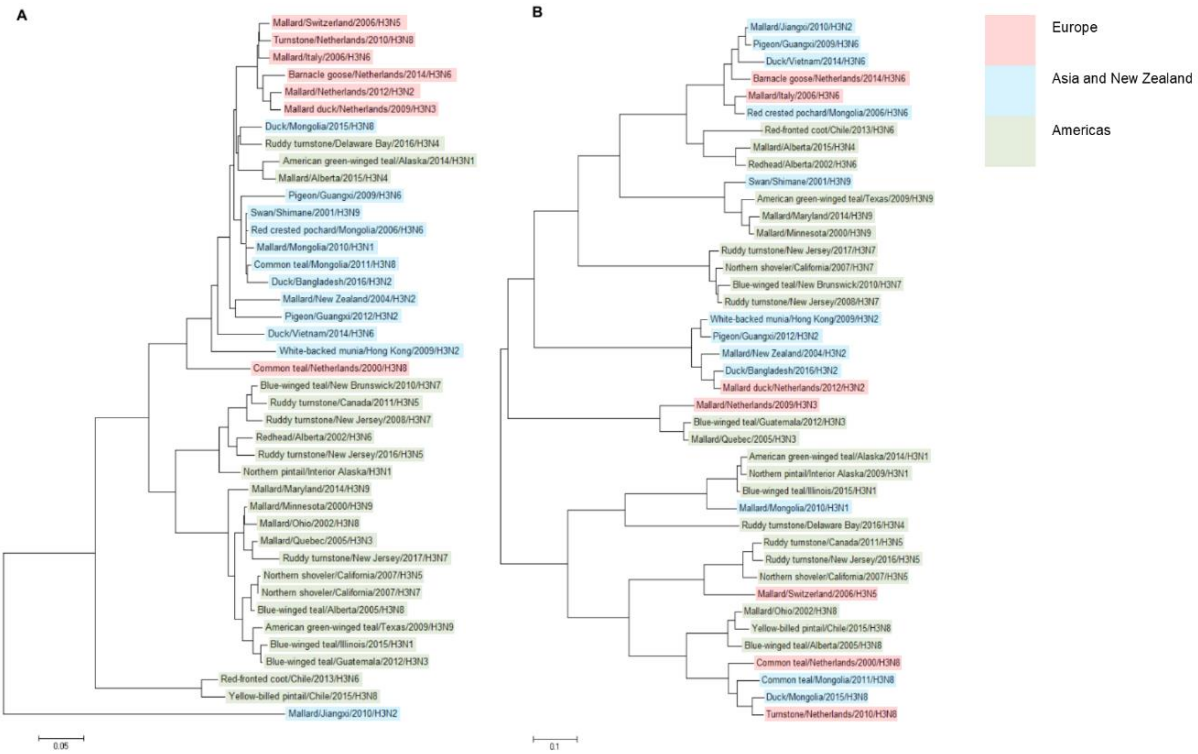


Figure 13: Phylogenetic analysis of (A) HA and (B) NA nucleotide sequences, selected from GISAID data analysis. The tree is drawn to scale, the branch lengths are measured in the number of substitutions per site.

The HA analysis in Figure 13 A, clearly presented two large clades of related H3 subtypes. The uppermost clade had isolates that were sampled in Europe, Asia and New Zealand, all of which had varied NA combinations and a variation of times when samples were taken. The second large clade represented viruses sampled in North and Central America, with much of the same variation in NA and time as observed in the first clade. South American samples were grouped separately. A single sample isolated from Jiangxi, China was the least related, acting as the 'root' for the analysis. The NA analysis (Figure 13, B) also presents two clusters of isolates, however these were grouped on NA subtype, which is consistent with the literature. It is thought NA typically divides into two groups of relatedness, these are N1, N4, N5, N8 and

N2, N3, N6, N7, N9 (Russell *et al.*, 2006). The uppermost cluster consisted of H3N2, H3N3, H3N6, H3N7, H3N9 and an instance of H3N4. With most of the grouping, NA appeared to be most related to NAs of the same subtype. It is observed that the isolates with a higher degree of relatedness, had geographical similarities (e.g. Blue-winged teal/New Brunswick/2010/H3N7 and Ruddy turnstone/New Jersey/2008/H3N7), however this is not consistent throughout. The second grouping consisted of H3N1, H3N5, H3N8 and an individual H3N4. As with the first group, the NA subtypes are clustered together with the same NA type, and with the most related were from the same continent, despite a variation in when samples were taken.

3.2. PCR amplification of viral genes

Attempts were made to amplify NA genes of A/mallard duck/England/7277/06 (H2N3) and to optimise the PCR which was unsuccessful at the PCR stage despite optimisation. Universal primers were used as specified in Inoue *et al*, (2010) in an attempt to amplify all genes of interest. We established RNA extraction was successful and the reverse transcription product contained cDNA (products measured by Nanodrop). Troubleshooting of PCR included:

- Replaced Taq DNA polymerase to high fidelity polymerase (Q5® High-Fidelity DNA Polymerase and Phire Hot Start II DNA Polymerase)
- Replaced dNTPs with fresh stock
- Altered cycle by increasing and decreasing cycle length (or to high fidelity polymerase as per manufacturer's guidelines)
- Annealing temperate gradient applied because stock primer annealing temperatures were high (SIGMA: forward TM = 84.3°C, reverse TM = 75.5°C)
- Replaced dilutions of primers from the stock stored in -20°C to ensure primers were of the correct concentration (10 pmol/µl)
- Replaced AMV reverse transcriptase and fresh RNA template extracted

- RT performed with non-specific primers to amplify

The primers described in section 2.2.2. were designed and ordered to replace the universal primers however, in the meantime, the focus of the project was switched to use existing plasmids.

