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The Genetic Compatibility of Neuraminidase Gene Segments (N1-9) of Wild Bird Origin with Chicken H9N2 Avian Influenza Virus

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Master Degree Project in Infection Biology, 45 credits.
Spring 2021
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Abstract

Avian influenza viruses (AIVs) circulate in wild birds, predominantly wild waterfowl, and shorebirds. Due to the segmented structure of their genome, influenza viruses can undergo drastic genetic changes when two viruses infect the same cell, a process called reassortment. The two surface proteins, hemagglutinin (HA) and neuraminidase (NA) are the major antigens of AIVs. The HA and NA have opposite functions, receptor-binding, and receptor-destroying. As a consequence, these functions need to be in balance. If HA or NA is exchanged during reassortment, this balance can become shifted, impairing the fitness of the new progeny virus. H9N2 is an AIV subtype that is endemic in poultry in large parts of the world. Furthermore, H9N2 has been shown to frequently reassort with other AIVs, giving rise to new highly pathogenic AIVs that potentially could spill over to humans.

The aim of this study was to investigate the reassortment dynamics of H9N2 from chicken, more specifically its genetic compatibility with NA segments from wild birds using an eight plasmid reverse genetics system. NAs representing all known subtypes (N1-9), were cloned into the pHW2000 plasmid, and transfected with seven plasmids containing the remaining H9N2 gene segments. All NA subtypes resulted in reassortant viruses with the H9N2 backbone, demonstrating genetic compatibility between the segments *in vitro*. Secondly, the NA activity of all rescued viruses was measured to estimate the functional balance between HA/NA. All reassortant viruses had a lower NA activity compared to H9N2, implying a shift in the functional balance. In conclusion, this shows that H9N2 from chicken can create reassortant viruses with representatives of all nine NA subtypes from wild birds.

Popular scientific summary

Every year millions of people are infected with the respiratory disease commonly known as "the flu". For many, the symptoms resolve after about a week, but in some cases, it can become more severe and up to 650 000 people die of the infection each year. The flu is caused by the influenza viruses. A virus is a small particle composed of genetic material and proteins, but not much more. Due to this, the virus needs to infect us in order to survive and propagate. It invades the cells in our respiratory tract and "hijacks" the replication machinery, eventually destroying the cells. This causes inflammation which is why we have symptoms such as a sore throat and coughing.

Influenza viruses do not only infect humans, but many different subtypes of influenza virus also circulate in birds, both domestic poultry and wild birds. These are characterised as avian influenza viruses (AIVs). In fact, almost all influenza viruses including those infecting humans originate from wild birds. In wild birds the infection is mostly mild, but in poultry it can become serious and also spill-over to humans working in close contact with the birds.

AIVs can change and mutate very rapidly, causing concern that a new subtype could emerge in birds that eventually would infect humans. Since no natural immunity would exist against this "new virus" in the human population it could have pandemic potential. This fast change occurs when two different influenza viruses infect the same cell. In the cell they can exchange genetic material with each other resulting in the generation of a new influenza virus subtype. This event is called reassortment and occurs on a regular basis in birds. Therefore, many researchers study the AIVs that circulate in birds and if they could possibly exchange genetic material with each other.

The aim of this project was to study one of these AIVs termed H9N2, which circulates in poultry populations in large parts of the world. It is not very harmful to poultry, but it can infect humans and has also been shown to be very prone to exchange genetic material with other AIV subtypes. We wanted to explore if H9N2 from chicken could exchange genetic material with wild birds, more specifically if a genetic part, which creates a protein called neuraminidase, could be exchanged. On the surface of the influenza virus there are two major proteins, the hemagglutinin (HA) and the neuraminidase (NA). HA makes it possible for the virus to attach and infect our cells and the NA releases the viruses from the cell after replication. Both are very important for the virus and that is why we chose to investigate the NA.

Our study showed that the NA from H9N2 could be exchanged with all the NA from wild birds that we picked in this study. This was done in a laboratory setting and the result could be different if it happened in nature. However, this shows that AIVs from poultry and wild birds have the potential to reassort and create new viruses. The knowledge created in this project contributes to our understanding on how different influenza A viruses can exchange genetic material between each other and how the acquisition of different NA segments affects the fitness of the new viruses.

1. Introduction

1.1 Avian Influenza Viruses

Influenza viruses, members of the family *Orthomyxoviridae,* are a group of single stranded negative-sense RNA viruses. The influenza viruses can be divided into four genera; A, B, C and D, where all except influenza D viruses are known to infect humans (1,2). Out of the four, influenza A viruses (IAVs) are the most well studied and have a wide variety of hosts including humans, mammals, and different species of birds (3). Both influenza A and B viruses circulate in the human population where they cause seasonal epidemics of respiratory disease, commonly known as "the flu". The symptoms can be both mild and severe and most frequently include fever, cough, sore throat, fatigue, and myalgia (4). Millions of people are infected each year and the annual number of respiratory deaths due to influenza has been estimated to be between 290 000-650 000 (5).

IAVs can infect a broad range of species, but have their natural reservoir in wild birds, more specifically wild waterfowl, and shorebirds. The IAVs that circulate in wild birds are characterised as avian influenza viruses (AIVs) (6). The different subtypes of AIVs are defined by the glycoproteins hemagglutinin (HA) and neuraminidase (NA) found on the surface of the virion. The HA is a receptor binding protein involved in virus attachment to the host cell, while NA primarily facilitates the release of progeny virions after replication. In birds, 16 different HA (H1-16) circulate together with 9 different NA (N1-9), creating a large diversity of AIV subtypes (1). In recent years two additional subtypes of influenza viruses have been discovered in fruit bats, H17N10 and H18N11. However, studies have shown that the structures and functions of the HA and NA of these subtypes differ significantly from previously studied IAVs, and they are yet to be found in any other species than fruit bats (7). AIVs mainly infect the epithelial cells of the intestinal tract in wild birds and the virus is shed via the faeces out into the surrounding environment where it spreads through the population via the faecal-oral route. The majority of infections are asymptomatic and classified as low pathogenic avian influenza viruses (LPAIV). Some subtypes may undergo mutations when introduced in poultry, producing highly pathogenic avian influenza viruses (HPAIV). Both low and highly pathogenic AIVs can infect humans who are in close contact with poultry, raising concern for the emergence of new pandemic strains (3,8,9).

1.2 Interspecies Transmission

In general, AIVs circulate within the wild waterbird fauna, most often confined to a single or a few related bird species. However, occasionally they spread to domestic animals and to humans (10). These novel strains might have pandemic potential since no immunity against them exists in the human population. Throughout history there have been four influenza pandemics caused by IAVs, which all were genetically related to influenza viruses found in the avian population, the most severe one being the H1N1 pandemic of 1918, known as the Spanish flu (11).

It was long hypothesized that an interspecies jump from avian hosts to humans required a mixing vessel for the virus to adapt to a mammalian host (12). Pigs have both "avian" and "human" receptors in their respiratory tract, enabling them to be infected by both human and avian subtypes, allowing for emergence of novel viruses through exchange of genetic material (12,13). However, recent research shows that receptors for AIVs are not exclusive to birds and pigs, but have also been found in the human respiratory tract (14). Similarly, human influenza virus receptors, have been observed in birds (15). Furthermore, virus histochemistry studies

indicate that AIVs can bind more extensively to human tissues rather than pig tissues, questioning the mixing vessel theory (16). Lastly, direct transmission of HPAIV and LPAIV from poultry to humans also supports the theory that an intermediate mammalian host is not always required for AIVs to infect humans. Nonetheless, transmission of AIVs from wild birds to humans is still a rather rare occurrence and the interspecies transmission is a complex process depending on many factors.

1.3 Genome and Virion Structure

AIVs are enveloped single stranded, negative-sense RNA viruses with a segmented genome. The virion is generally described as spherical in shape with a diameter of ~100 nm but it can also appear in filamentous elongated shapes during natural infections (1,17). Embedded into the viral envelope are the HA and NA glycoproteins, and the 8 separate viral RNA (vRNA) segments coated with nucleoprotein are found within the viral nucleocapsid.

The segments are numbered by decreasing nucleotide length with the three longest segments encoding for the three separate subunits of the RNA-dependent RNA polymerase (RdRP). Segment 1 for polymerase basic 2 (PB2), segment 2 for polymerase basic 1 (PB1), and finally segment 3 for polymerase acidic (PA). The nucleoprotein (NP) that coats the vRNA is encoded by segment 5 and together with the RdRP and vRNA they form the viral ribonucleoprotein (vRNP) complex which serves as a template for production of new virions during infection (18). The two final internal proteins are encoded by segment 8, which expresses both non-structural protein 1 (NS1) and non-structural protein 2 (NS2) by mRNA splicing. NS1 is an multifunctional protein working as an interferon-antagonist and as a post-transcriptional regulator (19,20), whilst NS2, also called the nuclear export protein (NEP,) mainly is involved in exporting vRNPs from the host cell nucleus (21). Finally, the surface glycoproteins HA and NA are encoded by segment 4 and 6, and segment 7 codes for the matrix protein (M1), which encloses the virion core, and two variants of the matrix ion channels (M2, M42) by splicing of the mRNA (1,22).