3.3. Optimisation of transfection reagent using green fluorescent protein

Figure 14 displays the outcome of the transfection reagent comparison. Cells transfected with each reagent were imaged with and without the emission filter. Lipofectamine and PEI 1:5 were the most effective with the least cell death (floating cells). JetPRIME performed relatively well however there was some toxicity to the cells, unlike lipofectamine. PEI 1:10 proved to be the most toxic, while the 1:1 dilution emitted the least fluorescence signal. Lipofectamine was selected for subsequent transfections.

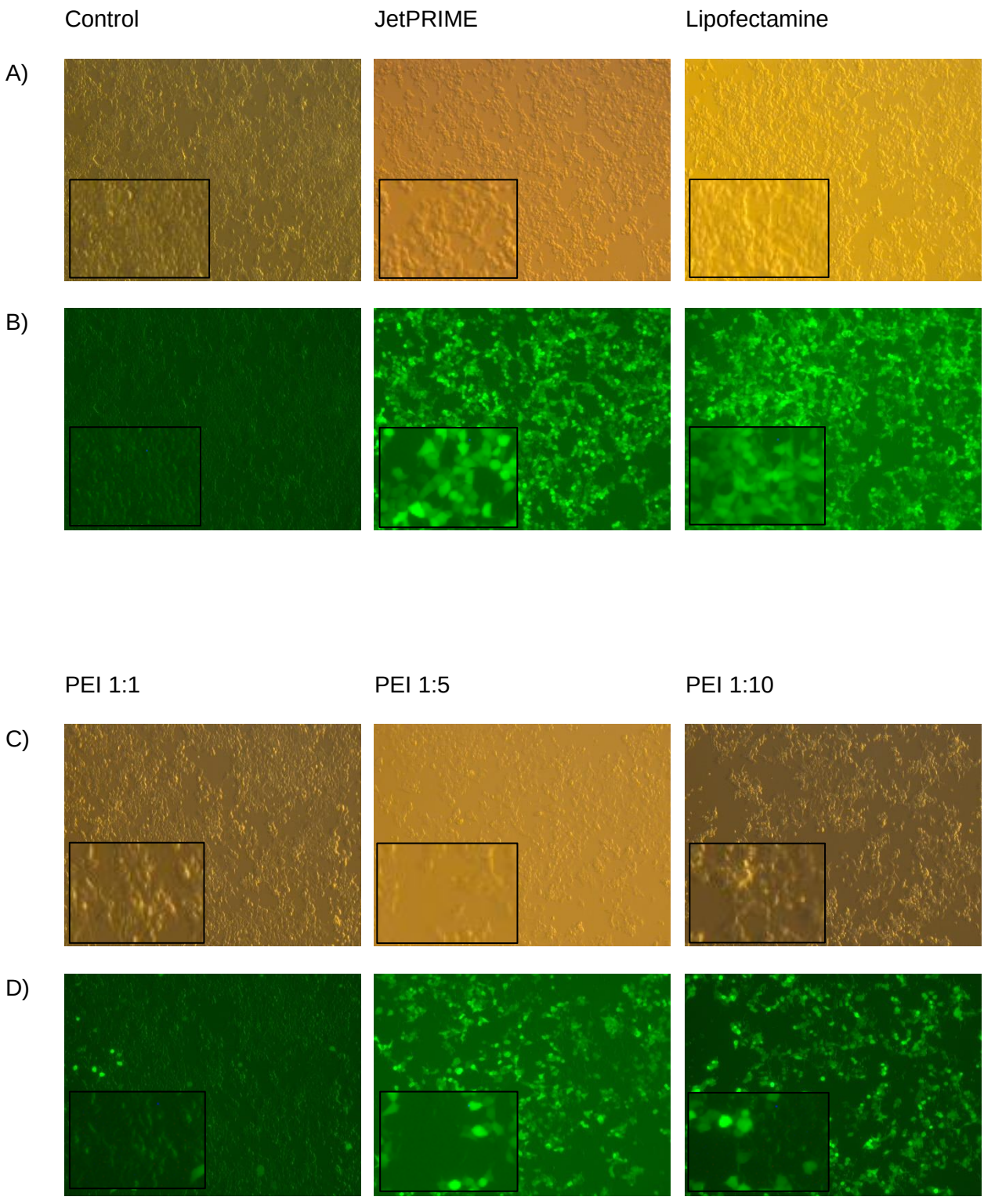


Figure 14: Transfection of 293T cells with PSGFP-NI plasmid containing GFP using three transfection reagents; Lipofectamine, Jetprime and PEI Max. A) and C) transmission microscopy B) and D) fluorescence microscopy

3.4 Generation of PVs

Using pre-generated plasmid stock from A/equine/Sussex/1989 (H3N8) a PV was generated using pI.18-HA (H3) and pI.18-NA (N8). Concentrations of plasmids were described in table 2. psPAX2, pCSFLW and pCAGGS-HAT were used in a five-plasmid system were used to generate a PV with both required surface proteins to mimic H3N8 (figure 9).

A OPV expressing NA alone was generated using the same system as in 2.3.1. omitting the pI.18-HA, to generate a novel PV. All concentrations were as before.

Vesicular stomatitis virus envelope glycoprotein (VSV-G) and neuraminidase PV were generated as in the original construct detailed in 2.3.1., using the same plasmids and concentrations, but replacing the pI.18-HA with VSV-G to see if a working PV could be generated this way.

3.4.1. PV titration

The PV titres of two separate preparations of H3N8 PV were 2.2±0.7 x 10^6 and 4.2±0.3 x 10^6 RLU/ml. PV generation was successful, however the titre of A/equine/Sussex/1989 (H3N8) was below the titre of previously generated A/equine/Sussex/1989 (H3N8) at 9.8 x 10^6 RLU/ml (Kinsley, 2017) using the same titration methodology and quantity of plasmid.

3.5. MUNANA NA activity assay

The newly generated H3N8 PV was tested in the MUNANA NA activity assay and did not appear to generate signal above the negative control. H5N8 PV was used as a positive control as this PV had previously been shown to work (Figure 15). PV generated with no HA/NA was included as a negative control.

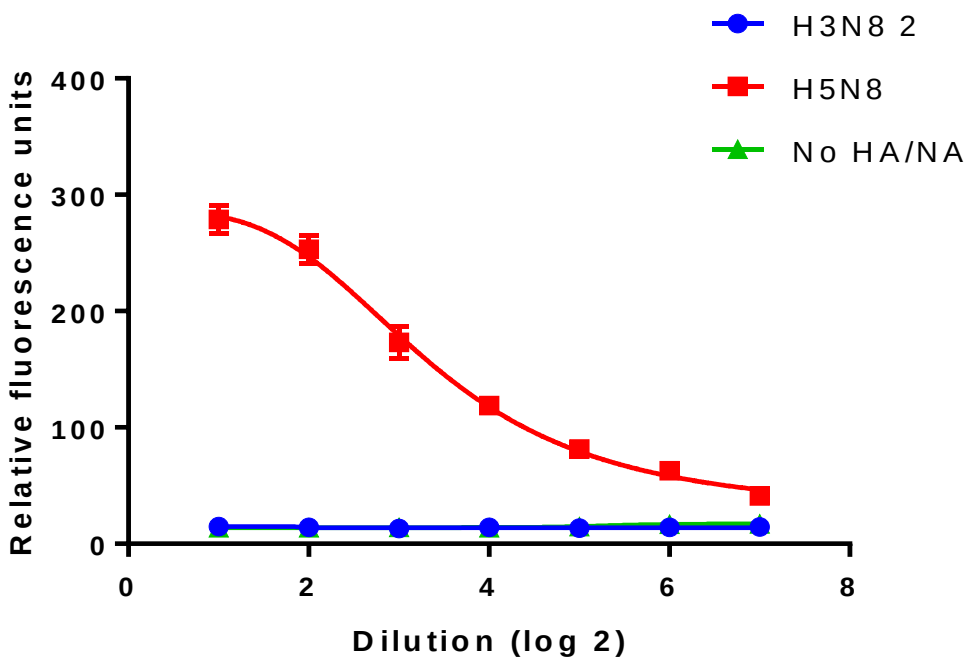


Figure 15: Neuraminidase activity of the second preparation of H3N8 PV (H3N8 2). Positive control H5N8, and negative control no HA/NA (mean ± SD)

H3 PVs H3N1 to H3N9 generated previously by Kinsley (2017) were tested in the MUNANA assay. PV H3N1 was the only PV for which an EC50 value could be determined reliably (Figure 16; EC_{50} = 2.4, R^2 = 0.97)

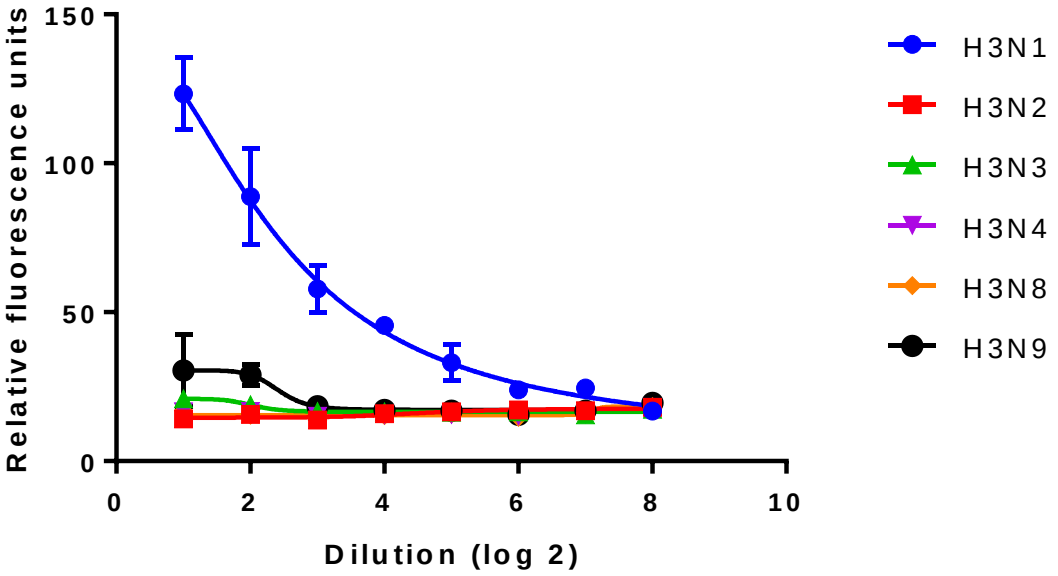


Figure 16: Neuraminidase activity generated by PVs H3N1-N9 which were produced by Kinsley (2017)

This prompted the decision to use the live virus; A/equine/Sussex/1989 as a means of direct comparison with these data. As previous MUNANA NA activity assays had displayed little NA activity with the PVs, the assay was only titrated up to the fourth dilution factor (1:160). The results (Figure 17a) showed the NA activity of the original virus. Interestingly the removal of the clear plate cover increased the NA activity result by approximately 50% (Figure 17b). The EC_{50} for the live virus was 5.0 with and 5.7 without the cover. This should be considered for further development of this protocol.