1.4 Surface Glycoproteins

Found in the envelope of the virus are the surface glycoproteins HA and NA, giving rise to the budded appearance of the virion (1). The two proteins are the major antigens of AIVs, and they play a large part in transmission, virulence, and host specificity of the viruses. They both recognise the molecule sialic acid (SA), a derivate of neuraminic acid found on the host cell. Binding to SA by HA and later release of virions from SA mediated by NA enables AIVs to infect and disseminate from the host cell (23). Due to their antigenic properties and vital role in the virus life cycle they have become the research target for both vaccines and antivirals.

1.4.1 Hemagglutinin

HA is a trimeric membrane glycoprotein that is responsible for both attachment to the host cell and fusion of the viral and host endosomal membrane. HA has three identical monomeric subunits forming a globular domain and a stem region (24). Polypeptide chain HA_1 forms the globular domain containing the receptor binding site and antigenic sites. The stem region of HA consists of polypeptide chain HA_2 and contains the fusion-peptide in the N-terminus. The two polypeptide chains are made from a precursor, HA_0, which requires cleavage by host proteases to form HA_1 and HA_2. Furthermore, temperature and low pH is important for the

conformational change of HA, exposing the fusion-protein on the N-terminus enabling fusion of the viral and host endosomal membranes (25,26).

HA recognises SA moieties, more specifically SA *N-5-acetylneuraminic acid* (Neu5Ac) or SA *N-5-glycolylneuraminic acid (*Neu5Gc) found at the terminal of different glycans in the host cell. Depending on the binding to the galactose of the glycans the SA can form either an α2,3- or α2,6-linkage (27). Different HA have either a predilection towards the α2,3- or α2,6-linked SAs, dependent on the virus host tropism. Subtypes that circulate in humans bind more specifically to α2,6-linked SAs found on the epithelial cells of the respiratory tract, whereas subtypes found in birds bind α2,3-linked SAs, present on the intestinal epithelial cells (28,29).

Depending on their genetic similarities the 16 HA of IAVs can be divided into two groups and four clades. Group 1 consists of clade H1 (H1, H2, H5, H6, H11, H13, H16) and clade H9 (H8, H9, H12), whilst group 2 consists of the H3 clade (H3, H4, H14) and the H7 clade (H7, H10, H15) (30).

1.4.2 Neuraminidase

The second surface glycoprotein of AIVs is the NA, a tetrameric transmembrane protein consisting of a cytoplasmic and transmembrane domain connected to the stalk with a globular head domain (31). NA has a receptor-destroying enzymatic function, where bonds between SA and the glycans on the host cell are hydrolysed. The active site of the enzyme sits in the globular domain of the protein and is highly conserved, although the overall amino acid sequence for NA can vary greatly between different subtypes. The nine NA of the AIVs are divided into two phylogenetic groups: group 1 consisting of N1, N4, N5 and N8, and group 2 of N2, N3, N6, N7 and N9 (32). Just like the HA the NA recognises both α2,3- and α2,6- SA linkages but has a higher specificity for the SA with an α2,3-linkage in avian subtypes. A change in specificity towards α2,6-linkage can be seen in human subtypes. However, this change is rather minor and the NA still preferentially cleaves α2,3-bonds (33).

During infection, NA helps to facilitate the virion's movement through the mucus towards the epithelial cells. The mucus contains sialylated mucins that bind to the HA preventing the virion from reaching its target cells. By cleaving the mucins bound to the HA receptors, NA promotes the continuous movement of the virus through the mucus layer (34,35). Secondly, the NA is essential for the release of progeny virions from the infected cells. After infection, the newly formed virions can aggregate at the host cell membrane via linkage to the terminal SAs present on the cell surface and the newly formed virus particles. These bonds are cleaved by NA allowing release of the virions from the cell surface. More recent research also implicates that NA is involved in more steps of the viral life cycle than previously thought, both enabling a rolling-motility of the virus and taking part in viral entry (36,37).

1.5 Functional Balance of HA and NA

Having the opposite functions, receptor-binding and receptor-destroying, the activities of HA and NA have to be balanced for productive infection. Previously, the functions of the proteins have been studied separately but the importance of the dynamics and interactions of the proteins is becoming more evident. The balanced action of the two proteins enables movement by alternating binding and cleaving of receptors, resulting in a NA-dependent rolling motion across the mucus. Furthermore, the balance is important for more efficient entry into the host cell and release of new virus particles (37). Several studies have shown that alterations to the balance

between HA and NA can significantly impair the fitness of the virus. It has also been shown that functional imbalances, for example through reassortment events, can be restored by mutations in the HA and NA, suggesting that the balance is flexible and adjustable (reviewed in 23,34,37). The balance is also hypothesised to be important for host tropism and virulence. One example of this is the short NA stalk associated with chicken H5 and H7 subtypes. A shortened stalk decreases the NAs ability to cleave SAs, lowering the activity of NA. The HA of H5 and H7 has an additional glycosylation lowering the affinity for its receptor, indicating that changes in both the HA and NA can be important for adaptation from wild birds to poultry (38,39). Furthermore, another study observed the potential importance of functional balance for virulence. A low pathogenic H7N3 (A/chicken/British Columbia/CN0006/2004) was reassorted with H5 from a highly pathogenic H5N1 (A/swan/Germany/R65/2006), containing the classical polybasic cleavage site in the HA associated with HPAIV, generating a H5N3 reassortant virus. However, the highly pathogenic phenotype was not observed in the reassortant. When passaged in chickens the reassortant H5N3 acquired a mutation in the NA associated with decreased NA activity and simultaneously the virulence of the reassortant increased, indicating that the functional balance plays a role in virulence and expression of a highly pathogenic phenotype (40).

As of today, there is no golden standard approach in measuring the functional balance of HA and NA simultaneously. Instead, the binding affinity (K_d) of HA and the enzymatic activity (K_m or K_{cat}) of NA are measured separately using different assays and then correlated to one another (23). The HA binding affinity can be measured with glycan binding assays (41), and the enzymatic activity of NA can be estimated by fluorescent and chemiluminescent assays (42,43). This gives an estimation of the balance between HA and NA but more methods to measure the balance on a multivalent level are needed to fully understand the interactions of the two proteins (23,37). In conclusion, the balance of HA and NA plays a major role in many parts of the virus life cycle, but how the balance works on a molecular level and how it changes depending on different hosts still remains largely unknown.

1.6 The Evolution and Changes of AIVs

With 16 HA and 9 NA present in the avian population there is a large diversity of antigenic subtypes (3). Furthermore, changes in the internal segments and mutations acquired over time has led to a vast multitude of lineages and genotypes. AIVs are distinguished by their ability to rapidly mutate and undergo large genetic and antigenic alterations, enabled by two separate processes: antigenic drift and antigenic shift (44).

1.6.1 Antigenic Drift

Antigenic drift is the accumulation of mutations in the HA and NA over time. AIVs are inclined to mutate due to their error prone RNA-polymerase, which in contrast to other polymerases lacks proof-reading activity, so if an error occurs this will remain after replication. A new study has shown that the polymerase is more error-prone than previously thought, resulting in an average of 2 mutations per new virus particle. With a fast replication cycle, accumulation of new mutations can alter the antigenic properties of the virus, allowing adaption to selective pressures such as vaccines, antivirals, and new hosts (44,45).

1.6.2 Antigenic Shift and Reassortment

Influenza viruses can undergo drastic changes in their antigenic structure as a result of the segmented structure of their genome. This process is referred to as antigenic shift. Antigenic shift is mediated by reassortment, which can take place when two different influenza viruses infect the same cell resulting in exchange of genetic segments (44,46). All segments can undergo reassortment, but exchange of the HA and NA segments will have the most profound effect on alteration of antigenic properties. Reassortment might lead to the emergence of new potential pandemic strains; this has been shown in the past H2N2 and H3N2 pandemics and most recently the 2009 H1N1 pandemic, which emerged from reassortment between the H1N1 European "avian-like" swine lineage, H1N2 triple reassortant swine lineage, and a Eurasian avian H1N1 lineage (47,48).

Most reassortment events are not successful due to segment mismatch. These segment incompatibilities can be divided into two categories, RNA mismatch and protein mismatch. RNA mismatch can occur between the vRNAs of two influenza strains during packaging. It is hypothesised that the segments interact via their packaging signals during assembly of the vRNPs, and if the mismatch is too substantial no progeny virus will be formed. Mismatch on protein level occurs later, after assembly of the virus. Two commonly observed protein mismatches are between the polymerase subunits, hindering transcription and replication, and between the surface glycoproteins HA and NA (49). As described earlier a balance between HA/NA is important for both movement and entry, and finally release of new viruses. If unbalanced this will have a large impact on the virus ability to infect and replicate.