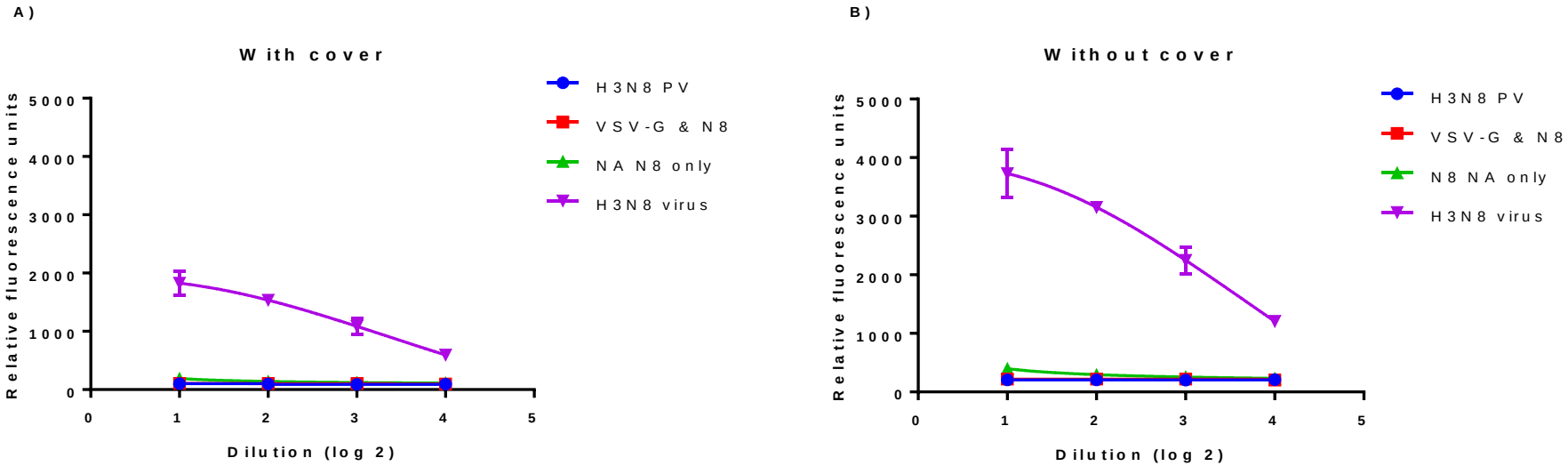


Figure 17: Neuraminidase activity measured by MUNANA assay in PV H3N8, PV VSV-G & N8, PV NA/N8 only and infectious H3N8 virus. A) with clear plate cover B) without clear plate cover. Each point is the mean of 3 replicates.

3.6. Pseudotype-based enzyme linked lectin assay

As the majority of the PVs generated did not produce sufficiently high signals in the MUNANA assay for EC_{50} values to be reliably calculated, the reportedly more sensitive ELLA assay was used. High OD values were obtained for the majority of the PVs, H3N1 to H3N9 tested with the exception of H3N2 (Figure 18). Performing the assay with two-fold dilutions of PVs allowed EC_{50} values to be calculated for the H3N3 and H3N8 PVs. However, at the lowest dilutions, the other five PVs produced OD values above the acceptance criteria for the plate reader (>2.75) and EC_{50} values could not be calculated.

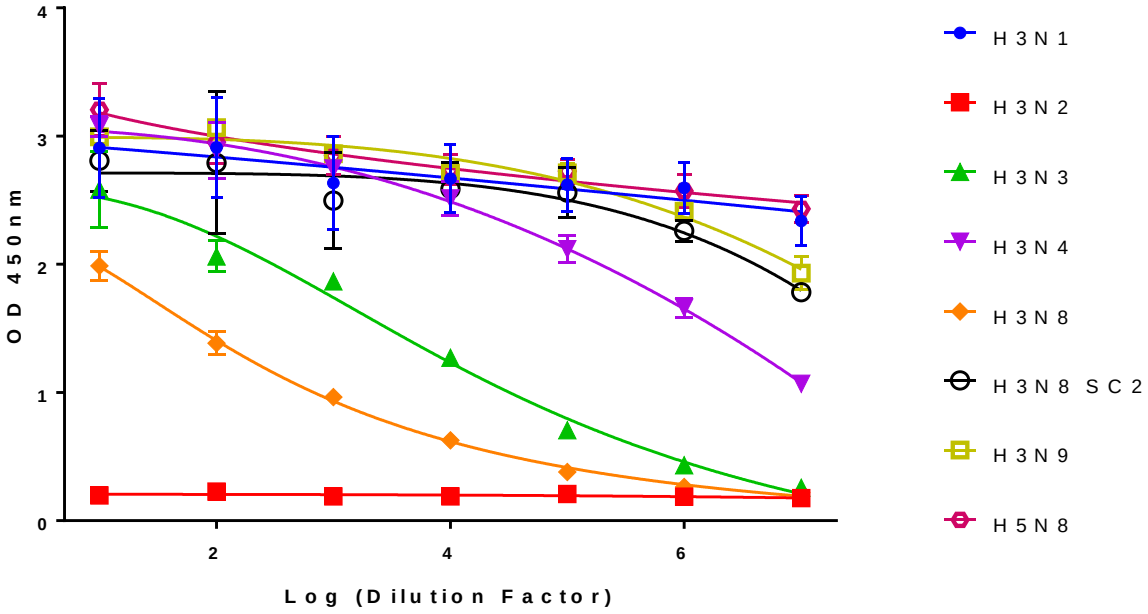


Figure 18: Neuraminidase activity in PVs H3N1 to H3N9 as measured by ELLA assay

The NA-only PV did not generate a signal in the ELLA assay (Figure 19). However, the PV with the VSV-G protein instead of HA and the IAV N8 NA produced a titration curve within the accurate range of the plate reader using doubling dilutions, enabling an EC_{50} value of 3.6 to be calculated.

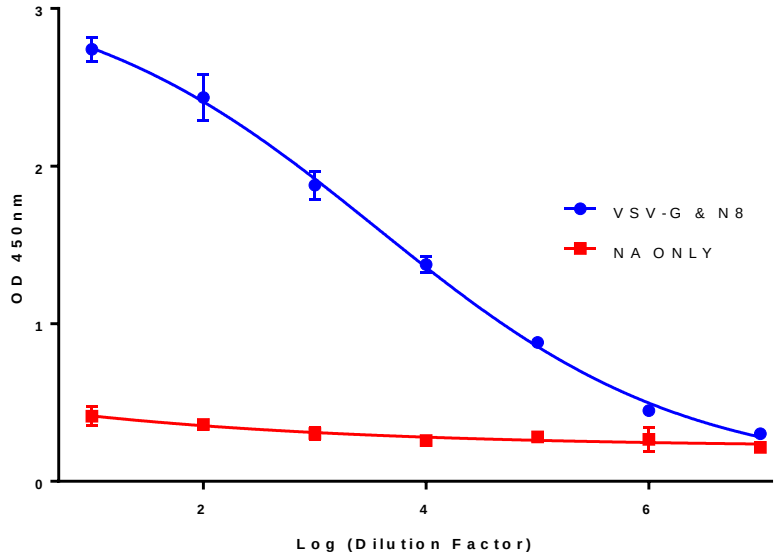


Figure 19: Neuraminidase activity of PV NA only and PV VSV-G N8 as measured by ELLA assay