1.7 AIVs in Wild Birds and Poultry

As mentioned earlier, AIVs have their natural reservoir in wild birds, more specifically *Anseriformes* (waterfowl) and *Charadriiformes* (gulls and shorebirds) (3). Out of these, the mallard is believed to be the main reservoir, with an AIV prevalence of up to 60 % in certain populations at particular time points (6). In wild birds, the virus is shed via the faeces in high titres, often to lake water where the virus has been shown to be very stable, remaining infectious for 4 days at 22 ºC, and for more than 30 days at 0 ºC (50). As a result of the migratory behaviour of many bird species, different subtypes of AIVs can spread geographically and be transmitted to new populations when the birds aggregate at certain stop sites during their migration (6).

During spill-over events to domestic poultry, the AIVs can undergo genetic changes resulting in the more pathogenic phenotype of HPAIV. The HA subtypes associated with HPAIV are H5 and H7 (51). In highly pathogenic strains, the HA contains a polybasic cleavage site in the precursor HA_0, enabling cleavage of the precursor by furin proteases instead of trypsin-like serine proteases, resulting in a more severe infection. The serine proteases are mainly localised in the epithelium of the gastrointestinal- and respiratory tract limiting the infection by LPAIV, whereas the furin proteases are present in more tissues and cell types giving rise to a systemic infection by HPAIV. (52,53). The spread of LPAIV from wild birds directly to humans is extremely rare, but outbreaks of AIVs in domestic poultry have resulted in both infections and fatalities in humans (54). In 1997, the first human case of the HPAIV H5N1 was documented in Hong Kong and has been followed by additional outbreaks of H5N1, but also other highly and low pathogenic subtypes such as H7N9, H7N7 and H9N2 (55–58).

1.8 H9N2

First discovered in turkeys in the United States in 1966 the LPAIV H9N2 has now become endemic in poultry in many parts of the world, primarily in South East Asia, West Africa, and the Middle East (59,60). Phylogenetically, H9N2 can be divided into two branches: the American lineage which is mostly found in wild birds, and the Eurasian lineage which has three established lineages in poultry: A/quail/Hong Kong/G1/1997 (G1), A/chicken/Beijing/1/94 (BJ94), and A/Chicken/Hong Kong/Y439/1997 (Y439) (61). Although it is classified as an LPAIV, H9N2 is associated with higher mortalities during outbreaks in poultry. This has been linked to co-infection with other pathogens and environmental factors such as poor living conditions for the poultry (60). The first cases of humans infected with H9N2 were recorded in Hong Kong in 1999 and since then more people have been infected. However, the disease in humans is often mild and only one fatal case has been registered (58,60).

H9N2 co-circulates with other AIV subtypes in poultry, often H5 and H7 subtypes, posing a risk for potential reassortment events and emergence of new subtypes. In recent years, novel reassortments of HPAIV with H9N2 have emerged, for example H7N9 and H5N6, both having received their internal gene cassette (all genes except HA and NA) from G1 H9N2 strains (62–64). The 1997 H5N1 strain causing outbreak in humans has also subsequently been shown to have all of its internal genes from co-circulating G1 lineages of H9N2 (65). An important trait of the G1 lineage is that it has a substitutional mutation in the HA, Q226L, which increases the receptor specificity towards α2,6-linked SAs (66). Altogether, this suggests that H9N2 is an AIV that could possibly play an important role in the emergence of new viruses capable of infecting humans, either by itself or by donating genes to co-circulating strains.

1.9 Eight Plasmid System for Reverse Genetics

In 2000 Hoffman *et. al* developed an eight-plasmid system for IAVs that result in rescue of complete viral particles, a reverse genetics technique that allows for the manipulation and rescue of synthetic vRNA from cDNA. The method uses a plasmid known as pHW2000 which is roughly ~2900 bp long containing an ampicillin resistance gene for selection, human Pol I/II promoters and a BsmBI restriction site for insertion of a selected gene (67). Eight plasmids, each containing the cDNA of one of the viral segments of the IAV genome are assembled and then used to transfect a co-culture of Human Embryonic Kidney 293T cells (HEK293T) and Madin-Darby Canine Kidney cells (MDCK). In the HEK293T cells cellular RNA polymerase I starts transcribing vRNAs from the cDNA and RNA polymerase II mRNAs. When the viral polymerase complex (PB1, PB2, PA and NP) has been translated by the cell the viral replication starts and the translated proteins and vRNAs are assembled into new viral particles. The newly produced virions can continue to propagate in the HEK293T cells, but MDCK cells are used in the culture to ensure proper viral growth, being the standard cell line used for IAVs (Figure 1) (67). This method can be used to investigate the compatibility of different segments and subtypes by exchanging segments during transfection. Furthermore, the relevance of different point mutations and other genetic changes can be studied (68).

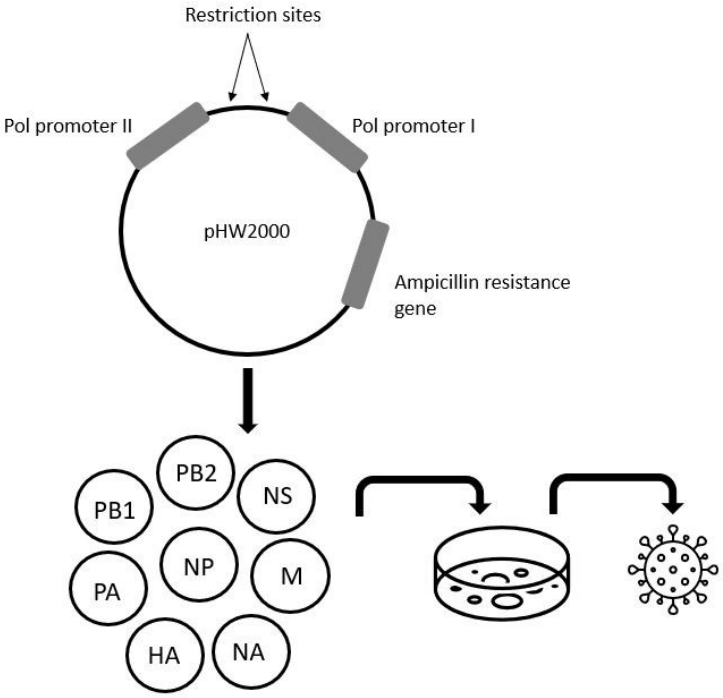

Figure 1. Simplified schematic picture showing the eight plasmid system. A gene of interest is inserted between the restriction sites of the pHW2000 plasmid. Once all gene segments have been cloned into separate plasmids, they can be transfected into a co-culture of HEK293T/MDCK cells. RNA polymerases will starts transcribing the vRNA and mRNAs from the two promotors, resulting in the assembly of a fully functional virus particle.

With naturally co-circulating AIVs in poultry able to infect humans, such as H9N2 and H5N1, it is of great importance to study the phenotypes of potential new subtypes that could possibly emerge. By using the reverse genetics method to better understand the reassortment dynamics of AIVs, more accurate predictions and surveillance could be made to prevent emergence and spread of new influenza viruses. Not only because of their pandemic potential but also because of the large negative impact they can have on the poultry industry, risking both the economy and health of the affected farmers.

2. Aim

The aim of this study was to assess the genetic compatibility of all avian NA subtypes (N1-9) from wild bird origin with the LPAIV H9N2 from chicken. The study was divided into three parts:

- Cloning of the nine NA segments into the pHW2000 plasmid.
- Rescue trials of reassortant viruses using transfection based on the eight plasmid system.
- Measure and compare the enzymatic activity of the NA of the obtained reassortant viruses with the parental LPAIV H9N2.

Investigating the compatibility between H9N2 and NA segments from other AIVs could hopefully increase the knowledge of the reassortment dynamics of AIVs in poultry and wild birds. A greater insight in this subject could result in better predictions of potential reassortants that could pose a risk for animals and humans.

3. Material and Methods

3.1. Viral Strains

The viruses (Table 1) were collected from mallards at the Ottenby Bird Observatory on Öland, Sweden, and isolated through passage in specific pathogen free (SPF) embryonated chicken eggs. The viral titer was determined by the egg infectious dose 50 (EID_{50}). The viruses were kindly provided by Professor Jonas Waldenström, Linneaus University, to the Zoonosis Science Centre (ZSC), Department of Medical Biochemistry and Microbiology, Uppsala University, Sweden. The different subtypes were chosen to represent all the nine different NA segments found in AIVs.

Table 1. The viruses used for cloning of the NA segments.

Subtype	Isolate
H10N1	A/Mallard/Sweden/102087/2009
H6N2	A/Mallard/Sweden/99825/2009
H9N2	A/Mallard/Sweden/67860/2007
H16N3	A/Black-headed Gull/Sweden/74340/2008
H8N4	A/Mallard/Sweden/58256/2006
H15N5	A/Mallard/Sweden/139647/2012
H4N6	A/Mallard/Sweden/80148/2008
H7N7	A/Mallard/Sweden/1690/2002
H3N8	A/Mallard/Sweden/101487/2009
H11N9	A/Mallard/Sweden/102103/2009

3.2. Cloning

All viruses in Table 1 were used in the cloning process. The NA segment of H9N2 (A/Mallard/Sweden/67860/2007) was cloned by Anishia Wasberg (ZSC, Uppsala University, Sweden) and the NA segment from H6N2 (A/Mallard/Sweden/99825/2009) was cloned by Emma Brodin and Klara Martinovic (ZSC, Uppsala University, Sweden). The remaining NA segments (Table 1) were generated in this study as described below:

3.2.1. RNA Extraction

Extraction of viral RNA was done using the Direct-zol™ RNA Miniprep Kit (Zymo Research Corporation, USA) for the H10N1(A/Mallard/Sweden/102087/2009) virus and the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) for the remainder of the virus subtypes. All extractions were performed according to manufacturer's protocol with one modification, the RNA was eluted in 50 µL of elution buffer.

3.2.2. RT-PCR

RT-PCR of the extracted RNA was performed using the SuperScript™ IV One-Step RT-PCR System (Invitrogen by Thermo Fisher Scientific, CA, USA) according to manufacturer's protocol with 10 µL of RNA. Specific forward and reverse primers were designed (Supplementary Table 1) for each NA segment. The primers consisted of four random extra nucleotides at the 5' end, followed by the coding sequence for the restriction enzyme, a small part of the plasmid and 20-25 nucleotides of the gene of interest. Amplification was set at 35

cycles using the Applied Biosystems™ 2720 Thermal Cycler (Applied Biosystems™ by Thermo Fisher Scientific, CA, USA). The amplified product was analysed on a 1.2 % agarose gel containing 1.5 % GelRed® (Biotium, CA, USA) and then extracted and purified using Thermo Scientific GeneJET Gel Extraction Kit (Thermo Fisher Scientific, MA, USA) according to the DNA extraction from gel using centrifuge protocol. The DNA was eluted in 20 µL of elution buffer and the concentration was measured using the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, MA, USA).

3.2.3. Digestion of the PCR Products and pHW2000 Plasmid

The restriction enzymes BsaI-HFv2 (New England Biolabs, MA, USA) and BsmBI (New England Biolabs, MA, USA) were used depending on the internal restriction sites of the NA segments. Both restriction enzymes created the same overhang sequence to ensure proper ligation. The pHW2000 was provided by Martin Schwemmle & Team (University of Freiburg, Germany). The digestion of the plasmid was carried out only using BsmBI. The digestion was performed according to manufacturer's protocol for 500 ng of DNA (for both the plasmid and NA segments) using the Bio-Rad T100™ Thermal Cycler (Bio-Rad Laboratories, CA, USA). The digested products were analysed on a 1.2 % agarose gel containing 1.5 % GelRed® (Biotium, CA, USA) and then purified using the NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel, Düren, Germany) according to the PCR clean-up protocol. The DNA was eluted in 20 µL of elution buffer and the concentration was measured using the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, MA, USA).

3.2.4. Ligation

Ligation of the digested product and pHW2000 plasmid was done using the T4 DNA Ligase Kit (New England Biolabs, MA, USA) according to the manufacturers protocol. A 3:1 ratio of insert and plasmid was calculated using the NEBioCalculator and the ligation mix was incubated at 16 ºC for 16 hours in the Bio-Rad T100™ Thermal Cycler (Bio-Rad Laboratories, CA, USA).

3.2.5. Transformation and Glycerol Stocks

1-5 µL of ligation mix was added to 50 µL of competent DH5α *Escherichia coli* (Thermo Fisher Scientific, MA, USA) followed by 30 minutes incubation on ice. The cells were then transformed using heat-shock for 30 seconds at 42 ºC and then placed immediately back on ice for 2 min. 200 µL of SOC media (Invitrogen by Thermo Fisher Scientific, CA, USA) was then added to all cells and then incubated at 37 ºC with shaking at 200 rpm for 1 hour. 100 µL of transformed cells was plated on LB plates containing 100 µg/mL ampicillin and incubated at 37 ºC for 14-16 h. Single colonies from the plates were picked separately and inoculated in 3 mL of LB media containing 100 µg/mL ampicillin followed by incubation at 37 ºC with shaking for 12-18 h. 500 µL of the overnight culture was added to 500 µL of 50 % glycerol and then stored at -80 ºC. The rest of the culture was used for plasmid extraction as described in the next step.

3.2.6. Mini Prep of Plasmid DNA

Plasmid DNA was purified from 2 mL of transformation overnight culture using the QIAGEN Plasmid Mini Kit (Qiagen, Hilden, Germany) or the Zyppy Plasmid Miniprep Kit (Zymo Research, CA, USA) according to manufacturer's protocol. DNA concentration was measured using the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, MA, USA) and then sent to Eurofins Genomics (Germany) for sequencing. The sequences were then analysed using Geneious software (Biomatters Ltd., Auckland, New Zealand).

3.2.7. Midi Prep of Plasmid DNA

5 µL of the selected glycerol stock was inoculated in 50 mL of LB media (100 µg/mL ampicillin) for 16-18 h. The cells were harvested by centrifugation for 10 min at 4700 x g. The plasmid DNA was purified using the GeneJET Plasmid Midiprep Kit (Thermo Fisher Scientific, MA, USA) according to manufacturer's protocol using low speed centrifuges. This was used to obtain both a higher and more pure concentration of the plasmid containing the N7 segment. The purified plasmid DNA was eluted in 250 µL elution buffer and the DNA concentration was measured using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, MA, USA) and then sent to Eurofins Genomics (Germany) for sequencing. The sequences were then analysed using Geneious software (Biomatters Ltd., Auckland, New Zealand).

3.2.8. Maxi Prep of Plasmid DNA

200 µL of the selected glycerol stock was inoculated in 250 mL of LB media (100 µg/mL ampicillin) for 16-18 h. The overnight culture was centrifuged at 4700 x g for 20 min at 4 ºC (adapted from the kit instruction, which was 6000 x g for 15 min, due to the speed limit of our centrifuge). The supernatant was discarded, and this was repeated until all overnight culture had been used. Plasmid DNA was then purified from the bacterial pellet using the QIAGEN Plasmid Maxi Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol with all centrifugation steps carried out at 4700 x g. This was used to obtain both a higher and more pure concentration of the plasmids containing the NA segments (except N7) and the backbone of chH9N2. The purified plasmid was eluted in 300 µL elution buffer and the DNA concentration was measured using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, MA, USA) and then sent to Eurofins Genomics (Germany) for sequencing. The sequences were then analysed using Geneious software (Biomatters Ltd., Auckland, New Zealand).

3.3. Cell Culture Maintenance

HEK293T (Human Embryonic Kidney 293 cells that contain the SV40 T-antigen) and MDCK (Madin-Darby Canine Kidney) (ATCC®, VA, USA) cells were maintained in Dulbecco Modified Eagle Medium (DMEM, Gibco™, Life Technologies, UK) supplemented with 5 % (v/v) fetal bovine serum (FBS, Gibco™, Life Technologies, UK) and 1 % (v/v) Antibiotic-Antimycotic (Anti-Anti, Gibco™, Life Technologies, UK) at 37 ºC. A co-culture of the MDCK and HEK293T cells (1:2 ratio) was seeded in a treated 6-well plate and used for transfection when at ~90 % confluency. The cell cultures were managed by Anishia Wasberg (ZSC, Uppsala University, Sweden).

3.4. Transfection

Transfection was done according to the eight plasmid system as described earlier (67), using the A/chicken/Egypt/S12568C/2016(H9N2) (chH9N2) plasmids (PB1, PB2, PA, NP, NS, M, HA, NA) provided by Dr. Ahmed Elsayed (Justus Liebig University Giessen, Germany), and the NA plasmids created in the cloning process (as described in section 3.2). A 6-well plate with a co-culture of MDCK and HEK293T cells was washed two times with serum-free DMEM (Gibco™, Life Technologies, UK) and serum-starved for 1 hour at 37 ºC in 1 mL of Opti-MEM (Gibco™, Life Technologies, UK) to remove any components that could potentially interfere with the transfection reagents. Plasmids were diluted to ~500 ng/µL and for the positive control all chH9N2 plasmids were used. For rescue of reassortants all plasmids of the chH9N2 were used with the exception of the NA plasmid, this was exchanged for the different NA plasmids from the mallard and black-headed gull viruses. Transfection mixtures were prepared according to the Lipofectamine™ 3000 reagent (Invitrogen by Thermo Fisher Scientific, CA, USA) protocol for 4 µg of DNA in a 6-well plate. 250 µL of transfection-mixture was added dropwise to the corresponding well and the plate was re-incubated in 37 ºC. 6-24 h post transfection the media was changed to 3 mL of infection media per well containing Opti-MEM (Gibco™, Life Technologies, UK), 0,2 % (v/v) Bovine Serum Albumin solution 35% (BSA, MpBio™, CA, USA), 0.5 µL/mL TPCK-Trypsin (Sigma-Aldrich, USA), and 100 I.U./mL Penicillin & 100 µL/mL Streptomycin (Gibco™, Life Technologies, UK). Supernatants were collected 48 h and 72 h post transfection, centrifuged at 4000 rpm for 10 min and stored at -20º C until further usage.

3.5. Egg Infection

SPF embryonated chicken eggs were obtained from Håtuna Lab AB, Sweden and kept in a 37 ºC incubator. To obtain a higher virus titer the eggs were infected 10 days post embryonation. A small hole was made in the shell and 200 µL of transfection supernatant was injected into the allantoic fluid using a syringe. The eggs were re-incubated in 37 ºC and the allantoic fluid was collected 72 h post infection. Virus titers were measured using the hemagglutination assay as described below.