In an attempt to measure neuraminidase activity in PVs H3N1, H3N3, H3N8 (2) and H3N9, ELLA assays were performed with further dilutions. EC50 calculations were successful for H3N1 (5.2), H3N8 (3), H3N9 (3.5) and H3N3 (0.76) (figure 20).

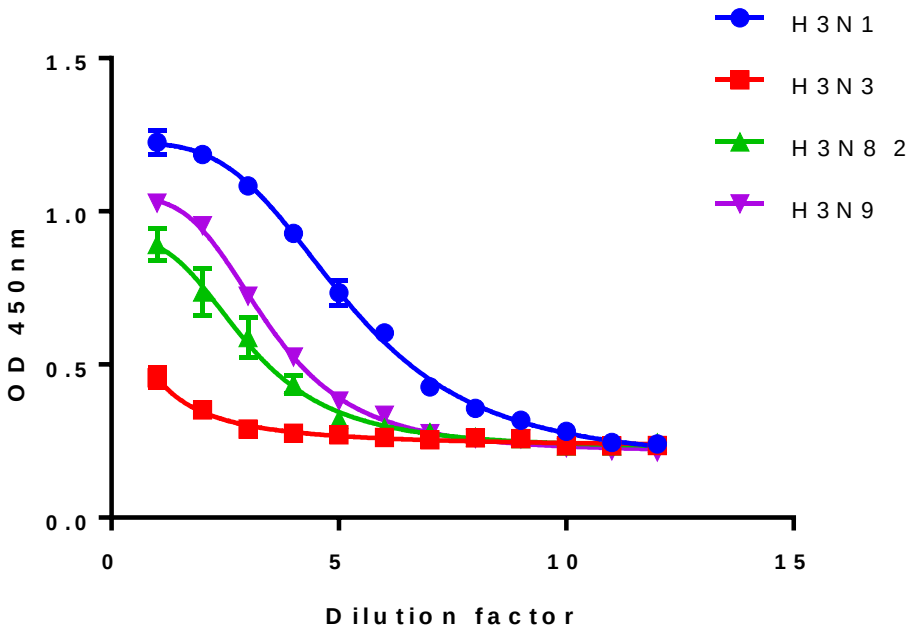


Figure 20: Neuraminidase activity of H3N1, H3N3, H3N8 2, H3N9 at a 1:10 dilution as measured by ELLA assay

4. Discussion

Viral pseudotyping offers a promising platform for the serosurveillance of influenza viruses and thus monitor the effectiveness of antibodies against viral combinations of HA and NA derived from birds and other animals. Pseudotyping is not a new concept, however new pseudotyping methodologies are enhancing our understanding of enveloped viruses, this has particular importance for new emerging viruses. Pseudotyping offers the opportunity to dissect and analyse highly contagious vectors, in a safe environment (Steffen and Simmons, 2016). The first objective of this study was to determine the compatibility of HA and NA surface proteins, with the aim of understanding how IAV may evolve in the wild bird reservoir. The initial analysis of the current literature highlighted certain combinations that are frequently observed from wild bird sampling, namely H3N2, H3N6 and H3N8. These subtypes are common in the literature and the analysis of the GISAID database confirmed these findings. The literature confirmed the interspecies transmission of H3N2 and H3N8 and suggested H3N6 has the potential to cross species barriers, despite only occurring in wild birds at this time. This suggested these combinations of HA and NA surface proteins are compatible. Additionally, the GISAID analysis suggested these subtypes can circulate in the reservoir simultaneously, although at times their occurrence appeared to alternate. Possibile explanations for this are that the viruses are occupying different niches or out competing each other due to lower viral fitness. Unsurprisingly, the most sampled subtypes were those that had made the species jump. The geographical location data suggested a high prevalence of H3N8, with occurrences of H3N2 and equal occurrences of H3N6, irrespective of location.

The interpretation of these data is subjective. One perspective is that isolates with these combinations of HA and NA are occupying ecological niches, challenging the innate and adaptive immunity of the avian host by presenting previously naïve host with novel NA. As a result the avian host may shed virus, enabling spill over into other avian hosts to enable persistence. Homosubtypic and heterosubtypic immunity are thought to be the main reason

why the mallard has decreased probability for reinfection with the same subtype and closely related subtypes. Data to support this hypothesis have~~This hypothesis has~~ been replicated in challenged mallards (Latorre-Margalef *et al.*, 2013). However, homosubtypic protection against reinfection of the same subtype is not always necessarily present. In a further study it was demonstrated that homosubtypic protection was not apparent when mallards were naturally challenged with H3 viruses, after receiving a H3 vaccine (H3N8) (Wille *et al.*, 2017). The H3 vaccine was cultured, administered and the H3 vaccine group demonstrated serum anti-NP antibodies. The study proceeded with naturally challenging the mallards in a duck trap, 14 days postvaccination. The natural challenge took place over a 21-day period and the mallards were sampled daily for new infections. H3 infections were amongst the detected subtypes in the H3N8 vaccinated mallards, of which a different H3N8 subtype was detected both as primary and secondary infection, although it was not detected twice in the same individual. H3N6 was more commonly observed in secondary infections in compared to H3N8 in primary infections. This suggests the NA subtype is switching from N8 to N6 to avoid complete immunity and maintain infectivity (Wille *et al.*, 2017). As a result of H3 being present in the H3 vaccinated group, despite the presence of specific H3 serum antibodies, this demonstrates a failure of homosubtypic immunity. However, the data and literature could be considered as biased, particularly because the samples taken at the time were relevant to the projects operating and the funding available. True conclusions cannot be drawn on these findings alone. In an ideal setting, sample extraction from wild bird populations would be a statutory requirement in all countries, with funding available to gain an accurate understanding of IAV subtype frequency and distribution.