3.6. Hemagglutination Assay

The hemagglutination assay was used to determine the concentration of virus by number of hemagglutination units. Influenza viruses binds to erythrocytes, by hemagglutination, leaving them suspended. In samples with no or too little virus the red blood cells sink to the bottom giving a characteristic red dot. The assay was performed using a 96-well V-bottom microtiter plate. 25 µl of 1x phosphate-buffered saline (PBS) was added to each well and 25 µL of allantoic fluid was added into the first-row wells as duplicates and serial diluted ½ in each well leaving the final row as a negative control. Chicken blood obtained from Håtuna Lab AB, Sweden was washed with 1x PBS and centrifuged at 1000 rpm for 5 minutes. The washing and centrifugation were repeated twice, and the red blood cells (RBCs) were collected after the final centrifugation. The RBCs were diluted to 1 % in 1x PBS and 25 µL was added to each well of the microtiter plate. The plate was incubated for ~20-30 min at room temperature until a clear dot could be observed in the bottom of the negative control wells. All wells where no dot could be seen in the bottom were determined as hemagglutination positive. The plate was tilted and the dots in the negative wells formed a teardrop-like shape. Wells with a dot that did not form a teardrop were also considered positive for hemagglutination.

3.7. Real-Time Reverse Transcriptase qPCR

To confirm that the correct subtype had been obtained during transfection the NAs of the rescued reassortant viruses were analysed using real-time reverse transcriptase qPCR. RNA was extracted from the viruses using the Maxwell® 16 Viral Total Nucleic Acid Purification Kit (Promega, Nacka, Sweden) according to manufacturer's protocol for viral samples. The viral RNAs were analysed by the AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystems™ by Thermo Fisher Scientific, CA, USA) targeting the NA using specific primers/probes for each NA subtype. The RT-qPCR was performed using the CFX Connect ™ Real-Time System (Bio-Rad Laboratories, CA, USA).

3.8. Neuraminidase Activity Chemiluminescent Assay

The NA-XTD™ Influenza Neuraminidase Assay (Applied Biosystems™ by Thermo Fisher Scientific, CA, USA) was used to measure the neuraminidase enzymatic activity of the reassortant viruses and chH9N2. The assay is based on a previously described method (43) and determines the cleaving activity of the neuraminidase by measuring the chemiluminescent units released when the enzyme cleaves the substrate (sodium (3-chloro-5-(4methoxyspiro{1,2-dioxetane-3,2'-(5-chloro)tricyclo[3.3.1.13,7]decan}-4-yl-phenyl 5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranoside)onate). Before performing the assay, the viruses were normalised by their HA titer as described earlier (69) and diluted to the same starting concentration of 32 HAU/25 µL. The assay was performed according to manufacturer's protocol without addition of neuraminidase inhibitors. The chemiluminescent signal was read (1s/well) using the infinite® M200 plate reader (Tecan, Männedorf, Switzerland) and Magellan™ software (Tecan, Männedorf, Switzerland). The data was analysed using Rstudio (Rstudio, MA, USA). The relative NA activity was calculated as described earlier (69). The significance of the relative activities was calculated using one-way ANOVA ($p=0.05$).

3.9. Alignment and Phylogenetic Tree

The sequences of all NAs (including chH9N2) used in the thesis (Table 1) were assembled and aligned using Geneious alignment (Geneious Prime, Biomatters Ltd., Auckland, New Zealand) and a phylogenetic tree from the alignment was created using Geneious Tree Builder (Geneious Prime, Biomatters Ltd., Auckland, New Zealand). NA from influenza B was included to outgroup the phylogenetic tree.

4. Results

4.1. Cloning of the NA Segments

To investigate the reassortment dynamics of the avian NA segments with H9N2 from chicken, representative viruses for each NA (1-9) were chosen (Table 1). Viral RNA was successfully extracted from all viruses and cDNA of the NA segments were obtained using specific forward and reverse primers designed for each NA segment. Primers designed for all NA segments successfully generated PCR products of expected size (~1500 bp) (Figure 2 A). After digestion of the pHW2000 plasmid and the insert they migrated to the expected fragment size on 1.2 % agarose gel (Figure 2 B). All cloned NA segments resulted in bacterial colonies after transformation and plating on LB agar plates containing ampicillin (100 µg/mL), demonstrating both successful ligation and transformation (Figure 2 C). Sequence analysis of all cloned NA segments showed that the gene had been successfully inserted into the plasmid and retained the correct nucleotide sequence as compared to the cDNA sequence of the corresponding isolate (Figure 2 D).

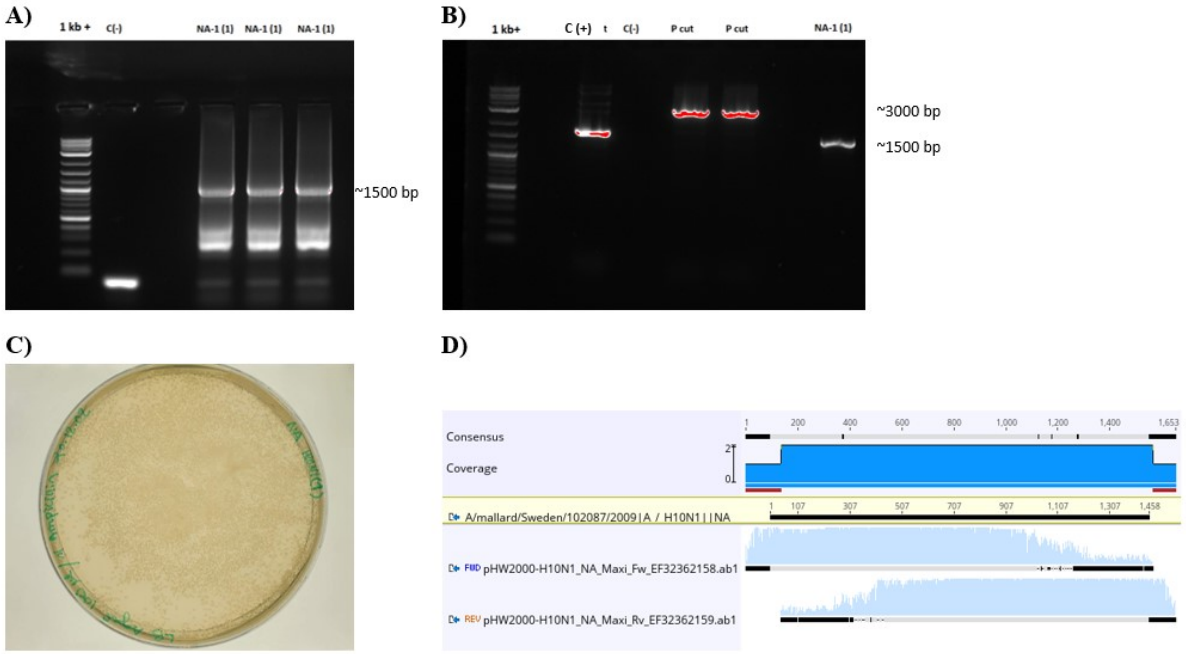

Figure 2. Cloning process of the NA segments, showing the cloning of N1 as an example. A) RT-PCR product showing the N1 segment at ~1500 bp. **B)** Digestion of the plasmid and N1 segment, showing the digested plasmid at ~3000 bp and the digested N1 segment at ~1500 bp. Undigested plasmid as positive control. **C)** Successfully transformed cells plated on LB (100 µg/mL ampicillin). **D)** Sequencing of the N1 segment in the pHW2000 plasmid aligned against the reference sequence of H10N1, showing both the forward and reverse sequence.

A second confirmation of the segments after extraction using maxi or midi prep by sequencing confirmed that no alterations had occurred to the segment during propagation in *E. coli* (Figure 2 D).

4.2. Rescue of Reassortant Viruses

4.2.1 Transfection and Hemagglutination Assay

Transfection based on the eight plasmid system (see section 3.4) was done to rescue reassortant viruses. The chH9N2 plasmids (PB1, PB2, PA, NP, NS, M, HA) were transfected together with one selected NA plasmid in a co-culture of HEK293T cells and MDCK cells. All NA plasmids (N1-9) created reassortant viruses with the chH9N2 backbone as shown by hemagglutination assay after propagation of the cell supernatant in embryonated chicken eggs (Table 2). The rescued viruses showed varying HA titers in chicken RBCs, ranging from 32 to 256 HAU. chH9Nx$_{mH7N7}$ showed the lowest HAU and chH9Nx$_{mH16N3}$, chH9Nx$_{mH15N5}$ chH9Nx$_{mH4N6}$ the highest HAU.

Table 2. Rescued reassortant viruses and the hemagglutination units (HAU) in chicken RBCs.

Reassortant viruses	HAU in chicken RBCs
chH9Nx$_{mH10N1}$	128
chH9Nx$_{mH6N2}$	64
chH9Nx$_{mH9N2}$	128
chH9Nx$_{gH16N3}$	256
chH9Nx$_{mH8N4}$	64
chH9Nx$_{mH15N5}$	256
chH9Nx$_{mH4N6}$	256
chH9Nx$_{mH7N7}$	32
chH9Nx$_{mH3N8}$	128
chH9Nx$_{mH11N9}$	64

m= Mallard, g= Black-headed gull. This represents the wild bird origin of the NA subtype.

4.2.2 RT-qPCR

To confirm that the correct NA was present in the corresponding reassortant virus and to ensure that no cross-contamination had occurred during transfection the samples were analysed using RT-qPCR. Primers targeting a specific NA subtype was used and each sample was cross-checked against all subtypes present in the transfection plate. The RT-qPCR showed that all reassortant viruses contained the correct NA subtype and that no samples had been cross-contaminated (Figure 3. A-J). Since the primers could not distinguish between variants of the same NA subtype the chH9Nx$_{mH9N2}$ and chH9Nx$_{mH6N2}$ were sent for sequencing to confirm that the correct N2 subtype was present. Sequencing showed the correct N2 in both chH9Nx$_{mH9N2}$ and chH9Nx$_{mH6N2}$ (data not shown).

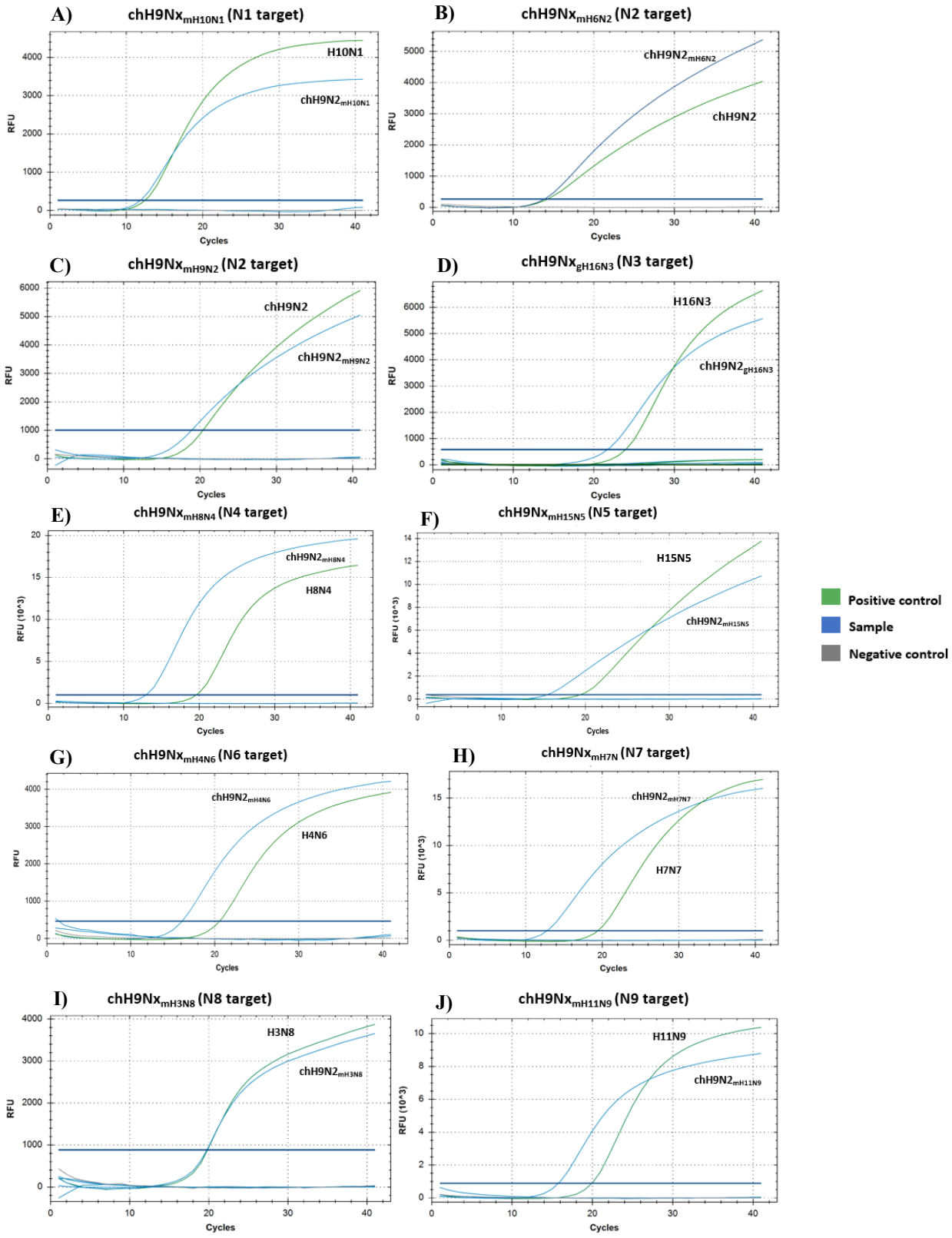

Figure 3. RT qPCR targeting the specific NA subtypes of the reassortant viruses. A) N1 of H10N1, chH9N2x$_{mH10N1}$ and negative control. **B)** N2 of chH9N2, chH9Nx$_{mH6N2}$ and negative control. **C)** N2 of chH9N2, chH9Nx$_{mH9N2}$ and negative control. **D)** N3 of H16N3, chH9Nx$_{gH16N3}$ and negative control. **E)** N4 of H8N4, chH9Nx$_{mH8N4}$ and negative control. **F)** N5 of H15N5, chH9Nx$_{mH15N5}$ and negative control. **G)** N6 of H4N6, chH9Nx$_{mH4N6}$ and negative control. **H)** N7 of H7N7, chH9Nx$_{mH7N7}$ and negative control. **I)** N8 of H3N8, chH9Nx$_{mH3N8}$ and negative control. **J)** N9 of H11N9, chH9N$_{xmH11N9}$ and negative control.

4.3. Neuraminidase Activity

To get an estimate of the functional balance between HA and NA of the reassortant viruses and the parental chH9N2, the enzymatic activity of each NA was measured using the NA-XTD chemiluminescent assay. The viruses where normalised by their HA titer so all had the same starting concentration of 32 HAU/25 µL. The assay showed that the parental chH9N2 had the highest NA activity, followed by chH9Nx$_{mH8N4}$. The lowest activity was seen for chH9Nx$_{mH4N6}$, chH9Nx$_{mH6N2}$, chH9Nx$_{mH11N9}$, chH9Nx$_{mH3N8}$ and chH9Nx$_{mH7N7}$. Finally, chH9Nx$_{gH16N3}$, chH9Nx$_{mH9N2}$, chH9Nx$_{mH15N5}$ and chH9Nx$_{mH10N1}$ had activities in the mid-range (Figure 4). Furthermore, the NA assay showed that relative to the NA activity of chH9N2, all reassortant viruses had significantly lower NA activities ($p<0.05$) (Figure 5).