Subtypes N1, N3, N4, N5, N7 and N9 were reported in the literature and also reported in the database analysis. The frequency of times observed could be compared to the literature. H3N1 appears more often than the other remaining NA combinations in Asia. This is unsurprising because the literature review identified Asia as the origin of H3N1, as well as the source of a novel reassortant of canine IAV H3N2 and pandemic H1N1 (Song *et al.*, 2012).

N3, N4, N5, N7 and N9 were less frequently observed in the extracted GISAID dataset and again, appeared to a lesser extent in the literature. The sequences selected for phylogenetic analysis originated from the GISAID data set. To increase diversity among the data, many additional isolates were selected from the database for analysis. However, this had limitations. Often the database did not contain the full nucleotide sequence encoding both the desired surface proteins. Many of the seemingly interesting samples (e.g. other species, unusual locations or subtypes) were not complete. Furthermore, the analysis raised some questions regarding the legitimacy of some of the samples entered into the database. For example, the NA sequences usually fits into two clusters; N1, N4, N5, N8 and N2, N3, N6, N7, N9 (Russell *et al.*, 2006). The analysis showed the occurrence of H3N4 in the incorrect cluster (A/mallard/Alberta/203/2015). This may be an error of the individual who entered the data into the database. However, if the H3N4 sequence is correct, it does not fit with the NA groupings described. Despite these factors, the data did demonstrate that the H3 relatedness is significant for geographical location and appear to divide into two groups (Eurasian and North American lineage) (Cui *et al.*, 2016). Interestingly, three of the North American samples were most related to European and Asian strains. This could be an indication of IAV transmission via migratory movements and a possible reassortment event. Unsurprisingly, the NA analysis demonstrated the NA types were most closely related to the same subtype, with some grouping toward geographical location. Ideally, in addition to the current phylogenetic analysis, an additional analysis on the NA per subtype, this would detail the NA also divides into the two groups of Eurasian and North American lineage (Cui *et al.*, 2016). Due to the mix of NA subtypes in this data set, the geographical location appears mixed. The phylogenetic NA analysis did enhance our understanding of likely reassortment events, which suggested that differing NA subtypes may be related to one another (e.g. Mallard/Jiangxi/2010/H3N2 and Pigeon/Guangxi/2009/H3N6). Additional data may have given an indication of when the reassortment occurred. On completion of these analyses, it became clear which H3NX combinations were most compatible in the environment, namely H3N8, H3N2 and H3N6.

The generation and serological testing of PVs was initiated to observe the HA and NA compatibility and NA activity how these compared with the field data. Subtype A/mallard duck/England/7277/06 (H2N3) was selected for cloning as the virus originated from a wild duck. Unfortunately, numerous failed PCRs resulted in a time delay in amplifying the NA gene, despite the trouble shooting attempts. These included using fresh enzymes, buffers and reagents. Fresh template RNA was extracted, and a gradient PCR cycle was used with varying cycle length. One potential solution may have been different primers. These had been ordered and would have been used if there was more time. To progress, existing plasmids were grown and used to generate a PV with the HA and NA from A/equine/Sussex/1989 (H3N8). This was then compared with PVs previously generated by Kinsley (2017) that were available for use. Optimisation of transfection reagents was performed to lessen toxicity during the transfection. Despite the optimisation, the titre of newly generated H3N8 PV was lower than previously generated H3N8 PV (4.38×10^6 vs 9.8×10^6 RLU/ml, respectively). This was possibly because the confluency of the cells used was not optimal for transfection, therefore a low titre was achieved. Ideally, the 293T cells would have been 90% confluent however they were 60% - 70% confluent. This may explain why the titre was lower. H3 PVs were available to test NA activity, initially the 2′-(4-Methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA) NA activity assay was performed. The MUNANA activity and inhibition assay is typically applied to live virus subtypes to measure NA enzyme cleaving the substrate to release the fluorescent product 4-methylumbelliferone (4-MU) (Leang and Hurt, 2017). This assay is quick to perform, easily reproduced and buffers can be made in advance. The fluorescence signal strength was low, for all the PVs tested except H3N1. When compared against the activity of the live virus H3N8, this assay could not be used for measuring NA activity in PVs reliably (additionally confirmed by Kinsley, 2017). This could be due to PVs being assembled with less NA protein on the surface than the infectious whole virus (Kolokoltsov, Weaver and Davey, 2005; Oh *et al.*, 2009). The results were similar to those obtained by Kinsley (2017) who found H3N1 PV had measurable activity, H3N9 displayed some activity whereas H3N2, H3N3, H3N4 and

H3N8 equally displayed a low proportion of activity. These results showed the requirement for a more sensitive assay to measure NA activity in PVs.

A PV-based ELLA (Enzyme-Linked Lectin Assay) NA activity assay was substituted to measure NA activity. This is a variation of the standard ELLA, which relies on fetuin as the NA substrate measuring the residual terminal galactose exposed after fetuin de-sialylation using peanut agglutinin (PNA) (Couzens *et al.*, 2014; Prevato *et al.*, 2015; Gao, Couzens and Eichelberger, 2016). This assay is designed to show far better sensitivity and should be suitable for use with influenza pseudotypes (Prevato *et al.*, 2015). The process of the assay is slightly more laborious than the MUNANA assay, due to the additional time required, although the initial plate coating can be completed in advanced and stored at 4°C. This ELLA appears to be much more sensitive to measure NA activity in PVs. Overall the assay was successful. Most of the other PVs resulted in EC_{50} values, H3N8 and H3N3 were measured by ELLA but the values were unreportable. However, this assay yielded improved results over the MUNANA assay. The PV dilutions resulted in measurable signals for H3N1, H3N8 and H3N9, other dilutions required further optimisation. Interestingly, the lowest activity was found for the H3N2 PV, which is in agreement with the literature (Yan *et al.*, 2016). The VSV-G and N8 NA PV performed well in the ELLA and may have a useful application to measure the activity of different NA proteins, without the necessity of a compatible HA protein. However, these results will need to be replicated using the PV based ELLA to ensure efficacy. The 1:10 dilution produced improved results for the PVs with the previously unreportable EC_{50} values. This demonstrated the necessary to titrate some PVs from 1:2 and some from 1:10. In the practical application of the ELLA it may be advantageous to pre-screen the PV dilutions or carry out 1:2 and 1:10 in tandem to ensure that a suitable curve is generated.