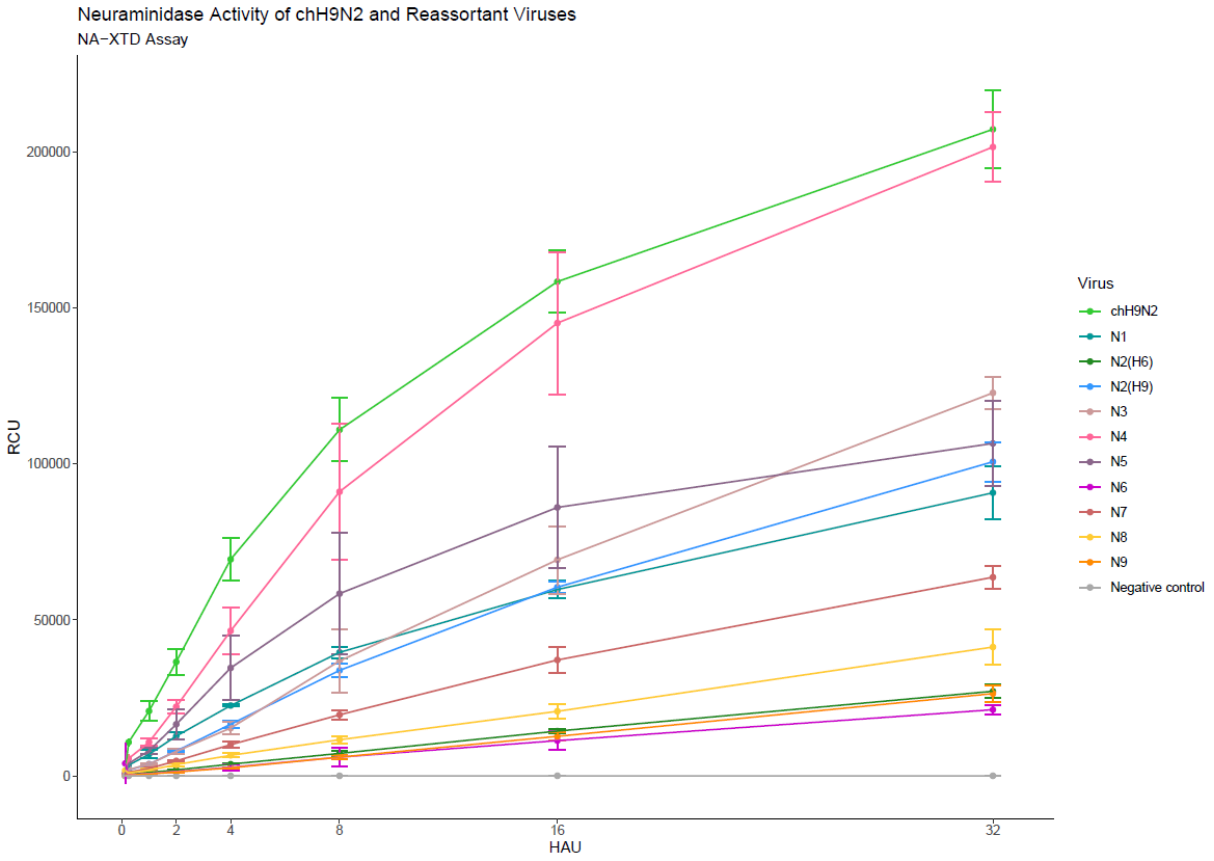

Figure 4. The NA activity of chH9N2 and the reassortant viruses. The NA activity was measured in relative chemiluminescent units (RCU) and plotted against hemagglutination units (HAU). Viruses: chH9N2, chH9N2x$_{mH10N1}$ (N1), chH9Nx$_{mH6N2}$ (N2(H6)), chH9Nx$_{mH9N2}$ (N2(H9)), chH9Nx$_{gH16N3}$ (N3), chH9Nx$_{mH8N4}$ (N4), chH9Nx$_{mH15N5}$ (N5), chH9Nx$_{mH4N6}$ (N6), chH9Nx$_{mH7N7}$ (N7), chH9Nx$_{mH3N8}$ (N8) and chH9N$_{xmH11N9}$ (N9).

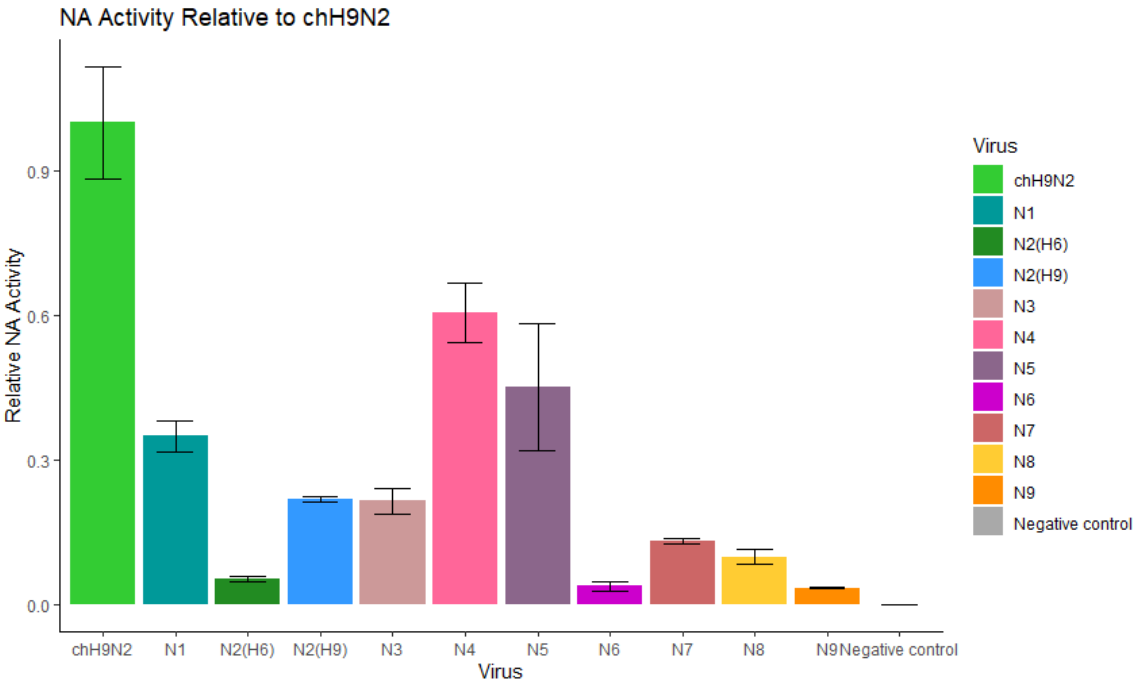

Figure 5. The NA activity relative to chH9N2. Viruses: chH9N2, chH9N2x$_{mH10N1}$ (N1), chH9Nx$_{mH6N2}$ (N2(H6)), chH9Nx$_{mH9N2}$ (N2(H9)), chH9Nx$_{gH16N3}$ (N3), chH9Nx$_{mH8N4}$ (N4), chH9Nx$_{mH15N5}$ (N5), chH9Nx$_{mH4N6}$ (N6), chH9Nx$_{mH7N7}$ (N7), chH9Nx$_{mH3N8}$ (N8) and chH9N$_{xmH11N9}$ (N9).

4.4. Phylogeny of the NAs

Alignment of the NA of chH9N2 and the NAs of the viruses in Table 1 (out grouped by NA from influenza B) showed that the NAs form two distinct groups as previously shown, with N1, N4, N5 and N8 belonging to group 1, and N3, N7, N6, N9 and N2 belonging to group 2 (Figure 6). The three N2 subtypes, form a separate clade within group 2. The two N2 from mallard (H6N2 and H9N2) show a closer evolutional relationship than H9N2 from mallard and H9N2 from chicken. All bootstrap values are >80 % showing good support for the nodes in the phylogenetic tree (70).

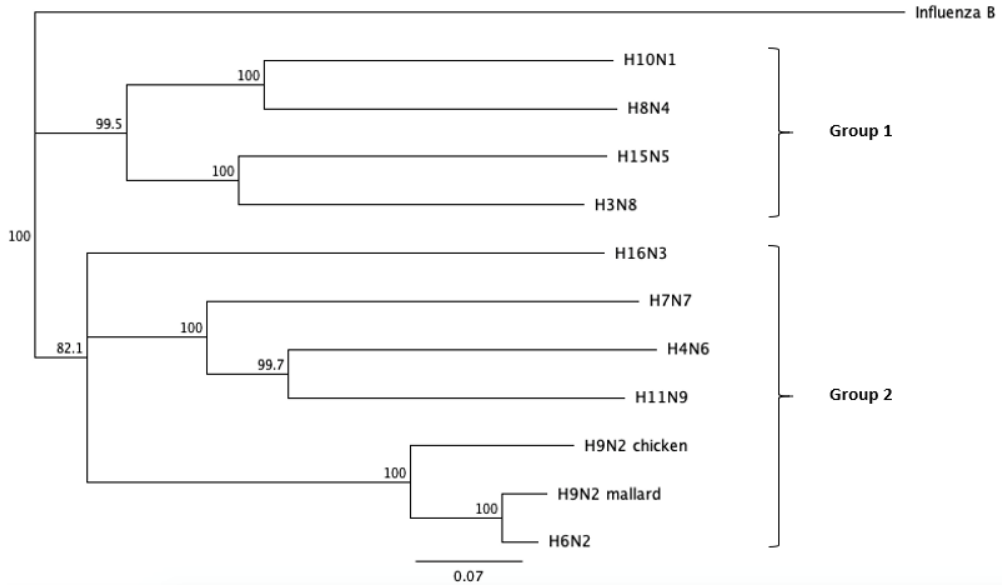

Figure 6. Phylogenetic tree of the NAs used in the project. N1, N4, N5 and N8 belonging to group 1, and N3, N7, N6, N9 and N2 belonging to group 2. The tree is out grouped by NA from influenza B.

5. Discussion

The LPAIV H9N2 lineage from chicken has been found to frequently reassort with other highly, and low pathogenic AIV strains. This lineage is endemic in poultry populations, and through contact with wild birds more reassortment events will most likely arise. Furthermore, H9N2 is capable of infecting humans, with some lineages containing a mutation causing a predilection towards α2,6-linked SAs. Together this suggests that H9N2 is a possible candidate for a potentially new pathogenic IAV, either by itself or though reassortment with other strains. By investigating the compatibility between chH9N2 and NAs from wild birds, the reassortment dynamics of AIVs can be better understood. In this study the genetic compatibility of nine different NA segments (N1-9) of wild bird origin with the LPAIV H9N2 from chicken was investigated.

The phylogenetic tree of the NAs in this study (Figure 6) showed the same evolutionary relationships as previous studies (32), with two separate groups, 1 and 2, and the three N2 subtypes forming a separate clade within group 2. Selecting NA segments from both groups and having representatives for each subtype provides a broader insight into the reassortment dynamics between H9N2 and the different NA segments.

The first objective of the present study was to clone the selected NAs into the pHW2000 plasmid. Sequencing revealed that all NAs from Table 1 were successfully cloned into the pHW2000 plasmid, showing that it is a compatible vector for the NA segments in the project. The cDNA of all the NA segments was ligated into the plasmid without any substantial mutations or deletions occurring in the sequence, creating accurate representatives of the NAs to be used in the eight plasmid transfection system. Successful implementation of the reverse genetics eight plasmid system provides a versatile technique that will enable many more aspects of reassortment to be investigated. This includes exchange of other segments, introduction of genes from other species and pathotypes, and site-directed mutagenesis in selected gene segments.

All of the NA plasmids, which were derived from wild birds, created reassortant viruses when transfected together with the chH9N2 backbone (Table 2). RT-qPCR confirmed that the correct NA was present in all reassortant viruses, and that no cross-contamination with other NA plasmids had occurred (Figure 3). Together, this shows that all of the NA subtypes used in this study (N1-9) are compatible with chH9N2 when transfected *in vitro.* All NAs, except for N3 which came from black-headed gull, where derived from mallards, indicating that there are no host-restriction factors in either mallard or black-headed gull derived viruses preventing reassortment with chH9N2 *in vitro*. However, there could be other barriers preventing successful reassortment or viral replication of the rescued viruses *in vivo*. For example, wild bird derived LPAIV have been shown to have inefficient replication when experimentally infected in poultry, indicating that an adaptation is needed for successful replication and maintenance (71,72). Furthermore, only four out of the nine NA subtypes have been recorded in combination with H9 in chicken (73). This suggests a possible mismatch or barrier with the other NA segments, either preventing reassortment or resulting in a low prevalence, limiting detection of their presence in chickens. Experimental studies in bird and mammalian models could provide insight into any potential barriers and also describe the virulence, pathogenicity, and transmission of the reassortant viruses generated in this study.

However, H9 is found together will all nine NA subtypes in wild birds, supporting the results of this study, that all NA subtypes can create successful reassortant viruses with the chH9N2

backbone. Still, N2 is by far most frequently combined with H9, especially in chickens (73), suggesting a certain compatibility between H9 and N2 that is not found with the other NA subtypes. In this study all the NAs were compatible with chH9N2 when no other NA subtype could compete with the one used in the transfection system. By investigating the reassortment of H9N2 with other NA segments through co-infection one could possibly observe the reassortment dynamics during conditions that resemble those in nature, when two viruses infect the same cell. This could give further insight in why all NA except N2 occur so rarely together with H9.

There are different factors that can affect the reassortment compatibility between different IAV strains and segments. These factors can result in segment mismatch, occurring on RNA or protein level. Mismatches on RNA level are believed to be caused by incompatibilities between the packaging signals of the vRNAs. If the mismatch between the RNA segments is too substantial the assembly of the vRNAs will be restricted, preventing formation of the progeny genotypes (49). In this study reassortant viruses with all NA (N1-9) were created, suggesting no major mismatch on RNA level between the segments. However, this does not rule out incompatibilities between the packaging signals that could result in suboptimal interactions, affecting the packaging during co-infection, when competition between segments take place (74).

Functional balance between HA and NA is necessary for segment compatibility on protein level. In this study the NA activity of the reassortant viruses was measured and compared to the NA activity of chH9N2 to assess any differences and possible shifts in the functional balance. The activity was measured using the NA-XTD assay, based on previously described methods (43,69). The assay showed that the chH9N2 had the most active NA, with all other reassortant viruses having significantly lower NA activities (Figure 4, Figure 5). The high activity of the chH9N2 NA correlates with results from a previous study, where Egyptian H9N2 viruses were shown to have higher NA activities than H9N2 viruses not belonging to the G1-lineage (75). Out of the reassortant viruses, chH9Nx_{mH8N4} had the highest NA activity, most similar to chH9N2. The decrease in activity of the reassortant viruses compared to chH9N2 suggests that there is a shift in the functional balance when the NA is exchanged. Interestingly, the chH9Nx_{mH6N2} and chH9Nx_{mH9N2} showed significantly lower NA activities than both chH9N2 and some of the other reassortant viruses, even though being most genetically similar to chH9N2 (Figure 6). Deeper genetical analysis of the reassortant viruses could perhaps provide more insight into the observed difference in NA activity. Comparing the NA activity of the reassortant viruses and each subtype's parental virus could show if the activity is the same or if it changes during rescue of the new reassortant subtype. Furthermore, by additional measurements of the HA binding affinity (K_d) and NA enzymatic activity (K_m/K_{cat}) as described in earlier studies (69,76), a more precise estimation of the functional balance could be made.

Previous studies have reported that imbalances between the two proteins impairs the fitness of the affected virus (76), and also that the balance is important for infection in human cells (69). Reassortant viruses with a balanced HA/NA should theoretically replicate more efficiently than those with an imbalance. Investigation of the replication kinetics in cell types from different species could provide further insight of the viral fitness of the reassortant viruses in this study, and any possible predilections towards certain host species. It would also be of interest to investigate if the lowered NA activity in the reassortant viruses could have a correlation with the fitness, both in cells and animal models.

In conclusion, this study shows that H9N2 from chicken is compatible with all nine NA subtypes when transfected *in vitro*. This correlates with the confirmed subtypes circulating in wild birds, where H9 is found in combination with all NA. It also demonstrates a compatibility between the chicken derived H9N2 and all nine NA, something that has not been recorded in nature. The impact of the lower NA activity in the reassortant viruses is yet to be determined. The genetical compatibility indicates that there is a possibility for these subtypes to emerge through reassortment between chickens and wild birds, generating an H9Nx subtype not previously recorded in chicken, calling for further research on the reassortment dynamics of H9N2.

Acknowledgements

First, I would like to thank my supervisors Patrik Ellström and Mahmoud Naguib for letting me do my master thesis as a part of their ongoing influenza project. Thank you for guiding and supporting me throughout my project and for inspiring me to hopefully continue with science in the future. This project has increased my theoretical knowledge about influenza and different techniques, as well as improved my practical laboratory skills, an experience I am very thankful for. Secondly, a big thank you to Anishia Wasberg for all the help in the lab and interesting discussions. I will forever be grateful for having had such a competent colleague and also great friend with me during the project. I would also like to thank Steinar Mannsverk for all your help and for always making the days in the lab fun and enjoyable, even during our longest days of cloning. I am also very grateful for having been a part of the Zoonosis Science Center, a group where you feel welcomed, and everyone is very helpful. Finally, I would like to thank my boyfriend Carl and my family, you always support and encourage me, and without you I would not be where I am today.

Supplementary Data

Supplementary Table 1. Forward and reverse primers designed for all NA subtypes used in the cloning process. Green shows the four random nucleotides, blue the restriction site, orange the plasmid sequence, and black the specific nucleotides from the NA segment of interest.

NA subtype	Primers
H10N1	**Fw:** 5' – GATCGGTCTCAGGGAGCAAAAGCAGGAGTTCAAAATGA – 3' **Rv**: 5' – GATCGGTCTCGTATTAGTAGAAACAAGGAGTTTTTTGAA – 3'
H6N2	**Fw:** 5' **–** GATCCGTCTCAGGGAGCGAAAGCAGGAGTGAAAATG – 3' **Rv:** 5' **-** GATCCGTCTCGTATTAGTAGAAACAAGGAGTTTTTTTCTA – 3'
H9N2	**Fw:** 5' – GATCCGTCTCAGGGAGCGAAAGCAGGAGTGAAAATG – 3' **Rv:** 5' - GATCCGTCTCGTATTAGTAGAAACAAGGAGTTTTTTTC – 3'
H16N3	**Fw:** 5' – GATCCGTCTCAGGGAGCAAAAGCAGGTGTGAAATGAATC– 3' **Rv:** 5' – GATCCGTCTCGTATTAGTAGAAACAAGGTGTTTTTTCTATTAC– 3'
H8N4	**Fw:** 5' – GATCCGTCTCAGGGAGCGAAAGCAGGAGTTTCATAATG – 3' **Rv:** 5' – GATCCGTCTCGTATTAGTAGAAACAAGGAGTTTTTTCG – 3'
H15N5	**Fw:** 5 ′- GATCCGTCTCAGGGAGCAAAAGCAGGAGTTTAAAATGAATCC - 3′ **Rv:** 5′- GATCCGTCTCGTATTAGTAGAAACAAGGAGTTTTTTCACAAGC - 3′
H4N6	**Fw:** 5' – GATCCGTCTCAGGGAGCAAAAGCAGGGTGAAAATGAATCC – 3' **Rv:** 5' – GATCCGTCTCGTATTAGTAGAAACAAGGGTGTTTTTCCT – 3'
H7N7	**Fw:** 5' – GATCCGTCTCAGGGAGCAAAAGCAGGGTGATTGAGAATG – 3' **Rv:** 5' – GATCCGTCTCGTATTAGTAGAAACAAGGGTGTTTTTGCA – 3'
H3N8	**Fw:** 5' – GATCCGTCTCAGGGAGCAAAAGCAGGAGTTTAAAATGAATC – 3' **Rv:** 5' – GATCCGTCTCGTATTAGTAGAAACAAGGAGTTTTT – 3'
H11N9	**Fw:** 5' – GATCCGTCTCAGGGAGCAAAAGCAGGGTCAAGATGAATC – 3' **Rv:** 5' – GATCCGTCTCGTATTAGTAGAAACAAGGGTCTTTTTTGC – 3'

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