Primarily, the study has focused on NA which is an important component of IAV. It was recognised in the 1970s as a potential drug target. Once the crystal structure of NA was realised, anti-viral drugs (namely oseltamivir and zanamivir) were developed to inhibit the

enzyme activity/viral release (Air, 2012). These anti-viral drugs, particularly oseltamivir (Tamiflu™), have been used around the world during 'flu season to treat severe influenza (Anna *et al.*, 2013). Due to the function of NA to cleave the sialic acid binding site, NA inhibitors do not prevent influenza, however they can significantly reduce the severity of the virus infection. Observational data from the H1N1 pandemic in 2009 suggest NI inhibitors reduced mortality in hospitalised adults by 25% (Nguyen-Van-Tam *et al.*, 2015). However, overuse of NI inhibitors is creating resistant viruses. In some influenza patients, H1N1 NA has undergone amino acid substitutions H275Y and I223V causing oseltamivir resistance (Paradis *et al.*, 2015). Most concerningly, oseltamivir is appearing in the wider environment. Following the seasonal H1N1 outbreak in Japan, oseltamivir carboxylate (metabolised oseltamivir phosphate) was detected in sewage treatment plants and surrounding rivers. The discharge from the sewage treatment plant was measured at 293.3 ng/L, in the river water samples, it was detected at 6.6–190.2 ng/L (Ghosh *et al.*, 2010). One study actually cited the drug production facility as a cause of NI inhibitor discharge into the environment (Prasse *et al.*, 2010). Evidently, rivers are prime locations for dabbling ducks and other waterfowl. Considering the faecal/oral transmission of avian IAV, it is thought NA inhibitors in the digestive system of ducks will create NA inhibitor resistance in the avian IAV reservoir. In addition to oseltamivir other antiviral drugs such as zanamivir, peramivir and lanamivir have been found in the environment in other countries during influenza outbreaks (Järhult, 2018). *In vivo* research has taken place in mallards challenged with avian IAV (mutant and wild-type strains), to assess resistances, to NA inhibitors. More recent findings have demonstrated the ability of viruses carrying the oseltamivir mutation H275Y to gain secondary mutations, thus boosting oseltamivir resistance when exposed to zanamivir (Nykvist *et al.*, 2017). An additional study relating to the H274Y mutation in LPAI H1N1, demonstrated that the removal of oseltamivir carboxylate from the Mallards' water did not alter the persistence of the oseltamivir resistance (Gillman *et al.*, 2015). In contrast, an earlier study focusing on the R292K mutation

found that in H6N2, resistance did not increase when drug pressure was reduced (Anna *et al.*, 2013).

It is also known that not all NA are made equal. For instance the N9 subtype possess haemagglutinin (HA) activity which can bind sialic acids (Air and Laver, 1995). The haemabsorbing (HB) site on the novel avian H7N9 was reported to enhance binding to human-like sialic acid receptors (Wan *et al.*, 2017). In 2013, humans were reported to be infected with an avian H7N9 virus. Within 2 months there had been 132 confirmed human infections and 37 deaths. Sequence and phylogenetic analysis revealed H7N9 is closely related to H9N2 subtype viruses that had circulated in poultry in China (Watanabe *et al.*, 2013). These findings demonstrate the genetic variability of IAV and also highlight the importance of inter-disciplinary collaboration (e.g. virologists, behavioural scientists and veterinarians) and periodically carrying out sampling and serosurveillance to monitor NA inhibitor resistance in avian influenza.

In addition to NA inhibitors, the HA protein is also a target for the development of anti-influenza drugs. Much like NA, HA elicits a protective antibody response from the hosts' immune system. As the HA is responsible for initial stage of infection due to the fusion of the cell membrane, it is often targeted for vaccine production and anti-influenza medication. However, rapid antigenic drift of the target virus often alters the efficacy of vaccines developed each 'flu season. It is thought the amino acid changes in the HA are more frequent than in the NA, particularly under immune pressure, thus decreasing the effectiveness of the antibody response produced by vaccinating against the new seasonal IAV (DeDiego, Chiem and Topham, 2018). This creates a necessity to periodically update vaccine strains with those circulating during the 'flu season.

In addition to the vaccines, the development of HA inhibitors is a current area of research. Tert-butylhydroquinone (TBHQ) binds to and form a hydrophobic pocket between HA monomers, inhibiting the virus from initially binding to the stem loop of HA and stabilising the

neutral pH of HA, thus disrupting the membrane fusion step. This has been particularly effective in the H3 subtype (Russell *et al.*, 2008; Antanasijevic *et al.*, 2013). Although the long-term effects of using HA inhibitors are unknown, if developed for wider use they could provide an alternative to medicating with antiviral drugs for which IAV is resistant, i.e. currently used NA inhibitors and M2 proton channel blockers such as amantadine and rimantadine (Hussain *et al.*, 2017). In relation to the wild bird reservoir, it is of utmost importance to monitor the effects of existing and newly developed influenza drugs within the environment and what impact that has on influenza of avian lineage.

To summarise, although limited by project time constraints, progress in optimising the use of PVs in serological studies was made. It highlighted the necessity for sensitive assays to fully manipulate the PV platform. Once optimised, these assays and PVs can be used as a substitute for which require high containment viruses. PV methodologies are becoming more sophisticated and robust, allowing those that could not work with the virus before to effectively study it. This could revolutionise in-field surveillance, presenting opportunities for less financially fortunate counties to monitor disease outbreaks (Wright *et al.*, 2009). Additionally, PVs are being explored for their potential as a broad subtype influenza A virus vaccine (Powell *et al.*, 2012).

This project emphasised the requirement for international serosurveillance of the wild duck reservoir and how PVs can be generated to aid this and identify mutant subtypes. Further work would be to optimise the PV-based ELLA NA activity assay and carry out NA inhibition assays using known NI inhibitors and sera from experimentally challenged birds and animals. Furthermore, a library of PVs representing existing avian IAV strains currently circulating in the wild could be assembled. Dr. Latorre-Margalef, Lund University has offered to provide duck serum samples from their experimental infections and such resources are invaluable (Latorre-Margalef *et al.*, 2017). Other work would be to explore deeper into the phylogeny of

IAV and generate antibody landscapes of other HA subtypes circulating in the wild duck reservoir.

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6. Appendix

Appendix 1. Reagents

MUNANA NA activity assay master and working stock solutions

Master stock solutions

- 325mM MES: 31.72g MES in 500ml ddH_2O - pH to 6.5 with concentrated NaOH
- 100mM $CaCl_2$: 5.55g $CaCl_2$ in 500ml ddH_2O
- 1M Glycine: 37.5g in 500ml ddH_2O - pH to 10.7 with concentrated NaOH
- 1mM MUNANA: 25mg in 51ml MES assay buffer - stored -20°C

Working solutions and buffers

- MES assay buffer: 32.5mM - 50ml MES 325mM, 20ml 4mM $CaCl_2$, ddH_2O 430ml, pH to 6.5
- 100µM MUNANA (per plate): 300µl 1mM MUNANA, 2.7ml MES assay buffer
- Stop solution (500ml): 25% ethanol - 125ml absolute ethanol, ddH_2O 325ml pH to 10.7

pH amended with concentrated NaOH

Appendix 2. Raw data

Titration results from PV generation of A/equine/Sussex/1989 H3N8 (measured in RLU)

Well	Multiplication factor	H3N8 1	H3N8 1	H3N8 2	H3N8 2
A	20	458800	429800	824420	771680
B	40	707760	616360	1316880	1196560
C	80	1144640	1038480	2057760	1658800
D	160	2073920	1383680	3393600	2745600
E	320	1953600	2608000	4962880	6668160
F	640	2013440	1335040	6699520	5025280
G	1280	7345920	2648320	9315840	7159040
H	2560	6282240	3678720	6456320	6584320

Average		2747540	1717300	4378403	3976180
Titre	10^6	2.75	1.72	4.38	3.98

Results of Kinsley, 2017 PVs tested in MUNANA NA activity assay (measured in relative fluorescence units RFU)

Well	H3N1	H3N1	H3N2	H3N2	H3N3	H3N3	H3N4	H3N4	H3N8	H3N8	H3N9	H3N9
A	114.6	131.9	14.25	14.15	20.19	21.65	17.42	15.77	16.7	17.22	21.89	39.08
B	100.2	77.43	15.72	15.69	18.72	19.08	16.88	16.56	16.54	14.44	26.34	31.58
C	63.27	52.36	15.38	12.36	16.66	16.65	14.87	15.33	15.31	13.69	18.53	18.25
D	46.39	44.82	15.82	16.08	16.88	16.31	15.31	15.83	15.67	15.03	16.87	17.5
E	37.37	28.87	16.89	16.18	16.97	15.98	15.41	16.03	15.81	15.83	16.5	17.37
F	22.83	25.12	17.28	17.15	16.89	16.26	15.02	15.46	15.06	14.32	15.15	16.07
G	26.52	22.69	17.41	16.56	16.04	15.19	15.55	15.97	16.33	16.18	16.37	17.42
Buffer	15.21	18.44	17.93	18.21	17.41	17.57	17.9	18.22	18.66	18.07	21.2	17.94

Newly generated PV, positive and negative control in MUNANA NA activity assay (measured in relative fluorescence units RFU)

Well	H3N82	H3N82	H5N8	H5N8	NO HA/NA	NO HA/NA
A	12.97	16.82	287.4	270	14.02	13.93
B	13.67	14.59	244.5	261.5	13.92	14.07
C	12.05	14.34	163	183	13.92	14.15
D	13.82	14.34	115.6	122	14.03	13.93
E	13.65	13.66	81.72	81.35	14.74	15.17
F	13.92	14.35	57.09	68.31	15.96	17.53
G	14.3	14.74	38.36	44.2	16.37	17.87
Buffer only	15.8	16.07	17.03	18.26	16.17	17.25

NA activity with and without clear plate cover in MUNANA NA activity assay (measured in relative fluorescence units RFU)

H3N8 (13/08/18)	H3N8 (13/08/18)	VSV-G	VSV-G	NA ONLY	NA ONLY	H3N8 VIRUS	H3N8 VIRUS
100.6	105.1	106	106.3	191.7	190.5	1973	1682
95.59	99.86	99.03	103.8	139.4	141.6	1549	1522
84.2	97.67	101	105.7	121.1	123.6	1187	986
87.47	96.17	98.05	96.61	112.5	112.7	589.2	599.5

Without plate cover

H3N8 (13/08/18)	H3N8 (13/08/18)	VSV-G	VSV-G	NA ONLY	NA ONLY	H3N8 VIRUS	H3N8 VIRUS
201.5	209.8	219.9	219.4	402.5	393.9	4019	3438
196.6	215.3	216.7	220.5	294.3	297.2	3199	3108
193.2	211.8	219.4	220.5	254.4	257.4	2411	2087
204.7	216.5	203.9	198.5	233.4	231.3	1212	1200